



Luiz Gustavo Dufner de Almeida

# Mutational and functional study of Tuberous Sclerosis Complex 1 and 2 genes (*TSC1* and *TSC2*)

*Estudo mutacional e funcional dos genes 1 e 2 do Complexo da Esclerose Tuberosa  
(TSC1 e TSC2)*

SÃO PAULO

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*“Eu não tenho ídolos. Tenho admiração por trabalho, dedicação e competência”*

*Ayrton Senna da Silva*

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## Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by pathogenic variants in either *TSC1* or *TSC2* tumor suppressor genes. It affects more often the brain, skin, kidneys, heart, lungs, and retina. The protein products of both genes, TSC1 (hamartin) and TSC2 (tuberin), interact, assembling a complex that inhibits mTORC1. Cells with bi-allelic inactivation of either *TSC1* or *TSC2* genes present hyperactivation of mTORC1, which phosphorylates downstream targets, up-regulating cell proliferation and growth. Moreover, a functional role as heat-shock protein (HSP) co-chaperone has been assigned to TSC1 protein. The first aim of the thesis was to analyze the nature, distribution and functional effects of *TSC1* and *TSC2* DNA variants from 100 patients with definite clinical diagnosis of TSC. We analyzed leukocyte DNA of 117 TSC patients from three Brazilian tertiary referral hospitals. Pathogenic DNA variants were detected in 102 (87.2%) unrelated individuals; 18 (17.6%) in *TSC1* and 84 (82.4%) in *TSC2*. Clear loss-of-function variants were detected in 89 patients, of which frameshift (29.4%) and nonsense (29.4%) were the most common types. In-frame deletions, missense and putative splicing DNA variants with uncertain clinical significance (VUS) have been functionally assessed. Eleven variants significantly increased phosphorylation of the reporter residue S6K Thr<sup>389</sup>. Forty-two novel pathogenic DNA variants and 19 novel single nucleotide variants have been detected. Among the 15 individuals with no mutation identified, ten rare alterations classified as VUS were detected and were putative missense, splicing, large deletion or in the 3'-UTR. To understand the regulatory relationship of *TSC1/2* gene expression, we aimed to evaluate *TSC1* and *TSC2* mRNA and protein levels in human cell lines with bi-allelic inactivation of each gene. We employed high-throughput transcriptome analysis (RNA-Seq) and Western blotting of HEK293T and other six HEK293T-derived cell lines that had the genomic sequence of the *TSC1* and or *TSC2* genes edited by the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 system. In lack of either TSC1 or TSC2 protein, a significant reduction of the respective mRNA was observed, inferring no positive transcriptional feedback. Serum-deprived cell lines without TSC1 decreased *TSC2* mRNA levels. Under these conditions, *TSC1* mRNA levels were not negatively affected by the lack of TSC2. In one cell line with loss of TSC1 (1C2) *TSC1/2* mRNA and TSC2 protein levels were consistently decreased independently on serum. RNA-Seq gene ontology analyses comparing 1C2 to HEK293T reference cell line disclosed down-regulation of genes from translational pathways independently on serum; and up-regulation of genes related to protein folding and stability pathways upon serum withdrawal. Our data are consistent with the role of TSC1 as HSP co-chaperone, and suggest that *TSC1* mRNA may be regulated at both transcriptional and decay levels.

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## Resumo

O complexo da esclerose tuberosa (TSC) é um distúrbio autossômico dominante causado por variantes patogênicas em um de dois genes supressores de tumor *TSC1* ou *TSC2*. Afeta mais frequentemente o cérebro, a pele, os rins, o coração, os pulmões e a retina. Os produtos proteicos de ambos os genes - TSC1 (hamartina) e TSC2 (tuberina) – interagem e formam um complexo que inibe o mTORC1. Células com inativação bi-alélica dos genes *TSC1* ou *TSC2* apresentam hiperativação de mTORC1, a qual fosforila alvos a jusante e regula positivamente proliferação e crescimento celular. Um papel funcional como co-chaperona da proteína de choque térmico (HSP) foi atribuído à proteína TSC1. O primeiro objetivo desta tese foi analisar a natureza, distribuição e os efeitos funcionais das variantes de DNA de *TSC1* e *TSC2* de 100 pacientes com diagnóstico clínico definitivo de TSC. Analisamos o DNA de leucócitos de 117 pacientes com TSC acompanhados em três hospitais brasileiros de atenção terciária. Variantes patogênicas de DNA foram detectadas em 102 (87,2%) indivíduos não relacionados; 18 (17,6%) em *TSC1* e 84 (82,4%) em *TSC2*. Variantes patogênicas de perda de função foram detectadas em 89 pacientes, dos quais as variantes *frameshift* (29,4%) e *nonsense* (29,4%) foram os tipos mais comuns. Deleções em fase, variantes missense e variantes de *splicing* com significância clínica incerta (VUS) foram avaliadas funcionalmente. Onze variantes aumentaram significativamente a fosforilação do resíduo Thr<sup>389</sup> da proteína repórter S6K. Quarenta e duas novas variantes patogênicas e 19 novas variantes de nucleotídeo único foram detectadas. Identificaram-se entre os 15 NMI indivíduos, dez alterações raras, classificadas como VUS, do tipo grande deleção, missense, *splicing* ou na região 3'-UTR. Para entender a relação regulatória da expressão dos genes *TSC1* e *TSC2*, o segundo objetivo foi avaliar os níveis de RNAm e proteína de TSC1 e TSC2 em linhagens de células humanas com inativação bi-alélica de cada gene. Empregamos uma análise de transcriptoma de alto rendimento (RNA-Seq) e *Western blotting* de HEK293T e outras seis linhagens celulares derivadas de HEK293T que possuíam a sequência genômica dos genes *TSC1* e/ou *TSC2* editados por CRISPR-Cas9. Na falta da proteína TSC1 ou TSC2, foi observada uma redução significativa do respectivo RNAm, inferindo ausência de feedback transcricional positivo. Linhagens celulares *TSC1*<sup>-/-</sup>, privadas de soro diminuíram os níveis de RNAm de *TSC2*. Sob estas condições, os níveis de RNAm de *TSC1* não foram afetados negativamente pela falta de *TSC2*. Numa linhagem celular com a perda de TSC1 (1C2), RNAm de *TSC1/2* e proteína TSC2 foram consistentemente diminuídos independentemente do soro. Análise de ontologia gênica de dados de RNAm comparando a linhagem celular de 1C2 com a referência HEK293T revelou uma regulação negativa de genes de vias de tradução independentemente de soro; e, após a retirada do soro, regulação positiva de genes das vias de dobramento e estabilidade de proteínas. Nossos dados são consistentes com o papel do TSC1 como co-chaperona de HSP, e sugerem que o RNAm de *TSC1* pode ser regulado ao nível da transcrição.

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This thesis has three chapters. The first one reviews the genetics and diagnostics aspects of tuberous sclerosis complex (TSC). The second chapter presents the results of DNA variant analyses in *TSC1* and *TSC2* genes in a cohort of 117 patients with definite clinical diagnosis of TSC. The last chapter is centered in the analysis of *TSC1* and *TSC2* gene expression in human cell lines lacking either protein. As the two aims of the thesis were non-overlapping, chapters 2 and 3 have each a final section where conclusions are presented.

# Chapter I

Genetics and diagnostics of tuberous  
sclerosis complex (TSC)

## List of Acronyms

- ADPKD:** Autosomal dominant polycystic kidney disease
- AML:** Angiomyolipomas
- eIF4E:** Eukaryotic translation initiation factor 4E
- GAP:** GTPase-activating protein
- HSP:** Heat-shock proteins
- LAM:** Lymphangioleiomyomatosis
- MLPA:** Multiplex ligation-dependent probe amplification
- MMPH:** Multifocal, micronodular, type II pneumocyte hyperplasia
- mTORC1:** Mechanistic target of rapamycin complex 1
- PECs:** Perivascular epithelioid cells
- PKD1:** Polycystic Kidney Disease 1 gene
- RHEB:** RAS-homology enriched in brain
- S6K1:** p70 40S ribosomal protein S6 kinase B1
- SEGA:** Subependymal giant cell astrocytoma
- SEN:** Subependymal nodule
- TSC:** Tuberous sclerosis complex
- TSC1:** Tuberous sclerosis complex 1 gene
- TSC2:** Tuberous sclerosis complex 2 gene
- UTR:** Untranslated region

## List of Tables

Table I-1: First clinical cases reported with signs of TSC in the 1860s to 1880s.....	18
Table I-2: Revised criteria for the diagnosis of TSC according to the International Conference on Consensus on Tuberous Sclerosis Complex (modified from Northrup et al. (2013)). .....	19

## List of Figures

Figure I-1: Map of the human chromosome 16p13.3 zoomed into 4,130 bp of TSC2 and PKD1 gene 3'-end overlapping region.....	25
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## Summary

I.	Chapter I .....	11
I.	Background .....	16
A.	TSC genetics .....	16
B.	Historical aspects of TSC diagnosis .....	17
C.	TSC lesion classification according to the cell of origin: clinical manifestations and outcomes	19
II.	TSC molecular diagnosis and genotype-phenotype correlations .....	24
III.	TSC1 and TSC2 proteins .....	26
A.	TSC2 GAP domain and the mechanistic target of rapamycin (mTOR) pathway .....	26
B.	Tsc1 as Hsp90 co-chaperone .....	27
IV.	References .....	29

## I. Background

Tuberous sclerosis complex (TSC) is a clinical disorder characterized as hereditary, multiple system hamartomatosis. TSC hamartomas can affect any organ, although most commonly the brain, heart, kidneys, lungs, retina and skin. It has an estimated incidence between 1:10,000 and 1:6,000 live born infants, and no ethnic bias (Sampson, Scahill *et al.* 1989, O'Callaghan, Shiell *et al.* 1998, Au, Williams *et al.* 2007).

The term hamartoma, suggested by Albrecht in 1904, defines slow-growing benign tumors of cells that normally occur in the tissue of origin, arranged in a disorganized, though circumscribed, non-encapsulated manner (Cooper 1971). TSC specific organ hamartomas classify as infrequent histological types, and in general have onset in a defined developmental period. Clinical manifestations usually occur due to organ dysfunction as a result of tissue architecture rupture or compressive or vascular phenomena (Roach and Sparagana 2004, Franz, Bissler *et al.* 2010, Grajkowska, Kotulska *et al.* 2010, Krueger, Northrup *et al.* 2013, Northrup, Krueger *et al.* 2013). Although neoplasms are not TSC hallmarks, they may uncommonly occur at earlier age than in the general population, in specific organs, notably the kidneys.

Two characteristics are frequently shared by TSC hamartomas: (i) the increased size of the affected cells, which are commonly described as giant cells; and (ii) the challenge to classify the cell type by immunolabeling with specific antibodies (Huttenlocher and Wollmann 1991). Hence, it has been proposed that TSC hamartomas stem from deficiencies in cell size, proliferation and differentiation control.

### A. TSC genetics

TSC is an autosomal dominant disorder caused by germline pathogenic variants in either the *TSC1* or *TSC2* tumor suppressor genes (Consortium 1993, van Slegtenhorst, de Hoogt *et al.* 1997). Molecular studies of TSC hamartomas resected from mouse models of the disease or from patient surgery specimens have mostly agreed with Knudson's second-hit hypothesis (Niida, Stemmer-Rachamimov *et al.* 2001), which has been established as a genetic model for tumor suppressor genes (Knudson 1971). It currently predicts that a somatic cell harboring a germline mutation will present tumor clonal properties if the wild-type allele is inactivated by a pathogenic DNA variant or epigenetic alteration. The randomness and uncertainty of the second allele-inactivating hit are believed to contribute to decrease the penetrance of the disease. TSC incomplete penetrance has been reported (Connor, Stephenson *et al.* 1986, Webb and Osborne 1991). However, recent technological developments have estimated the penetrance of this disease to be, though incomplete, significantly higher than previously observed (Hasbani and Crino 2018). As presented below, TSC clinical diagnostics is based on lesion identification and not on symptomatic clinical presentation (Northrup *et al.*, 2013). Therefore, the increase in population coverage of medical imaging has allowed for the definite clinical diagnosis in asymptomatic patients. Moreover, molecular analysis breakthroughs and the availability of

parental DNA testing have contributed to increase the estimates of the disease penetrance rates (Caylor, Grote *et al.* 2018). On the other hand, factors that may negatively influence the penetrance of TSC are its clinical heterogeneity, later age at onset for specific organ hamartomas of the adulthood, and *TSC1* and *TSC2* genotype-phenotype correlations (Northrup, Wheless *et al.* 1993, Camposano, Greenberg *et al.* 2009, von Ranke, Faria *et al.* 2017). Remarkably, nearly 70% of TSC cases have been reported as *de novo*, due to a novel mutation that may be in somatic mosaicism. Consequently, inconclusive diagnosis may implicate in difficulty to reconcile clinical data and mutation segregation studies in families. Finally, familial cases tend to have higher penetrance (Kwiatkowski 1994).

## B. Historical aspects of TSC diagnosis

TSC was first described in 1862 by Friedrich D. von Recklinghausen after the autopsy of a newborn patient disclosed cardiac tumors and malformations of the brain surface. A similar case was later reported in 1864 by Rudolph Virchow. However, it was only in 1880 that the French physician, Désiré Magloire Bourneville, coined the name of the disease as tuberous sclerosis of the cerebral convolutions upon description of autopsied cerebro-cortical malformations that presented tuber-like morphology and consistency firmer than the adjacent parenchyma. The patient had epilepsy, as well as facial and renal lesions (Bourneville 1880, Gomez, Sampson *et al.* 1999, Jansen, van Nieuwenhuizen *et al.* 2004) (Table I-1).

Taken together, these descriptions and four other clinical cases reported until 1890 (Table I-1) present clinic-pathological aspects of a disorder that appeared (i) to be systemic, affecting the brain, skin, heart and kidneys; (ii) to be presented as a variable combination of signs, not necessarily observed in all patients; and (iii) to have cortical tubers as the commonest lesion. TSC is also classified as a phacomatosis (from the Greek word *phakos*, birthmark), a clearly congenital condition associated with a pathological birthmark (McCall, Chin *et al.* 2006), or as neurocutaneous syndrome, an inherited condition with major involvement of both, brain and skin.

Table I-1: First clinical cases reported with signs of TSC in the 1860s to 1880s.

Physician	Year	Cardiac tumor	Cortical tubers	Periventricular nodules	Angiofibroma	Fibrotic plaques	Kidney injuries
Friedrick D. von Recklinghausen	1862	✓	✓				
Rudolph Virchow	1864	✓	✓				
Désiré Magloire Bourneville	1880		✓		✓		✓
Hartdegen	1881		✓	✓			
Bourneville and Brissaud	1881	✓	✓	✓			✓
François H. Hallopeau and Émile Leredde	1885		✓		✓		
John J. Pringle	1890		✓		✓	✓	

✓: Observed phenotype

In 1908, Vogt proposed a clinical triad consisting of sebaceous adenoma (angiofibroma), intellectual disability and epilepsy for the diagnosis of TSC. The development and improvement of medical imaging techniques expanded the knowledge on the disease, leading to the first classification of lesions into major and minor criteria for TSC diagnosis by Gómez in 1979. TSC diagnosis criteria were later reviewed twice by medical experts (Roach, Gomez *et al.* 1998, Northrup, Krueger *et al.* 2013). The currently adopted diagnostics criteria are presented in (Northrup, Krueger *et al.* 2013). The combination of major and minor criteria may yield a definite or possible clinical diagnosis. It is important to notice that each lesion defined as a criterion can occur isolate in a patient, due to two somatic mutation hits. Thus, the adherence to these guidelines is of utmost importance in the clinical setting. Moreover, the latest review board has included the genetic diagnostic criteria, by which the detection of a pathogenic variant in either *TSC1* or *TSC2* genes is sufficient for the definite diagnosis of TSC. Genetic variants of *TSC1* or *TSC2* not confirmed as pathogenic are not a criterion for the diagnosis (Table I-2).

Table I-2: Revised criteria for the diagnosis of TSC according to the International Conference on Consensus on Tuberous Sclerosis Complex (modified from Northrup et al. (2013)).

<b>A. Genetic diagnostic criteria</b>	
The identification of a pathogenic mutation in the <i>TSC1</i> or <i>TSC2</i> gene in normal tissue is enough for the definitive diagnosis of TSC *.	
<b>B. Clinical diagnostic criteria</b>	
<b>Major features</b>	
1.	Hypomelanotic macules (>3, at least 5-mm diameter)
2.	Angiofibromas (≥3) or fibrous cephalic plaque
3.	Ungual fibromas (>2)
4.	Shagreen patch
5.	Multiple retinal hamartomas
6.	Cortical dysplasias**
7.	Subependymal nodules (SEN)
8.	Subependymal giant cell astrocytoma (SEGA)
9.	Cardiac Rhabdomyoma
10.	Lymphangioleiomyomatosis (LAM)***
11.	Angiomyolipomas (>2)*** (AML)
<b>Minor features</b>	
1.	“Confetti” skin lesions
2.	Dental enamel pits (>3)
3.	Intraoral fibromas (≥2)
4.	Retinal achromic patch
5.	Multiple renal cysts
6.	Nonrenal hamartomas
<b>Definite diagnosis:</b> Two major features; or one major feature with ≥2 minor features.	
<b>Possible diagnosis:</b> One major feature; or ≥2 minor features.	

\* A pathogenic DNA variant is defined as an alteration that inactivates the function of the proteins expressed by *TSC1* or *TSC2*, prevents protein synthesis, **or** is a missense variant affecting protein function if demonstrated by a functional assay ([www.lovd.nl/TSC1](http://www.lovd.nl/TSC1), [www.lovd.nl/TSC2](http://www.lovd.nl/TSC2)).

\*\* Cortical dysplasia includes cortical tubers and/or radial migration lines in cerebral white matter.

\*\*\* A combination of the two main criteria - LAM and angiomyolipomas - in the absence of another criterion does not fit the definite diagnosis of TSC, being considered as a single main criterion, due to the co-occurrence of the two lesions in at least 30% of patients with isolated form of LAM<sup>1</sup>.

## C. TSC lesion classification according to the cell of origin: clinical manifestations and outcomes

TSC lesions can be classified in different ways; according to organ or system affected, histological diagnosis subtyping, following clinical manifestations, etc. Here we divide TSC into five large groups according to the pathogenesis and cell of origin.

### 1. Central nervous systems (CNS) lesions: TSC as a neurodevelopmental disorder

Nearly 80% of patients with TSC will present subependymal nodules (SEN, (Houser and Gomez 1992)), 5-mm nodules in the subependymal layer of the lateral cerebral ventricles protruding into their cavity, with a smooth, firm and hardened surface, due to frequent calcification. They are lined by a relatively intact ependymal epithelium. Giant cells, with large, convoluted nuclei tend to be observed inside the nodule. They are highly vascular lesions, with vessels prone to hyalinization (Trombley and Mirra 1981, Scheithauer and

Reagan 1999). Internal hemorrhage and necrosis are rare but may be observed in larger nodules. In 5% to 15% of patients with TSC, SENs may progress to subependymal giant cell astrocytoma (SEGA), especially in the first two decades of life, a hypothesis supported by follow-up imaging tests, histological similarities between these hamartomas and their encephalic location (Roach and Sparagana 2004, Grajkowska, Kotulska *et al.* 2010). The majority of SEGAs are solitary, discrete masses, larger than 5 mm in diameter and located near the foramen of Monro.

SENs and SEGAs are hamartomas formed by dysplastic astrocytes and neuronal cells (Gomez, Sampson *et al.* 1999, Jozwiak, Jozwiak *et al.* 2005). Despite debates on their origins (Davidson, Yoshidome *et al.* 1991, Johnson, Yoshidome *et al.* 1991), and although classified as astrocytomas, it is now recommended they should be classified as tumors of mixed origin due to the neuroglial composition of SEGAs (Buccoliero, Franchi *et al.* 2009). This composition suggests that SEN and SEGA should originate from neuroglial progenitors of the subventricular zone of the brain (Crino 2013). Although SEGA is a tumor classified as grade I astrocytoma (World Health Organization), it may maintain a slow growth rate, at most 2 mm per year. This growth and its location may contribute to hydrocephalus and acute intracranial hypertension, due to obstructive phenomena. Vascular calcification and hyalinization contribute to the rare occurrence of intramural hemorrhage. Calcification of the stroma is more common in SEN than in SEGA (Huttenlocher and Wollmann 1991, Shepherd, Scheithauer *et al.* 1991, Stefansson 1991, Scheithauer and Reagan 1999).

Cortical tubers are lesions present at birth in most patients with TSC. However, its observation by magnetic resonance imaging may be impaired in the first two years of life, a period necessary for myelination, which increases the contrast for the identification of these lesions (Houser and Gomez 1992, Shepherd, Houser *et al.* 1995). Cortical tubers consist of circumscribed enlargement of cerebral gyri in the cerebral parenchyma, with cortical and subcortical location, presenting itself firmer than the adjacent parenchyma. Their slight protrusion in the cerebral parenchyma creates tubercle-like appearance (tuber). Therefore, the term tuberous sclerosis was coined to name the disease.

Neuronal heterotopias result from dissynchrony in neuronal migration along the radial glial fibers (Roach and Sparagana 2004). They present as radial migration lines of the white matter, possibly formed by groups of post-mitotic neuron soma that did not complete the radial migration process (Northrup, Krueger *et al.* 2013, van Eeghen, Teran *et al.* 2013). Cortical tubers and neuronal heterotopias are collectively named dysplasias. Since they are composed of non-proliferative cells, they classify as hamartias, and not hamartomas (Huttenlocher and Wollmann 1991, Scheithauer and Reagan 1999).

Cortical tubers and neuronal heterotopias are associated with epilepsy, often refractory to anti-convulsant medication. They may associate with intellectual disability, autism and other types of behavioral disorders. In most patients younger than two years, seizures manifest as infantile spasms, which if not fully

controlled, may progress to other types of seizures, and associate with autism and intellectual disability (Chiron, Dumas *et al.* 1997, Hancock and Osborne 1998, Curatolo, Seri *et al.* 2001, Thiele 2004, Adriaensen, Schaefer-Prokop *et al.* 2009, Chu-Shore, Major *et al.* 2010). The lack of therapeutic control of epilepsy in the first five years of life (60-80% of patients with TSC - (Orlova and Crino 2010)) appears to relate to later severity of intellectual disability (Joinson, O'Callaghan *et al.* 2003, Humphrey, Williams *et al.* 2004). Although a correlation between the number and location of cortical tubers and clinical severity has not been convincingly established, patients with temporal distribution of tubers seem to develop more severe epilepsy and behavioral problems (Goodman, Lamm *et al.* 1997, Hosoyaa, Naitoa *et al.* 1999, Holmes, Stafstrom *et al.* 2007). The absence of clinical manifestations due to cortical tubers in the first five years of life is related to a better cognitive and social function in this period and in successive phases of development (Joinson, O'Callaghan *et al.* 2003, Humphrey, Williams *et al.* 2004).

The location of each of these four brain lesions (SEN, SEGA, neuronal heterotopias and cortical tubers), commonly observed in the same patient with TSC, suggested they may have a common origin. It has been demonstrated that SEN and SEGA present cell proliferation and growth control deficiency, and potentially originate after the embryonic corticogenesis period from subventricular zone progenitor cells, in which a second, somatic *TSC1* or *TSC2* mutation is expected. Early in embryonic development (end of the first third of human gestation), post-mitotic neurons generated from neuroglial progenitors start migrating to initiate corticogenesis. Post-mitotic migrating neurons, possibly with cell dysfunction in adhesion and migration processes (Lamb, Roy *et al.* 2000, Goncharova, Goncharov *et al.* 2004) may prematurely abrogate cell migration, and accumulate, forming neuronal heterotopias. Neurons that may reach the appropriate cortical layer could form cortical tubers due to synaptogenesis defects (Johnson, Yoshidome *et al.* 1991, Mizuguchi and Takashima 2001, Nishio, Morioka *et al.* 2001, Crino 2013).

## 2. PEComas

The World Health Organization histological, including immunohistochemical, definition of perivascular epithelioid cell neoplasms (PECs) is mesenchymal tumors of distinct perivascular epithelioid cells. The family of tumors of the PEComa type is diverse. In patients with TSC, PEComas occur in the form of angiomyolipomas (AML) and pulmonary lymphangioleiomyomatosis (LAM) (Martignoni, Pea *et al.* 2008, Folpe and Kwiatkowski 2010).

PEComas are characterized by their perivascular localization and are often arranged radially around the vascular lumen. Careful analysis of PEComas reveals blood vessels compressed by the tumor wall. They present eosinophilic, clear to transparent cytoplasm, different from smooth muscle cells, which are intensely eosinophilic. Perivascular epithelioid cells can accumulate large amounts of lipids, mimicking adipocytes or lipoblasts, cells normally found distant from the vascular wall (Folpe and Kwiatkowski 2010).

AML is the most common PEComa, with a prevalence of 0.13% in adults, being more frequently diagnosed in women than in men. Renal AMLs are seen in 60% to 80% of adult patients with TSC. Displaying a higher frequency in adulthood, AMLs notably have age-dependent frequency (Rakowski, Winterkorn *et al.* 2006). Despite the high frequency among patients with TSC, about 80% of patients with AML do not have TSC; hence, isolate AML is more common than associated with TSC (Eble 1998).

These lesions are highly vascularized hamartomas of mixed composition, whose cells have characteristics of adipocytes and smooth muscle, also presenting dysmorphic blood vessels. It is now believed that AMLs are derived from perivascular epithelioid cells (Weeks, Malott *et al.* 1991, Ashfaq, Weinberg *et al.* 1993), although the nature of these cells is still a matter of debate, suggestive of pericytes or a cell type originating from the neural crest (Fernandez-Flores 2011).

AMLs are usually expansive, infiltrating masses, which can undergo vascular complications such as microthrombi, embolism and bleeding. These tumors are most commonly seen in the kidneys, and may occur in other location, such as in the liver, adrenal glands, pancreas, and pelvis (Crundwell 2004, Orlova and Crino 2010, Yang, Feng *et al.* 2012, Northrup, Krueger *et al.* 2013). Although a large proportion of patients with AML are asymptomatic (Folpe and Kwiatkowski 2010), there is a risk of local painful bleeding due to rupture of tumor aneurysms or compression and/or invasion of the renal parenchyma. The likelihood of progression to chronic renal failure is high if AML is not treated (Northrup, Krueger *et al.* 2013).

Pulmonary LAM is a progressive and often fatal disease caused by proliferation of perivascular epithelioid cells in both lungs, in muscle fibers, distributed around lymphatic vessels, bronchi, interlobar septa, and pleura (Smolarek, Wessner *et al.* 1998, Folpe and Kwiatkowski 2010). The (i) diffuse and bilateral distribution of LAM in the lungs, (ii) the co-occurrence of AML and isolate LAM, (iii) the demonstration of somatic mutation in the *TSC2* gene in both lesions, AML and LAM, from the same patient, and (iv) the identification of circulating cells with the same aspect of perivascular epithelioid cells found in these hamartomas are all evidences suggesting that LAM results from AML metastasis to the lung (Carsillo, Astrinidis *et al.* 2000).

Non-TSC pulmonary LAM (70%) is more common than LAM in association with TSC (30%) (Smolarek, Wessner *et al.* 1998, Carsillo, Astrinidis *et al.* 2000, Franz, Bissler *et al.* 2010). The first studies indicated that 1% to 5% of patients with TSC had LAM (Castro, Shepherd *et al.* 1995). After the increased use of computed tomography of the thorax, it was revealed that between 25% and 40% of female patients with TSC have LAM (Moss, Avila *et al.* 2001). More recent studies indicate that the frequency of LAM in patients with TSC increases with age and can reach 80% of female patients at the end of the fourth decade of life. Patients with AML present progressive dyspnea on exertion and recurrent pneumothorax, typically in the third and fourth decades of life (Northrup, Krueger *et al.* 2013).

### 3. TSC cutaneous signs

Hypomelanotic macules are frequent signs in patients with TSC, since they occur in 90% of the patients, often at birth, and are thus useful for the clinical diagnosis. At least three hypomelanotic macules with 5 mm or more in diameter consist a major criterion for the clinical diagnosis (Northrup, Krueger *et al.* 2013).

Facial angiofibromas, characterized as erythematous, painful papules, commonly in the malar processes occur in approximately 75% of patients with TSC. Usually these lesions develop around 2 to 5 years of age, being very evident in the pubertal phase (Northrup, Krueger *et al.* 2013). Over time, these lesions become larger, with no significant increase in total number, and may extend to the nasolabial folds (Roach and Sparagana 2004).

Ungual fibroids are nodules in the nail bed, usually painful, that can appear upon minor trauma. They have a frequency of 20% in young patients, and higher than 80% in adults with TSC (Roach and Sparagana 2004, Northrup, Krueger *et al.* 2013).

Fibrotic plaques usually occur on the scalp or face in 25% of TSC patients, or in the form of Shagreen plaques in 50% of patients. The latter appear as lumbar plaques with an irregular surface with appearance of leather or orange peel, and its clinical presentation most frequently relate relate to TSC (Northrup, Krueger *et al.* 2013).

### 4. Cardiac Rhabdomyomas

Two-thirds of the fetuses and newborns with TSC have one or more cardiac rhabdomyomas. Although these tumors usually regress in the postnatal period, there may be a second peak of frequency in adolescence. These hamartomas can cause cardiac arrhythmia often in newborns or do not present any clinical sign. Their prenatal or neonatal identification is an early sign of TSC, very useful for the diagnosis as it is highly suggestive of TSC (Roach and Sparagana 2004, Northrup, Krueger *et al.* 2013).

### 5. Epithelial cells hyperplasia

Hyperplasia is defined as proliferation of cells at rates higher than the average expected for the tissue. In patients with TSC, they occur most commonly in the lungs and kidneys, although they may affect other organs, such as the liver.

Multifocal, micronodular, type II pneumocyte hyperplasia (MMPH) is a possible pulmonary manifestation in TSC patients. MMPH is characterized by small nodules (1 mm to 10 mm), with clear margins, on the wall of the alveoli in both lungs, at a frequency between 2% and 3% among TSC patients. MMPH can

occur independently of pulmonary LAM (Muir, Leslie *et al.* 1998, Maruyama, Seyama *et al.* 2001, Hayashi, Kumasaka *et al.* 2010).

Although renal epithelial cell hyperplasia as single, unilateral or bilateral cysts may occur in patients with TSC, their isolate presentation is very common in the general population, and increases with aging. On the other hand, 5% of patients with TSC present a genomic deletion of chromosome 16 involving the 3' portions of the contiguous genes, *TSC2* and *PKD1* (Polycystic Kidney Disease 1 gene). In these patients, with the TSC-ADPKD contiguous deletion syndrome, there is coexistence of TSC with ADPKD, a condition characterized by bilateral development of multiple renal cysts (Bastos and Onuchic 2011).

## II. TSC molecular diagnosis and genotype-phenotype correlations

The *TSC1* (NG\_012386.1, NM\_000368.4) gene maps to 9q34.1, has 23 exons and approximately 55 kb. The coding sequence for the 1,164-amino acid TSC1 protein, also known as hamartin (130 kiloDaltons - KD), lies in exons 3 through 23 (van Slegtenhorst *et al.*, 1997). Exon 23 is the largest (5,407 bp) *TSC1* exon, contributing to the long length of the 3'-untranslated region (UTR) of its transcript. *TSC1* intron 1 is the longest (9,447 bp) intron, followed by introns 8 (8,905 bp) and 2 (6,080 bp). The average exon size is 374 bp, while the average intron size is 2030 bp.

The *TSC2* (NG\_005895.1, NM\_000548.3) gene maps to 16p13.3, has 42 exons and nearly 46 kb in length. Its coding sequence resides in exon 2 through exon 42, and is responsible for the synthesis of a 1,807-amino acid protein (tuberin or TSC2, 180 kiloDaltons). The largest *TSC2* exon is 488-bp long (exon 34), and the largest intron has 4,820 bp (intron 16). The average exon size is 134 bp, while the average intron size is 855 bp ((Consortium 1993, Jones, Daniells *et al.* 1997, van Slegtenhorst, de Hoogt *et al.* 1997, Dabora, Jozwiak *et al.* 2001, Sancak, Nellist *et al.* 2005, Au, Williams *et al.* 2007)).

Since the cloning of the *TSC1* and *TSC2* genes ((Consortium 1993, van Slegtenhorst, de Hoogt *et al.* 1997)), TSC mutation detection has been performed by Sanger sequencing of PCR products from different exons of each gene ((Wilson, Ramesh *et al.* 1996, Jones, Daniells *et al.* 1997, Au, Rodriguez *et al.* 1998, Niida, Lawrence-Smith *et al.* 1999, van Slegtenhorst, Verhoef *et al.* 1999, Dabora, Jozwiak *et al.* 2001)). Techniques such as MLPA (multiplex ligation-dependent probe amplification) and microarray comparative genome hybridization have additionally been used to search for *TSC1* and *TSC2* genomic deletions ((Rendtorff, Bjerregaard *et al.* 2005, Kozlowski, Roberts *et al.* 2007, Oyazato, Iijima *et al.* 2011, van den Ouweland, Elfferich *et al.* 2011)), which correspond to approximately 7% of TSC-causing mutations (Kozlowski, Roberts *et al.* 2007). In the last decade, next-generation sequencing (NGS) of leukocyte DNA has been employed to search for *TSC1* and *TSC2* mutations. Most commonly, both gene coding sequences have been captured for NGS library constructions of whole exome or customized multigene panels (Qin, Kozlowski *et al.* 2010, Tyburczy, Wang *et al.* 2014, Nellist, Brouwer *et al.* 2015, Tyburczy, Dies *et al.* 2015, Ismail, Rani *et al.* 2017).

Sanger or next-generation DNA sequence efforts generally detect pathogenic DNA variants in 80% to 95% TSC patients with definite clinical diagnosis. Nearly 70% of all pathogenic variants are *de novo* germline or post-zygotic alterations. These novel mutations occur approximately four times more often in the *TSC2* than the *TSC1* gene. Among the familial cases, *TSC1* and *TSC2* pathogenic DNA variants have similar frequencies (Wilson, Ramesh *et al.* 1996, Au, Rodriguez *et al.* 1998, Jones, Shyamsundar *et al.* 1999, Niida, Lawrence-Smith *et al.* 1999, van Slegtenhorst, Verhoef *et al.* 1999, Astrinidis, Khare *et al.* 2000, Dabora, Jozwiak *et al.* 2001, Rendtorff, Bjerregaard *et al.* 2005, Sancak, Nellist *et al.* 2005, Hung, Su *et al.* 2006, Au, Williams *et al.* 2007, Kozlowski, Roberts *et al.* 2007, Sasongko, Wataya-Kaneda *et al.* 2008).

A genotype-phenotype correlation is observed in patients with a contiguous *TSC2* and *PKD1* gene deletion (Brook-Carter *et al.*, 1994; Longa *et al.*, 1997). The two genes lie at 16p13.3 in a tail-to-tail fashion, displaying their polyadenylation signals 60 bp apart (Figure I-1). The development of polycystic kidney disease in TSC patients is due to gross deletions involving both genes; thus, the phenotype has been termed *TSC2/PKD1* contiguous gene deletion syndrome (Brook-Carter *et al.*, 1994).

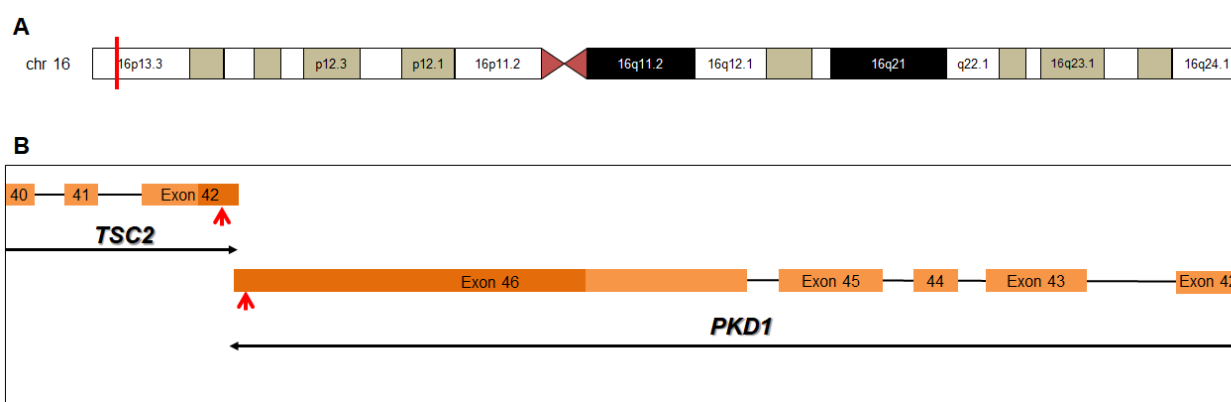


Figure I-1: Map of the human chromosome 16p13.3 zoomed into 4,130 bp of *TSC2* and *PKD1* gene 3'-end overlapping region. (A) Human chromosome 16. The red bar indicates the location of both genes *TSC2* and *PKD1* on p13.3 band. (B) Zoom into 4,130 bp relative to the red bar from (A). The direction of *TSC2* and *PKD1* genes are indicated by thin opposite arrows, as their coding sequences are in different strands of the chromosome 16 DNA. Rectangles represent exons, and lines, introns. Exon numbers from each gene 3'-end are indicated. Dark orange are untranslated region, and light orange rectangles correspond to the coding sequence. Polyadenylation signals (red thick, short arrows) are 60 bp apart. The drawing of the figure was fully based on and in scale to data retrieved from the genome browser at the University of California in Santa Cruz (Santa Cruz, CA, USA; <http://genome.ucsc.edu>), according to the GRCh38/hg38 human genome version. Accessed in April, 2019.

Although TSC patients without *PKD1* gene alterations may develop renal cysts, the histopathological features appear different in the contiguous gene deletion syndrome (Consugar, Wong *et al.* 2008). Information on kidney imaging data is helpful for genetic service decision on the choice of DNA test, as MLPA analysis has currently a relatively lower cost, and should be sufficient to detect the genetic cause of the contiguous gene deletion syndrome (Consugar, Wong *et al.* 2008).

TSC cases classified as mild phenotypes tend to be diagnosed at older age, have more frequently normal cognition, less epilepsy episodes, SEN, SEGA, and sclerotic bone lesions than patients with *TSC2* pathogenic variants. Additionally, these individuals tend to show more frequent bilateral and larger renal

angiomyolipomas than patients with *TSC1* mutations. TSC mild phenotypes are more frequently associated with patients with no mutation identified (NMI) or with familial cases (Camposano, Greenberg *et al.* 2009, Boronat, Barber *et al.* 2017, Peron, Au *et al.* 2018).

### III. *TSC1* and *TSC2* proteins

*TSC1* (NP\_000359.1) protein product of *TSC1* is a 130-kDa (1164 amino acid) hydrophilic protein with a predicted coiled-coil region of 266 amino acids (residue 730 - 996) close to the C-terminus and an N-terminal domain required for the interaction with *TSC2*. *TSC1* has the ability to form homodimers, demonstrated by the yeast two-hybrid system. *TSC1* homodimers are prone to self-aggregation in detergent resistant complexes in the absence of the *TSC2* gene product. In the absence of *TSC2* overexpression, *TSC1* overexpression produces insoluble *TSC1*, presenting a dotted cytoplasmic distribution at immunofluorescence analysis. If both genes are co-overexpressed, *TSC1* becomes soluble and has a more homogeneous cytoplasmic distribution (Nellist, van Slegtenhorst *et al.* 1999).

*TSC2* (NP\_000539.2) is a 200-kDa (1,807 amino acid) protein with a short 183-residue region close to the C-terminus that is homologous to the GTPase-activating protein (GAP) for the small GTPase RAP1. This C-terminus domain is encoded by exons 34 to 38 (Maheshwar, Cheadle *et al.* 1997) and has been shown to act as a GAP for the GTPase RAS Homolog Enriched in Brain (RHEB), catalyzing the RHEB-dependent hydrolysis of GTP to GDP. The N-terminal domain of *TSC2* is required for the interaction with *TSC1*, enabling the two proteins to form a stable complex (Plank, Yeung *et al.* 1998, van Slegtenhorst, Nellist *et al.* 1998).

Initially, *TSC2* was thought to be localized to the Golgi apparatus, while *TSC1* was detected in a punctate pattern in the cytoplasm of cultured cells and localized to a membrane fraction (Plank, Yeung *et al.* 1998). However, because *TSC1* and *TSC2* associate physically *in vivo*, it seemed likely that *TSC1* and *TSC2* function together in the same complex rather than in separate pathways (van Slegtenhorst, Nellist *et al.* 1998). It had been suggested that *TSC2* could act as *TSC1* chaperone, preventing its self-aggregation (Nellist, van Slegtenhorst *et al.* 1999) maintaining the *TSC1*–*TSC2* complex in a soluble form. The quaternary structure of the TSC complex is not yet clear, but gel filtration and co-immunoprecipitation experiments suggest that the complex contains multiple subunits of both *TSC1* and *TSC2* (Hoogeveen-Westerveld, van Unen *et al.* 2012).

#### A. *TSC2* GAP domain and the mechanistic target of rapamycin (mTOR) pathway

*TSC1* and *TSC2* form a tumor suppressor complex, the TSC complex, which acts as mediator of intracellular and extracellular signals to inhibit the mechanistic target of rapamycin (mTOR, NP\_004949.1) complex 1 (mTORC1) kinase (Garami, Zwartkruis *et al.* 2003, Tee, Manning *et al.* 2003). *TSC2* acts as GAP

toward RHEB (Garami, Zwartkruis *et al.* 2003, Inoki, Li *et al.* 2003). Inactivation of the TSC complex results in increased levels of RHEB-GTP, that is required for mTORC1 to phosphorylate downstream targets, including p70 40S ribosomal protein S6 kinase 1 (S6K1, NP\_003152.1) and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1, NP\_004086.1). Indeed, co-expression of TSC1 and TSC2 represses S6K1 phosphorylation at Thr<sup>389</sup>, therefore repressing its activity. mTORC1-dependent phosphorylation of 4E-BP1 causes dissociation of 4E-BP1 from eIF4E, enabling the configuration of the 48S pre-initiation translational complex. Mutants of *TSC2* are defective in repressing phosphorylation of 4E-BP1 (Tee, Fingar *et al.* 2002, Tee, Manning *et al.* 2003). Taken together, the results implicate the TSC complex in the mTORC1-mediated effects on S6, S6K1 and 4E-BP1, leading in the absence of the active TSC complex to cell over-growth and proliferation, frequent aspects of cell dysfunction in TSC patients. Understanding TSC as an mTOR dysfunction has allowed for clinical trials of rapamycin or analogues (rapalogs) to treat SEGAs, AML and LAM. Rapamycin or rapalogs have been clinically approved to control tumor size for these indications in Brazil.

The stability of TSC1-TSC2 complex is central in the control of mTOR signaling. Understanding the regulatory complex of the *TSC1* and *TSC2* genes is important and may contribute to the selection of vectors for gene therapies in specific TSC lesions (Prabhakar, Zhang *et al.* 2015). One interesting open question is if the expression regulation of one TSC gene impacts the other TSC gene.

## B. Tsc1 as Hsp90 co-chaperone

Misfolded proteins may aggregate due to the exposure of hydrophobic amino acid residues to the cellular environment. Nearly 30% of newly synthesized proteins are targeted for degradation due to improper folding (Schubert, Anton *et al.* 2000). Most proteins require assistance to achieve their correct tertiary structure. Among the systems that have evolved to regulate protein turnover, the most well-known are chaperones. To achieve the correct tertiary and quaternary structure, protein folding factors (chaperones) are required for many proteins (Carlisle, Prill *et al.* 2017).

When improper protein folding occurs, the heat shock response is activated, and the synthesis of a class of inducible chaperones known as heat-shock proteins (HSP) is increased (Calderwood and Murshid 2017). The molecular chaperone heat-shock protein HSP 90- $\alpha$  (Hsp90, NP\_001017963.2) is an essential component of the cellular homeostatic machinery in eukaryotes. Purified Hsp90 binds to denatured proteins and displays ATP-dependent anti-aggregate properties (Panaretou, Prodromou *et al.* 1998). Most Hsp90 clients are medically relevant, including the nuclear receptors for steroid hormones and several proto-oncogenic kinases and non-kinases.

The ATP-binding pocket in the Hsp90 central domain (residue 272 – 617) is essential for chaperone activity and to bind and hydrolyze ATP, changing the N-terminal open state to a closed conformation

(Prodromou and Pearl 2003). The Aha1 co-chaperone aids in high-energy conformational modulations necessary for Hsp90 ATPase competence (Panaretou, Siligardi *et al.* 2002, Lotz, Lin *et al.* 2003).

Hsp90 may interact with TSC1 even in the absence of TSC2. The association between TSC2 and TSC1 decreases under thermal shock, which does not affect the association of TSC1-HSP90 (Inoue, Uyama *et al.* 2010).

Tsc1 has a higher binding affinity for Hsp90 than Aha1. The C-terminal domain of Tsc1 (residue 998 - 1,164) forms a homodimer that inhibits ATPase activity and stabilizes the closed conformation of the Hsp90 central domain (residues 284 – 620 and 728 – 732) (Brown, Foreman *et al.* 2015, Woodford, Dunn *et al.* 2016, Woodford, Sager *et al.* 2017). Conversely, phosphorylation of Aha1 Y<sup>223</sup> increases its affinity for Hsp90 and displaces Tsc1. Because Aha1 and Tsc1 compete for the same domain, Tsc1 may prevent the conformational changes in the catalytic loop of Hsp90 that reduces the release of R400 from its retracted, inactivating conformation, consequently inhibiting Hsp90 ATPase activity (Woodford, Sager *et al.* 2017). Thus the Hsp90 system switches between an ATP-bound closed conformational state mediated by Tsc1, with low affinity for kinase and non-kinase clients, and an ADP-bound conformational state with high affinity for substrate (Bukau and Horwich 1998, Woodford, Sager *et al.* 2017).

TSC1 p.L117P naturally occurring human variant located within the N-terminal domain of TSC1 was shown to be pathogenic (Nellist, van den Heuvel *et al.* 2009). The L117P substitution destabilizes Tsc1 and therefore prevents Tsc2 binding to Hsp90, leading to ubiquitination and degradation by the proteasome (Woodford, Sager *et al.* 2017), and resulting in increased mTORC1 activity.

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# Chapter II

*TSC1* and *TSC2* pathogenic variants in  
patients with a clinical diagnosis of TSC

## List of Acronyms

- ACMG:** American College of Medical Genetics and Genomics
- DMSO:** Dimethyl sulfoxide
- GADPH:** Glyceraldehyde-3-phosphate dehydrogenase
- GFI1B:** Growth Factor-Independent 1B
- GRAACC:** Grupo de Apoio ao Adolescente e à Criança com Câncer
- HC-UFPR:** Hospital de Clínicas of the Universidade Federal do Paraná
- LOVD:** Leiden Open Variation Database
- mTORC1:** Mechanistic target of rapamycin kinase complex 1
- MLPA:** Multiplex ligation-dependent probe amplification
- NGS:** Next generation sequencing
- panel-NGS:** Next Generation Sequencing on a customized gene panel chip
- NMI:** No mutation identified
- ORF:** Open reading frame
- PKD1:** Polycystic Kidney Disease 1 gene
- PKDTS:** Polycystic Kidney Disease, Infantile Severe, with tuberous sclerosis
- PCR:** Polymerase chain reaction
- Q-PCR:** Quantitative PCR
- RHEB:** Ras homologue enriched in brain
- S6K1:** S6 kinase 1
- HSCM-SP:** Santa Casa de Misericórdia
- SFs:** Secondary findings discoveries
- SINE:** Short interspersed nuclear element
- SNV:** Single nucleotide variants
- TSC:** Tuberous sclerosis complex
- VUS:** Variant of unclear significance
- WT:** Wild-type

## List of Tables

Table II-1: <i>TSC1</i> and <i>TSC2</i> pathogenic variant classification and worldwide distribution. ....	44
Table II-2: Monoclonal and polyclonal antibodies used to functional assessment of <i>TSC2</i> DNA variants. ....	52
Table II-3: <i>TSC1</i> and <i>TSC2</i> pathogenic DNA variants according to hospital of patient origin. ....	54
Table II-4: <i>TSC1</i> pathogenic DNA variants in a cohort of 117 TSC patients. ....	58
Table II-5: <i>TSC2</i> pathogenic DNA variants in a cohort of 117 TSC patients. ....	59
Table II-6: Pathogenicity criteria for classification of <i>TSC2</i> missense (N=20) and in-frame deletion (N=5) DNA variants with low population frequency (Richards, Aziz et al. 2015) .....	66
Table II-7: MLPA results and Q-PCR validation of <i>TSC1</i> and <i>TSC2</i> CNV. ....	74
Table II-8: <i>TSC2</i> internal deletion breakpoint mapping .....	80

## List of Figures

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Figure II-1: Workflow of <i>in silico</i> and <i>in vivo</i> analysis of DNA variants detected on <i>TSC1</i> and <i>TSC2</i> . ....	50
Figure II-2: <i>TSC2</i> missense and in-frame deletion functional assessment workflow. BDT: Big Dye terminator reaction .....	52
Figure II-3: Overview of the distribution of <i>TSC1</i> and <i>TSC2</i> pathogenic DNA variants according to frequency and alteration type. (A) Ratio of <i>TSC1</i> and <i>TSC2</i> pathogenic variants.....	55
Figure II-4: Schematic representation of <i>TSC1</i> protein segments and distribution of all <i>TSC1</i> pathogenic variants according to the amino acid number.....	56
Figure II-5: Schematic representation of <i>TSC2</i> protein segments and distribution of all <i>TSC2</i> pathogenic variants according to the amino acid number. Splicing variants are not represented. ....	57
Figure II-6: Workflow of the pathogenicity classification of 20 <i>TSC2</i> missense DNA variants. ....	64
Figure II-7: Functional assessment of the <i>TSC2</i> (NM_000548.3) variants.....	69
Figure II-8: Schematic representation of distribution of pathogenic <i>TSC2</i> splice variants.....	70
Figure II-9: Functional evaluation of a substitution DNA variant in the <i>TSC2</i> gene (c.4493G>T p.Ser1498Ile variant). ....	71
Figure II-10: Functional evaluation of a deep intronic DNA variation in the <i>TSC2</i> gene (c.1361+54_1361+57del variant). ....	73
Figure II-11: MPLA and Q-PCR results for <i>TSC1</i> or <i>TSC2</i> deletions and duplication. Left panel is MLPA report and right panel the Q-PCR results.....	75
Figure II-12: Breakpoint mapping of the <i>TSC2</i> intragenic deletion c.(975+_976-)_ (1716+_1717-)del.....	81
Figure II-13: Schematic representation of <i>TSC1</i> and the segmental deletion c.-234-?_-144+?del of exon 1 on patient#25 c.(737+1_738-1)_(*1+_?)del from exon 9 to exon 23 found on patient#29.....	83
Figure II-14: Schematic representation of <i>TSC2</i> and the segmental deletion c.(975+628)_ (1716+41)del from exon 11 to exon 16, c.(5068+1_5069-1)_(*102_?)del from exon 39 to exon 42, c.(1599+1_1600-1)_ (2545+1_2546-1)del from exon 16 to exon 22, c.(?-30)_(*102_?)del whole <i>TSC2</i> , c.(1716+1_1717-1)_ (2545+1_2546-1)del from exon 17 to exon 22, c.(774+1_775-1)_ (848+1_849-1)del from exon 22 to exon 42, and duplication c.(2355+1_2356-1)_(*102_?)dup from exon 22 to 42.....	84
Figure II-15: Global alignment of <i>TSC2</i> , phosphorylation site of GAP Domain and positioning of <i>TSC2</i> :p.Tyr1608del, p.Ile1614del and p.Arg1743Gly variants on predicted <i>Homo sapiens</i> GAP domain structure (PDB: 1SRQ).....	89
Figure II-16: Global alignment of <i>TSC2</i> , phosphorylation site of <i>TSC1</i> binding domain and positioning of <i>TSC2</i> :p.Trp167Arg variant on predicted <i>Chaetomium thermophilum</i> <i>TSC1</i> binding domain structure (PDB:5HIU).....	91

Figure II-17: Breakpoint mapping of the *TSC2* intragenic deletion c.(975+\_976-)\_ (1716+\_1717-)del . (A) Sanger sequencing of the DNA from the patient with the intragenic *TSC2* deletion c.(975+\_976-)\_ (1716+\_1717-)del. ....94

## Summary

II. Chapter II .....	35
I. Introduction .....	42
II. Aim and Objectives .....	45
A. Aim.....	45
B. Objectives .....	45
III. Subjects and Methods .....	46
A. Patients .....	46
B. Sanger Sequencing and Next Generation Sequencing .....	46
C. Multiplex ligation-dependent probe amplification (MLPA) .....	48
D. Quantitative polymerase chain reaction.....	48
E. Pipeline of <i>in silico</i> and <i>in vivo</i> analysis of DNA variants .....	48
F. Functional assessment of TSC2 VUS missense and in-frame deletion .....	50
G. Splicing effect of TSC2 missense and deep intronic variants.....	52
IV. Results .....	54
A. Pathogenic DNA variants .....	54
B. TSC2 missense and in-frame deletion variants .....	63
C. Variants that affect splicing .....	70
D. Large segmental gene deletions and duplications .....	73
E. Single nucleotides variants .....	85
V. Discussion .....	86
A. TSC2 missense and in-frame deletion effect on mTOR .....	87
B. ‘Miscalled’ missense DNA variants?.....	92
C. TSC1 and TSC2 CNV and genetic counseling implications .....	92
D. NMI patients .....	95
VI. Conclusions.....	97
VII. References .....	98
VIII. Supplementary Tables .....	104

IX.	Supplementary Figures .....	119
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## I. Introduction

Tuberous sclerosis complex (TSC) is a genetic disease affecting 1 in every 6,000 to 10,000 live-born infants, showing no ethnic bias. It is characterized by hamartoma growth more often in the brain, skin, kidneys, heart, lungs, and retina (Sampson, Scahill *et al.* 1989, O'Callaghan, Shiell *et al.* 1998, Au, Williams *et al.* 2007, Northrup, Krueger *et al.* 2013). Renal manifestations, epilepsy and intellectual disability are important sources of morbidity (Rakowski, Winterkorn *et al.* 2006, Moavero, Pinci *et al.* 2011, Northrup, Krueger *et al.* 2013, Kwiatkowski, Palmer *et al.* 2015). Northrup *et al.* (2013) presented an up-to-date classification of TSC lesions considered for the clinical diagnosis of the disease.

TSC is an autosomal dominant disorder due to pathogenic variants in either *TSC1* or *TSC2* tumour suppressor genes (Consortium 1993, van Slegtenhorst, de Hoogt *et al.* 1997). The *TSC1* gene (NG\_012386.1, Ch:9q34.1, MIM#605284), with approximately 55 kb comprising 23 exons, codes for the TSC1 protein (hamartin, NP\_000359.1) with 130 kDa (van Slegtenhorst, de Hoogt *et al.* 1997). The *TSC2* (NG\_005895.1, Ch:16p13.3, MIM#191092) gene has 42 exons and nearly 40 kb, and encodes the TSC2 protein (tuberin, NP\_000539.2) with 180 kDa (Consortium 1993, van Slegtenhorst, de Hoogt *et al.* 1997, Jones, Shyamsundar *et al.* 1999, Dabora, Jozwiak *et al.* 2001, Sancak, Nellist *et al.* 2005, Au, Williams *et al.* 2007). TSC1 and TSC2 physically interact as a cytosolic heterodimer (TSC1/2 complex), which acts as mediator of intracellular signals to inhibit the mechanistic target of rapamycin (mTOR) kinase complex 1 (mTORC1) through the small GTPase RHEB (Ras homologue enriched in brain; (Garami, Zwartkuis *et al.* 2003, Tee, Manning *et al.* 2003). Inactivation of the TSC1/2 complex results in increased levels of RHEB-GTP, required for activation of mTORC1, which phosphorylates downstream targets. Inactivation of the TSC1/2 complex increases mTORC1 phosphorylation of, for instance, the S6 kinase 1 (S6K1) at residue Thr<sup>389</sup> (Goncharova, Goncharov *et al.* 2002, Tee, Fingar *et al.* 2002) causing excessive cell growth and augmented cell proliferation.

Gómez (1979) first established a broad set of major and minor clinical criteria for the definite, probable or possible diagnosis of TSC. Since then TSC diagnosis criteria have been reviewed and updated (Roach, Gomez *et al.* 1998, Northrup, Krueger *et al.* 2013). Besides updating the TSC clinical criteria, the 2012 working group implemented the genetic diagnostic criterion, by which the detection of a pathogenic variant in either *TSC1* or *TSC2* genes is sufficient for the definite molecular diagnosis of TSC (Northrup, Krueger *et al.* 2013), following the American College of Medical Genetics and Genomics (ACMG) guidelines for pathogenicity report of DNA variants (Richards, Aziz *et al.* 2015). Nearly 90% of patients with definite clinical diagnosis of TSC have a *TSC1* or *TSC2* pathogenic DNA variant identified by genetic testing (Peron, Au *et al.* 2018). The ratio between *TSC1* and *TSC2* pathogenic variant frequencies appears close to 1:1 among TSC familial cases, and may vary from 1:3 to 1:6 among *de novo* cases (Jones, Shyamsundar *et al.* 1999, Dabora, Jozwiak *et al.* 2001, Rendtorff, Bjerregaard *et al.* 2005, Au, Williams *et al.* 2007). *De novo* TSC cases with more severe angiomyolipomas

and/or intellectual disability tend to have germline mutations more frequently on the *TSC2* gene than the *TSC1* gene (Dabora, Jozwiak *et al.* 2001, Sancak, Nellist *et al.* 2005, Au, Williams *et al.* 2007). In approximately 10% of patients with definite clinical diagnosis of TSC, no pathogenic DNA variant is identified in *TSC1* or *TSC2* by current molecular techniques (Kozlowski, Roberts *et al.* 2007, Camposano, Greenberg *et al.* 2009, Qin, Kozlowski *et al.* 2010, Tyburczy, Wang *et al.* 2013, Nellist, Brouwer *et al.* 2015). *TSC1* or *TSC2* somatic mosaicism and deep intronic DNA variants have recently been reported in those patients with no mutation identified (NMI) in the coding region of either gene (Nellist, Brouwer *et al.* 2015, Tyburczy, Dies *et al.* 2015). Worldwide studies detected pathogenic DNA variants on *TSC1* and *TSC2* by gold-standard methodologies, such as Sanger sequencing and MLPA, as well as next-generation sequencing (NGS), disclosing similar results (Table II-1).

The detection of a pathogenic DNA variant in the *TSC1* or *TSC2* genes as an independent diagnostic criterion rendered it sufficient for the diagnosis or prediction of TSC regardless of the clinical findings (Northrup, Krueger *et al.* 2013). It allows in some cases for early diagnosis notably in young individuals who may not yet fulfil the criteria for a definite clinical diagnosis. Although health economics studies are needed to evaluate the cost-effectiveness of NGS testing in TSC patients that present with isolate lesions, it is evident that gene panel NGS costs have been declining over the years (Schwarze, Buchanan *et al.* 2018). Genetic test-based early TSC diagnosis is likely to accelerate implementation of clinical surveillance and selection of patients for treatments that will potentially lead to better clinical outcomes.

In a previous work, we have evaluated by Sanger sequencing the *TSC1* coding sequence in a cohort of 28 TSC patients (Dufner-Almeida 2014). In the present study, we extended the cohort to a total of 117 patients from the states of São Paulo and Paraná, Brazil, and employed their leukocyte DNA to search for *TSC1* and *TSC2* germline pathogenic DNA variants and segmental deletions/duplications. We disclosed a pathogenic variant detection rate of 87,18% (102/117), with 84 (82.35%) pathogenic variants in the *TSC2* and 18 (17.64%) in the *TSC1*.

Table II-1: *TSC1* and *TSC2* pathogenic variant classification and worldwide distribution.

Reference	Country	Patient s	Pathogenic Variants	TSC1/TSC2 ratio	<i>TSC1</i>							<i>TSC2</i>						
					Total	Nonsense	Frameshift	Missense	Splicing	Inframe	Duplication/ Deletion	Total	Nonsense	Frameshift	Missense	Splicing	Inframe	Duplication/ Deletion
Jones, A. C. et. al. (1997) *	UK	171	21 (12.3%)	-	21 (100%)	7	12	1	2	-	-	-	-	-	-	-	-	-
Ali, J. B., et. al. (1998) *	UK	83	16 (19.3%)	-	16 (100%)	6	8	-	2	-	-	-	-	-	-	-	-	-
Au, K. S., et. al. (1998) **	USA	90	22 (24.4%)	-	-	-	-	-	-	-	-	22 (100%)	2	8	10	-	2	-
Beauchamp, R. L. et. al. (1998) **	USA	40	21 (52.5%)	-	-	-	-	-	-	-	-	21 (100%)	6	5	6	3	1	-
Young, J. M. et. al. (1998) *	UK	79	27 (34.2%)	-	27 (100%)	13	13	-	1	-	-	-	-	-	-	-	-	-
Jones, A. C. et. al. (1999)	UK	150	120 (80%)	1:4.4	22 (18.3%)	8	12	1	2	-	-	98 (81.7%)	20	21	22	8	5	22
Niida, Y. et. al. (1999)	USA	126	74 (59%)	1:3.6	16 (21.6%)	7	7	-	2	-	-	58 (78.4%)	14	21	13	4	2	4
van Slegtenhorst, M. A. (1999) *	The Netherlands	225	29 (12.8%)	-	29 (100%)	11	17	-	1	-	-	-	-	-	-	-	-	-
Dabora, S. L. et. al. (2001)	USA	224	166 (83%)	1:4.9	28 (15.0%)	11	15	-	2	-	-	138 (85%)	37	43	31	27	-	-
Rendtorff, N. D. et. al. (2005)	Denmark	65	51 (78%)	1:3.6	11 (21.6%)	4	6	-	1	-	-	40 (78.4%)	10	10	10	6	2	2
Hung, C. C. et. al. (2006)	Taiwan	84	64 (76 %)	1:6.1	9 (14%)	5	3	-	-	1	-	55 (86%)	15	21	12	7	-	-
Au, K. S. et. al. (2007)	USA	325	243 (74.8%)	1:3	61 (25%)	21	35	1	4	-	-	182 (75%)	39	57	58	25	-	3
Kozlowski, P. et. al. (2007) ***	USA	261	54 (20.7%)	1:12.5	4 (7%)	-	-	-	-	-	4	50 (93%)	-	-	-	-	-	50
Sasongko, T. H. et. al. (2008)	Japan	21	16 (76.2%)	1.3:1	9 (56.3%)	1	8	-	-	-	-	7 (43.7%)	-	4	1	2	-	-
Chopra, M. et. al. (2011) ****	Australia	45	33 (73%)	1:2.7	9 (27.8%)	-	-	-	-	-	-	24 (72.2%)	-	-	-	-	-	-
Niida, Y. et. al. (2013)	Japan	57	31 (54.4%)	1:1.8	11 (35.5%)	2	5	1	2	-	1	20 (64.5%)	-	6	5&	4	4	1
Rosset, C. et. al. (2017)	Brazil	53	47 (88.7%)	1:3.4	11 (23.4%)	6	4	-	1	-	-	37 (76.6%)	12	7	2	8	3	5

\* *TSC1* study only

\*\* *TSC2* study only

\*\*\* Duplication and deletion study only

\*\*\*\* *TSC1* and *TSC2* variant distribution data only

& Including one stop codon variant

## II. Aim and Objectives

### A. Aim

To analyze the nature, distribution and functional effects of *TSC1* and *TSC2* DNA variants of 100 patients with clinical definite diagnosis of TSC.

### B. Objectives

1. To sequence the DNA coding sequence of *TSC1* and *TSC2* by the Sanger method or next generation sequencing (NGS);
2. To perform multiplex ligation-dependent probe amplification (MLPA) of TSC NMI patient DNA;
3. To confirm every MLPA alteration on *TSC1* or *TSC2* by quantitative PCR (qPCR);
4. To functionally evaluate the effect of identified missense *TSC1* or *TSC2* DNA variants of uncertain significance on *in vitro* hyperactivation of mTORC1 having S6K1 Thr<sup>389</sup> phosphorylation as reporter;
5. To functionally assess putative splicing DNA variants detected outside canonical splice sites, by overexpressing a minigene harboring the DNA variant and reporting its splicing output.

### III. Subjects and Methods

#### A. Patients

This study was approved by the Institutional Ethics Review Board from the three participating institutions (Protocol Number CAAE: 125729112.3.000.5464, Appendix 1 and 2): *Santa Casa de Misericórdia* (HSCM-SP), São Paulo, Brazil; *Hospital de Clínicas* of the *Universidade Federal do Paraná* (HC-UFPR), Curitiba, Brazil, and *Grupo de Apoio ao Adolescente e à Criança com Câncer* (GRAACC), São Paulo, Brazil. All 117 patients had informed consent signed by a parent or tutor. A definite clinical diagnosis of TSC (Northrup, Krueger *et al.* 2013) was the only inclusion criterion. Twenty-three patients were referred from the HSCM-SP, 75 from the HC-UFPR and 19 from GRAACC. Peripheral blood samples (4 mL) were harvested by venipuncture and sent to the Functional Genetics laboratory at the University of São Paulo (Instituto de Biociências, IBUSP, São Paulo, Brazil) for *TSC1* and *TSC2* testing. Genetic counselling has been offered to all patients after the genetic analysis had been concluded.

#### B. Sanger Sequencing and Next Generation Sequencing

Genomic DNA (DNA) was extracted from peripheral blood leukocytes using *Puregene chemistry* (QIAGEN, Hilden, Germany) and *Autopure LS* devices (Thermo Fisher Scientific, Waltham, Massachusetts, USA), at *Centro de Estudos do Genoma Humano e Células Tronco* (CEGH-CEL-IBUSP) or with *QIAamp DNA Blood Midi* and *Maxi Kits* (QIAGEN, Hilden, Germany) at the Functional Genetics laboratory, according to manufacturer recommendation. Quantification and quality control were measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for Sanger sequencing procedures, as well as agarose gel and Qubit 2.0 (Invitrogen, Carlsbad, California, USA) for Next Generation Sequencing on a customized gene panel chip (panel-NGS).

Oligonucleotides for polymerase chain reaction (PCR) and Sanger sequencing were designed with Primer3 (v. 0.4.0 (Rozen and Skaletsky 2000) or Primer-BLAST (NCBI), with 20-22 nucleotides (optimum at 21 nt), melting temperatures of 59-61°C (optimum at 60°C, and 1°C of maximal difference between pair oligonucleotides) and guanine and cytosine percentage of 40-60% (optimum at 50%). The ensemble of PCR amplicons covered all exons, intron boundaries and the core promoter regions of both *TSC1* (NG\_012386.1) and *TSC2* (NG\_005895.1) genes (Supplementary Table II-1 and Supplementary Table II-2). For *TSC1*, the sequenced region consisted of ~9.4 kb of the total genomic locus (60 kb; ~16%), including 517 bp of the core promoter (Gerstein, Bruce *et al.* 2007), and upstream sequences, 3.5 kb of exonic sequences and 5.4 kb of intronic sequences. For *TSC2*, the sequenced region consisted of ~17.8 kb (46 kb; ~39%): 485 bp of the core promoter and upstream sequence, 5.6 kb of exonic sequences and 11.7 kb of intronic sequences. Coding sequence annotation was according to transcript reference sequences NM\_000368.4 (*TSC1*) and

NM\_000548.3 (TSC2). Primer specificity was tested against the human genome build GRCh37/hg19 with the BLAT program (UCSC Genome Browser, Santa Cruz CA, USA) and nucleotide BLAST (NCBI, Bethesda MD, USA). Conditions for 25  $\mu$ L of PCR reaction were standardized with DNA samples from three unrelated non-TSC individuals (non-TSC): 1X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl; Invitrogen, Carlsbad, California, USA), 2 mM  $MgCl_2$  (Invitrogen, Carlsbad, California, USA), 5% dimethyl sulfoxide (DMSO), each deoxyribonucleotide at 0.2 mM, primer oligonucleotide at 0.2  $\mu$ M, 80 ng of DNA and 0.2 U of *Taq* polymerase (Invitrogen, Carlsbad, California, USA). For *TSC1* exon 1 plus the promoter segment, a region highly enriched in cytosines and guanines (66%), we adopted the slowdown PCR protocol (Supplementary Table II-3, Frey, Bachmann *et al.* (2008)); a standard PCR protocol was employed for the remaining reactions (Supplementary Table II-4). PCR products were electrophoresed on 1.5% agarose gels with 5  $\mu$ L of SYBRsafe (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 50 mL of 90 mM Tris and boric acid, 2 mM EDTA (TBE), and band images were captured on a Gel Doc™ EZ System using Image Lab™ software (Bio-Rad, Hercules, California, USA). Eight microliters of PCR products were treated with 1.5  $\mu$ L of exonuclease I : shrimp alkaline phosphatase (5U:1U; Affymetrix, Santa Clara, California, USA) for 30-60 minutes at 37°C, followed by enzymatic inactivation for 15 minutes at 60°C.

Sanger sequencing was performed on purified PCR products following the ABI BigDye terminator protocol (Applied Biosystems; Foster City, California, USA) having 4  $\mu$ L of enzyme-treated PCR product, the sequencing oligonucleotide primer at 0.3  $\mu$ M (Supplementary Table II-1 and Supplementary Table II-2), 2  $\mu$ L of BigDye terminator solution (v3.1), 1.5  $\mu$ L 5X BigDye buffer (BigDyeno kit® - Applied Biosystems, Carlsbad, CA) and 6.5  $\mu$ L of ultrapure water to a final reaction volume of 15  $\mu$ L. Products were purified in a *Sephadex* column (Amersham Biosciences; Little Chalfont, United Kingdom), and submitted to capillary electrophoresis on an ABI 3730xl DNA Analyzer (Amersham Biosciences; Little Chalfont, United Kingdom). The results were analyzed using Sequencher V.4.10.1 or early version (Gene Codes Corporation; Ann Arbor, Michigan, USA).

Twenty-three DNA samples were submitted to NGS-panel as they had no pathogenic DNA variant identified or had been partially assessed by Sanger sequencing (N=18), had a missense variant with unclear significance (N=4) or low concentration of DNA (N=1). Gene probes had been designed to specifically capture the coding sequence of 95 selected genes that compose a customized gene panel. This strategy comprehends capture of coding and intronic boundary sequences of both *TSC1* and *TSC2* genes with Nextera rapid capture (Illumina; San Diego, California, USA). The library was quantified using Bioanalyzer 2100 (Agilent Technologies; Santa Clara, California, USA), and sequencing was performed on the MiSeq platform (Illumina; San Diego, California, USA). All reads were aligned to the human genome (build GRCh37/hg19) using the Burrows-Wheeler Aligner (BWA) algorithm to efficiently align short sequencing reads against the human genome (Li and Durbin 2009), followed by Genome Analysis Toolkit (GATK - McKenna, Hanna *et al.* (2010)) and ANNOVAR variant calling and annotation (Open Bioinformatics - Wang, Li *et al.* (2010)).

### C. Multiplex ligation-dependent probe amplification (MLPA)

The SALSA MLPA kits P124-C1 *TSC1* and P046-C1 *TSC2* (MRC-Holland; Amsterdam, The Netherlands) were used for detection of duplications and deletions affecting *TSC1* and *TSC2*. (Rendtorff, Bjerregaard *et al.* 2005, Kozlowski, Roberts *et al.* 2007, Padma Priya and Dalal 2012). The three non-TSC control samples used for Sanger sequencing standardization were employed as reference for all procedures, following the manufacturer protocol. Ligated products were submitted to capillary electrophoresis on an ABI 3730xl DNA Analyzer (Amersham Biosciences; Little Chalfont, United Kingdom), and analyzed using GeneMarker (SoftGenetics; State College, Pennsylvania, USA) and Coffalyser.NET (MRC-Holland; Amsterdam, The Netherlands). Signal intensities between deleted or duplicated and normal genomic segments from the same subject were compared using Student's *t* test and considered significant if *p*-value < 0.05.

### D. Quantitative polymerase chain reaction

To validate the copy number variations detected by MLPA, quantitative PCR (Q-PCR) was performed. Primers were designed using Primer-BLAST (NCBI) and tested *in silico* with Beacon Designer Free Edition (Premier Biosoft; Palo Alto, California, USA), BLAT program (UCSC Genome Browser, Santa Cruz CA, USA) and nucleotide BLAST (NCBI, Bethesda, MD, USA) (Supplementary Table II-5). Prior to Q-PCR, all primers were tested by standard PCR and submitted to agarose gel electrophoresis, according to section II.B. Q-PCR was carried out with the SYBR Green system (Applied Biosystems; Foster City, California, USA) on a 7500 Fast Real-Time PCR System apparatus (Applied Biosystems; Foster City, California, USA), as described previously (Ambar and Chiavegatto 2009). Optimal DNA and primer concentrations were determined by titration: for a 24  $\mu$ L reaction, 25 ng DNA and 12.5 pmol of each primer were used with 12  $\mu$ L PCR SYBR Green master mix. A non-TSC control DNA sample was used as a reference. Amplification and melting step (dissociation curve) were performed following standard parameters from the manufacturer program (Supplementary Table II-6). The melting step followed each run to confirm product specificity and the absence of primer dimers (Supplementary Figure II-1).

For copy number calibration, the reaction was normalized to the amplification of an amplicon for the *GADPH* gene (Glyceraldehyde-3-phosphate dehydrogenase, NM\_002046.1, human chr:12). All samples were run in triplicates at least three times, and the data analyzed using the comparative  $\Delta\Delta C_t$  cycle threshold method (Applied Biosystems; Foster City, California, EUA). Student's *t* test was applied to compare test and reference samples.

### E. Pipeline of *in silico* and *in vivo* analysis of DNA variants

DNA variant nomenclature was according to Human Genome Variation Society (HGVS, Melbourne, Australia) and were verified using both Variant Validator (University of Leicester, Freeman, Hart *et al.* (2018))

and Mutalyzer (LUMC, Wildeman, van Ophuizen et al. (2008)) web tools. Pathogenic DNA variants were defined according to ACMG standards and guidelines. *In silico* tools that predict damaging effects of missense change over protein structures and those that predict effect on splicing output were considered as a single piece of evidence in sequence interpretation (Richards, Aziz et al. 2015).

To determine the pathogenicity of the DNA variants, the following data banks, web tools and software were consulted: Leiden Open Variation Database (Version 2; Leiden, The Netherlands), Ensembl (release 93 - July 2018; Wellcome Trust Sanger Institute; European Bioinformatics Institute, Hinxton, Cambridgeshire, United Kingdom, (Zerbino, Achuthan et al. 2018)), PolyPhen-2 (Adzhubei, Schmidt et al. 2010), Mutation Taster (Schwarz, Rodelsperger et al. 2010), SIFT and PROVEAN (Ng and Henikoff 2006, Kumar, Henikoff et al. 2009, Choi and Chan 2015), PhosphositePlus (Hornbeck, Zhang et al. 2015) and Alamut 2,7-1 (Sophia Genetics, Saint Sulpice, Switzerland).

*In silico* analysis of DNA variants of uncertain clinical significance (VUS) potentially regulating pre-mRNA splicing was performed using the bioinformatics web tools Acscan2, SpliceAid2 (Piva, Giulietti et al. 2012), Human Splicing Finder 3.1 (HSF -Desmet, Hamroun et al. (2009)) and Alamut 2,7-1 (Sophia Genetics, Saint Sulpice, Switzerland). To identify repetitive elements in the *TSC2* genomic sequence the RepeatMasker (Version open-3.0, Institute for Systems Biology, Seattle WA) web tool was used. Information on all identified DNA variants has been deposited at the *TSC1* and *TSC2* Leiden Open Variation Database (LOVD).

All variants detected by Sanger sequencing or NGS-Panel were submitted to VEP web tool (Figure II-1). First, the transcript reference and mutation code were concatenated (e.g. *TSC2* variant c.1832G>A: NM\_000548.3) and submitted to standard configuration of VEP, excepted for:

- Transcript database to use: RefSeq transcripts;
- Identifiers: Gene symbol, Transcript version, Protein, UniProt and HGVS;
- Variant and frequency data:
  - Frequency data for co-located variants: 1000 Genomes global minor allele frequency, 1000 Genomes continental allele frequencies, ESP allele frequencies and gnomAD (exomes) allele frequencies;
- Filters:
  - Restrict results: show one selected consequence per variant.

All variants with frequency above 1% were filtered out. The remaining variants were verified on LOVD data base for *TSC1* and *TSC2*. If the variant was not present, the impact on protein or splicing was accessed by *in silico* analysis and functional assessment of mTORC1 pathway activation and/or splicing aberrant edition.

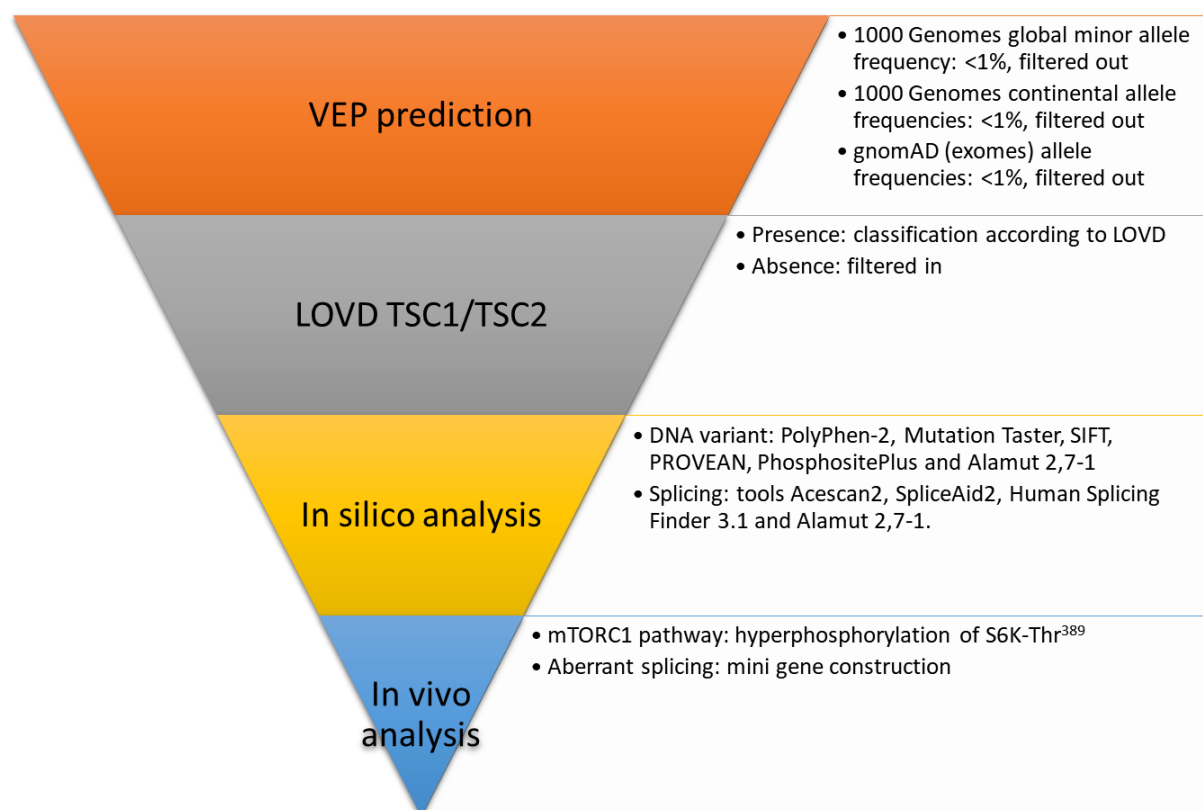


Figure II-1: Workflow of *in silico* and *in vivo* analysis of DNA variants detected on *TSC1* and *TSC2*.

Global alignment of *Homo sapiens* (NP\_000539.2), *Rattus norvegicus* (P49816), and *Mus musculus* (Q61037) *TSC2* C-terminus, and Rap1GAP catalytic domain (*Homo sapiens*, pdb|1SRQ|A) (Daumke et al., 2004) were performed using UniProtKB BLAST (EMBL-EBI,). The alignments among *TSC2* paralogues from the three organisms and Rap1GAP domain were set as reference to identify the position of *TSC2* variants. Structural predictions and spatial position of *TSC2* variant were generated with PyMOL molecular Graphic System Version 2.1.1 software (Schrödinger, Inc., Cambridge, Massachusetts, USA). Phosphorylation sites of the *TSC2* GAP domain was accessed using PhosphoSite Plus (NIH,) web tool. Supplementary Table II-7 contains a list of softwares and websites consulted.

## F. Functional assessment of *TSC2* VUS missense and in-frame deletion

*TSC2* missense and in-frame deletion functional assessment was performed according to Hoogeveen-Westerveld, Wentink *et al.* (2011) and Hoogeveen-Westerveld, Ekong *et al.* (2012), except for the usage in the present work of the cell line HEK293T that had been genetically modified to have both alleles from *TSC1* and *TSC2* genes inactivated by CRISPR/Cas9 genome editing, generating the 3H9-1B1 cell line (Dufner-Almeida et

al., submitted). Briefly, 3H9-1B1 cell line overexpressing full-length *TSC2* harboring the assayed DNA variant was tested against the control according to its ability to inhibit mTOR and thus activate S6K phosphorylation.

Full-length *TSC2* expression construct encoding the variant of interest was derived by site-directed mutagenesis of the DNA of the human wild-type (WT) *TSC2* (NM\_000548.3) expression construct previously cloned in the pcDNA3 vector (Hoogveen-Westerveld, Ekong *et al.* 2013). Procedures followed the manufacturer protocol (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies; Santa Clara, California, USA). Plasmidial DNA that had been submitted to site-directed mutagenesis was isolated and digested with *EcoRI*, in order to check for the presence of appropriate plasmid DNA. Clone sequence identity was verified by Sanger sequencing, and clones with successful mutagenesis were subcloned, and had the entire open reading frame (ORF) sequenced. Other constructs employed in the functional assay have been described before (Nellist, Sancak *et al.* 2005), including wild-type (WT) *TSC2*, *TSC2-GFP*, *TSC1-Myc* and WT *RPS6KB1* (*S6K*). Because of the low levels of endogenous expression of *S6K*, every transfection included the *S6K* clone DNA. Transfections were performed with  $0.5 \times 10^6$  to  $1.0 \times 10^6$  cells per well of 24-well dishes, and contained a combination of 0.2  $\mu$ g WT-*TSC2*, 0.2  $\mu$ g *TSC2-GFP*, 0.2  $\mu$ g mutant *TSC2*, 0.4  $\mu$ g *TSC1-Myc* and 0.2  $\mu$ g *S6K* plasmidial DNA, as well as 4.0  $\mu$ g polyethylenimine (PEI; Sigma-Aldrich, St. Louis, Missouri, USA). Eighteen hours after transfection, the transfection efficiency was verified according to the fluorescence emitted by *TSC2-GFP* cells on confocal microscopy. Only assays yielding transfection rates higher than 10% were considered for Western blotting (Figure II-2).

### SDM (Site-direct mutagenesis)

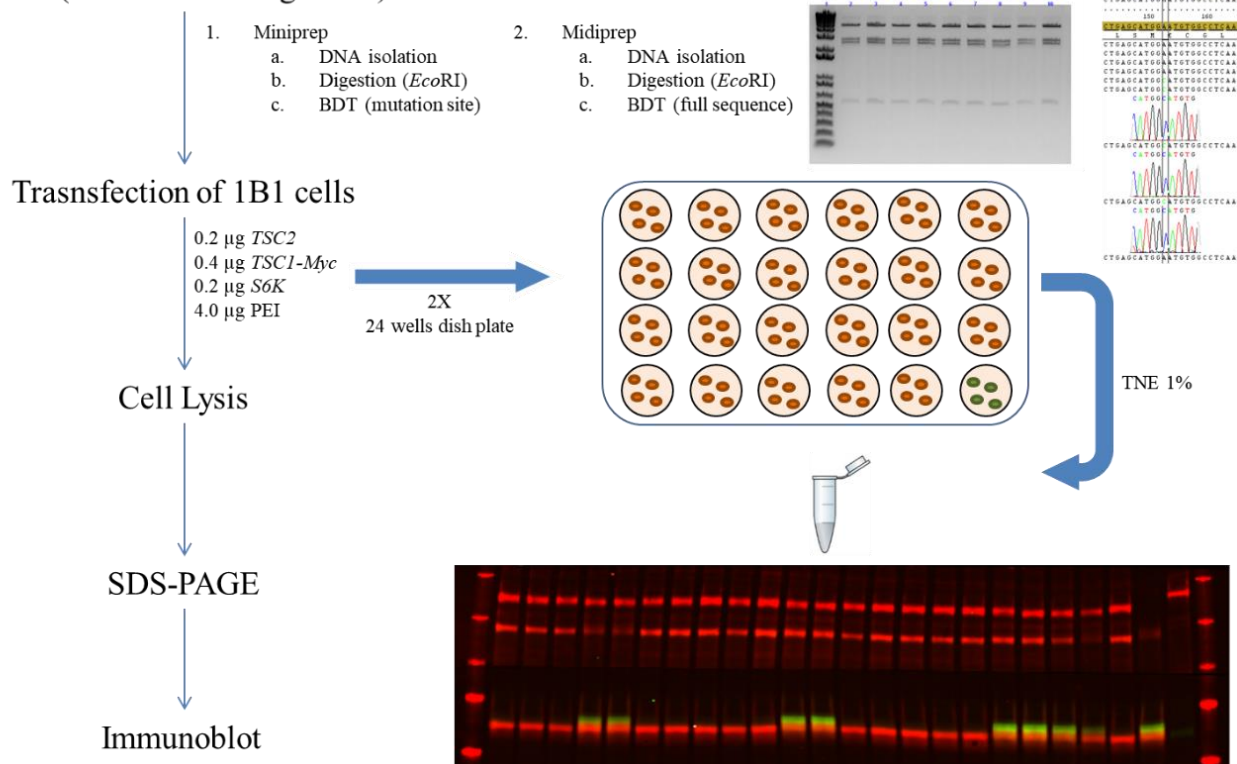


Figure II-2: *TSC2* missense and in-frame deletion functional assessment workflow. BDT: Big Dye terminator reaction

For immunoblotting, cells were lysed in 50 mM Tris-HCl (pH 7.6), 100 mM NaCl, 50 mM NaF, 1% Triton-X-100, 1 mM EDTA and complete protease inhibitor cocktail (Roche, Basel, Switzerland) on ice. After 10,000 X *g* centrifugation for 10 minutes at 4°C, proteins were submitted to SDS-PAGE in a Criterion™ TGX™ Precast Gel (4-15%) (Bio-Rad, Hercules, California, USA) and transferred to nitrocellulose filters (Bio-Rad, Hercules, California, USA). The membrane was blocked with 5% powder milk in PBS (Dulbecco's Phosphate Buffer Saline (0.0095M) – BioWhittaker - Radnor, Pennsylvania, USA) for 30 minutes. Antibodies were from Cell Signaling Technology (1A5 anti-Thr<sup>389</sup> phospho- p70 S6 kinase (S6K) mouse monoclonal; 9B11 anti-myc tag mouse monoclonal) or have been described previously (anti-TSC1 and -TSC2; van Slegtenhorst, Verhoef *et al.* (1999)). Secondary antibodies were from Li-Cor Biosciences (Lincoln, Nebraska, USA). Antibody dilutions were in PBS containing 0.1% Tween 20 (PBST) (Sigma-Aldrich Fine Chemicals, Poole, United Kingdom) according to Table II-2. Primary antibody was incubated for 4 hours or overnight at 8°C, secondary antibody incubations were for 60 minutes at room temperature. All washes were in PBST with tween. Blots were scanned using either the Odyssey scanner (Li-Cor Biosciences, Lincoln, Nebraska, USA) or the Gel Doc™ EZ System and Image Lab™ software (Bio-Rad, Hercules, California, USA). Signal intensities were measured and normalized to the signals corresponding to wild-type *TSC2*. The experiment was repeated at least three times and Student's *t* test was used for pairwise comparison of each variant to the WT-*TSC2* and the *TSC2* p.Arg611Gln variant, a positive control acknowledged to disrupt the TSC1-TSC2 dimer (Nellist, Sancak *et al.* 2005). Tested DNA variant effect was considered positive if the ratio of S6K-Thr<sup>389</sup>/total S6K is at least a four-fold compared to WT-*TSC2*.

Table II-2: Monoclonal and polyclonal antibodies used to functional assessment of *TSC2* DNA variants.

Color on Blot	Detected Protein	Mass (kDa)	Primary antibody/species in which it was generated	Dilution fold
Red	TSC2-GFP	225	Anti-TSC2 (1895*)/rabbit	1:10,000
Red	TSC2	200	Anti-TSC2 (1895*)/rabbit	1:10,000
Red	TSC1	130	Anti TSC1 (2197**)/rabbit	1:5,000
Green	Myc-Tag	130	9B11 anti-myc tag/mouse	1:5,000
Green	S6K-Thr389	70	1A5 anti-Thr <sup>389</sup> phospho- S6K/mouse	1:2,000
Red	Total S6K	70	Anti-myc tag/rabbit	1:2,000

\* Primary antibodies 1/15,000 dilution of 1895 (rabbit polyclonal against TSC2) from (van Slegtenhorst, Nellist *et al.* 1998)

\*\* Primary antibodies 1/5,000 dilution of 2197 (rabbit polyclonal against TSC1) from (van Slegtenhorst, Nellist *et al.* 1998)

## G. Splicing effect of *TSC2* missense and deep intronic variants

Partial genomic segments containing a DNA variant potentially affecting *TSC2* pre-mRNA splicing were cloned in the pcDNA3.1 vector, determining a mini-gene for overexpression in HEK293 cells that had not been genetically modified. The two *TSC2* variants tested, c.1361+54\_1361+57del in intron 13 and c.4493G>T

(p.Ser1498Ile) in exon 34, defined PCR amplicons containing genomic segments from exon 12 through exon 14, and from exon 33 through exon 35, respectively. Both mutated and wild-type *TSC2* alleles were separately cloned in the pcDNA3.1 vector following PCR amplification of DNA from the heterozygous individual for each DNA variant. Forward and reverse primers contained *Bam*HI and *Xho*I or *Not*I restriction sites (Supplementary Table II-8). Sanger sequencing of recombinant plasmidial DNA confirmed cloning of both wild-type alleles, the 4-bp deletion and c.4493G>T variant. HEK293 cells were transfected using lipofectamine 2000 (Thermo Fisher Scientific, Waltham MA, USA). Eighteen hours after transfection, total RNA was isolated with TRIzol, treated with AmpGrade DNase I (1U/ $\mu$ L; Invitrogen, Carlsbad, CA, USA), and reversely transcribed (SuperScript III; Invitrogen, Carlsbad CA, USA). PCR amplification of the cDNA from the mini-gene transcript, with primers specific for the pcDNA3.1 vector (Supplementary Table II-8), yielded products with expected sizes of 210 bp (non-recombinant clone); 596 bp (upon exon 12-14 mini-gene transcript constitutive splicing) or 458 bp (if internal exon is skipped due to the 4-bp deletion); and 896 bp (upon exon 33-35 mini-gene transcript constitutive splicing) or 408 bp (if internal exon is skipped due to the c.4493T mutant allele). Additionally, a nested PCR approach was adopted with primers annealing to *TSC2* exons 12 and 14, and 33 and 35 (Supplementary Table II-8). Moreover, primers annealing to exons 8 and 10 were used to amplify *TSC2* endogenous transcript (Supplementary Table II-8) as an internal control (327bp). RT-PCR products were verified after electrophoresis in 1.5% agarose gel, and the images captured by Gel Doc™ EZ System and Image Lab™ software (BioRad, Hercules, California, EUA). All bands were isolated from agarose gel and submitted to Sanger sequencing in order to identify the sequence alteration caused by any splicing defect.

## IV. Results

### A. Pathogenic DNA variants

Our cohort of 117 patients with definite clinical diagnosis of TSC, consisted of 63 male and 54 female individuals. Seventy-five patients were from HC-UFPR, 23 patients from HSCM-SP, and 19 patients from GRAACC-SP. Age at the time of enrolment varied between five months and 19 years (mean: 10.4 years, median: 11 years).

Among the 117 patients, 28 had DNA previously analyzed by Sanger sequencing of the *TSC1* gene, having pathogenic alterations detected in seven of them (Dufner-Almeida, 2014). In the present work, the patient cohort and experimental approach have been significantly extended. Overall pathogenic DNA variants were identified in 102 patients (87.2%), 18 affecting *TSC1* (17.6%), and 84 on *TSC2* (82.4%), and a *TSC1*:*TSC2* mutation ratio of 1:4.7 (Figure II-3A). The number of TSC pathogenic DNA variants is presented in Table II-3 according to the hospital of patient origin.

Table II-3: *TSC1* and *TSC2* pathogenic DNA variants according to hospital of patient origin.

Hospital	HC-UFPR	HSCM-SP	GRAACC-SP	Total
Number of patients enrolled	75	23	19	117
Patients with pathogenic DNA variants detected (percentage of total)	67 (89.33%)	22 (95.65%)	13 (68.42%)	102 (87.17%)
Patients with <i>TSC1</i> pathogenic DNA variants (percentage of total identified)	7 (10.77%)	9 (40.91%)	2 (15.38%)	18 (17.64%)
Patients with <i>TSC2</i> pathogenic DNA variants (percentage of total identified)	60 (89.55%)	13 (59.09%)	11 (84.61%)	84 (82.35%)
<i>TSC1</i> : <i>TSC2</i> ratio	1:8.6	1:1.4	1:5.5	1:4.7
NMI patients (analyses concluded)	4	1	2	7
VUS patients (awaiting functional analysis disclosure)	5	0	4	9

Nonsense (30 patients) and frameshift (29 patients) were the most frequent pathogenic variant categories. Complex mutation and large segmental gene duplication were observed once each in our cohort (Figure II-3B).

Distinct *TSC1* alterations comprehended eight frameshift and six nonsense variants, and one large deletion (Figure II-3, and Table II-4). *TSC1* exon 15 exhibited four nonsense and three frameshift variants; thus 41.2% of this gene pathogenic DNA variants (7/17). Two variants, Glu478Lysfs\*53 and Arg500\*, were identified in two unrelated patients (Table II-4).

Distinct *TSC2* pathogenic variants included 20 nonsense, 20 frameshift, 16 splicing and 12 missense DNA variants, six large deletions, four in-frame deletions, one large duplication and one complex mutation. To our knowledge, 52 variants have not been previously reported. Although four of these have been deposited at LOVD *TSC2* database by others, no further description is available (Table II-5). We observed a cluster of

pathogenic variants in TSC2 GAP domain (residues 1562 to 1764) (Figure II-3D), with 20 out of 84 pathogenic variants (23.8%) in this segment (Figure II-5).

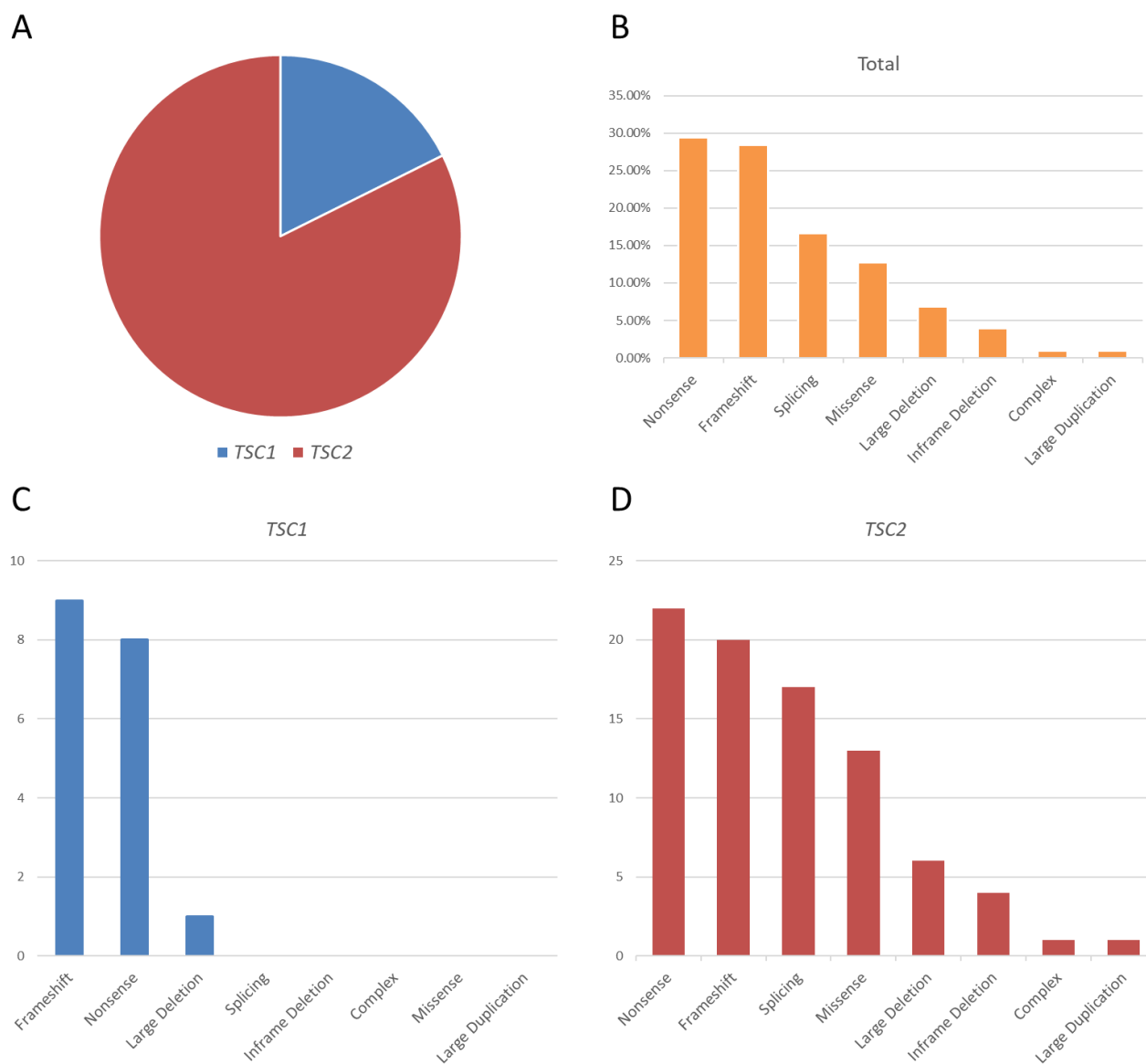


Figure II-3: Overview of the distribution of *TSC1* and *TSC2* pathogenic DNA variants according to frequency and alteration type. (A) Ratio of *TSC1* and *TSC2* pathogenic variants. (B) All classes of pathogenic variants found on *TSC1* and *TSC2*. (C) All classes of pathogenic variants found on *TSC1*. (D) All classes of pathogenic variants found on *TSC2*.

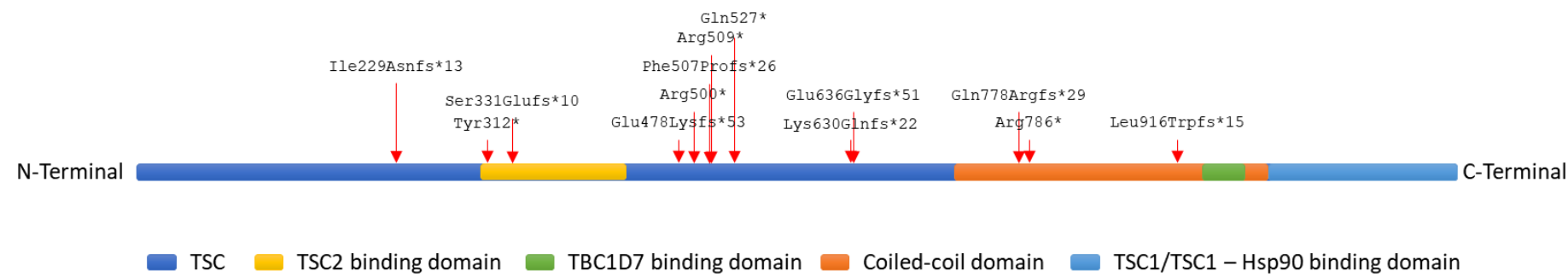


Figure II-4: Schematic representation of TSC1 protein segments and distribution of all *TSC1* pathogenic variants according to the amino acid number.

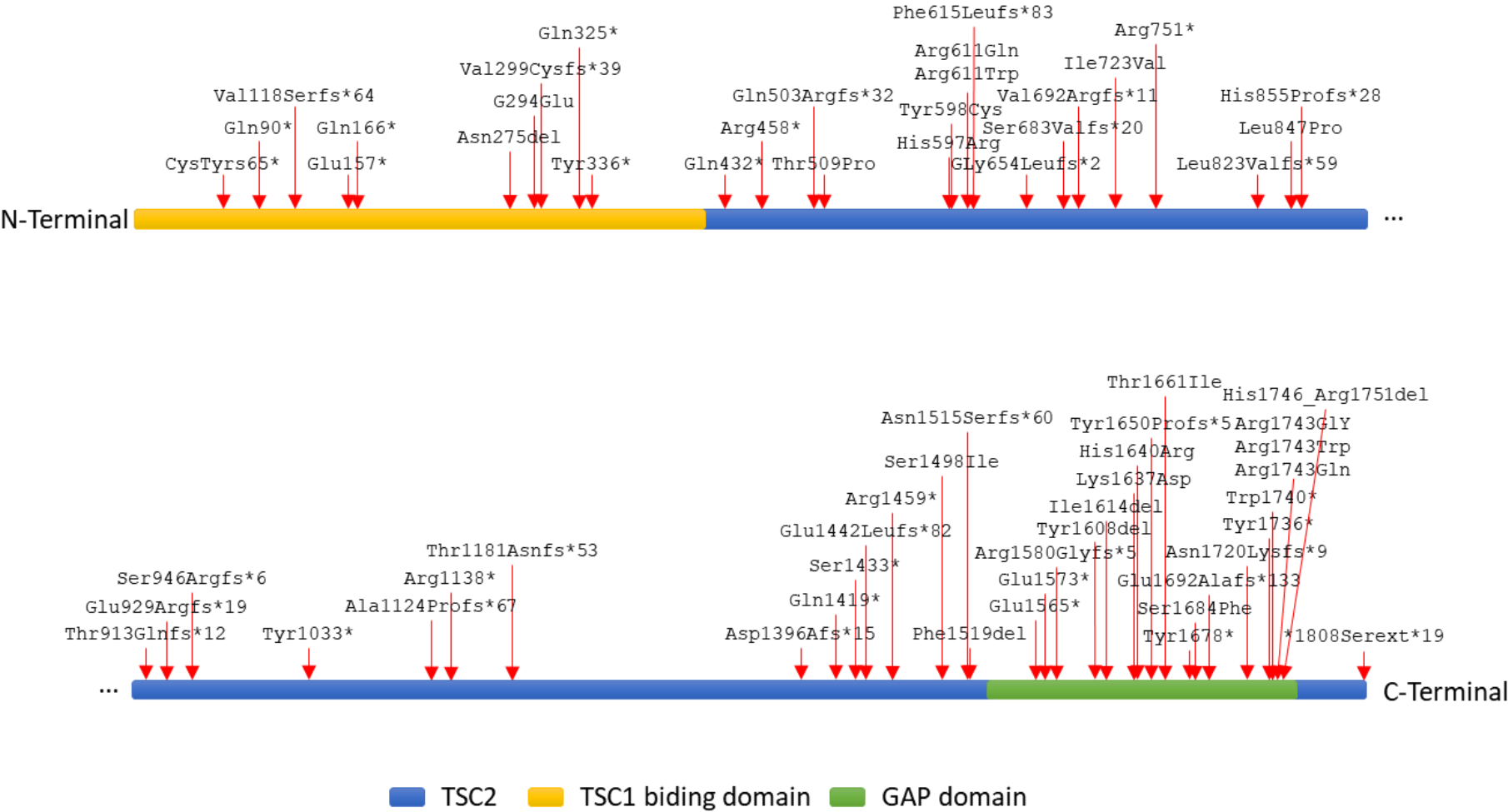


Figure II-5: Schematic representation of TSC2 protein segments and distribution of all TSC2 pathogenic variants according to the amino acid number. Splicing variants are not represented.

Table II-4: *TSC1* pathogenic DNA variants in a cohort of 117 TSC patients.

	DNA variant type	DNA Variant	Location	Patient #	References <sup>f</sup>
1.	Frameshift	c.683dupG (p.Ile229Asnfs*13) <sup>a</sup>	Exon 08	36	-
2.	Frameshift	c.989dupT (p.Ser331Glufs*10) <sup>a, g</sup>	Exon 10	19	-
3.	Frameshift	c.1431_1434del (p.Glu478Lysfs*53) <sup>c, d, g</sup>	Exon 14	13, 78	Au, 2007
4.	Frameshift	c.1517_1518dupCC (p.Phe507Profs*26) <sup>a, g</sup>	Exon 15	17	-
5.	Frameshift	c.1888_1891del (p.Lys630Glnfs*22) <sup>c, d</sup>	Exon 15	111	Rosset, 2017; Tyburczy, 2014; Tsai, 2011; Au, 2007; Hung, 2006
6.	Frameshift	c.1907_1908del (p.Glu636Glyfs*51)	Exon 15	122	Iyer, 2013; Muzykewicz, 2009; Dabora, 1998; Kwiatkowska, 1998; vanSlegtenhorst, 1997
7.	Frameshift	c.2332del (p.Gln778Argfs*29) <sup>a, c, d</sup>	Exon 18	79	-
8.	Frameshift	c.2746delC (p.Leu916Trpfs*15) <sup>a, g</sup>	Exon 21	10	-
9.	Nonsense	c.936C>G (p.Tyr312*) <sup>a, c, d</sup>	Exon 10	93	-
10.	Nonsense	c.1525C>T (p.Arg509*) <sup>g</sup>	Exon 15	5, 56	Kwiatkowska, 1998 and 2015; Suspitsin, 2018; Ismail, 2017; Yang, 2016; Lee, 2014
11.	Nonsense	c.1498C>T (p.Arg500*) <sup>g</sup>	Exon 15	8, 35	Tan, 2017; Tyburczy, 2015; Jansen, 2008; Au, 2007; Sancak, 2005
12.	Nonsense	c.1579C>T (p.Gln527*) <sup>c, d</sup>	Exon 15	106	Voss, 2014; Iyer, 2013
13.	Nonsense	c.2356C>T (p.Arg786*) <sup>g</sup>	Exon 18	16	Suspitsin, 2018; Lee, 2014; Au, 2007; Hung, 2006; Sancak, 2005
14.	Nonsense	c.2626G>T (p.Glu876*)	Exon 21	82	-
15.	Large Deletion	c.(737+1_738-1)_(*1+_?)del <sup>a, b, d</sup>	Exon 9-23	29	-
<b>Variants of unclear significance (VUS) in NMI patients</b>					
16.	Large deletion	c.-234-?-144+?del	Exon1-intron1	25	-
17.	Splicing	c.738-197T>A	Intron 8	60	-
18.	Stop loss	c.*1G>A	Exon 23	100	-

<sup>a</sup> First description of this variant.<sup>b</sup> Next-generation sequencing and MLPA performed for both genes, besides Sanger sequencing.<sup>c</sup> MLPA performed for both genes, besides Sanger sequencing<sup>d</sup> Deletion confirmed by Q-PCR.<sup>e</sup> At LOVD database without further information of submission.<sup>f</sup> Five most recent references.<sup>g</sup> Previously described (Dufner-Almeida 2014).

Table II-5: *TSC2* pathogenic DNA variants in a cohort of 117 TSC patients.

	DNA variant type	DNA Variant	Location	Patient #	References <sup>h</sup>
1.	Nonsense	c.195T>A (p.Cys65*) <sup>a</sup>	Exon 03	4	-
2.	Nonsense	c.268C>T (p.Gln90*)	Exon 04	105, 120	Kwiatkowski, 2015; Jansen, 2008; Au, 2007; Yuan, 2007; Hung, 2006
3.	Nonsense	c.469G>T(p.Glu157*)	Exon 05	42	-
4.	Nonsense	c.496C>T (p.Gln166*) <sup>c</sup>	Exon 06	94, 99	Niida, 1999
5.	Nonsense	c.973C>T (p.Gln325*)	Exon 10	52	Jansen, 2008
6.	Nonsense	c.1008T>G (p.Tyr336*)	Exon 11	115	Rosset, 2017
7.	Nonsense	c.1195G>T (p.Glu399*)	Exon 12	53	-
8.	Nonsense	c.1294C>T (p.Gln432*) <sup>a</sup>	Exon 13	28	-
9.	Nonsense	c.1372C>T (p.Arg458*)	Exon 14	108	Cai, 2017; Kwiatkowski, 2015; Tyburczy, 2015; Tsai, 2011; Au, 2007
10.	Nonsense	c.2251C>T (p.Arg751*)	Exon 21	1	Cai, 2017; Yang, 2016; Kwiatkowski, 2015; Yang, 2014; Kacerovska, 2012
11.	Nonsense	c.3099C>G (p.Tyr1033*) <sup>a</sup>	Exon 27	9	-
12.	Nonsense	c.3412C>T (p.Arg1138*)	Exon 30	92	Cai, 2017; Kwiatkowski, 2015; Tyburczy, 2014; Hung, 2006; Rendtorff, 2005
13.	Nonsense	c.4298C>A (p.Ser1433*) <sup>a</sup>	Exon 34	26	-
14.	Nonsense	c.4375C>T (p.Arg1459*) <sup>a</sup>	Exon 34	33	-
15.	Nonsense	c.4255C>T (p.Gln1419*)	Exon 34	67	Cai, 2017; Au, 2007; Roberts, 2004; Langkau, 2002; Jones, 1999
16.	Nonsense	c.4693G>T (p.Glu1565*) <sup>a</sup>	Exon 37	32	-
17.	Nonsense	c.4717G>T (p.Glu1573*) <sup>a</sup>	Exon 37	101	-
18.	Nonsense	c.5034C>A (p.Tyr1678*) <sup>a</sup>	Exon 39	18	-
19.	Nonsense	c.5208C>G (p.Tyr1736*) <sup>a</sup>	Exon 41	48	-
20.	Nonsense	c.5220G>A (p.Trp1740*)	Exon 41	90	Kwiatkowski, 2015; Roberts, 2002
21.	Frameshift	c.352del (p.Val118Serfs*64) <sup>a, c</sup>	Exon 05	83	-
22.	Frameshift	c.894dup (p.Val299Cysfs*39)	Exon 10	123	Giannikou, 2016
23.	Frameshift	c.1507del (p.Gln503Argfs*32) <sup>a, c</sup>	Exon 15	91	-
24.	Frameshift	c.1842del (p.Phe615Leufs*83) <sup>a</sup>	Exon 18	22	-
25.	Frameshift	c.2073dup (p.Val692Argfs*11) <sup>a</sup>	Exon 19	41	-
26.	Frameshift	c.2046dup (p.Ser683Valfs*20) <sup>a</sup>	Exon 19	71	-

27.	Frameshift	c.1959_1960del (p.Gly654Leufs*2)	Exon 19	103	Li, 2011
28.	Frameshift	c.2467_2476delinsGTGGATGA (p.Leu823Valfs*59) <sup>a, b</sup>	Exon 21	20	-
29.	Frameshift	c.2563dup (p.His855Profs*28) <sup>a</sup>	Exon 23	73	-
30.	Frameshift	c.2737_2739delinsC (p.Thr913Glnfs*12) <sup>a</sup>	Exon 24	3	-
31.	Frameshift	c.2784del (p.Glu929Argfs*19) <sup>a</sup>	Exon 25	39	-
32.	Frameshift	c.3370del (p.Alal124Profs*67) <sup>a</sup>	Exon 29	110	-
33.	Frameshift	c.3541dup (p.Thr1181Asnfs*53) <sup>a</sup>	Exon 30	86	-
34.	Frameshift	c.4324_4327delinsCTTCT (p.Glu1442Leufs*82) <sup>a</sup>	Exon 34	57	-
35.	Frameshift	c.4187del (p.Asp1396Alafs*15) <sup>a, c</sup>	Exon 34	95	-
36.	Frameshift	c.4544_4547del (p.Asn1515Serfs*60)	Exon 35	69	Kwiatkowski, 2015; Li, 2011; Au, 2007; Hung, 2006; Ali, 2005
37.	Frameshift	c.4738del (p.Arg1580Glyfs*5) <sup>a</sup>	Exon 37	112	-
38.	Frameshift	c.4947_4948insCCATTGT (p.Tyr1650Profs*5) <sup>a</sup>	Exon 38	31	-
39.	Frameshift	c.5159dup (p.Asn1720Lysfs*9) <sup>a</sup>	Exon 39	11	-
40.	Frameshift	c.5075_5078del (p.Glu1692Alafs*133)	Exon 40	49	Roberts, 2004
41.	Missense	c.1525A>C (p.Thr509Pro) <sup>g, j</sup>	Exon 15	118	-
42.	Missense	c.1790A>G (p.His597Arg)	Exon 17	68	Wang, 2016; Hardy, 2012; vanEeghen, 2012; Dunlop, 2011; Hoogeveen-Westerveld, 2013
43.	Missense	c.1793A>G (p.Tyr598Cys)	Exon 17	109	Suspitsin, 2018; Cai, 2017; Ishikawa, 2017; Kwiatkowski, 2015; Liu, 2015
44.	Missense	c.1832G>A (p.Arg611Gln) <sup>c</sup>	Exon 17	38	Kwiatkowski, 2015; Tyburczy, 2015; You, 2013; vanEeghen, 2012; Hoogeveen-Westerveld, 2011
45.	Missense	c.1831C>T (p.Arg611Trp)	Exon 17	58	Suspitsin, 2018; Hoogeveen-Westerveld, 2012; Li, 2011
46.	Missense	c.2540T>C (p.Leu847Pro) <sup>j</sup>	Exon 22	46	van Eeghen, 2012; Hoogeveen-Westerveld, 2013; Sancak, 2005
47.	Missense	c.4909_4911delinsGAC (p.Lys1637Asp) <sup>a</sup>	Exon 38	85	Zhang, 2013; Kwiatkowski, 2015
48.	Missense	c.4919A>G (p.His1640Arg) <sup>a</sup>	Exon 38	72	Papadopoulou, 2018; Suspitsin, 2018; Cai,
49.	Missense	c.5024C>T (p.Pro1675Leu)	Exon 39	64, 65	
50.	Missense	c.5227C>G (p.Arg1743Gly) <sup>a, c</sup>	Exon 41	43	
51.	Missense	c.5227C>T (p.Arg1743Trp)	Exon 41	77	

52.	Missense	c.5228G>A (p.Arg1743Gln)	Exon 41	124	2017; Yu, 2017; Bai, 2016 Papadopoulou, 2018; Ismail, 2017; Bai, 2016; Kwiatkowski, 2015; Tyburczy, 2015	
53.	In-frame deletion	c.824_826del (p.Asn275del)	Exon 09	88	Hoogeveen-Westerveld, 2011; Coevoets, 2009; Sancak, 2005	
54.	In-frame deletion	c.4842_4844del (p.Ile1614del) <sup>a</sup>	Exon 37	37	Kwiatkowski, 2015; van Eeghen, 2012, Sancak, 2005, Dabora, 2001	
55.	In-frame deletion	c.4823_4825del (p.Tyr1608del) <sup>a</sup>	Exon 37	40	Kwiatkowski, 2015; Hayashi, 2012; van Eeghen; 2012 Au, 1998 and 2007	
56.	In-frame deletion	c.5238_5255del (p.His1746_Arg1751del)	Exon 41	81	Hoogeveen-Westerveld, 2011; Qin, 2011; Tsai, 2011; Au, 2007; Choi, 2006	
57.	Large Deletion	c.(774+1_775-1)_(848+1_849-1)del <sup>a, d</sup>	Exon 9	97	-	
58.	Large Deletion	c.(975+628)_(1716+41)del <sup>a, b, d</sup>	Exon 11-16	12	-	
59.	Large Deletion	c.(1599+1_1600-1)_(2545+1_2546-1)del <sup>d</sup>	Exon 16 - 22	59	Longa, 2001	
60.	Large Deletion	c.(1716+1_1717-1)_(2545+1_2546-1)del <sup>a, d</sup>	Exon 17 - 22	84	-	
61.	Large Deletion	c.(5068+1_5069-1)_( <sup>*</sup> 102_?)del <sup>d, g</sup>	Exon 39 -	51	-	
62.	Large Deletion	c.(?-30)_( <sup>*</sup> 102_?)del <sup>a, d</sup>	<i>PKD1</i>			
63.	Large Duplication	c.(2355+1_2356-1)_( <sup>*</sup> 102_?)dup <sup>d</sup>	<i>TSC2</i>	61	-	
64.	Complex	c.5423G>C (p. <sup>*</sup> 1808Serext <sup>*</sup> 19) <sup>c</sup>	Exon 22 - 42	89	-	
	<b>Splicing variants</b>	<b>DNA Variant</b>	<b>Predicted effects: [r.spl?] <sup>e</sup> or [r.(spl?)] <sup>f</sup></b>	<b>Location <sup>g</sup></b>	<b>Patient #</b>	<b>References</b>
65.	Splicing	c.337-1G>A <sup>b</sup>	[r.spl?]: exon 5 skipping, p.(Gly113Leufs*20)	Intron 04	2	Niida, 2013
66.	Splicing	c.481+2T>C <sup>a</sup>	[r.spl?]: exon 5 skipping, p.(Gly113Leufs*20)	Intron 05	27	-
67.	Splicing	c.482-1G>A + c.482-3C>T <sup>a, i</sup>	[r.spl?]: exon 6 skipping, p.(Gly113Leufs*20)	Intron 05	114	-
68.	Splicing	c.600-2A>G	[r.spl?]: exon 7 skipping, p.(Met201Glyfs*18)	Intron 06	74	Suspitsin, 2018; Yu, 2017; Rendtorff, 2005; Sancak, 2005; Langkau, 2002
69.	Splicing	c.775-2A>G <sup>a</sup>	[r.spl?]: exon 9 skipping	Intron 08	75	-
70.	Splicing	c.848+4_848+9del <sup>a</sup>	[r.(spl?)]: exon 9 skipping, p.(Leu259Serfs*52)	Intron 09	6	-
71.	Splicing	c.1717-1G>A <sup>a</sup>	[r.spl?]: exon 17 skipping	Intron 16	102	-
72.	Splicing	c.1947-2_1947-1delinsCC <sup>a</sup>	[r.spl?]: exon 19 skipping, p.(Glu650Glyfs*3)	Intron 18	45	-
73.	Splicing	c.2355+2_2355+5del	[r.spl?]: exon 21skipping	Intron 21	98	Rosset, 2017; Kwiatkowski, 2015;

						LeCaignec, 2009; Sancak, 2005; Jones, 1999
74.	Splicing	c.2545+1G>A <sup>g</sup>	[r.spl?]: exon 22 skipping	Intron 22	62	-
75.	Splicing	c.2639+1G>A <sup>g</sup>	[r.spl?]: exon 23 skipping	Intron 23	121	-
76.	Splicing	c.2838-122G>A (p.Ser946Argfs*6) <sup>c</sup>	[r.(2837_2838ins2838-120_2838-1)], p.(Ser946Argfs*6)	Intron 25	34, 47	Nellist,2015
77.	Splicing	c.3132-3T>G (p.1044del50) <sup>a, c</sup>	[r.(spl?)]: in-frame exon 28 skipping, p.(1044del50)	Intron 27	7	-
78.	Splicing	c.4493+1G>C <sup>a</sup>	[r.spl?]: exon 34 skipping	Intron 34	70	-
79.	Splicing	c.5160+1G>C <sup>a</sup>	[r.spl?]: exon 40 skipping, p.(Asp1690Aspfs*6)	Intron 40	23	-
80.	Splicing	c.5160+1G>A	[r.spl?]: exon 40 skipping, p.(Asp1690Aspfs*6)	Intron 40	24	Sancak, 2005; Dabora, 2001
<b>Variants of unclear significance (VUS) in NMI patients</b>						
81.	Synonym	c.1119G>A (p.=)		Exon 11	104	-
82.	Missense	c.1443G>T (p.Glu481Asp)		Exon14	66, 68	-
83.	Missense	c.1663G>C (p.Ala555Pro) <sup>j</sup>		Exon 16	50	-
84.	Missense	c.4493G>T (p.Ser1498Ile)		Exon 34	30	
85.	Missense	c.5051C>T (p.Ser1684Phe)		Exon 39	116	
86.	Missense	c.5228G>C (p.Arg1743Pro)		Exon 41	116	
87.	Splicing	c.4005+153T>A		Intron 33	63	-
88.	Complex	c.*44_*45insGTCA		Exon 42	100	-

<sup>a</sup> First description of this variant.

<sup>b</sup> Next-generation sequencing and MLPA performed for both genes, besides Sanger sequencing.

<sup>c</sup> MLPA performed for both genes, besides Sanger sequencing.

<sup>d</sup> Deletion or duplication confirmed by Q-PCR.

<sup>e</sup> [r.spl?]: RNA was not analysed but the change is expected to affect splicing.

<sup>f</sup> [r.(spl?)]: RNA was not analysed but the change might affect splicing.

<sup>g</sup> At LOVD database without further information of submission.

<sup>h</sup> Five most recent references.

<sup>i</sup> Found in *trans*

<sup>j</sup> Under functional analysis

## B. *TSC2* missense and in-frame deletion variants

Initially, 20 distinct *TSC2* putative missense variants and five in-frame deletions had general population frequency lower than 0.1, and were predicted to be disease-causing variants by Mutation Taster, PolyPhen, PROVEAN, SIFT and Human Splicing Finder bioinformatics web tools. Among the missense variants, nine have been previously reported by other groups: His597Arg, Tyr598Cys, Arg611Trp, Arg611Gln, Leu847Pro, Pro1675Leu, Arg1743Gly, Arg1743Trp and Arg1743Gln (Table II-5). Arg611Trp, Arg611Gln, Arg1743Trp and Arg1743Gln have been reported in family cases (Wilson, Ramesh *et al.* 1996, Beauchamp, Banwell *et al.* 1998, Niida, Lawrence-Smith *et al.* 1999, Nellist, Sancak *et al.* 2005, Au, Williams *et al.* 2007, Hoogeveen-Westerveld, Wentink *et al.* 2011). Pathogenicity had been confirmed by functional assays for six cases: His597Arg, Arg611Trp, Arg611Gln, Lys1637Asp (M. Nellist, Erasmus Medical Centre, The Netherlands, personal communication), Arg1743Trp and Arg1743Gln (Nellist, Sancak *et al.* 2005, Hoogeveen-Westerveld, Wentink *et al.* 2011). Moreover, *TSC2* Tyr598Cys has been reported as likely pathogenic because its functional assay had yielded a S6K-Thr<sup>389</sup>/S6K ratio of intermediate value. It was significantly higher than that of wild-type *TSC2*, but significantly lower than that of pathogenic *TSC2* Arg611Gln (Hoogeveen-Westerveld, Ekong *et al.* 2013). DNA variants Leu847Pro and Arg1743Gly had been reported as pathogenic without further information (Li, Zhou *et al.* 2011, Hoogeveen-Westerveld, Ekong *et al.* 2012, Kwiatkowski, Palmer *et al.* 2015). Finally, variants Thr1661Ile and Ser1684Phe had yielded non-pathogenic evidence in the mTOR-based functional assay (M. Nellist, Erasmus Medical Centre, The Netherlands, personal communication). In summary, ten out of 19 missense DNA variants identified in our cohort with significantly low or null frequency in population databases had been functionally assessed, yielding six pathogenic, two non-pathogenic results, and one borderline evidence (Figure II-6).

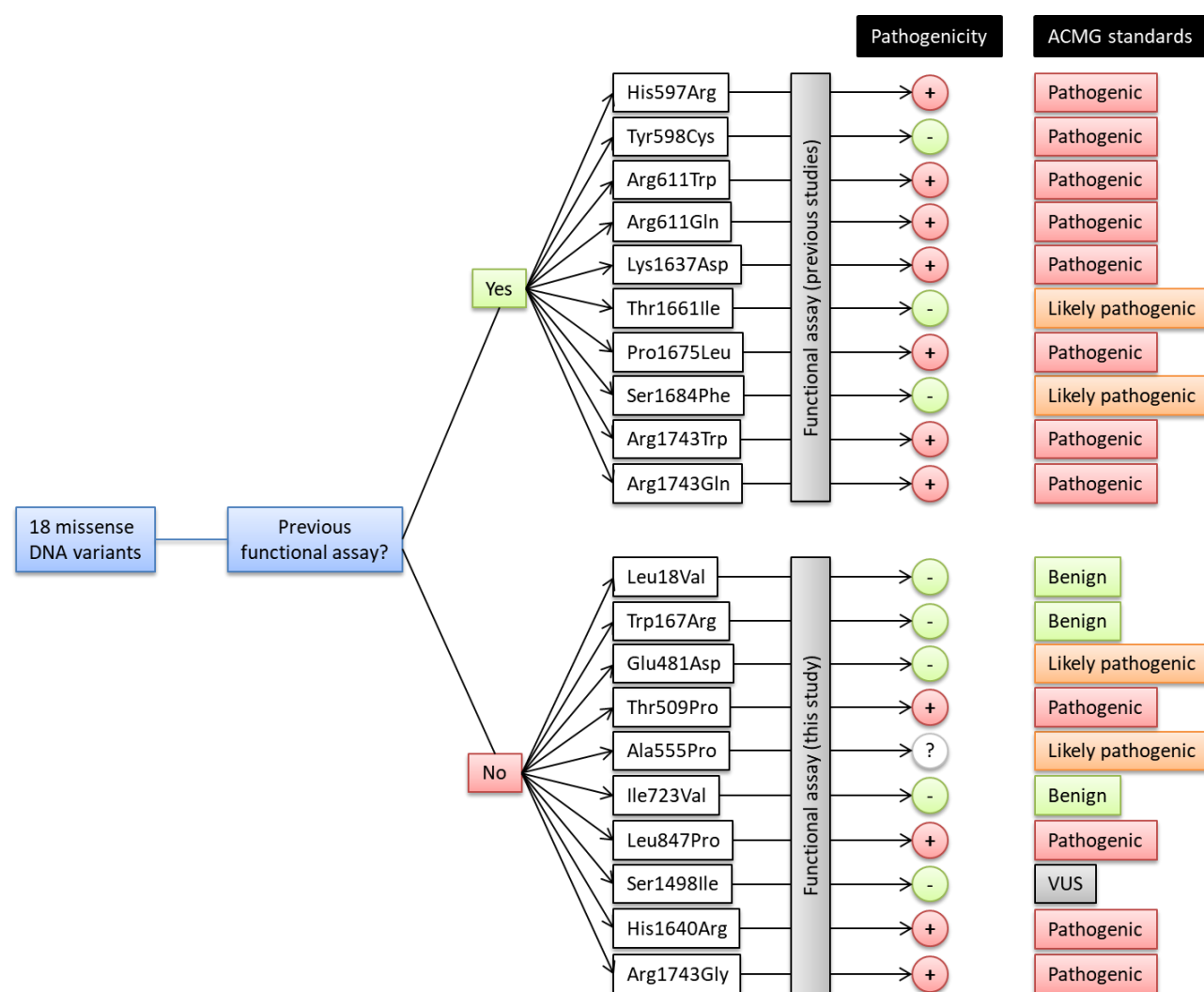


Figure II-6: Workflow of the pathogenicity classification of 20 *TSC2* missense DNA variants. VUS: variant of uncertain significance. ?: Variant under functional investigation. Classification according to ACMG standards before the functional assay of the present study

We adopted the American College of Medical Genetics and Genomics (ACMG, Bethesda, MD, USA) criteria for pathogenicity assessment of missense DNA variants and criteria combination (Richards, Aziz *et al.* 2015; Table II-6, Supplementary Table II-9 and 11). (Richards, Aziz *et al.* 2015) Besides the six variants that had kept mTORC1 activated in the functional assay, two others (Tyr598Cys and Arg1743Gly) fulfilled the criteria for pathogenicity (Supplementary Table II-9). Moreover, the two variants that inactivated mTOR in the functional assay (Thr1661Ile and Ser1684Phe) classified as likely pathogenic. Among the nine cases of missense DNA variants for which no functional data were available, four were likely benign (Leu18Val, Trp167Arg, Glu481Asp and Ala555Pro). Two of them (Trp167Arg and Ala555Pro) were from patients with *TSC1* or *TSC2* nonsense variants, one detected in this study and the other from a case available at LOVD. The third likely benign variant Leu18Val was from the same patient with the missense variant Ser1498Ile. Four additional missense DNA variants classified as likely pathogenic (Thr509Pro, Ile723Val, Leu847Pro and His1640Arg), and

Ser1498Ile was considered a variant of uncertain significance, according to ACMG criteria (Table II-6 and Figure II-6).

Five in-frame deletions identified by Sanger sequencing in the *TSC2* gene were not detected in any database consulted. All in-frame deletions have been reported before (Table II-5). Asn275del and His1746\_Arg1751del had been functionally assessed displaying T389/S6K phosphorylation ratios significantly higher than that of wild-type TSC2 (Sancak, Nellist *et al.* 2005, Hoogeveen-Westerveld, Wentink *et al.* 2011). DNA variants c.4842\_4844del (p.Ile1614del) and c.4823\_4825del (p.Tyr1608del) have been described as pathogenic (Au, Rodriguez *et al.* 1998, Dabora, Jozwiak *et al.* 2001, Sancak, Nellist *et al.* 2005, Au, Williams *et al.* 2007) but no functional assay had been conducted. These two in-frame deletion variants, Ile1614del and Tyr1608del, were predicted to be disease-causing variants by Mutation Taster and PROVEAN bioinformatics web tools. The fifth in-frame deletion, c.4527\_4529del (p.Phe1510del) on exon 35, was not present in 1000 Genomes Project neither gnomAD databases, but according to ClinVar it classifies as benign or likely benign. At TSC2LOVD database, this variant has been reported more than 25 times and classified as probable neutral as the functional assessment did not detect S6K-Thr<sup>389</sup> phosphorylation significantly higher than wild-type TSC2 (Sancak, Nellist *et al.* 2005, Hoogeveen-Westerveld, Wentink *et al.* 2011).

Table II-6: Pathogenicity criteria for classification of TSC2 missense (N=20) and in-frame deletion (N=5) DNA variants with low population frequency (Richards, Aziz et al. 2015)

			TSC2 DNA variants with significantly low frequency in the general population	Missense DNA variants																				In-frame deletions					
				c.52C>G (p.L18V)	c.499T>C (p.W167R)	c.1443G>T (p.Glu481Asp)	c.1525A>C (p.Thr509Pro)	c.1663G>C(p.Ala555Pro)	c.1790A>G (p.His597Arg)	c.1793A>G (p.Tyr598Cys)	c.1831C>T (p.Arg611Trp)	c.1832G>A (p.Arg611Gln)	c.2167A>G (p.Ile723Val)	c.2540T>C (p.Leu847Pro)	c.4493G>T (p.Ser1498Ile)	c.4909_4911delinsGAC (p.Lys1637Asp)	c.4919A>G (p.His1640Arg)	c.4982C>T (p.Thr1661Ile)	c.5024C>T(p.Pro1675Leu)	c.5051C>T (p.Ser1684Phe)	c.5227C>G (p.Arg1743Gly)	c.5227C>T (p.Arg1743Trp)	c.5228G>A (p.Arg1743Gln)	c.824_826del (p.Asn275del)	c.4527_4529del (p.Phe1510del)	c.4842_4844del (p.Ile1614del)	c.4823_4825del (p.Tyr1608del)	c.5238_5255del (p.His1746_Arg1751del)	
Evidence strength		Evidence description	Patient Number:	30	33	66, 68	118	50	68	109	58	38	53	46	30	85	72	113	64, 65	116	43	77	124	88	96	37	40	81	
Very strong	PVS1	Null variant	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	
			NO	NO	NO	NO	NO	YES	YES	YES	YES	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	YES	YES	YES	NO	NO	NO	YES	YES
Strong	PS1	Same amino acid change previously described as pathogenic regardless of nucleotide change	NO	NO	NO	NO	NO	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	
		De novo in patient (both maternity and paternity confirmed)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	
		In vitro or in vivo functional assay supportive of damaging effect	NO	NO	NO	YES	NI	YES	NO	YES	YES	NO	YES	YES	NO	YES	YES	NO	YES	NO	YES	YES	YES	YES	YES	NO	YES	YES	YES
		Prevalence of variant in affected individual significantly increased compared to controls	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Mo	PM1	Located in a mutational hot spot or critical domain*	NO	NO	YES	NO	YES	YES	YES	YES	YES	YES	YES	YES	NO	YES	YES	YES	YES	YES	YES	YES	YES	NO	NO	YES	YES	YES	

	PM2	Absent from controls**	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	NO	YES	YES	YES
	PM3	For recessive disorders, detected in trans with a pathogenic variant.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	PM4	Protein length changes as a result of in-frame del/ins in a non repeat	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
	PM5	Novel missense in residue with different missense previously reported as pathogenic.	NO	NO	NO	NO	NO	NO	YES	YES	YES	NO	NO	NO	NO	YES	NO	NO	YES	YES	YES	YES	YES	NO	NO	NO	NO	NO
	PM6	Assumed de novo, but without confirmation of paternity and maternity																										
Supporting	PP1	Cosegregation with disease in multiple affected family members	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
	PP2	Gene has low rate of benign missense variants	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
	PP3	Multiple lines of computational evidence support a deleterious effect on protein	NO	YES	YES	YES	NO	YES	YES	YES	YES	NO	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	NO	YES	YES	NO
	PP4	Patient phenotype or family history is highly specific for a disease with single gene etiology	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
	PP5	Reported as pathogenic by reputable source but evidence is unavailable	NO	NO	NO	YES	NO	YES	NO	YES	YES	NO	NO	NO	YES	NO	NO	NO	NO	NO	NO	YES	YES	NO	NO	NO	NO	NO
Criteria combined before functional assay:			LB	LB	LP	LP	LB	NA	NA	NA	NA	VUS	LP	VUS	NA	LP	NA		NA	P	NA	NA	NA	VUS	LP	LP	NA	
Criteria combined after functional assay:			B	B	LB	P	***	P	P	P	P	B	P	VUS	P	P	LP	P	LP	P	P	P	LP	B	P	P	P	
Functional assay performed by			DA	DA	FR	MN	MN	FR	FR	FR	FR	MN	MN	DA	MN	DA	MN	MN	MN	DA	FR	FR	MN	MN	DA	DA	MN	

NO: negative criterion; YES: positive criterion; NA: criterion does not apply; NI: criterion not investigated

P: pathogenic; LP: likely pathogenic; B: benign; LB: likely benign; VUS: variant of uncertain significance.

DA: Dufner-Almeida, this study. MN: Mark Nellist, personal communication. FR: former reports as referenced on the text.

\*TSC2 domains (approximate amino acid numbers): DUF3384 (55-469); Tuberin domain - hamartin binding (555-903); Rap-GAP domain (1562-1748).

\*\*1000 genomes, Exome Variation and EXAc.

The mTORC1 activity-based functional assay was employed to evaluate the pathogenicity of five missense variants - c.52C>G (p.Leu18Val), c.499T>C (p.Trp167Arg), c.4493G>T (p.Ser1498Ile), c.5227C>G (p.Arg1743Gly) and c.4919A>G (p.His1640Arg), a splicing variant leading to in-frame deletion of exon 27 - c.3132-3T>G (p.1044del50) - and two in-frame deletions identified in the present study c.4842\_4844del (p.Ile1614del) and c.4823\_4825del (p.Tyr1608del). *TSC2* variants Ser1498Ile, His1640Arg and 1608del had *TSC2* levels significantly decreased to WT-*TSC2* (Figure II-7B). No variant impaired *TSC1* levels as Arg611Gln did (Figure II-7C). For all variants tested, total S6K levels were not different from those of the WT-*TSC2* (Figure II-7D). Finally, 1044del50, 1614del, His1640Arg, 1608del and Arg1734Gly had relative S6K-Thr<sup>389</sup>/S6K signal ratio similar to the Arg611Gln and were significantly higher than *TSC2*-WT (Figure II-7E), thereby considered pathogenic variants. On the other hand, the relative S6K-Thr<sup>389</sup>/S6K signal for Ser1698Ile was significantly reduced comparatively to Arg611Gln ( $p = 0.002$ ; Student's t-test with Bonferroni correction), and nearly four-fold increased comparatively to WT-*TSC2* as although without statistic significance ( $p = 0.042$ ), hence remaining a variant of unclear significance (VUS). Three missense variants have been later tested by Dr Nellist group using the same functional assay. Two had pathogenicity confirmed (Thr509Pro and Leu847Pro) and one (Ile723Val) was confirmed as benign (data not shown).

In summary, the functional assay confirmed pathogenicity for a putative pathogenic DNA variant (Arg1743Gly), disclosed pathogenicity for three likely pathogenic missense DNA variant (His1640Arg, ), and did not change Ser1498Ile classification as VUS nor three likely benign variants (Leu18Val, Trp167Arg and Ile723Val). Two missense variants remain to be functionally assessed (Glu481Asp and Ala555Pro). Thus, 11 out of 18 functionally tested missense DNA variants in *TSC2* had pathogenicity confirmed. Among the twelve pathogenic missense variants ascertained according to the ACMG pathogenicity criteria (Table II-6), ten were confirmed by the functional assay and one had a borderline result (Figure II-6).

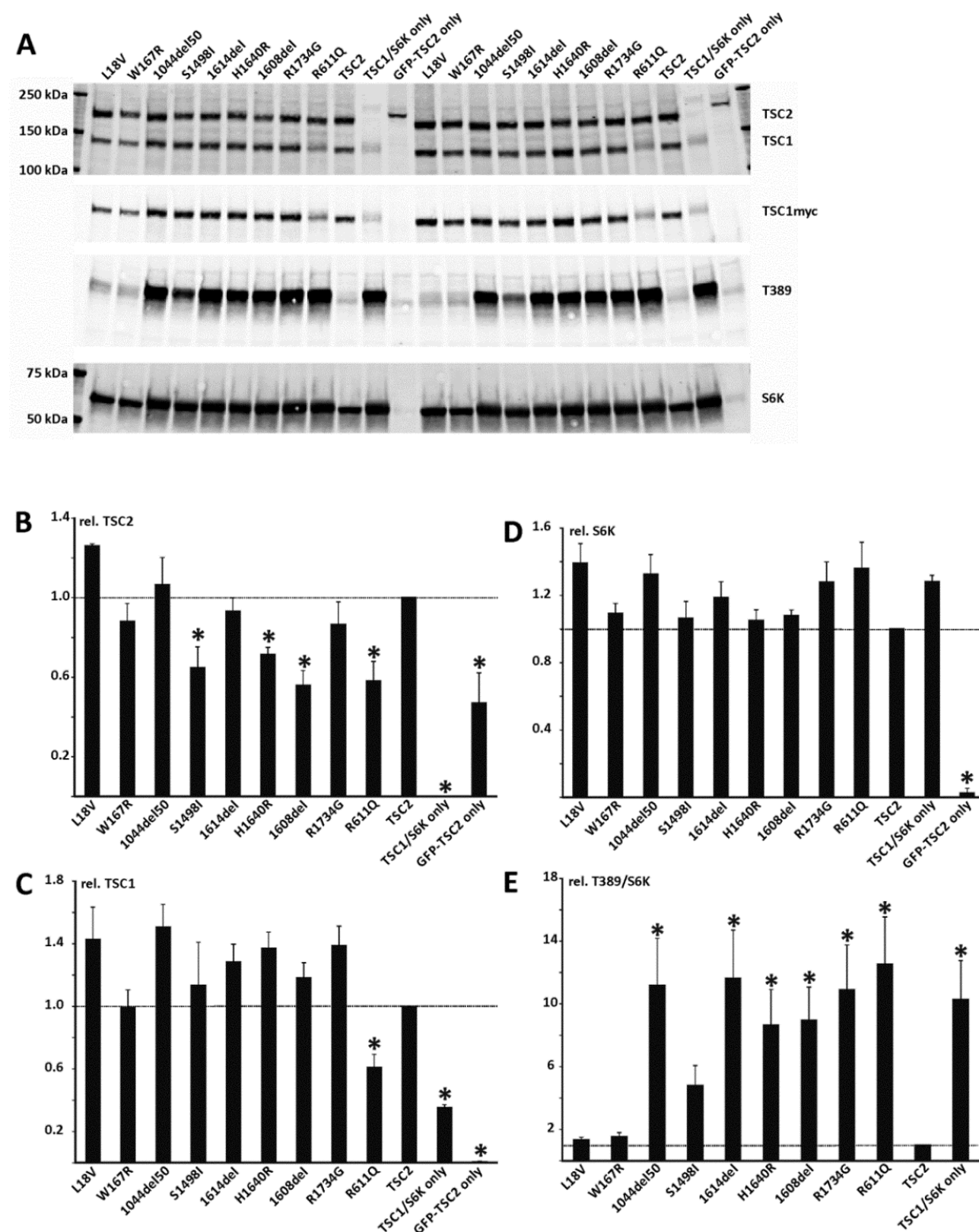


Figure II-7: Functional assessment of the TSC2 (NM\_000548.3) variants c.52C>G (p.Leu18Val), c.499T>C (p.Trp167Arg), c.3132\_3282del (p.1044\_1094del), c.4493G>T (p.Ser1498Ile), c.4840\_4842del (p.1614del), c.4919A>G (p.His1640Arg), c.4823\_4825del (p.1680del) and c.5227C>G (p.Arg1743Gly). 3H9-1B1 (TSC2/TSC1 double knockout HEK 293T) cells were transfected with the indicated TSC2 variant expression constructs, together with expression constructs for myc-tagged TSC1 and S6K. The known pathogenic TSC2 c.1832G>A (p.Arg611Gln) variant (R611Q) and cells not expressing any TSC2 (TSC1/S6K only) were included as controls. To monitor transfection efficiency, cells were transfected with an expression construct for GFP-TSC2 (GFP-TSC2 only). Twenty-four hours after transfection cells were harvested and the cleared cell lysates analyzed by immunoblotting (A). The signals for TSC2, TSC1, total S6K (S6K) and T389-

phosphorylated S6K (T389) were determined per variant, relative to the wild-type control (TSC2) in two independent, duplicate experiments. The mean TSC2 (B), TSC1 (C) and S6K (D) signals and mean T389/S6K ratio (E) are shown for each variant. In each case the dotted line indicates the signal/ratio for wild-type TSC2 (1.0). Error bars represent the standard error of the mean. TSC2 variants for which the TSC2, TSC1 or S6K signals were significantly reduced compared to wild-type TSC2, or for which the T389/S6K was significantly increased compared to wild-type TSC2, are indicated with an asterisk ( $P < 0.05$ ; Student's paired t-test). Amino acid changes are given according to TSC2 cDNA reference transcript sequence NM\_000548.3.

## C. Variants that affect splicing

Sixteen *TSC2* pathogenic splice variants were identified in the cohort. Fifteen variants mapped to canonical splice sites: eight at splice donor site and seven at splice acceptor site. Cloning and sequencing of c.482-1G>A and c.482-3C>T variants from the same patient disclosed they were in *trans*. Therefore, c.482-3C>T should be considered a rare SNV. One variant mapped to a deep intronic sequence (Figure II-8) and has been detected in two patients (Table II-5).

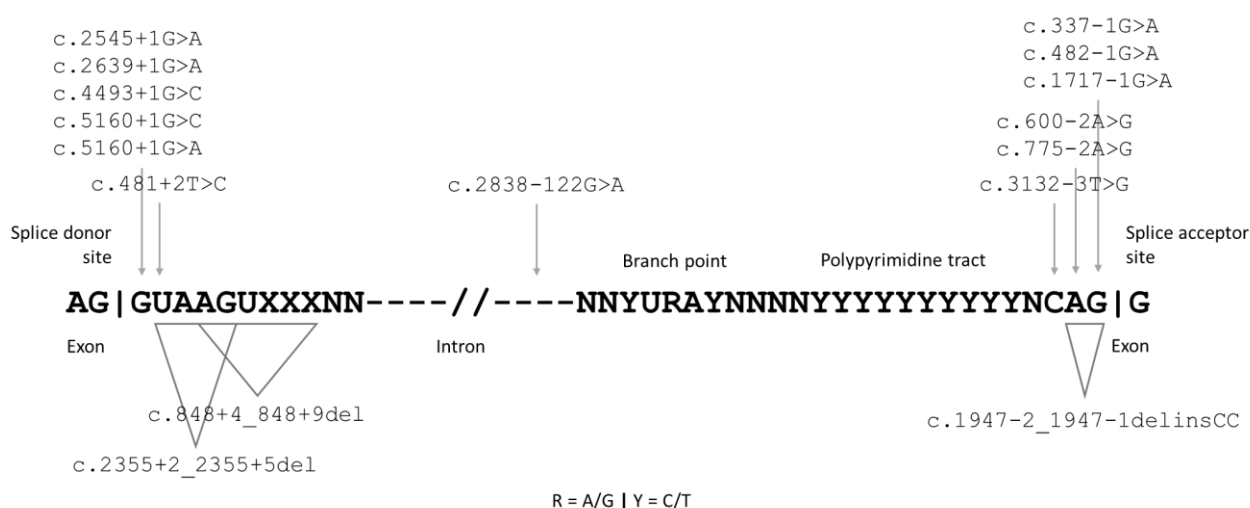


Figure II-8: Schematic representation of distribution of pathogenic *TSC2* splice variants. Intron canonical splice sites are indicated (donor and acceptor) as well as conserved branch point and downstream polypyrimidine tract. Vertical bar: border between exon and intron.

The DNA variant c.4493G>T (p.Ser1498Ile) disclosed a non-pathogenic result in the functional mTOR-based assay (Figure II-7). On the other hand, splicing-based softwares predicted the c.4493G>T variant could potentially impact on *TSC2* canonical splicing site choice leading to skipping of exon 34. Therefore, we interrogated whether c.4493G>T could affect splicing of *TSC2* exon 34. The genomic segment of *TSC2* exon 33 through the end of exon 34 from the heterozygous patient DNA was cloned in pCDNA3 vector, and each clone, wild-type or harbouring the nucleotide substitution, was used to transfect the human cell line HEK293. Twenty-four hours after transfection, RT-PCR was performed with vector primers. A 210-bp product was observed for the non-recombinant vector cDNA. If the interrogated exon 34 had been skipped upon splicing of the mini-gene primary transcript, the expected RT-PCR products would have 408 bp, whereas constitutive splicing products would be expected with 896 bp.

We did not detect products with the length corresponding to the skipped exon. The constitutive splicing product was present for the wild-type allele. Moreover, an additional RT-PCR reaction was included as an endogenous control, limiting a 327 bp product from *TSC2* exon 8 to 10 mRNA. The endogenous internal control was equally amplified in all samples, including the non-recombinant vector sample (Figure II-9). Slow-migrating bands were observed for all samples. All RT-PCR bands were isolated from agarose gel and Sanger sequencing confirmed them as the canonical splicing product of exon 34 mini-gene. We concluded that, in HEK293 cells, the *TSC2* variant c.4493T allele did not affect the splicing of the mini-gene. This variant found in patient 14 was observed in patient 50, classified as NMI. As there the frequency of the in the population and two homozygous individuals have already been observed according to the gnomAD database.

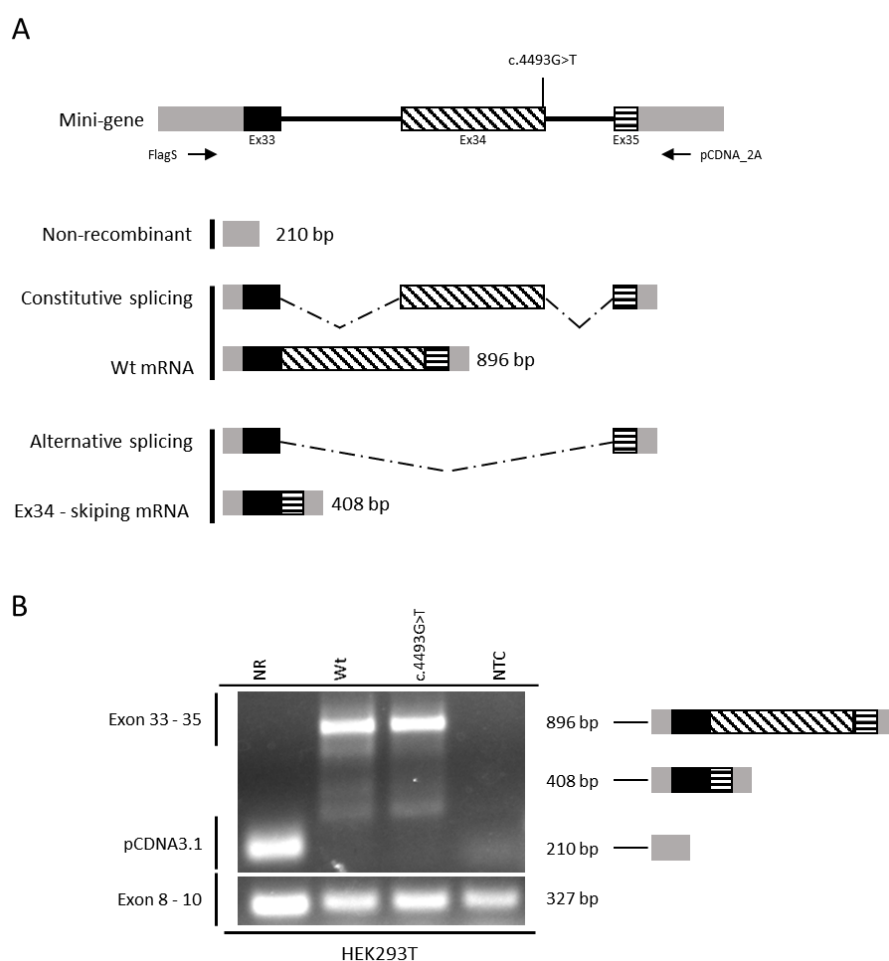


Figure II-9: Functional evaluation of a substitution DNA variant in the *TSC2* gene (c.4493G>T p.Ser1498Ile variant). (A) Scheme of predicted constitutive and alternative splicing skipping exon 34 of the mini-gene. The top segment refers to a scheme of the mini-gene: grey blocks are part of vector sequence flanking the mini-gene insert, black blocks are external exons, striped block is the interrogated exon, and black lines are introns. Primer location and identification are indicated by arrows. The location of patient DNA variant that originated the mutant clones, c.4493G>T, is indicated. The expected RT-PCR products of cDNA from cells transfected with either wild-type or deletion clones are indicated, as well as for the non-recombinant clone. (B) Electrophoresis in 1.5% agarose gel of RT-PCR products from HEK293 cell line transfected with non-recombinant vector (NR), wild-type mini-gene (WT), deletion clones and NTC (no-template control). 'Exon 33 – 35' indicates the gel region expected to contain the bands corresponding to the RT-PCR from constitutive and alternative splicing of the mini-gene. Exon 8 – 10 indicate the RT-PCR internal control.

As mentioned, one deep intronic DNA variant (c.2838-122G>A) was considered pathogenic as it had been functionally assessed. It altered splicing of the minigene primary transcript, consequently shifting the reading frame (p.Ser946Argfs\*6) (Nellist, Brouwer *et al.* 2015). In an NMI patient, a variant of uncertain significance (c.1361+54\_1361+57del) was identified in *TSC2* intron 13. This DNA variant has not, to our knowledge, been observed in the general population. In spite of the lack of prediction by the Human Splicing Finder tool, both computational tools Acescan2/Rescue and Spliceaid disclosed the 4-bp sequence c.1361+54\_1361+57 embedded within a putative intronic splicing enhancer. Of note, Spliceaid recognized it as a potential binding site for the trans-acting protein factor CUG triplet repeat RNA-binding protein 1 / ELAV-like family member 1 (CUGBP1/CELF1). The genomic segment of *TSC2* exon 12 through the end of exon 14 from the heterozygous patient DNA was cloned in pCDNA3 vector, and each clone, wild-type or harbouring the deletion, was used to transfect the human cell lines HEK293. If the exon 12 was skipped upon splicing of the mini-gene primary transcript, the expected RT-PCR products would be 430 bp, whereas constitutive splicing products would be expected to be 534 bp. We observed no products with the length corresponding to the skipped exon. The constitutive splicing product was present for the wild-type. The endogenous control from *TSC2* exons 8 to 10 mRNA was equally amplified in all samples (Figure II-10). All RT-PCR bands were isolated from agarose gel and Sanger sequencing confirmed it as the constitutive splicing product of exon 12-14 mini-gene. We conclude that in HEK293 cells, the *TSC2* variant c.1361+54\_1361+57del allele did not change the splicing output of the mini-gene. Therefore, that variant was considered benign.

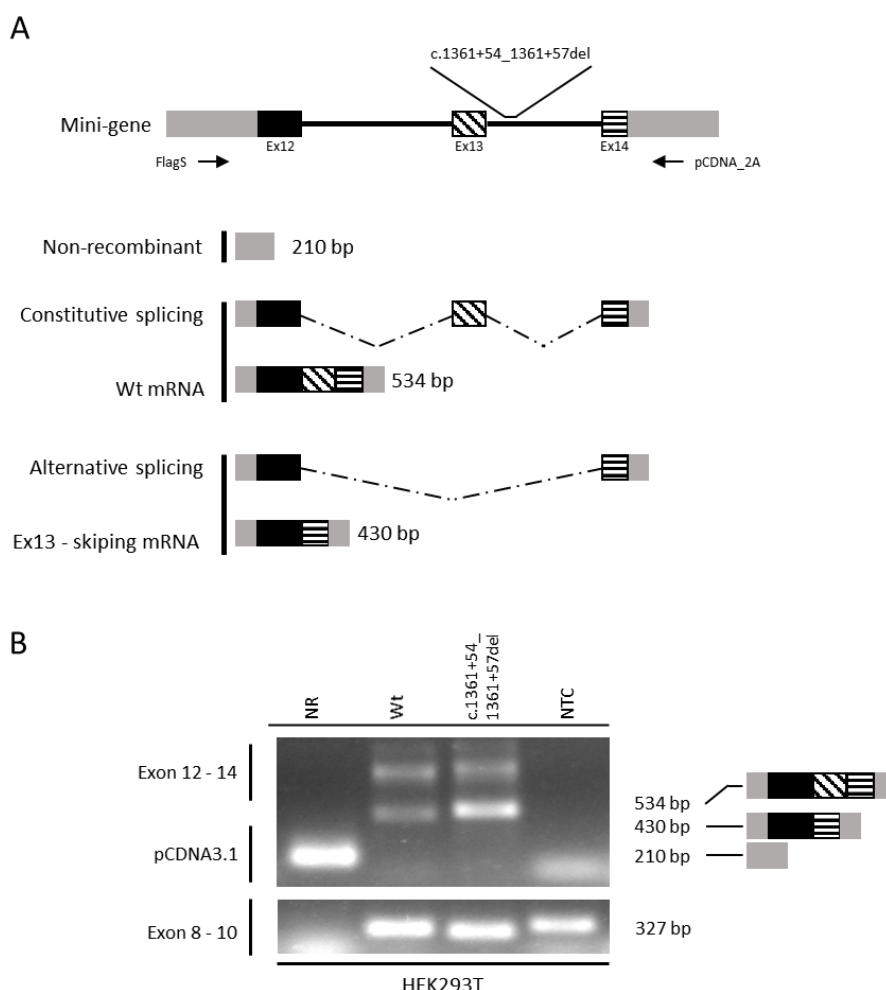


Figure II-10: Functional evaluation of a deep intronic DNA variation in the *TSC2* gene (c.1361+54\_1361+57del variant). (A) Scheme of predicted constitutive and alternative splicing, involving skipping of exon 12, of the c.1361+54\_1361+57del variant mini-gene. In The top segment refers to a scheme of the mini-gene is shown: grey blocks are part of vector sequence flanking the mini-gene insert, black blocks are external exons, the striped block is the interrogated exon to be tested, and black lines are introns. Primers location and identification are indicated by arrows. The location of the patient DNA variant that originated the mutant clones, c.1361+54\_1361+57del, is indicated. The expected RT-PCR products of cDNA from cells transfected with either wild-type or deletion clones are indicated, as well as for the non-recombinant clone. (B) Electrophoresis in 1.5% agarose gel of RT-PCR products from HEK293 cell line transfected with non-recombinant vector (NR), wild-type mini-gene (WT), deletion clones and NTC (no-template control). 'Exon 12 – 14' indicates the gel region expected to contain the bands corresponding to the RT-PCR from constitutive and alternative splicing of the mini-gene. Exon 8 – 10 indicate the RT-PCR internal control.

Data recently available at gnomAD (Massachusetts Institute of Technology, MIT, Cambridge, MA, USA) revealed the frequency of the c.1361+54\_1361+57del allele as 0.2%, as it has been detected in 467 out of 207,454 individuals.

## D. Large segmental gene deletions and duplications

After Sanger sequencing, DNA from 43 patients with missense variants, in-frame deletions or no mutation identified (NMI) was submitted to MLPA of both *TSC1* and *TSC2* genes. Nine copy number variations

(CNV) were identified in NMI patients: two in the *TSC1* and seven in the *TSC2* genes. All CNV were validated by quantitative PCR (Q-PCR). MLPA and corresponding Q-PCR results are presented in Table II-7 and Figure II-11. Both methods inferred somatic mosaicism for the deletions in patients #25 (*TSC1* c.-234-?-144+?del), #12 (*TSC2* c.(975+628)\_(1716+41)del), and #51 (*TSC2* c.(5068+1\_5069-1)>(\*102\_?)del) (Table II-7).

Table II-7: MLPA results and Q-PCR validation of *TSC1* and *TSC2* CNV.

		MLPA		Q-PCR	
Patient	CNV	Position	ps; p	Position	rq (sem); p
<i>TSC1</i>					
25	c.-234-?-144+?del	Exon 1	0.80; <0.01	Exon 1	0.79 (0.05); <0.01
29	c.(737+1_738-1)>(*1+_?)del a	Exon 9 - 23	0.60; <0.01	Exon 17	0.40 (0.01); <0.01
<i>TSC2</i>					
97	c.(774+1_775-1)_(848+1_849-1)del a	Exon 9	0.58; <0.01	Exon 9	0.45 (0.03); <0.01
12	c.(975+628)_(1716+41)del a	Exon 11 - 16	0.74; <0.01	Exon 12	0.62 (0.07); 0.03
59	c.(1599+1_1600-1)_(2545+1_2546-1)del	Exon 16 - 22	0.56; <0.01	Exon 19	0.54 (0.01); <0.01
84	c.(1716+1_1717-1)_(2545+1_2546-1)del	Exon 17 - 22	0.56; <0.01	Exon 19	0.63 (0.02); <0.01
89	c.(2355+1_2356-1)>(*102_?)dup	Exon 22 - 42	1.47; <0.01	Exon 41	2.43 (0.07); <0.01
51	c.(5068+1_5069-1)>(*102_?)del	Exon 39 - PKD1	0.68; <0.01	Exon 41	0.75 (0.02); <0.01
61	c.(?-30)>(*102_?)del	TSC2	0.54; <0.01	Exon 12	0.50 (0.03); <0.01

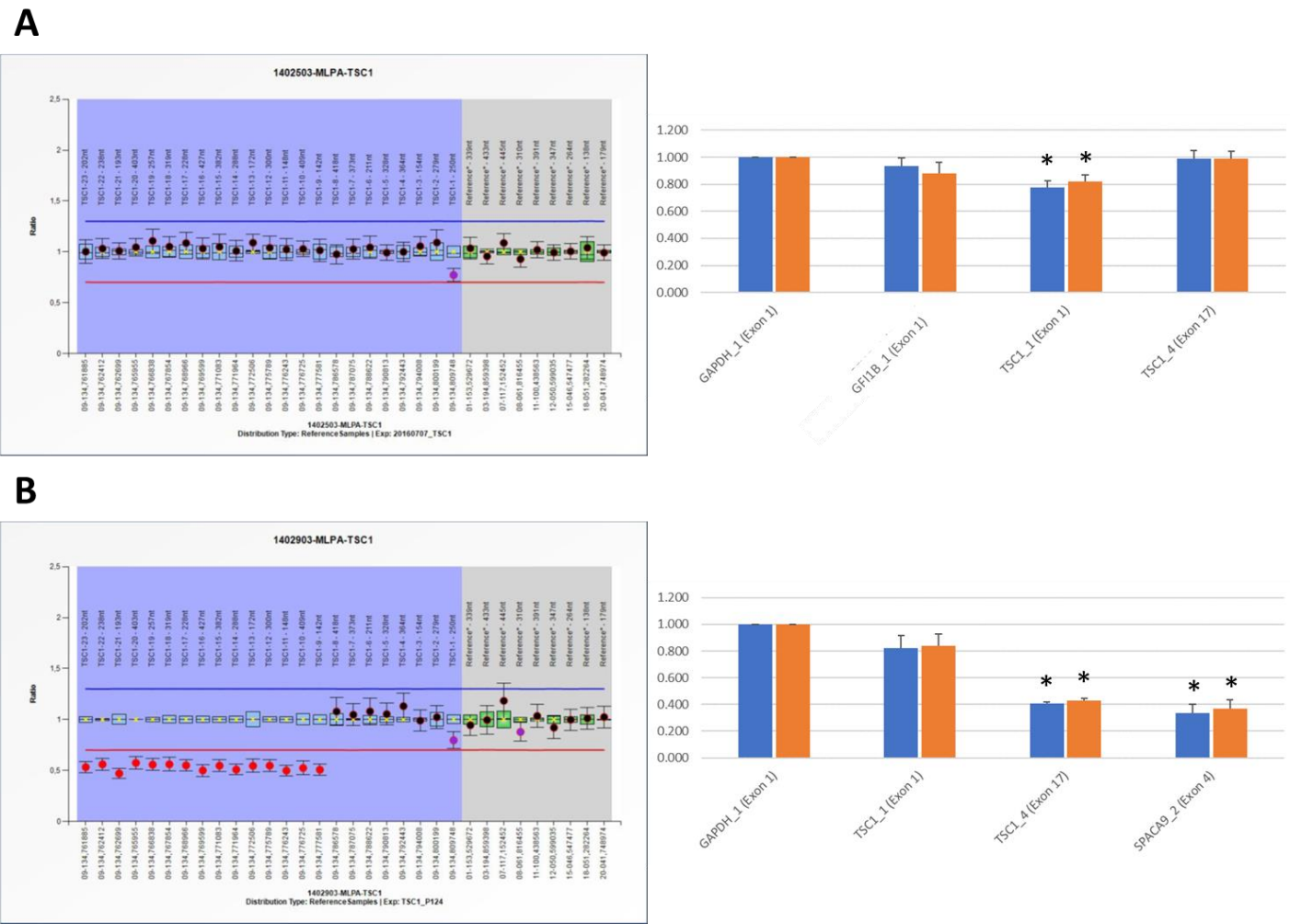
MLPA position refers to the extension of the detected deletion or duplication, while Q-PCR position refers to exon tested to validate MLPA.

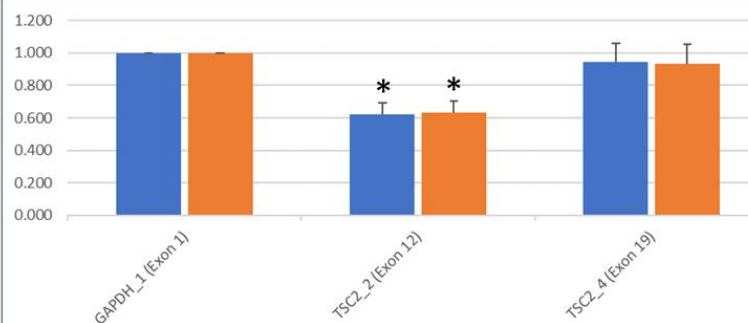
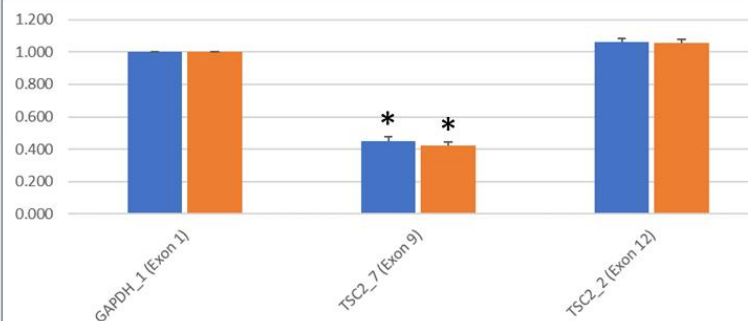
ps: mean probe signal; p: Student's t test p-value; rq: ratio coefficient; sem: standard error of mean.

<sup>a</sup> First description of this variant

NextGENe analysis of *TSC1* and *TSC2* NGS data from three patients with segmental deletions confirmed the large *TSC1* deletion encompassing exons 9 through 23 (patient #29), and the *TSC2* deletion between exons 11 and 16 (patient #12). The mosaic segmental deletion containing *TSC1* exon 1 (patient #25) was not identified as this exon was not covered by the Nextera probe set. In addition, no other DNA variant was identified by NGS in these patients.

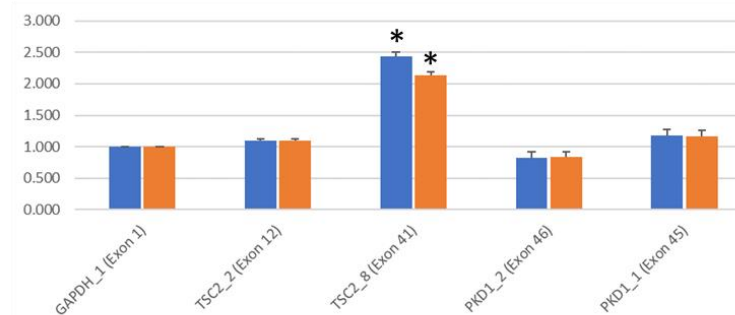
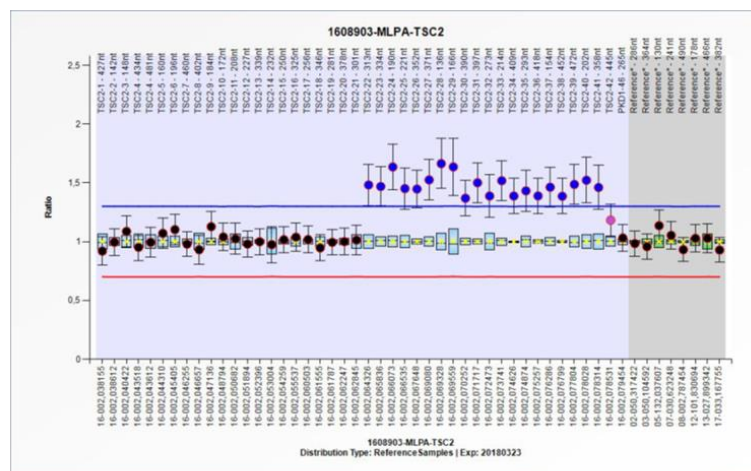
Due to the somatic mosaicism of the *TSC1* exon 1 deletion, the absence of coding region in this exon, and previous reports interrogating its clinical significance (van den Ouweland, Elfferich *et al.* 2011), it was not classified as pathogenic, and patient #25 remained with NMI. The other eight CNV classified as pathogenic.



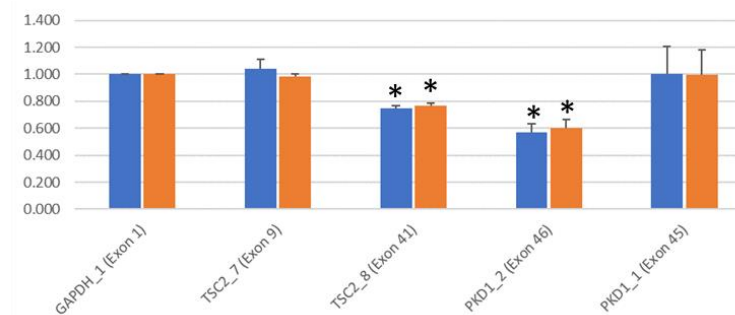
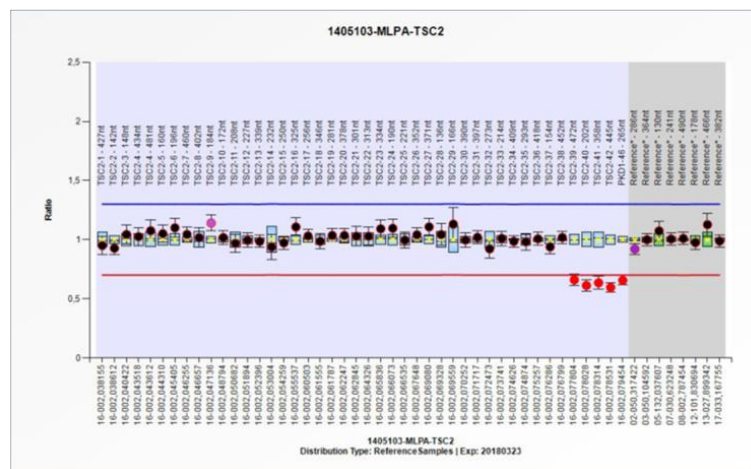




## G



H





We attempted to identify the extension of the eight segmental deletions and the *TSC2* duplication. *GFI1B* (Growth Factor-Independent 1B Transcription Repressor - NM\_004188.6), a gene positioned in a head-to-head fashion 34 kb upstream *TSC1*, was employed to investigate the boundary of patient #25 mosaic deletion of *TSC1* exon 1. *GFI1B* exon 1 deletion was ruled out by Q-PCR (rq = 0.93; sem = 0.06; p = 0.34) (Figure II-11A). The long extension between *TSC1* exon 1 and upstream *GFI1B* exon 1 (nearly 34 Kb) or downstream *TSC1* exon 2 (*TSC1* intron 1 of 6 Kb) impaired the identification of the breakpoints by PCR.

The extension of *TSC1* deletion c.(737+1\_738-1)\_(\*1+\_?)del from patient #29 was analyzed by Q-PCR. The *SPACA9* (Sperm acrosome-associated 9 - NM\_001316898.1) gene is approximately 2 kb downstream of *TSC1* polyadenylation signal. Q-PCR of *TSC1* 3'-UTR (rq = 0.49; sem = 0.03; p = 4.1x10<sup>-2</sup>) and of *SPACA9* exon 4 (rq = 0.38; sem = 0.06; p = 9.3x10<sup>-3</sup>) confirmed the deletion comprised both segments (Figure II-11B).

Four internal deletions have been detected by MLPA on *TSC2* (Table II-8; patients #97, 12, 59, and 84). Table II-8 indicates the expected locations of upstream and downstream breakpoints that generated those deletions.

Table II-8: *TSC2* internal deletion breakpoint mapping

Patient	CNV	Position	Breakpoint location			
			Expected		Observed	
			Upstream	Downstream	Upstream	Downstream
97	c.(774+1_775-1)_(848+1_849-1)del	Exon 9	Intron 8	Intron 9	Inc	Inc
12	c.(975+628)_(1716+41)del	Exon 11-16	Intron 10	Intron 16	Intron 10	Intron 16
59	c.(1599+1_1600-1)_(2545+1_2546-1)del	Exon 16-22	Intron 15	Intron 22	Inc	Inc
84	c.(1716+1_1717-1)_(2545+1_2546-1)del	Exon 17-22	Intron 16	Intron 22	Inc	Inc

Inc: inconclusive

Various forward and reverse PCR primers were designed to anneal respectively on upstream and downstream sites as an approach to sequence the junction of breakpoints. This strategy was successful to identify the breakpoints of *TSC2* intragenic deletion c.(975+628)\_(1716+41)del from patient #12. Sequencing of PCR products amplified with primers annealing to introns 9 and 17 disclosed the deletion spans 6.2 kb, from c.975+627 (intron 10) to c.1716+41 (intron 16). The breakpoint in intron 10 maps to an Alu repeat flanked by other Alu units, whereas the intron 16 breakpoint is 296 bp upstream of a L1 repeat with three Alu repeats downstream. *TSC2* intron 10 and intron 16 sequences, respectively 1,796 bp and 4,820 bp long, have only 11% similarity. However, the 909-bp segment encompassing the short interspersed nuclear element (SINE) sequences within intron 10 and 16 breakpoints are 66% identical (Figure II-12). A defined PCR product spanning the breakpoint junction was not obtained from DNA of patients #97, 59 or 84 (Table II-8).

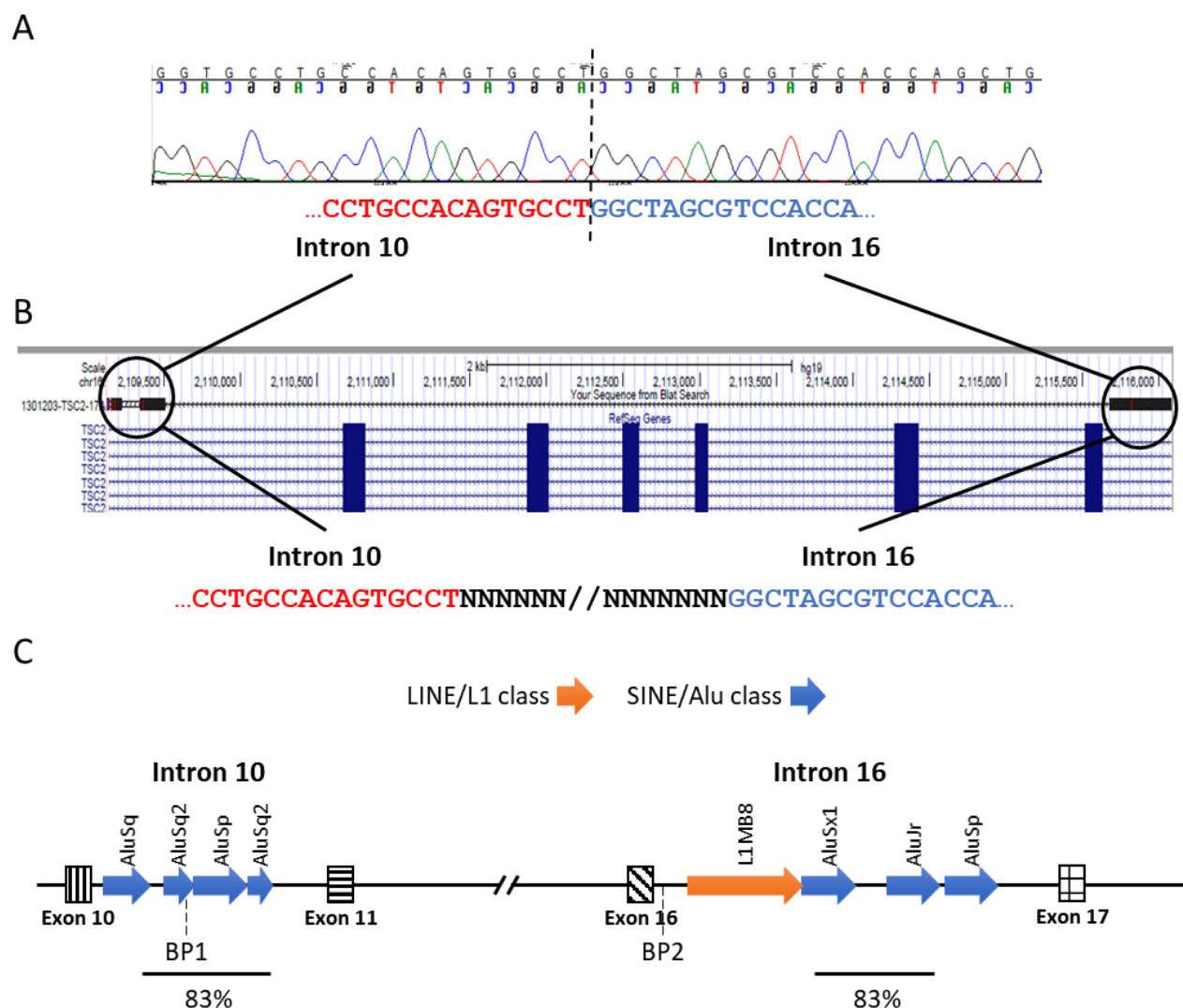


Figure II-12: Breakpoint mapping of the *TSC2* intragenic deletion c.(975+ \_976-)(1716+ \_1717-)del. (A) Sanger sequencing of the DNA from the patient with the intragenic *TSC2* deletion c.(975+ \_976-)(1716+ \_1717-)del. (B) UCSC genome browser mapping of the breakpoints in *TSC2* introns 10 and 16. (C) Diagram illustrating the positions of SINE/Alu and LINE/L1 repeat sequences in *TSC2* introns 10 and 16, respectively within and adjacent to each breakpoint. The regions of 83% similarity between introns are indicated.

One segmental duplication and two additional deletions were identified in the *TSC2* gene (Table II-7 and Figure II-11). *TSC2* is tail-to-tail to *PKD1* (Polycystic Kidney Disease 1 gene, NM\_001009944). In other to evaluate if the CNV spanned through *PKD1*, its most 3' exons, 45 and 46, were tested by Q-PCR in those three cases. In the case of c.(2355+1\_2356-1)(\*102\_?)dup (Patient #89), neither exon 45 or 46 was duplicated (exon 45: rq = 1.18, sem = 0.1; p = 0.14; exon 46: rq = 0.83; sem = 0.09; p = 0.19) (Figure II-11G).

Patient #51 *TSC2* deletion of exon 39 (c.(5068+1\_5069-1)(\*102\_?)del) extended through *PKD1* exon 46, as validated by Q-PCR (rq = 0.57; sem = 0.06; p = 0.02), though exon 45 was preserved (rq = 1.0; sem = 0.2; p = 0.99) (Figure II-11H). Hence, upstream and downstream breakpoints should be respectively at *TSC2* intron 38 and *PKD1* intron 45. Similarly, the deletion of full *TSC2* gene c.(?\_-30)(\*102\_?)del (patient #61) encompassed *PKD1* exon 46 as detected by Q-PCR (rq = 0.58; sem = 0.02; p = 2.2x10<sup>-3</sup>), preserving exon 45 (Q-

PCR;  $r_q = 0.92$ ;  $sem = 0.1$ ;  $p = 0.42$ ). The *NTHL1* (Endonuclease III-Like 1, MIM#602656) lies upstream *TSC2* in a tail-to-head fashion. Q-PCR of *NTHL1* exon 6 ( $r_q = 0.50$ ;  $sem = 0.05$ ;  $p = 8.6 \times 10^{-3}$ ) disclosed that the deletion affected the 3' end of this gene as well.

In summary, Figure II-13 and 16 illustrate the eight CNV identified as *TSC1* (1) or *TSC2* (7) pathogenic alterations, as well as the mosaic *TSC1* exon 1 deletion, which was considered an alteration of uncertain significance. Their estimated extension is presented according to the gene exon location.

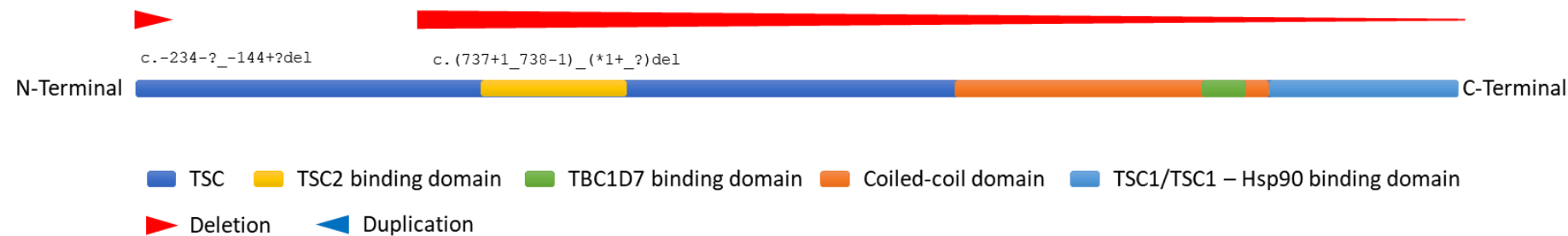


Figure II-13: Schematic representation of TSC1 and the segmental deletion c.-234-?-144+?del of exon 1 on patient#25 c.(737+1\_738-1)\_(\*1+\_?)del from exon 9 to exon 23 found on patient#29.

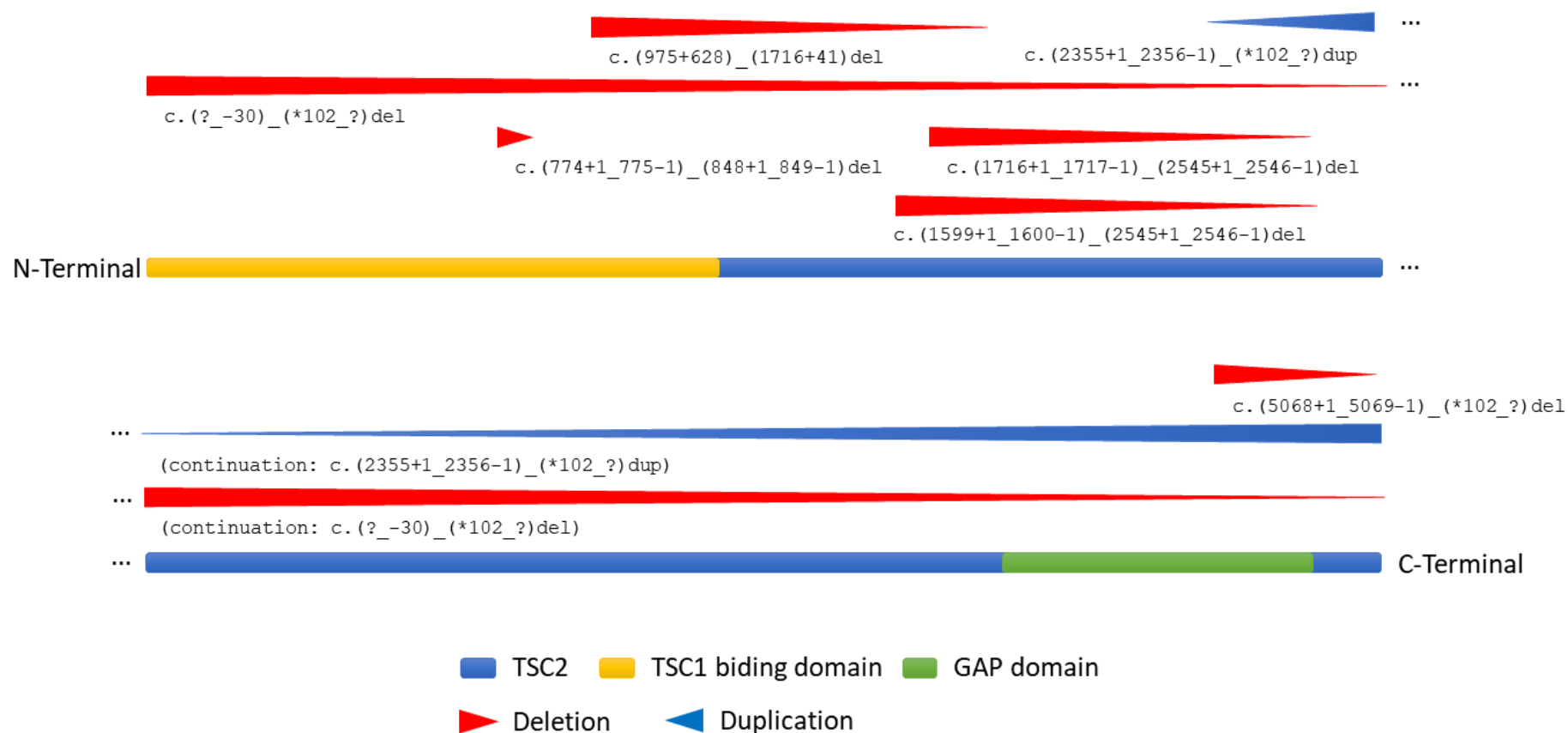


Figure II-14: Schematic representation of TSC2 and the segmental deletion  $c.(975+628)_{-}(1716+41)del$  from exon 11 to exon 16,  $c.(5068+1_{-}5069-1)_{-}(*102_{-}?)del$  from exon 39 to exon 42,  $c.(1599+1_{-}1600-1)_{-}(2545+1_{-}2546-1)del$  from exon 16 to exon 22,  $c.(?_{-}30)_{-}(*102_{-}?)del$  whole *TSC2*,  $c.(1716+1_{-}1717-1)_{-}(2545+1_{-}2546-1)del$  from exon 17 to exon 22,  $c.(774+1_{-}775-1)_{-}(848+1_{-}849-1)del$  from exon 22 to exon 42, and duplication  $c.(2355+1_{-}2356-1)_{-}(*102_{-}?)dup$  from exon 22 to 42.

## E. Single nucleotides variants

Finally, we identified 176 distinct, single nucleotide variants (SNV; frequency higher than 0.01) without clinical significance, 48 on *TSC1* and 128 on *TSC2*. Of those, 53 variants were identified within exonic segments (Supplementary Table II-11) and 113 in introns (Supplementary Table II-12). Nineteen variants were not present in 1000 Genomes, dbSNP and COSMIC data banks, and occurred in patients with pathogenic variants (Supplementary Table II-11 and 12).

NMI patients presented ten rare DNA alterations that classified as VUS, three in *TSC1* (c.-234-?-144+?del, c.738-197T>A and c.\*1G>A), and in *TSC2*: c.1119G>A (p.=), c.1443G>T (p.Glu481Asp), c.1663G>C (p.Ala555Pro), c.4493G>T (p.Ser1498Ile), c.5051C>T (p.Ser1684Phe), c.4005+153T>A and c.\*44\_\*45insGTCA (Table II-4 and 7).

## V. Discussion

We analyzed DNA from 117 patients with definite clinical diagnosis of TSC (Northrup, Krueger *et al.* 2013). They were from the states of São Paulo (SP), Paraná (PR) or Santa Catarina (SC), in the South and Southeast regions of Brazil; and clinically followed up at three large, tertiary referral University hospitals (HSCM-SP, GRAACC-SP, and HC-UFPR). The clinical evaluation of these patients has been the subject of other graduate research studies (Juliana P Almeida Master's dissertation – *Hospital Santa Casa de Misericórdia*, 2016, Laís Masulk Cardoso, Master's dissertation – *Complexo de Hospital de Clínicas da Universidade Federal do Paraná*, Laís Masulk ; and Nasjla Saba, unpublished), and will thus not be discussed here. The vast majority of samples has been first assessed by Sanger sequencing (N=116), whereas one sample was initially analyzed by NGS. Pathogenic DNA variants identified by NGS have been confirmed by Sanger sequencing. MLPA was carried out in a subset of patient DNA (N=38), and any altered result further assessed by Q-PCR.

We report a mutation detection rate of 87.2% (102/117), which is similar to previous reports that sequenced both *TSC1* and *TSC2* coding sequence and intronic boundaries, and assessed exonic copy number variation (Jones, Shyamsundar *et al.* 1999, Dabora, Jozwiak *et al.* 2001, Rendtorff, Bjerregaard *et al.* 2005, Sancak, Nellist *et al.* 2005, Hung, Su *et al.* 2006, Au, Williams *et al.* 2007). Despite the high number of distinct TSC-causing mutations already described worldwide in both *TSC1* and *TSC2* genes (LOVD-TSC1, LOVD-TSC2), we report 41 novel pathogenic DNA variants (Table II-4 and 5) as well as 19 novel SNV (Supplementary Table II-11 and 12).

It has been reported that patients with mutation identified, either on *TSC1* or *TSC2* genes, are generally more severely affected than patients with NMI, who present a lower incidence of brain findings on imaging studies, neurological features, renal findings and lower incidence of seizures than those with *TSC1* or *TSC2* mutations (Camposano, Greenberg *et al.* 2009). Our mutation detection rate by currently standard sequencing approaches lies in the average rate as other studies. Ninety-eight patients were from Neurological services from two tertiary referral hospitals (HC-UFPR and HSCM, SP), and the remaining 19 patients were from the Oncology service of another tertiary referral University hospital (UNIFESP, São Paulo, SP). Tertiary referral hospitals tend to retain the most severe cases.

In regard to TSC *de novo* cases, when patients with *TSC2* mutations are compared to those with *TSC1* mutations, they present higher mean number of cerebral cortical tubers and subependymal nodules, mean grade of kidney angiomyolipomas, as well as frequency of moderate/severe cases of intellectual disability and retinal hamartomas (Dabora, Franz *et al.* 2011). Accordingly, Sancak, Nellist *et al.* (2005) and Au, Williams *et al.* (2007) reported intellectual disability, angiomyolipoma and retinal phacoma more frequently among TSC patients with *TSC2* than *TSC1* mutations. As mentioned, all patients from our cohort came from tertiary referral hospitals, which tend to assist more severe cases. This could justify the higher numbers of *TSC2*

pathogenic variants observed by us. However, if we estimate this ratio specifically for each hospital, we observe that 71.4% (60/84) of all *TSC2* pathogenic variants in the present study are from HC-UFPR while *TSC1/TSC2* ratio from HSCM-SP is nearly 1:1.5 (Table II-3). At enrolment in this study, familial cases have been informed based solely on parental report. Among the 102 cases with pathogenic alteration identified, familial cases are expected to be 30% of all TSC cases (Jones, Shyamsundar *et al.* 1999, Dabora, Jozwiak *et al.* 2001, Au, Williams *et al.* 2007). It remains to be evaluated if more familial cases could be part of the HSCM-SP cohort of 23 patients, what could explain the 1:1.5 *TSC1:TSC2* partition ratio for pathogenic DNA alterations.

Frameshift and nonsense variants were the alteration types most commonly observed among *TSC1* and *TSC2* pathogenic DNA alterations. Altogether nonsense and frameshifting pathogenic variants corresponded to 94.4% (17/18) and 50% (42/84) of all genetic alterations, respectively for *TSC1* and *TSC2* genes. In our study, missense, in-frame deletions and splicing DNA variants have been detected only in the *TSC2* gene. Segmental gene deletions and duplications comprehended 7.8% (8/102) of all pathogenic DNA alterations, 5.5% (1/18) and 8.3% (7/84) respectively for *TSC1* and *TSC2*. Of note, 2.5% of TSC patients with a mutation identified had *TSC2* deletions extending through *PKD1* gene, defining cases with the contiguous gene deletion syndrome (Martignoni, Bonetti *et al.* 2002). Our data are similar to another Brazilian study (Rosset, Vairo *et al.* 2017) as well as to reports from many countries (Rendtorff, Bjerregaard *et al.* 2005, Hung, Su *et al.* 2006, Au, Williams *et al.* 2007, Kozlowski, Roberts *et al.* 2007, Sasongko, Wataya-Kaneda *et al.* 2008, Chopra, Lawson *et al.* 2011, Niida, Wakisaka *et al.* 2013). Because our study does not aim to estimate the frequency of TSC in Brazil, we should not compare our data to previous reports that reported on the lack of ethnic or geographical bias of TSC distribution (Wiederholt, Gomez *et al.* 1985, Sampson, Scahill *et al.* 1989, Osborne, Fryer *et al.* 1991, O'Callaghan, Shiell *et al.* 1998).

### A. *TSC2* missense and in-frame deletion effect on mTOR

Missense DNA variants cause amino acid substitution in the encoded protein. As missense variants are not considered *a priori* a loss-of-function variant, the interpretation of their clinical significance is not straight forward, and relies on several bioinformatics tools, although based on common biological aspects. PolyPhen-2 involves comparison of biochemical and genetic properties between the wild-type (ancestral, normal) allele and those of the mutant (derived, disease-causing) allele (Adzhubei, Schmidt *et al.* 2010). Mutation Taster integrates information from different biomedical databases and uses established analysis tools, comprising evolutionary conservation, splice-site changes, loss of protein features and changes that might affect the amount of mRNA (Schwarz, Rodelsperger *et al.* 2010). SIFT and PROVEAN predict whether a protein sequence variation affects protein function (Ng and Henikoff 2003, Choi and Chan 2015).

Among 20 missense variants and five rare in-frame deletion variants identified, at least two *in silico* bioinformatics tools predicted damaging effects, except for c.2167A>G (p.Ile723Val) for which Mutation Taster

was the only one to predict a deleterious output. Moreover, splicing analysis using Human Splicing Finder also predicted for almost all variants submitted to analysis the potential to disrupt splicing. The only exceptions were c.1663G>C (p.Ala555Pro), c.1831C>T (p.Arg611Trp) and c.2167A>G (p.Ile723Val).

Our *in silico* predictions agreed with previous functional assessments for variants c.1831C>T (p.Arg611Trp), c.1832G>A (p.Arg611Gln), c.5227C>T (p.Arg1743Trp) and c.5228G>A (p.Arg1743Gln) (Hoogeveen-Westerveld, Ekong *et al.* 2013). However, *TSC2* variants c.499T>C (p.Trp167Arg) and c.1793A>G (p.Tyr598Cis) had been predicted as pathogenic by all *in silico* analysis, but disclosed no inactivating effects on mTOR by the functional assessment. Moreover, our functional assay disagrees with *in silico* prediction for c.52C>G (p.Leu18Val) and c.499T>C (p.Trp167Arg), which classified as deleterious but did not activate the mTORC1 pathway (Figure II-7). Finally, c.4493G>T (p.Ser1498Ile), c.4909\_4911delinsGAC (p.Lys1637Asp), c.4919A>G (p.His1640Arg) and c.5227C>G (p.Arg1743Gly) were predicted to be deleterious by all *in silico* analysis, supported by our functional assessment (Figure II-7). The remaining variants are under investigation.

Well-established *in vitro* or *in vivo* functional assays supportive of a damaging effect on the gene product or pathway have been classified as a strong criterion for pathogenicity classification of missense DNA variants (Richards, Aziz *et al.* (2015), Supplementary Table II-9). Based on the recognized repressive role of TSC1/2 complex on mTORC1 kinase activity, a functional assay has been developed (Hoogeveen-Westerveld, Wentink *et al.* 2011, Hoogeveen-Westerveld, Ekong *et al.* 2012, Hoogeveen-Westerveld, Ekong *et al.* 2013, Kwiatkowski, Palmer *et al.* 2015) and further improved (Dufner-Almeida, L. G. *et al.* submitted). It relies on reporting the phosphorylation status of S6K-Thr<sup>389</sup>, a target of mTORC1 (Goncharova, Goncharov *et al.* 2002, Tee, Fingar *et al.* 2002). We employed the mTORC1 activity-based assay to functionally assess five *TSC2* missense variants, two *TSC2* in-frame deletions, and one splicing variant (Figure II-7). A genetically modified HEK293T cell line (3H9-1B1) having both *TSC1* and *TSC2* genes fully inactivated by CRISPR/Cas9 (Figure II-7; Dufner-Almeida *et al.*, manuscript submitted) was used in the assay. For each assay, the 3H9-1B1 cell line was transfected with three different full-length cDNA clones (WT or mutant *TSC2*, WT *TSC1*, and WT *RPS6KB1* – S6K). This additional step leads to higher efficiency in reporting S6K-Thr<sup>389</sup> phosphorylation due to higher stability of overexpressed TSC1/2 complex than its endogenous levels (Dufner-Almeida, L. G. *et al.* submitted). TSC1 and S6K protein levels were not affected by the overexpression of any *TSC2* variant tested in the present study (Figure II-7C and D). TSC2 levels were reduced by Ser1498Ile and 1608del variants (Figure II-7B) with no effect on TSC1 and S6K. Because all three proteins, TSC1, TSC2 and S6K, are co-overexpressed, TSC2 levels are unlikely to be solely affected by contaminants from transfection. Hence, variants Ser1498Ile and 1608del may render TSC2 unstable, possibly due to incorrect folding and faster degradation than wild-type TSC2. Moreover, as both variants are outside the TSC1-binding domain, the TSC1–TSC2 interaction should not be destabilized (Chong-Kopera, Inoki *et al.* 2006), thus preserving TSC1 levels.

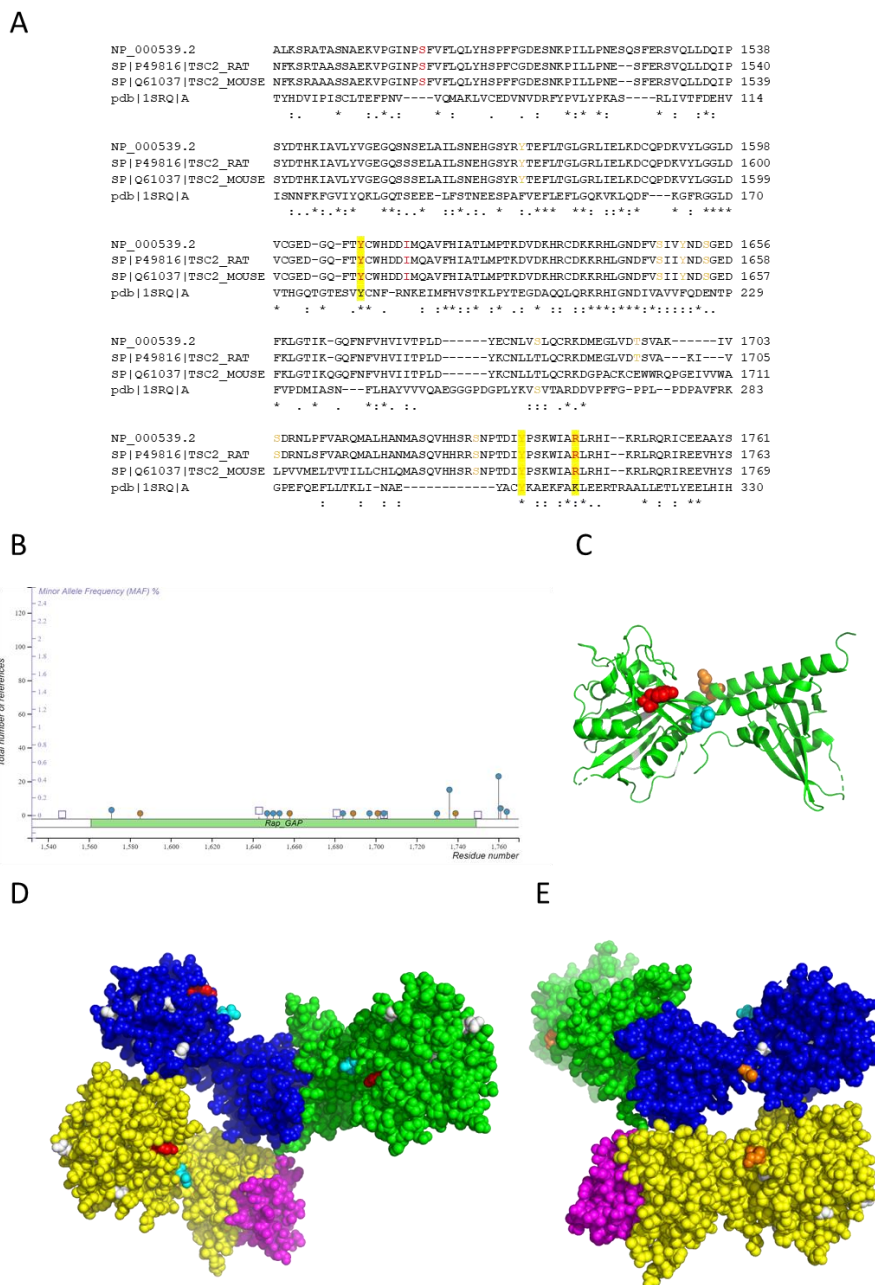
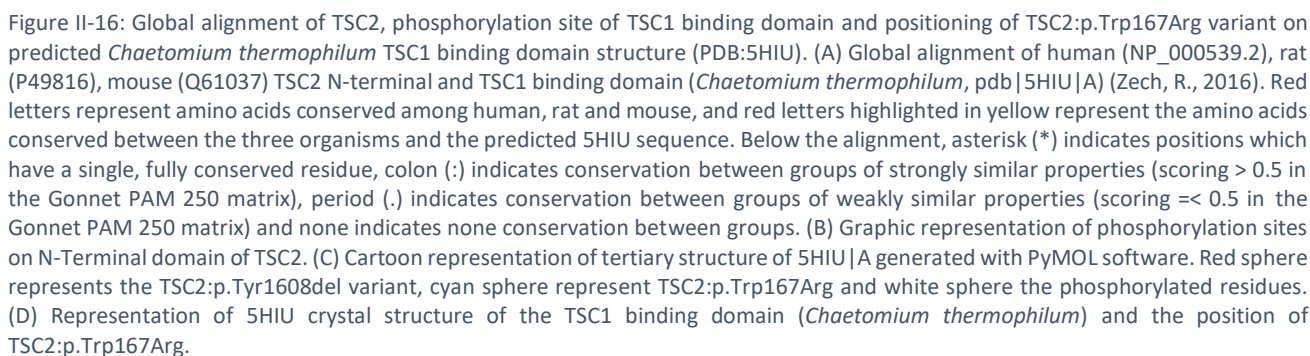


Figure II-15: Global alignment of TSC2, phosphorylation site of GAP Domain and positioning of TSC2:p.Tyr1608del, p.Ile1614del and p.Arg1743Gly variants on predicted *Homo sapiens* GAP domain structure (PDB: 1SRQ). (A) Global alignment of human (NP\_000539.2), rat (P49816), mouse (Q61037) TSC2 C-terminal and Rap1GAP catalytic domain (*Homo sapiens*, pdb|1SRQ|A) (Daumke et al., 2004). Red letters represent amino acids conserved among human, rat and mouse, and red letters highlighted in yellow represent the amino acids conserved between the three organisms and the predicted 1SRQ sequence. Below the alignment, asterisk (\*) indicates positions which have a single, fully conserved residue, colon (:) indicates conservation between groups of strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix), period (.) indicates conservation between groups of weakly similar properties (scoring < 0.5 in the Gonnet PAM 250 matrix) and none indicates none conservation between groups. (B) Graphic representation of phosphorylation sites on C-Terminal domain of TSC2. (C) Cartoon representation of tertiary structure of 1SRQ|A generated with PyMOL software. Red sphere represents the TSC2:p.Tyr1608del variant, cyan sphere represent TSC2:p.Ile1614del variant, orange sphere represent TSC2:p.Arg1743Gly variant and white sphere the phosphorylated residues. (D) and (E), respectively front and back schematic representation of 1SRQ crystal structure of the Rap1GAP catalytic domain (*Homo sapiens*) and the position of TSC2:p.Tyr1608del, p.Ile1614del and p.Arg1743Gly variants.

We identified five variants that significantly increased S6K-Thr<sup>389</sup>/S6K ratios, a result from reduced TSC1–TSC2 inhibition on mTORC1 activity (Tee, Fingar *et al.* 2002, Inoki, Li *et al.* 2003, Zhang, Cicchetti *et al.*

2003, Riemenschneider, Betensky *et al.* 2006). Two missense variants (His1640Arg and Arg1743Gly), one in-frame exon skipping due to splice variant (1044del50), and two in-frame deletions (Tyr1608del and Ile1614del) were thus classified as pathogenic (Figure II-7). TSC-causing missense variants have been described more often in the coding sequence for *TSC1* or *TSC2* domains, notably *TSC2* GAP domain and *TSC2/TSC1*-interacting domain, such as Arg611Gln in the latter (Nellist, Sancak *et al.* 2005, Hoogeveen-Westerveld, van Unen *et al.* 2012). The missense Arg1743Gly variant and the two in-frame deletions (1608del and 1614del) locate within *TSC2* GAP domain. Residues Ile1498, Thy1608, Ile1614 and Arg1743 are conserved among human, rat and mouse *TSC2* (Figure II-15A). None of those is close to phosphorylation sites (Figure II-15B). However, analysis of *TSC2* GAP domain (pdb:1SRQ) by pymol shows that Thy1608 and Ile1614 are close and both are towards the inner face of the structure, while the Arg1743 locates outside the domain folding and faces a probable catalytic pore (Figure II-15D and E). The spatial prediction of each variant and the possible function on activity of *TSC2* GAP together with functional assessment (Figure II-7) suggest these residues are important for the structural stability of *TSC2* GAP domain.

In two other cases (Leu18Val and Trp167Arg), the S6K-Thr<sup>389</sup>/S6K ratios were not significantly different from wild-type *TSC2*. The variants then classified as non-pathogenic. Variant c.499T>C (p.Trp167Arg) has been detected in an individual with a nonsense mutation. The functional assay corroborates it is a benign substitution. The missense Trp167Arg is conserved among human, rat and mouse *TSC2* (Figure II-16A) and are not close to phosphorylation site (Figure II-16B). Moreover, Trp167Arg variant are located within alpha-helix (Figure II-16C) but embedded inside the predicted *TSC1* binding domain (pdb|5HIU, Figure II-16D) of *Chaetomium thermophilum*. The spatial prediction and functional assessment suggests these residues are not important for *TSC1/2* complex.



## B. 'Miscalled' missense DNA variants?

One result of the mTOR-based functional assay, c.4493G>T (p.Ser1498Ile), disclosed S6K-Thr<sup>389</sup>/S6K ratio similar to both, wild-type and Arg611Gln TSC2. This borderline result led us to consider c.4493G>T with uncertain significance. As they did not significantly affect S6K phosphorylation (Figure II-7), we further evaluated the potential of c.4493G>T (p.Ser1498Ile) to affect pre-mRNA splicing. This substitution of unclear significance is on the last nucleotide of exon 34. Nucleotide substitutions, as in putative missense and synonymous variants, may lead to splicing abnormalities if they abolish exonic splicing enhancers (ESE) or silencers (ESS) or activate cryptic exon splice sites (reviewed by Dufner-Almeida *et al.* 2018). Variant c.4493G>T (p.Ser1498Ile) was predicted by ALAMUT to disrupt splice site (Supplementary Figure II-2). ALAMUT employs different softwares to predict effect on pre-mRNA splicing. Acescan2 identifying candidate *cis*-elements in alternative and constitutive splicing in mammalian exons and within the flanking introns. SpliceAid2 searches for motifs versus a database of experimentally assessed target RNA sequences, that can predict the effect of the DNA mutations at the level of the target sequences of the RNA-binding proteins that determine the pattern of mRNA splicing. Finally, HSF combines algorithms based on PWM matrices, Maximum Entropy principle or Motif Comparison to identify and predict the variant effect on splicing RNA motifs.

To functionally assess the effect of c.4493G>T on the splicing of *TSC2* exon 34, the mini-gene strategy was employed by cloning and expressing the genomic DNA segment from exon 33 through exon 35. The internal exon (34) was interrogated to contain an element that regulates its splicing. The expression of the mini-gene in transfected eukaryotic cells produces the processed mRNA. If internal transcribed elements of the mini-gene are recognized by the cell nuclear proteome, differential splicing may take place. We did not obtain evidence for an effect of the c.4493G>T variant on splicing on HEK293 cells. However, as transcription output depends on the combinatorial effect of trans-acting elements, splicing variants may have as well different effects according to tissue.

A 4-bp deletion (*TSC2*:c.1361+54\_1361+57del) from one NMI patient was not identified on 1000 Genomes, but twice on dbSNP (rs137854304) and once on ClinVar (ID 49694) without clinical significance reported. The four bases are embedded within a putative intronic splicing enhancer potentially recognized by a trans-acting protein factor CUGBP1/CELF1 that is widely expressed in over 20 human tissues (Fagerberg, Hallstrom *et al.* 2014). Tissue-specific transcriptional factors could change the splicing pattern of this segment, then the deletion of 4 bp could disrupt the canonical splicing in some tissues, whereas other remain normal.

## C. *TSC1* and *TSC2* CNV and genetic counseling implications

Seven segmental deletions and one segmental duplication have been identified in this study as pathogenic alterations (8/102; 7.84%). One deleted segment extended from *TSC1* intron 8 to at least the

downstream gene (*SPACA9*), having a length of more than 23 Kb. Although its breakpoints have not been sequenced, the segment overlaps part of a deletion reported by van den Ouweland, Elfferich et al. (2011) with a breakpoint mapping to nearly 1,400 bases downstream intron 8 and exon 9 junction, and extending downstream for at least 119,000 kb.

The deletion of full-length *TSC2* (*TSC2*:c.(?\_-30)\_(\*102\_?)del) extended downstream to *PKD1* exons 46 but not exon 45, and upstream to exon 6 of *NTHL1*. Homozygosity pathogenic DNA variants in this gene relate to an autosomal recessive cancer predisposition syndrome, often with progression to colorectal cancer (Weren, Ligtenberg *et al.* 2015). In this report, the most frequent *NTHL1* mutation had a frequency of 1:250, constituting a high risk for the patient that already has one allele deleted. Therefore, sequencing the most frequent *NTHL1* pathogenic DNA variant should be an alternative for homozygosity verification in this patient.

Four intragenic deletions were identified on *TSC2*. In only one case the two breakpoints could be sequenced. Of those that break point were not sequenced, the length of flanking introns prevented the sequencing of break point, even in the deletion of exon 9. However, the *TSC2* internal deletion which had both breakpoints sequenced mapped to introns 10 and 16. Each intron breakpoint had SINE nearby (Figure II-17). Alu high copy number in the human genome and similarity with members from this SINE family allow for unequal recombination (Gu, Yuan *et al.* 2015). Alu repeats from *TSC2* introns 10 and 16 are 66% identical. The relatively long distance between them (deletion size of 6.2 Kb) rules out the possibility of DNA replication-mediated deletion, and reinforces unequal recombination as a likely pathway to have generated the deletion (Abeyasinghe, Chuzhanova *et al.* 2003, Abo-Dalo, Kutsche *et al.* 2010, Gu, Yuan *et al.* 2015). Moreover, deletion breakpoints from both cancer and inherited disease are commonly associated to AT-rich sequences (Abeyasinghe, Chuzhanova *et al.* 2003), as found in Alu sequences such as those from *TSC2* introns 10 and 16.

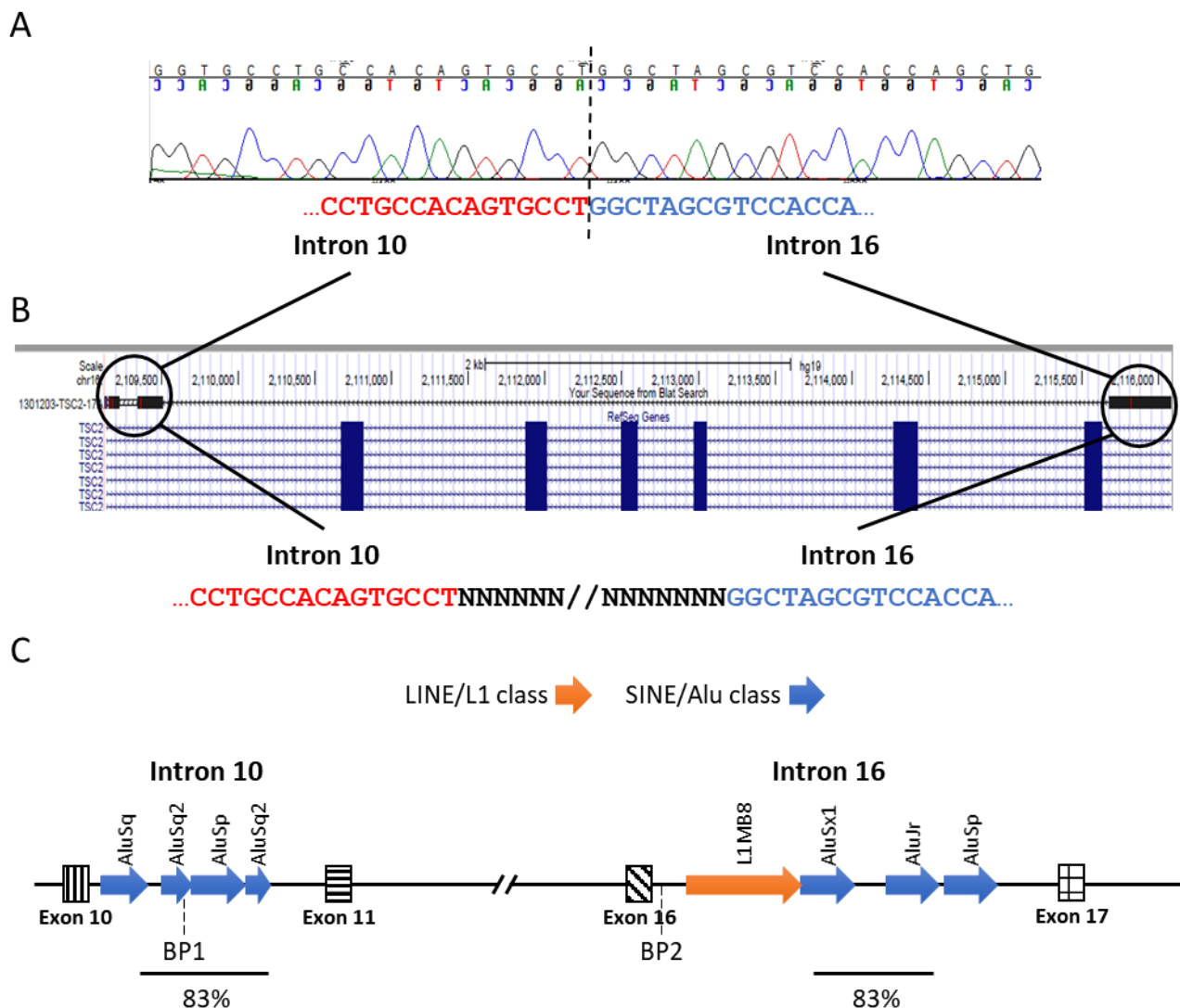
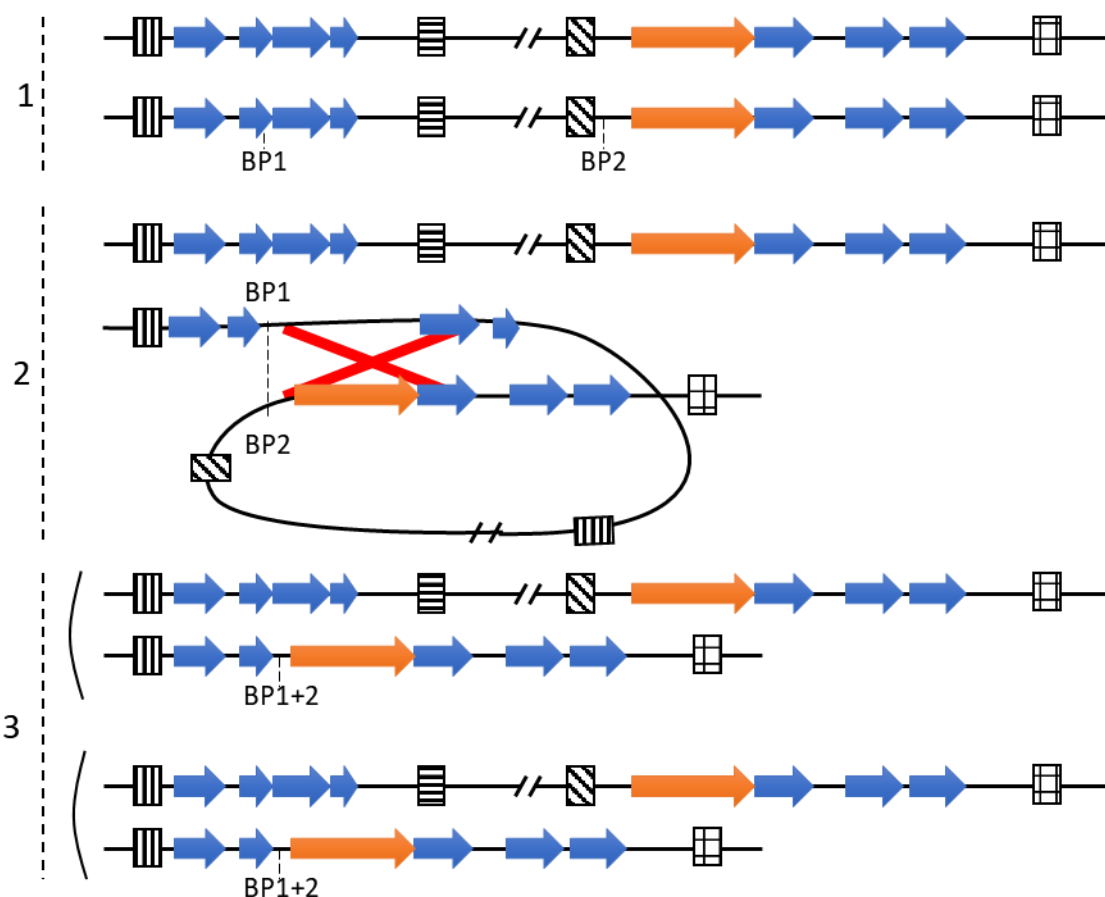


Figure II-17: Breakpoint mapping of the *TSC2* intragenic deletion c.(975+\_976-)\_ (1716+\_1717-)del . (A) Sanger sequencing of the DNA from the patient with the intragenic *TSC2* deletion c.(975+\_976-)\_ (1716+\_1717-)del. (B) UCSC genome browser mapping of the breakpoints in *TSC2* introns 10 and 16. (C) Diagram illustrating the positions of SINE/Alu and LINE/L1 repeat sequences in *TSC2* introns 10 and 16, respectively within and adjacent to each breakpoint. The regions of 66% similarity between introns are indicated. (D) Steps representing the predicted intra-chromosomal crossing-over leading to deletion. 1: normal chromosomal pairing; 2: intra-chromosomal pairing. Red cross represents the crossing over. 3: Daughter cells with normal and *TSC2* intragenic deleted chromosome (continue on next page).

D



## D. NMI patients

An NMI patient presented a mosaic *TSC1* exon 1 deletion. The mosaic deletion was confirmed by Q-PCR, and deletion of upstream gene *GFI1B* has been ruled out. MLPA false-positive deletion of only one exon can be due to SNV impairing probe hybridization (van Dijk, Rombout et al. 2005, Kim, Cho et al. 2016). However, no DNA variant has been detected by Sanger sequencing of the patient *TSC1* exon 1. Due to the long extent of *TSC1* intron 1 (9,447 bp) and intergenic segment upstream (nearly 34 Kb), we could not map the breakpoints of *TSC1* exon 1 deletion from the patient by a PCR-based approach with different primer pairs (data not shown). A study by van den Ouweland, Elfferich et al. (2011) identified five out of 327 TSC patients with deletions affecting *TSC1* exon 1, preserving exon 2. Upstream breakpoints from four deletions mapped differently between *TSC1* and *GFI1B* genes. Likewise, downstream breakpoints from the four cases mapped on *TSC1* intron 1 between 300 and more than 4 Kb away the exon-intron junction. In one case of non-mosaic *TSC1* exon 1 deletion, only the wild-type allele was expressed, suggesting the deletion inactivated transcription of the gene (van den Ouweland, Elfferich et al. 2011). As we could not evaluate the *TSC1* mRNA levels from our patient, the patient classified as NMI and the mosaic *TSC1* exon 1 deletion of uncertain significance. If this

is definitely not the TSC-causing mutation, it remains a possibility that the patient may have a pathogenic alteration in mosaicism that could not have been detected by sequencing due to limited sensitivity.

The second NMI patient had the c.1361+54\_1361+57del variant in *TSC2* gene intron 13, which did not affect the splicing of the minigene primary transcript in HEK293 cell line (Figure II-10). Since the patient mother has facial angiofibromas, we ruled out the possibility that the disease in the affected child is due to a novel mutation in mosaicism. As the patient had no segmental alteration on *TSC1* or *TSC2* detected by Sanger sequencing, NGS-Panel and MLPA, it is more likely that a DNA variant belongs to deep intronic sequence do not cover by our primer designed or to sequence that potentially altering *TSC1/2* gene expression regulation causes the disease.

NMI patients tend to correspond to less severe TSC cases (Camposano, Greenberg *et al.* 2009). This class of patient should be submitted to whole gene-targeted NGS to access the deep intronic regions and possible mosaics (Nellist, Brouwer *et al.* 2015, Tyburczy, Dies *et al.* 2015). Currently, six VUS are under functional investigation: c.1119G>A (p.=), c.1443G>T (p.Glu481Asp), c.1663G>C (p.Ala555Pro), c.4493G>T (p.Ser1498Ile), c.5051C>T (p.Ser1684Phe) and c.5228G>C (p.Arg1743Pro).

Of note, one splicing mutation is a deep intronic variant (c.2838-122G>A) previously reported as pathogenic by a functional assay (Nellist, Brouwer *et al.* 2015) and appear in two unrelated patients. As demonstrated by recent reports (Nellist, Brouwer *et al.* 2015, Tyburczy, Dies *et al.* 2015), deep intronic DNA variants affecting splicing along with somatic mosaicism explains part of the NMI cases. Identification of deep intronic variants was possible because in our study many primers designed for Sanger sequencing annealed on intronic sequences at least 200 bp away the exon/intron junctions.

Somatic mosaicism has been described in many individuals with TSC. The likely overestimate of the percent of mosaicism in some studies because are caused by the sensitivity of the pathogenic variant screening methods and the difficulty of detecting low-level mosaicism. It has been estimated that levels of mosaicism greater than 20% in lymphocyte DNA can be detected with confidence by Sanger sequencing and next-generation sequencing methods (Qin, Kozlowski *et al.* 2010). Using targeted deep next-generation sequencing of *TSC1* and *TSC2*, somatic mosaic pathogenic variants at 9.5% in an affected individual who did not have a pathogenic variant identified by exon sequence analysis or gene-targeted deletion/duplication analysis was reported (Nellist, Brouwer *et al.* 2015). However, it has not yet been determined whether levels of somatic mosaicism below 9.5% can be detected by deep next-generation sequencing. Although detection of somatic mosaicism between 1% and 9.5% has been reported (Tyburczy, Dies *et al.* 2015).

## VI. Conclusions

We analyzed DNA from 117 patients with definite clinical diagnosis of TSC. We report a mutation detection rate of 87,2% (102/117, 41 novel pathogenic DNA variants as well as 19 novel SNV. Nonsense and frameshifting pathogenic variants corresponded altogether to 94.4% and 50%, respectively, of *TSC1* and *TSC2* pathogenic DNA alterations. Missense, in-frame deletion and splicing DNA variants have been detected only in the *TSC2* gene. Segmental gene deletions and duplications comprehended 7.8% of all pathogenic DNA alterations. Primers designed more deeply in introns could identify four deep intronic DNA variants. These variants may explain part of NMI patients but require experimental assessment for pathogenicity confirmation. Our rate of pathogenic alteration detection could have been lower without a deeper analysis of intronic DNA sequence.

Among 20 rare missense variants and five rare in-frame deletion variants, at least two *in silico* bioinformatics tools predicted damaging effects. The mTOR-based functional assay disclosed non-overlapping results for *in silico* prediction of two missense variants but confirmed pathogenicity of five variants.

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## VIII. Supplementary Tables

Supplementary Table II-1: PCR and Sanger sequencing primers for the *TSC1* (NG\_012386.1).

Targeted segment	Name	Oligonucleotide sequence (5' – 3')
<b>Promoter + exon 1</b>	gTSC1_1S	AAATGTTTAGCCCAGGAAGGA
	gTSC1_1A	GCCGGAGATAGCGTGTAAATA
	gTSC1_2A	CATCTTGGACGTACAGCACCT
	gTSC1_2S	CCGTCTATCCTTCCTTTCGAG
<b>Exon 2</b>	gTSC1_3S	TTGGATTTTAACCCGGAAGTC
	gTSC1_3A	TCAGGCACTGAATACAAGCAA
<b>Exon 3</b>	gTSC1_4A	GGGGTTCACTGCATGATTCT
	gTSC1_4S	CCTCTTCATAAACTCGCCAAAG
<b>Exon 4</b>	gTSC1_5S	CAGAACTGTAATGCTGCACAAA
	gTSC1_5A	TTCAAGAATCATGGGTCCTACA
<b>Exon 5</b>	gTSC1_6S	TTTTATCTGCATGACCCTTGC
	gTSC1_6A	CCATACTTGCATGGACAAGGT
<b>Exon 6</b>	gTSC1_7S	CAGTAGAGTTGGGGCTCAGTG
	gTSC1_7A	GCACCCAAGATATTCCCTCA
<b>Exons 7+8</b>	gTSC1_8S	CTGAAGAGGAGGGCAGAAGTT
	gTSC1_8A	ATTAGTCCTCCGCCTGTGAA
	gTSC1_9A	AATTTCCCTGTCTGCCGTTA
	gTSC1_9S	CAATCCCTAGGCAGCCACTA
<b>Exon 9</b>	gTSC1_10S	TTTCCATTTTGAGGCTACACC
	gTSC1_10A	TTCCAGAGACAAAGTTGCAAAA
<b>Exon 10</b>	gTSC1_11S	ACCTAAAACACACACTAAGCC
	gTSC1_11A	GGAATGCTAAGTCATCCACGA
<b>Exons 11+12</b>	gTSC1_12S	GGGAAAATTTACACTGCTCA
	gTSC1_12A	CACACCTTGAGAGCAGCTTGT
	gTSC1_13A	CCCAGGGATTGCAATAAGT
	gTSC1_13S	CGGCAGTTTTTCTAATAGTTGG
<b>Exons 13+14</b>	gTSC1_14S	CATCCCAACAATTTGAGAATCA
	gTSC1_14A	GGCATCACTTTACCTGGCATA
	gTSC1_15A	TCCCAGAATTTCTTGTCTTCC
	gTSC1_15S	CCATGTCCAGCCTTCTCTGT
<b>Exon 15</b>	gTSC1_16S	GGATGCCACTTTTTCTCTCT
	gTSC1_16A	TCCCAATTTAGGTGCACAGAG
	gTSC1_17A	GATGACAAAATGATGGGCTGT
	gTSC1_17S	CACACCAAAGCAAGCCTTTAC
<b>Exons 16+17</b>	gTSC1_18S	TTATGCCATTGCAGATTTTGAC
	gTSC1_18A	GGAAGGACTGGGAAGCTGTAC
	gTSC1_19A	ACTTGGCAACACTTGAGATCCT
	gTSC1_19S	AAGCTAACAACACATGGGAAGG
<b>Exon 18</b>	gTSC1_20S	GCAAACTGATCCCTGAGAAGA
	gTSC1_20A	AGTTGGGGAACCTCTGTCCTA
<b>Exon 19</b>	gTSC1_21S	CAGAACTCTTCTGCAGCATCC
	gTSC1_21A	CAGCACCAAAAACATGAACCT
<b>Exon 20</b>	gTSC1_22S	CCATTATGTGAGGACTGTGAA
	gTSC1_22A	TAGCTGGACCACGGAGTAGTG
<b>Exons 21+22</b>	gTSC1_23S	GCTTGGGGATAGATTTCAAGG
	gTSC1_23A	ACACGGAGTGAGCTGAGTGTT
	gTSC1_24A	TGCAGCTGTCCTCTGAAAGAT
	gTSC1_24S	GTCAAACCTCCAGGCAAGGTAA
<b>Exon 23</b>	gTSC1_25S	CATATGGCCACAGGAAGTGTT
	gTSC1_28A	CCGTCCCATTTCACACATG
	gTSC1_26A	CAGAAAGGCTACTGGTCATGC
	gTSC1_26S	GGGAGACGACTATGGGAGAAG

Supplementary Table II-2: PCR and Sanger sequencing primers for the *TSC2* (NG\_005895.1).

Targeted segment	Name	Oligonucleotide sequence (5' – 3')
Promoter + exon 1	gTSC2_1S	CGAGGACAGCAAGTTCCTACTG
	gTSC2_1A	GTTTGCCGTCTCTCCTCTACC
	gTSC2_2A	GAGCTTGCTGGGAGTTGTAGTT
	gTSC2_2S	CTACCTGCTGCAGCCTCTCT
Exon 2	gTSC2_3S	GGTAGAGGAGAGACGGCAAAC
	gTSC2_3A	AAGTGTGCCTGAACCAGGTC
Exon 3	gTSC2_4S	CGGCTCGTCAAGTGAATCTT
	gTSC2_4A	GTCAGCTGTCAACCATGTTCC
Exon 4	gTSC2_5S	TGAGACTGTCCCATGACTTCC
	gTSC2_5A	AGGGCAAAACAACACCGTAG
Exon 5	gTSC2_43S	CCTGCCCTGTACAATGCTGA
	gTSC2_43A	CAAGCCCCAGAGACTCACAG
Exon 6	gTSC2_44S	GATCCTAGTGTCCGTGCGTAG
	gTSC2_44A	CGGAGCTGAACCTTAGGACCAT
Exon 7	gTSC2_8S	GCTCTCATCTGATGTCTTGTT
	gTSC2_8A	GTCATTGATGCTGTCATCCAC
Exon 8	gTSC2_9S	GTCCCCCATGTAAGTCAGGAT
	gTSC2_9A	ATCTCCTCCCAAAGACAGAGG
Exon 9	gTSC2_10S	CTGTCTCCCATGAATGGTTGT
	gTSC2_10A	GGCTAAGTAGTTGGGGAGCAC
Exon 10	gTSC2_45S	GTGTTACTGCTGGCCTCTGT
	gTSC2_11A	CAGCTCACTGCACACAGAAAC
Exon 11	gTSC2_12S	GGATTGAGTTGCTGGTCTGTC
	gTSC2_12A	ACTAATGCGGTCTCCAAAGT
Exon 12	gTSC2_46S	CCTCTGGTGCCAAGTCCATG
	gTSC2_45A	CCCTAAGCTGAGTGTTCCTGG
Exon 13	gTSC2_14S	CAGTTTCCTCCACCTGTGT
	gTSC2_14A	GGAGCATCTCTCCAGACGAC
Exon 14	gTSC2_15S	GTGCTAGCTTGCTTTCCAGTC
	gTSC2_15A	AGACTGGCTGAAACGAACTCA
Exon 15	gTSC2_16S	GCTGCTCCTTGTTGAGTTGTG
	gTSC2_16A	ACTGTGCAGAAACCAAAATGC
Exon 16	gTSC2_17S	CTCAGAACCATGAGCCTGTGT
	gTSC2_17A	AGCGTGTGCTACTGGTATGCT
Exon 17	gTSC2_18S	GTTGATGACTGCCCTGATGAT
	gTSC2_18A	TTAGAGCGACAAGCCACAGAT
Exon 18	gTSC2_19S	CAGAGTCCTGTTTCAGCCTGTC
	gTSC2_19A	GAAGCAAGAGAAGCAGCTGAG
Exon 19	gTSC2_20S	CTACATGTACGCGGGACCTC
	gTSC2_20A	GCCTTCTGGACCCTAGAGACA
Exon 20	gTSC2_21S	GTGCCCTACTCCCTGCTCTT
	gTSC2_46A	GCTCGCAGTCTTTTGGGGAA
Exon 21	gTSC2_47S	GTGTGTTACTTGGCAGGCAC
	gTSC2_22A	GTGGACAGGGAACACTGGAT
Exon 22	gTSC2_23S	GAGTCTGCTCGGGTAGCTCA
	gTSC2_23A	ACCTGAGCTCCTGAAGTCACA
Exon 23	gTSC2_24S	TCACGGATCACACAAATGGTA
	gTSC2_24A	GAGCCACCTTAGTGATGAAA
Exon 24	gTSC2_25S	CGCACCTCTACAGGAACCTTG
	gTSC2_25A	GAGTGAGCACACCCAGACAGT
Exon 25	gTSC2_26S	TCATCACTAAGGTGGGCTCAG
	gTSC2_26A	AACCCCAATTCCACAAGTAG
Exon 26	gTSC2_27S	ACCCACACACGTTTAAATTTGC
	gTSC2_27A	GAATACGAAAAGGCCAAAACC
Exons 27 + 28	gTSC2_28S	AATGTGGTCCACGTGATTCTC
	gTSC2_28A	GACTTAGTCCCCAGGCTGGTA
Exon 29	gTSC2_29S	CGCTCCCTGTCTTCTAGGTCT
	gTSC2_29A	CAGAGAAGGGCTCCAGGACT
Exon 30	gTSC2_48S	CTTGAGGCTGGTGGTTTTGC
	gTSC2_47A	AGAGGGCCAAGTCTGCAATC
Exon 31	gTSC2_31S	TGAGGGGTGCAAAGAGTAGG
	gTSC2_31A	GGAGAACAAATGGTGTGAGG

<b>Exon 32</b>	gTSC2_32S	GACGTCTATTACGGGAGGA
	gTSC2_32A	CTAAACAGCTGCCACCCATC
<b>Exon 33</b>	gTSC2_33S	GTTACGAGGGCTGGTTTCAG
	gTSC2_33A	ACACTGCGTGAGCAGAGGTAT
<b>Exon 34</b>	gTSC2_34S	ATACCTCTGCTCACGCAGTGT
	gTSC2_34A	AGCTGCAGGAACACGAAACT
	gTSC2_35A	CTCTTTAAGGCGTCCCTCTCT
	gTSC2_35S	CTGTGGACCTCTCCTTCCAG
<b>Exon 35</b>	gTSC2_36S	AGCCTCCAATGCAGAGAAAGT
	gTSC2_36A	GTGTCGTATGATGGGATCTGG
<b>Exon 36</b>	gTSC2_37S	TGTTCTCTGCAGCTCTACCATT
	gTSC2_37A	TGTCAGCTCACTGACCAACAG
<b>Exon 37</b>	gTSC2_38S	GAGGGAAGAGAGGGAGTCAAG
	gTSC2_38A	GGCACCTCCTGATTACTCCA
<b>Exon 38</b>	gTSC2_39S	CTCCCATCCAGTCCTGCTAC
	gTSC2_39A	TCTGCACTTGCCAGTTACTCC
<b>Exons 39 + 40</b>	gTSC2_49S	CAGAGGGGAAAGTTCAGGGG
	gTSC2_40A	GTAGATATCGGTGGGGTTGGA
<b>Exon 41</b>	gTSC2_41S	AAGTCTCCCCAGACATGGAG
	gTSC2_41A	CACAAACTCGGTGAAGTCCTC
<b>Exon 42</b>	gTSC2_42S	CCGATATCTACCCCTCCAAGT
	gTSC2_48A	CTTCTAGAGCCTCGACACCC

Supplementary Table II-3: *TSC1* exon 1 slowdown PCR cycle program.

Cycles	Time	Temperature	Ramp
1X	5min	95°C	2,5°/s
	30sec.	95°C	2,5°/s
53X	30sec.	70°C (-0,3°/cycle)	1,5°/s
	40sec.	72°C	2,5°/s
	30sec.	95°C	2,5°/s
15X	30sec.	58°C	1,5°/s
	40sec.	72°C	2,5°/s
1X	7min	72°C	-
1X	∞	10°C	-

Supplementary Table II-4: Standard PCR cycle program.

Cycles	Time	Temperature
1X	5 min	95°C
	1 min	95°C
30X	1 min	60°C
	1 min	72°C
1X	8 min	72°C
1X	∞	4°C

Supplementary Table II-5: Q-PCR primers for *TSC1* (NM\_000368.4), *TSC2* (NM\_000548.3), *GAPDH* (NM\_002046), *GFI1B* (NM\_004188.6), *SPACA9* (NM\_001316898.1), *NTHL1* (NM\_002528) and *PKD1* (NM\_001009944).

Segment	Name	Oligonucleotide sequence (5' – 3')	Product size (bp)	DNA concentration (ng/μL)	Efficiency
TSC1					
Exon 1	qPCRgTSC1_1S	AGGGACTGTGAGGTAAACAGC	85	3.125	2.0111
	qPCRgTSC1_1A	AGGAAGCCCCCATAAAAAGGAG			
Exon 17	qPCRgTSC1_4S	CAGATGAGATCCGCACCCTC	131	3.125	2.0324
	qPCRgTSC1_4A	AGCTGCTGCTTTGATCACCT			
TSC2					
Exon 9	qPCRgTSC2_7S	CCACAGCGCCATCTACAACA	195	3.125	1.9019
	qPCRgTSC2_7A	AAGTCATGGGCCTCGCTCTA			
Exon 12	qPCRgTSC2_2S	TCCATGACCTGTTGACCACG	95	3.125	2.0527
	qPCRgTSC2_2A	CGCACATCTCTCCACCAGTT			
Exon 19	qPCRgTSC2_4S	CTGAGAAGAAGACCAGCGGC	107	6.25	1.9124
	qPCRgTSC2_4A	GAAGAGCAGGGAGTAGGGCA			
Exon 41	qPCRgTSC2_8S	TCACAGGTGCATCATAGCCG	107	3.125	2.2134
	qPCRgTSC2_8A	CCCATATTCCCTACCCGCTG			
GADPH					
Intron 1	gGAPDH_1S	GCTCCCACCTTTCTCATCC	147	6.25	2.0545
	gGAPDH_1A	CTGCAGCGTACTCCCCAC			
GFI1B					
Exon 1	qPCRgGFI1B_1S	TATTGCGAGGCTGAGTATGATGG	96	3.125	1.9485
	qPCRgGFI1B_1A	TTCATATCCCACAGTTCGACTTCA			
SPACA9					
Exon 4	qPCRgSPACA9_2S	GAGGCCCGGAACCACTAC	111	3.125	2.1000
	qPCRgSPACA9_2A	GAGTGGGCGATCCATTCTTTC			
NTHL1					
Exon 6	qPCRgNTHL1_1S	TCAATGGACTCTTGGTGGGC	163	6.25	2.1350
	qPCRgNTHL1_1A	AAAGCCACTTCACAGACGGT			
PKD1					
Exon 45	qPCRgPKD1_1S	GGCTCTCTACCCTGTGTCCT	112	3.125	2.1407
	qPCRgPKD1_1A	CGGAGAATAACAGCCCCCAG			
Exon 46	qPCRgPKD1_2S	TAGGTGTGGTGGCGTTATGG	107	6.25	2.1537
	qPCRgPKD1_2A	CTCTGGGGTGATGAGAGTGC			

Supplementary Table II-6: Q-PCR cycle program.

	Cycling	Time	Temperature	Ramp
<b>Holding</b>	1X	2 min	50°C	100%
	1X	10 min	95°C	100%
<b>Cycling</b>	35X	15 seg	95°C	100%
		1 min	60°C	100%
<b>Melting</b>	1X	15 seg	95°C	100%
	1X	1 min	60°C	100%
	1X	30 seg	95°C	1%
	1X	15 seg	60°C	100%

Supplementary Table II-7: List of softwares and websites consulted.

Program	Web site
Primer3	<a href="http://primer3.ut.ee/">http://primer3.ut.ee/</a>
Primer-BLAST	<a href="https://www.ncbi.nlm.nih.gov/tools/primer-blast/">https://www.ncbi.nlm.nih.gov/tools/primer-blast/</a>
BLAST	<a href="https://blast.ncbi.nlm.nih.gov/Blast.cgi">https://blast.ncbi.nlm.nih.gov/Blast.cgi</a>
Beacon Designer Free Edition	<a href="http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1">http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1</a>
BLAT	<a href="http://genome.ucsc.edu/cgi-bin/hgBlat?command=start">http://genome.ucsc.edu/cgi-bin/hgBlat?command=start</a>
Human Genome Variation Society	<a href="http://varnomen.hgvs.org/">http://varnomen.hgvs.org/</a>
Variant Validator	<a href="https://variantvalidator.org/">https://variantvalidator.org/</a>
Mutalyzer	<a href="http://www.lovd.nl/mutalyzer/">http://www.lovd.nl/mutalyzer/</a>
Leiden Open Variation Database TSC1	<a href="http://chromium.lovd.nl/LOVD2/TSC/home.php?select_db=TSC1">http://chromium.lovd.nl/LOVD2/TSC/home.php?select_db=TSC1</a>
Leiden Open Variation Database TSC2	<a href="http://chromium.lovd.nl/LOVD2/TSC/home.php?select_db=TSC2">http://chromium.lovd.nl/LOVD2/TSC/home.php?select_db=TSC2</a>
Ensembl	<a href="http://www.ensembl.org/index.html">http://www.ensembl.org/index.html</a>
PolyPhen-2	<a href="http://genetics.bwh.harvard.edu/pph2/">http://genetics.bwh.harvard.edu/pph2/</a>
Mutation Taster	<a href="http://www.mutationtaster.org/">http://www.mutationtaster.org/</a>
SIFT and PROVEAN	<a href="http://provean.jcvi.org/genome_submit_2.php">http://provean.jcvi.org/genome_submit_2.php</a>
PhosphositePlus	<a href="https://www.phosphosite.org/homeAction.action?sessionId=D937EFB1B37CAF55C7289FE52C813AA1">https://www.phosphosite.org/homeAction.action?sessionId=D937EFB1B37CAF55C7289FE52C813AA1</a>
Acscan2	<a href="http://genes.mit.edu/acscan2/index.html">http://genes.mit.edu/acscan2/index.html</a>
SpliceAid2	<a href="http://193.206.120.249/splicing_tissue.html">http://193.206.120.249/splicing_tissue.html</a>
Human Splicing Finder 3.1	<a href="http://www.umd.be/HSF3/">http://www.umd.be/HSF3/</a>
RepeatMasker	<a href="http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker">http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker</a>
UniProtKB BLAST	<a href="https://www.uniprot.org/help/uniprotkb">https://www.uniprot.org/help/uniprotkb</a>

Supplementary Table II-8: Primer pairs for function assessment of splicing variant.

Segment	Name	Oligonucleotide sequence (5' – 3')
<b>Mini-gene Exon 12-14</b>	gTSC2_51S	GCCCATGGGGATCCACCTTGGACAGCCCGGAGC
	gTSC2_50A	TCTAGAACTCGAGCTCATAGAACTGCCTGTTGATGAGC
<b>Mini-gene Exon 13-35</b>	gTSC2_53S	GCCCATGGGGATCCACTCCGCCGTGGTCATGGAG
	gTSC2_52A	TCTAGAAGCGGCCCGCCTCATTGGGCAGCAGGATTGGC
<b>Endogenous Exons 8-10</b>	cTSC2_2S	TGGTCTGCTACAACCTGCCTG
	cTSC2_1A	CGGCGAGTTCCTGAGAGAAT

BamHI restriction site: GGATTC  
XhoI restriction site: CTCGAG  
NotI restriction site: GCGCCGC

Supplementary Table II-9: Rules for combining criteria to classify sequence variants according to ACMG (Richards, Aziz *et al.* 2015).

Rules for combining criteria to classify sequence variants		
<b>Pathogenic</b>	(i) 1 Very strong (PVS1) AND	(a) $\geq 1$ Strong (PS1–PS4) OR (b) $\geq 2$ Moderate (PM1–PM6) OR (c) 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) OR (d) $\geq 2$ Supporting (PP1–PP5) OR
	(ii) $\geq 2$ Strong (PS1–PS4) OR	
	(iii) 1 Strong (PS1–PS4) AND	(a) $\geq 3$ Moderate (PM1–PM6) OR (b) 2 Moderate (PM1–PM6) AND $\geq 2$ Supporting (PP1–PP5) OR (c) 1 Moderate (PM1–PM6) AND $\geq 4$ supporting (PP1–PP5).
<b>Likely pathogenic</b>	(i) 1 Very strong (PVS1) AND 1 moderate (PM1– PM6) OR	
	(ii) 1 Strong (PS1–PS4) AND 1–2 moderate (PM1–PM6) OR	
	(iii) 1 Strong (PS1–PS4) AND $\geq 2$ supporting (PP1–PP5) OR	
	(iv) $\geq 3$ Moderate (PM1–PM6) OR	
	(v) 2 Moderate (PM1–PM6) AND $\geq 2$ supporting (PP1–PP5) OR	
	(vi) 1 Moderate (PM1–PM6) AND $\geq 4$ supporting (PP1–PP5)	
<b>Benign</b>	(i) 1 Stand-alone (BA1) OR	
	(ii) $\geq 2$ Strong (BS1–BS4).	
<b>Likely benign</b>	(i) 1 Strong (BS1–BS4) and 1 supporting (BP1– BP7) OR	
	(ii) $\geq 2$ Supporting (BP1–BP7).	
<b>Uncertain significance</b>	(i) Other criteria shown above are not met OR	
	(ii) the criteria for benign and pathogenic are contradictory.	

Supplementary Table II-10: Evidence of benign impact according to ACMG (Richards, Aziz *et al.* 2015).

Evidence of benign impact	
Stan-alone	BA1: Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium
Strong	BS1: Allele frequency is greater than expected for disorder (see Table 6)
	BS2: Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age
Supporting	BS3: Well-established <i>in vitro</i> or <i>in vivo</i> functional studies show no damaging effect on protein function or splicing
	BS4: Lack of segregation in affected members of a family
Supporting	BP1: Missense variant in a gene for which primarily truncating variants are known to cause disease
	BP2: Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern
Supporting	BP3: In-frame deletions/insertions in a repetitive region without a known function
	BP4: Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)
Supporting	BP5: Variant found in a case with an alternate molecular basis for disease
	BP6 Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation
Supporting	BP7 A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved

Supplementary Table II-11: *TSC1* and *TSC2* exonic single nucleotide variants.

Variant	Exon	Existing variation	1000 Genomes								gnomAD								
			AF	AFR	AMR	EAS	EUR	SAS	AA	EA	AF	AFR	AMR	ASI	EAS	FIN	NFE	OTH	SAS
TSC1																			
c.-99C>T	02/23	rs114755636	0.004	0.015	0.001	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.-129A>T	02/23	rs116951280	0.005	0.000	0.013	0.000	0.014	0.001	-	-	-	-	-	-	-	-	-	-	-
c.840A>G (p.=)	09/23	rs1171852730	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
c.965T>C (p.Met322Thr)	10/23	rs1073123	0.135	0.234	0.094	0.074	0.131	0.098	0.220	0.138	0.125	0.217	0.086	0.141	0.088	0.102	0.138	0.126	0.104
c.1342C>T (p.Pro448Ser)	14/23	rs118203518	0.004	0.015	0.001	0.000	0.000	0.000	0.011	0.000	0.001	0.011	0.001	0.000	0.000	0.000	0.000	0.000	0.000
c.1335A>G (p.=)	14/23	rs7862221	0.139	0.233	0.097	0.074	0.144	0.101	0.219	0.146	0.132	0.218	0.088	0.150	0.088	0.115	0.148	0.135	0.107
c.1760A>G (p.Lys587Arg)	15/23	rs118203576	0.018	0.000	0.128	0.000	0.002	0.000	0.002	0.000	0.026	0.002	0.175	0.000	0.000	0.012	0.000	0.026	0.001
c.2829C>T (p.=)	22/23	rs4962081	0.062	0.095	0.097	0.023	0.064	0.032	0.091	0.082	0.079	0.089	0.154	0.053	0.028	0.046	0.081	0.076	0.051
c.*4437G>A	23/23	rs11553763	0.103	0.214	0.123	0.040	0.068	0.043	-	-	-	-	-	-	-	-	-	-	-
c.*3679G>A	23/23	rs1050700	0.726	0.595	0.648	0.825	0.749	0.832	-	-	-	-	-	-	-	-	-	-	-
	23/23	rs739441	-	0.660	0.752	0.821	0.830	0.834	-	-	-	-	-	-	-	-	-	-	-
c.*1488C>T	23/23	rs739442	0.451	0.056	0.496	0.581	0.612	0.651	-	-	-	-	-	-	-	-	-	-	-
c.*1362G>T	23/23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.*1275T>G	23/23	rs2809244	-	0.778	0.738	0.682	0.753	0.780	-	-	-	-	-	-	-	-	-	-	-
c.*289delT	23/23	rs11323835	0.439	0.137	0.473	0.566	0.515	0.616	-	-	-	-	-	-	-	-	-	-	-
c.*191dupC	23/23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.*107T>C	23/23	rs116917669	0.017	0.002	0.020	0.002	0.044	0.024	-	-	-	-	-	-	-	-	-	-	-
c.*1G>A	23/23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.3324C>T (p.=)	23/23	rs35593170	0.016	0.003	0.095	0.006	0.002	0.001	0.001	0.001	0.018	0.002	0.125	0.000	0.003	0.002	0.001	0.013	0.001
c.3282G>A (p.=)	23/23	rs116747861	0.005	0.017	0.001	0.000	0.000	0.000	0.013	0.001	0.002	0.014	0.002	0.005	0.000	0.000	0.001	0.002	0.000

c.3127_3129del (p.Ser1043del)	23/23	rs397514812	-	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<b>TSC2</b>																			
c.52C>G (p.Leu18Val)	02/42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.499T>C (p.Trp167Arg)	06/42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.881G>A (p.Gly294Glu)	10/42	rs45487497	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1100G>A (p.Arg367Gln)	11/42	rs1800725	0.009	0.002	0.016	0.000	0.014	0.017	0.005	0.020	0.014	0.003	0.011	0.027	0.000	0.007	0.018	0.015	0.017
c.1443G>T (p.Glu481Asp)	14/42	CS091000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1578C>T (p.=)	15/42	rs34012042,	0.035	0.002	0.046	0.000	0.073	0.070	0.015	0.070	0.057	0.011	0.033	0.100	0.000	0.086	0.067	0.064	0.070
c.1731C>T (p.=)	17/42	rs144122318	0.000	0.000	0.000	0.000	0.001	0.000	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
c.2073C>T (p.=)	19/42	rs45512398	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.011	0.000	0.000	0.000	0.001	0.001
c.2580T>C (p.=)	23/42	rs13337626	0.063	0.093	0.042	0.002	0.088	0.074	0.087	0.086	0.070	0.088	0.035	0.077	0.001	0.111	0.082	0.069	0.063
c.3126G>C (p.=)	27/42	rs36078782	0.013	0.047	0.003	0.000	0.000	0.000	0.037	0.000	0.003	0.043	0.003	0.000	0.000	0.000	0.000	0.003	0.000
c.3431T>A (p.Val1144Glu)	30/42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.3557A>G (p.Tyr1186Cys)	30/42	rs137854421	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
c.3768G>A (p.=)	31/42	rs201599540	0.000	0.000	0.000	0.001	0.000	0.000	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
c.3889G>A (p.Ala1297Thr)	33/42	rs45517319	0.001	0.000	0.000	0.000	0.003	0.002	0.002	0.004	0.003	0.001	0.001	0.004	0.000	0.005	0.004	0.002	0.002
c.3914C>T (p.Pro1305Leu)	33/42	rs45517320	0.009	0.033	0.003	0.000	0.000	0.000	0.021	0.000	0.002	0.026	0.002	0.000	0.000	0.000	0.000	0.000	0.000
c.3915G>A (p.=)	33/42	rs11551373	0.022	0.077	0.006	0.001	0.002	0.000	0.066	0.000	0.005	0.068	0.004	0.000	0.000	0.000	0.000	0.002	0.000
c.3986G>A (p.Arg1329His)	33/42	rs45517323,	0.020	0.070	0.007	0.001	0.002	0.000	0.048	0.001	0.005	0.054	0.002	0.001	0.001	0.011	0.001	0.004	0.000
c.4316G>A (p.Gly1439Asp)	34/42	rs150397923	0.005	0.020	0.000	0.000	0.000	0.000	0.013	0.000	0.001	0.017	0.001	0.000	0.000	0.000	0.000	0.000	0.000
c.4536C>T (p.=)	35/42	rs35986575	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.005	0.003	0.000	0.001	0.021	0.000	0.002	0.004	0.005	0.002
c.4716G>T	37/42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(p.=)																			
c.4908C>T	38/42	rs115200071	0.002	0.008	0.000	0.000	0.000	0.000	0.007	0.001	0.001	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000
(p.=)																			
c.4959C>T	38/42	rs45517384	0.003	0.002	0.009	0.000	0.008	0.000	0.003	0.015	0.010	0.003	0.007	0.001	0.000	0.020	0.014	0.010	0.001
(p.=)																			
c.4982C>T	38/42	rs776541842	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
(p.Thr1661Ile)																			
c.5010C>T	39/42	rs376306544	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
(p.=)																			
c.5025G>A	39/42	rs35118875	0.004	0.014	0.003	0.000	0.000	0.000	0.016	0.000	0.001	0.014	0.001	0.000	0.000	0.000	0.000	0.001	0.000
(p.=)																			
c.5051C>T	39/42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(p.Ser1684Phe)																			
c.5202T>C	41/42	rs1748	0.276	0.748	0.199	0.000	0.184	0.074	0.634	0.178	0.178	0.662	0.112	0.210	0.000	0.207	0.177	0.179	0.081
(p.=)																			
c.5312C>T	42/42	rs137854214	0.001	0.004	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000
(p.Pro1771Leu)																			
c.5397G>C	42/42	rs1051771	0.041	0.002	0.055	0.000	0.101	0.064	0.019	0.099	0.073	0.015	0.033	0.094	0.000	0.144	0.096	0.075	0.058
(p.=)																			
c.*26G>A	42/42	rs13332015	0.039	0.138	0.016	0.000	0.000	0.000	0.110	0.000	0.009	0.122	0.006	0.000	0.000	0.000	0.000	0.005	0.000
c.*35G>A	42/42	rs200025534	0.002	0.006	0.000	0.000	0.000	0.000	0.008	0.000	0.001	0.008	0.000	0.000	0.000	0.000	0.000	0.001	0.000
c.*61_*62delAA	42/42	rs36032671	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Table II-12: *TSC1* and *TSC2* intronic single nucleotide variants.

Variant	Intron	Existing variation	1000 Genomes										gnomAD							
			AF	AFR	AMR	EAS	EUR	SAS	AA	EA	AF	AFR	AMR	ASJ	EAS	FIN	NFE	OTH	SAS	
TSC1																				
c.2976-43G>C	22/22	rs7853849	0.025	0.084	0.016	0.000	0.003	0.000	0.066	0.001	0.007	0.077	0.009	0.000	0.000	0.000	0.001	0.008	0.000	
c.2976-54G>A	22/22	rs118203740	0.007	0.019	0.007	0.000	0.003	0.000	-	-	-	-	-	-	-	-	-	-	-	
c.2625+68G>A	20/22	rs1076160	0.412	0.055	0.450	0.559	0.511	0.618	-	-	-	-	-	-	-	-	-	-	-	
c.2502+131C>T	19/22	rs7020175	0.082	0.107	0.059	0.072	0.086	0.073	-	-	-	-	-	-	-	-	-	-	-	
c.2502+51A>G	19/22	rs75802666	0.080	0.004	0.042	0.198	0.067	0.099	0.009	0.050	0.073	0.009	0.079	0.024	0.197	0.124	0.054	0.067	0.080	
c.2392-35T>C	18/22	rs11243931	0.175	0.363	0.108	0.074	0.146	0.101	0.346	0.148	0.142	0.348	0.094	0.150	0.088	0.117	0.149	0.138	0.107	
c.2392-222C>T	18/22	rs141011030	0.005	0.000	0.013	0.000	0.014	0.000	-	-	-	-	-	-	-	-	-	-	-	
c.2391+59G>C	18/22	rs117301441	0.003	0.000	0.004	0.000	0.012	0.000	-	-	-	-	-	-	-	-	-	-	-	
c.2391+34G>A	18/22	rs116518821	0.010	0.027	0.013	0.001	0.004	0.000	0.022	0.003	0.004	0.025	0.008	0.017	0.000	0.000	0.002	0.008	0.000	
c.2041+268A>G	16/22	rs12345576	0.175	0.364	0.108	0.074	0.146	0.101	-	-	-	-	-	-	-	-	-	-	-	
c.1439-37C>T	14/22	rs10901220	0.139	0.234	0.097	0.074	0.144	0.101	0.204	0.132	0.133	0.224	0.090	0.152	0.090	0.118	0.153	0.135	0.107	
c.1334-55C>G	13/22	rs7872606	0.082	0.106	0.059	0.072	0.086	0.073	-	-	-	-	-	-	-	-	-	-	-	
c.1334-174T>C	13/22	rs117147322	0.017	0.002	0.020	0.001	0.042	0.024	-	-	-	-	-	-	-	-	-	-	-	
c.1333+209C>T	13/22	rs7872860	0.139	0.235	0.097	0.074	0.144	0.101	-	-	-	-	-	-	-	-	-	-	-	
c.1333+187A>G	13/22	rs114683952	0.010	0.027	0.013	0.000	0.004	0.000	-	-	-	-	-	-	-	-	-	-	-	
c.1142-26_1142-25del	11/22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
c.1142-33A>G	11/22	rs6597586	0.135	0.232	0.094	0.074	0.131	0.098	0.218	0.138	0.124	0.217	0.086	0.142	0.088	0.102	0.138	0.126	0.103	
c.1141+63G>A	11/22	rs77891974	0.011	0.038	0.006	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-	
c.738-197T>A	08/22	rs1336366611	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
c.-80-71T>A	02/22	rs45607538	0.001	0.000	0.004	0.000	0.002	0.001	-	-	-	-	-	-	-	-	-	-	-	
TSC2																				
c.226-101C>T	03/41	rs144408387	0.008	0.002	0.027	0.000	0.017	0.001	-	-	-	-	-	-	-	-	-	-	-	

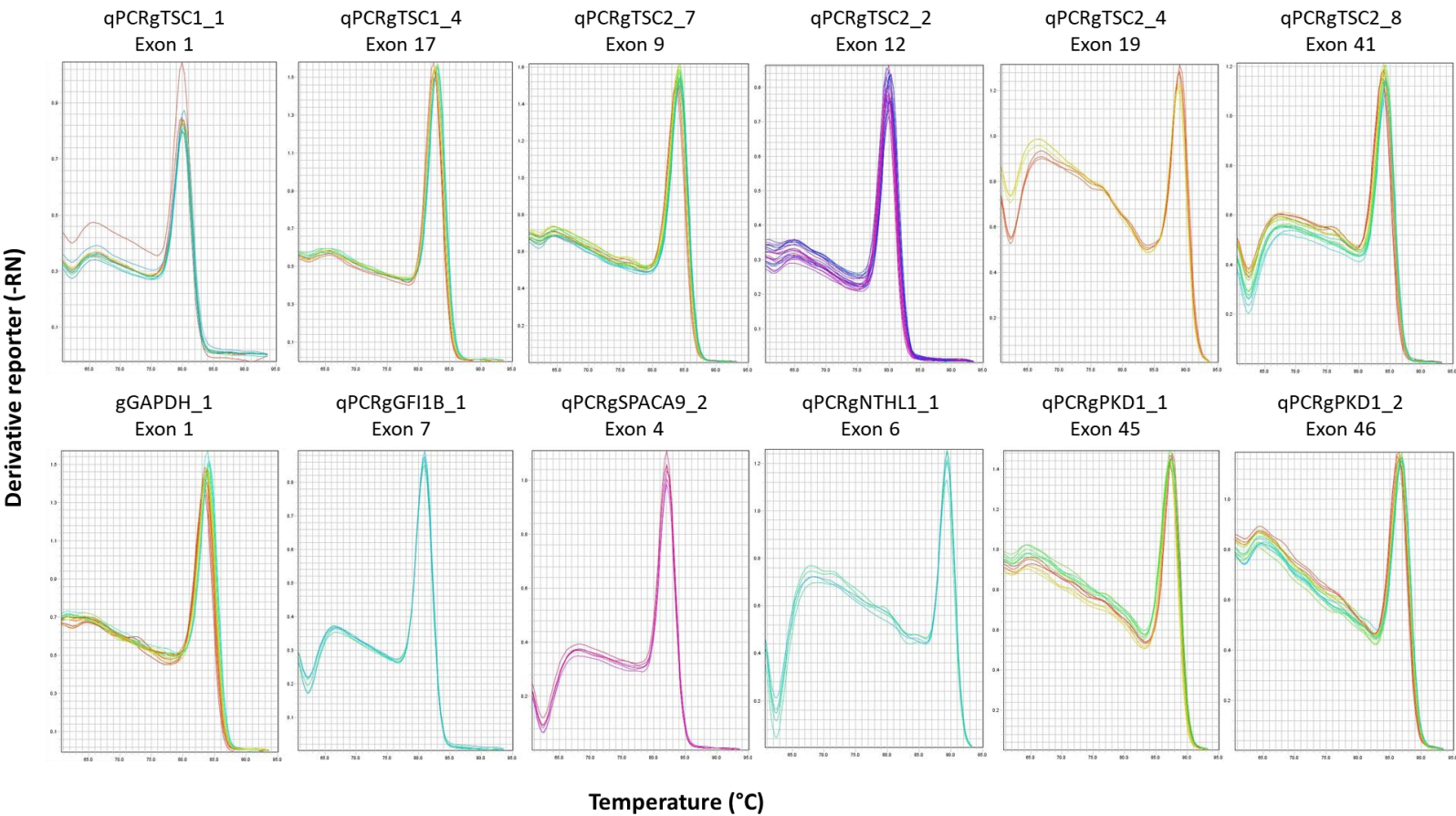
c.226-82C>T	03/41	rs7185742	0.003	0.000	0.003	0.001	0.011	0.003	-	-	-	-	-	-	-	-	-	-	-
c.336+33G>T	04/41	rs45517104	0.009	0.034	0.001	0.000	0.000	0.000	0.033	0.000	0.002	0.040	0.001	0.000	0.000	0.000	0.000	0.002	0.000
c.336+66G>C	04/41	rs920782009	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.482-114T>C	05/41	rs77037371	0.012	0.041	0.009	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.482-68C>G	05/41	rs2516734	0.153	0.374	0.151	0.000	0.136	0.032	-	-	-	-	-	-	-	-	-	-	-
c.482-59G>A	05/41	rs200833855	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.600-84C>T	06/41	rs35364892	0.007	0.008	0.001	0.000	0.003	0.020	-	-	0.009	0.007	0.003	0.036	0.000	0.006	0.008	0.010	0.019
c.648+12C>A	07/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.848+113A>G	09/41	rs56077830	0.011	0.038	0.004	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.848+143G>T	09/41	rs78160478	0.046	0.042	0.076	0.000	0.099	0.022	-	-	-	-	-	-	-	-	-	-	-
c.976-100C>G	10/41	rs2074968	-	0.398	0.499	0.714	0.547	0.665	-	-	-	-	-	-	-	-	-	-	-
c.976-63G>A	10/41	rs12927333	0.035	0.002	0.046	0.000	0.075	0.064	-	-	-	-	-	-	-	-	-	-	-
c.1257+109dupG	12/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1258-223G>A	12/41	rs924724434	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1361+54_1361+57del	13/41	rs137854304	0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1362-151G>T	13/41	rs545796760	0.000	0.000	0.000	0.000	0.002	0.000	-	-	-	-	-	-	-	-	-	-	-
c.1443+65T>G	14/41	rs115599333	0.007	0.027	0.000	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.1444-201G>A	14/41	rs60763712	0.051	0.181	0.017	0.000	0.002	0.000	-	-	-	-	-	-	-	-	-	-	-
c.1599+85_1599+87del	15/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1599+162C>T	15/41	rs74819416	0.009	0.030	0.006	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.1599+216T>G	15/41	rs17654678	0.038	0.003	0.042	0.001	0.112	0.044	-	-	-	-	-	-	-	-	-	-	-
c.1599+282_1599+283insCTGGGG	15/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1716+183A>G	16/41	rs8063461	0.615	0.350	0.716	0.839	0.590	0.698	-	-	-	-	-	-	-	-	-	-	-
c.1717-121G>A	16/41	rs1275330311	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.1717-55T>C	16/41	rs7187438	0.393	0.714	0.297	0.221	0.371	0.229	-	-	-	-	-	-	-	-	-	-	-
c.1840-172G>A	17/41	rs532002248	0.000	0.000	0.001	0.001	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.1840-136T>C	17/41	rs113749674	0.013	0.046	0.006	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2097+43A>G	19/41	rs186681035	0.003	0.001	0.001	0.000	0.010	0.001	0.001	0.010	0.009	0.003	0.003	0.012	0.000	0.026	0.013	0.009	0.005
c.2098-114A>G	19/41	rs6600185	0.110	0.393	0.040	0.000	0.002	0.000	-	-	-	-	-	-	-	-	-	-	-

c.2221-28A>G	20/41	rs7196184	0.109	0.392	0.040	0.000	0.002	0.000	0.330	0.001	0.026	0.354	0.019	0.005	0.000	0.000	0.001	0.017	0.001
c.2356-63A>G	21/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.2545+26G>A	22/41	rs45517242	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.005	0.003	0.001	0.002	0.001	0.000	0.004	0.004	0.005	0.000
c.2545+31C>T	22/41	rs45517243	0.005	0.014	0.007	0.000	0.000	0.000	0.015	0.000	0.001	0.017	0.001	0.000	0.001	0.000	0.000	0.000	0.000
c.2545+95dupG	22/41	rs397515216	0.063	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.2545+135C>T	22/41	rs139579913	0.004	0.012	0.003	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2545+157G>C	22/41	rs9941195	0.056	0.201	0.020	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2545+194A>G	22/41	rs7185909	0.035	0.123	0.013	0.000	0.002	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2545+240C>T	22/41	rs9921591	0.056	0.201	0.020	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2545+244C>G	22/41	rs143553665	-	0.012	0.001	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2546-12C>T	22/41	rs13331451	0.206	0.620	0.107	0.000	0.085	0.052	0.521	0.074	0.095	0.552	0.063	0.074	0.000	0.098	0.072	0.083	0.048
c.2639+44C>G	23/41	rs1800715	0.215	0.624	0.115	0.000	0.097	0.074	0.526	0.087	0.107	0.557	0.069	0.102	0.000	0.111	0.085	0.099	0.063
c.2639+44C>T	23/41	rs1800715	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
c.2640-74G>A	23/41	rs143128054	0.004	0.014	0.003	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2640-65C>T	23/41	rs138967036	0.004	0.014	0.003	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.2640-26G>T	23/41	rs45483797	0.003	0.012	0.001	0.000	0.000	0.000	0.013	0.000	0.001	0.011	0.001	0.000	0.000	0.000	0.000	0.000	0.000
c.2743-40A>G	24/41	rs45517265	0.004	0.000	0.006	0.000	0.015	0.002	0.002	0.010	0.007	0.002	0.007	0.004	0.000	0.001	0.010	0.010	0.007
c.2837+93G>A	25/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.2838-4A>G	25/41	rs45517272	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.001	0.001	0.000	0.000	0.003	0.000	0.000	0.001	0.001	0.001
c.2966+92_2966+94dup	26/41	rs397515128	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.2966+217G>T	26/41	rs118124428	0.008	0.002	0.009	0.000	0.030	0.001	-	-	-	-	-	-	-	-	-	-	-
c.2967-41C>T	26/41	rs972958530	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
c.3131+34C>T	27/41	rs45487103	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.002	0.000	0.000	0.001	0.001	0.000
c.3132-30A>G	27/41	rs45517283	0.003	0.012	0.001	0.000	0.000	0.000	0.013	0.000	0.001	0.012	0.001	0.000	0.000	0.000	0.000	0.000	0.000
c.3397+126C>T	29/41	rs115243225	0.051	0.184	0.019	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.3610+46dupC	30/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.3611-136C>T	30/41	rs376053649	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.3611-100T>C	30/41	rs370512781	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.3611-28C>T	30/41	rs45517303	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

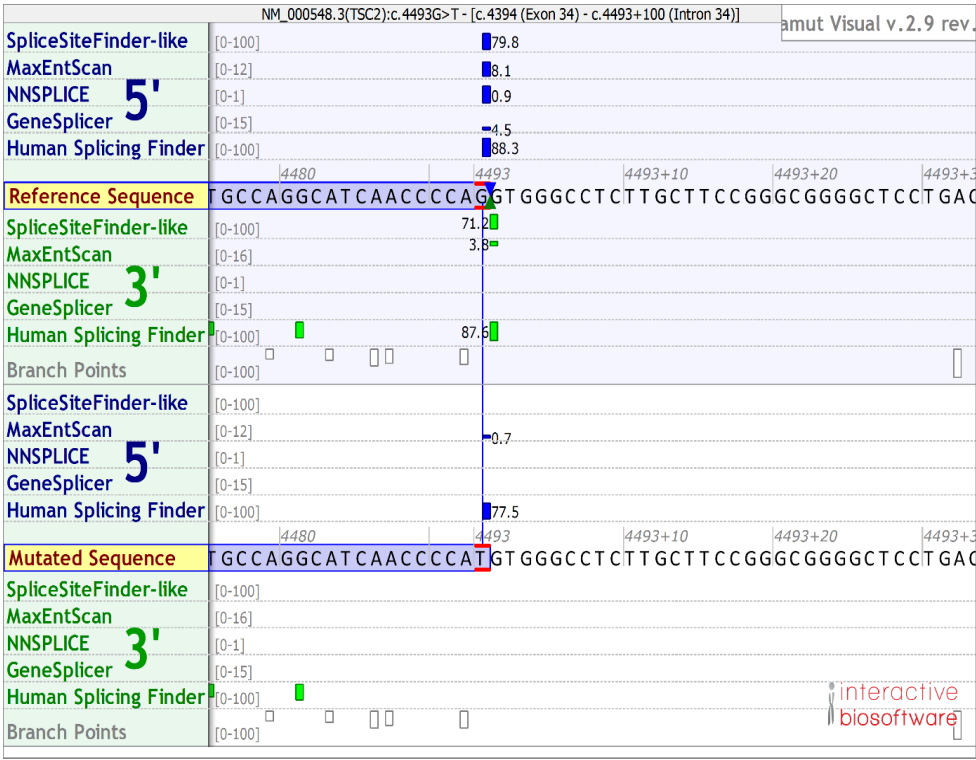
c.3815-124C>T	31/41	rs143001593	0.003	0.009	0.001	0.000	0.001	0.000	-	-	-	-	-	-	-	-	-	-	-
c.3815-38C>T	31/41	rs371592734	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
c.3883+8C>G	32/41	rs45517316	0.012	0.043	0.004	0.000	0.000	0.000	0.033	0.000	0.003	0.039	0.003	0.000	0.000	0.000	0.000	0.002	0.000
c.3883+66G>A	32/41	rs74002774	0.011	0.042	0.003	0.000	0.000	0.000	0.035	0.000	-	-	-	-	-	-	-	-	-
c.3883+78G>A	32/41	rs1800705	0.011	0.003	0.017	0.000	0.027	0.011	-	-	-	-	-	-	-	-	-	-	-
c.3883+127G>A	32/41	rs562317274	0.001	0.004	0.000	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.3883+226G>A	32/41	rs9928737	0.188	0.409	0.180	0.000	0.188	0.088	-	-	-	-	-	-	-	-	-	-	-
c.3884-68C>T	32/41	rs114209035	0.004	0.014	0.003	0.000	0.000	0.000	-	-	0.001	0.012	0.001	0.000	0.000	0.000	0.000	0.001	0.000
c.3884-56C>G	32/41	rs1800724	0.036	0.001	0.038	0.000	0.078	0.079	-	-	0.060	0.009	0.029	0.092	0.000	0.110	0.071	0.059	0.066
c.4005+84C>T	33/41	rs30259	0.040	0.002	0.053	0.046	0.074	0.041	-	-	-	-	-	-	-	-	-	-	-
c.4005+153T>A	33/41	rs28535326	0.019	0.070	0.006	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.4006-60C>A	33/41	rs376519405	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.4006-8C>T	33/41	rs45517325	0.002	0.000	0.004	0.000	0.003	0.002	0.000	0.004	0.002	0.001	0.004	0.000	0.000	0.000	0.004	0.004	0.001
c.4493+110G>A	34/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.4569+44C>T	35/41	rs142227748	0.010	0.039	0.000	0.000	0.000	0.025	0.000	0.002	0.032	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000
c.4569+46C>T	35/41	rs45482793	0.004	0.001	0.003	0.000	0.018	0.001	0.003	0.018	0.010	0.002	0.005	0.010	0.000	0.008	0.017	0.011	0.003
c.4569+47G>A	35/41	rs45517351	0.019	0.065	0.013	0.000	0.000	0.001	0.059	0.001	0.005	0.059	0.003	0.006	0.000	0.000	0.000	0.004	0.000
c.4570-38G>A	35/41	rs770778907	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
c.4662+199G>A	36/41	rs1379483764	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.4663-88C>T	36/41	rs146465188	0.006	0.017	0.009	0.000	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.4663-56C>G	36/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.4849+75C>T	37/41	rs76029733	0.028	0.011	0.026	0.043	0.031	0.035	-	-	-	-	-	-	-	-	-	-	-
c.4849+76G>A	37/41	rs144647393	0.015	0.057	0.001	0.000	0.001	0.000	-	-	-	-	-	-	-	-	-	-	-
c.4849+97delA	37/41	rs142421783	0.056	0.201	0.020	0.001	0.000	0.000	-	-	-	-	-	-	-	-	-	-	-
c.4850-133C>T	37/41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.4850-109T>C	37/41	rs13335638	0.241	0.595	0.195	0.000	0.198	0.090	-	-	-	-	-	-	-	-	-	-	-
c.4990-7C>T	38/41	rs45457095	0.009	0.027	0.012	0.000	0.000	0.000	0.028	0.001	0.002	0.025	0.002	0.006	0.000	0.000	0.000	0.002	0.001
c.5160+37C>T	40/41	rs45517400	0.001	0.000	0.001	0.000	0.003	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.000
c.5160+43C>T	40/41	rs45517402	-	-	-	-	-	-	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

c.5161-28_5161-25delTGAG	40/41	rs1799758	0.005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c.5161-10A>C	40/41	rs1800718	0.257	0.616	0.213	0.000	0.236	0.090	0.543	0.219	0.200	0.563	0.128	0.250	0.000	0.258	0.214	0.217	0.097
c.5260-49C>T	41/41	rs13332221	0.168	0.458	0.140	0.000	0.116	0.020	0.397	0.102	0.106	0.410	0.083	0.142	0.000	0.114	0.103	0.125	0.028
c.5260-25C>G	41/41	rs13332222	0.201	0.576	0.153	0.000	0.118	0.020	0.478	0.105	0.114	0.507	0.089	0.142	0.000	0.115	0.106	0.130	0.029
c.5260-15C>T	41/41	rs45517416	0.002	0.004	0.000	0.000	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.000	0.000	0.001	0.002	0.002	0.002

IX. Supplementary Figures



Supplementary Figure II-1: Melt curve of pair of primers used to in Q-PCR. The first row there are primers for *TSC1* and *TSC2* sorted according to exon position on gene. On second row, *GAPDH* was the reference for Q-PCR, *GFI1B* and *SPACA9* respectively upstream and downstream genes for *TSC1*, and *NTHL1* and *PKD1* respectively upstream and downstream genes for *TSC2*. They are sorted according to exon position on gene



Supplementary Figure II-2: ALAMUT splicing site prediction analysis for TSC2 (NM\_000548.3) c.4493G>T substitution.

# Chapter III

Assessment of regulatory relationships  
between *TSC1* and *TSC2* mRNA and protein

## List of Acronyms

**3'-UTR:** 3'- untranslated region

**5'-UTR:** 5'-untranslated regions

**DMEMplus:** DMEM supplemented with 5% calf serum and penicillin-streptomycin

**DMEMminus:** DMEM with no supplementation

**DPBS:** Dulbecco's Phosphate Buffered Saline

**DMEM:** Dulbecco's Modified Eagle's Medium

**EDTA:** Ethylenediamine tetra-acetic acid

**FDR:** False discovery rate

**GO:** Gene ontology

**HSP90:** Heat shock protein 90-alpha

**O/N:** Overnight

**TPM:** Transcript per million

**UTR:** Untranslated region

## List of Tables

Table III-1: Total of samples per cell line for each treatment - DMEMplus or DMEMminus. ....	134
Table III-2: Observed <i>TSC1</i> and <i>TSC2</i> mRNA and protein levels for each knock-out cell line comparatively to HEK293T. ....	143
Table III-3: Number of genes down- or up-regulated in 1C2 cell line in regard to HEK293T in DMEMplus or DMEMminus groups. ....	143
Table III-4: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMminus group. ....	144
Table III-5: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMplus group. ....	144
Table III-6 Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMminus group. ....	144
Table III-7: Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMplus group. ....	145
Table III-8: Number of genes observed when groups assigned in Table 13 were intersected. ....	145
Table III-9: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, independently on the serum presence. ....	146
Table III-10: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, detected in DMEMplus but not in DMEMminus samples. ....	146
Table III-11: Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, detected in DMEMminus but not in DMEMplus samples. ....	147
Table III-12: Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, detected in DMEMplus but not in DMEMminus samples. ....	147
Table III-13: Gene sets significantly enriched in down-regulated genes in DMEMminus group in regard to DMEMplus, in the 1C2 cell line. ....	148
Table III-14: Gene sets significantly enriched in up-regulated genes in DMEMminus group in regard to DMEMplus, in the 1C2 cell line. ....	148

## List of Figures

Figure III-1: <i>TSC1</i> locus and mRNA expression.....	126
Figure III-2: <i>TSC2</i> locus and mRNA expression.....	127
Figure III-3: Cladogram and genotype of wildtype cell line and six edited cell lines by the CRISPR-Cas9. ....	132
Figure III-4: Workflow of cell culture, DNA isolation, RNA Isolation and protein isolation from all seven cell lines. O/N: overnight. ....	133
Figure III-5: <i>TSC1</i> , <i>TSC2</i> , <i>AKT</i> and <i>S6</i> protein levels assessed by Western blotting. ....	137
Figure III-6: Specific protein profile from genetically modified cell lines.....	139
Figure III-7: <i>TSC1</i> and <i>TSC2</i> mRNA RPKM upon DMEMplus and DMEMminus treatments.....	140
Figure III-8: Log2FoldChange of <i>TSC1</i> (A) and <i>TSC2</i> (B) transcript amount upon DMEMplus treatment. Asterisk = $p$ -value<0.05. ....	141
Figure III-9: Log2FoldChange of <i>TSC1</i> (A) and <i>TSC2</i> (B) transcript amount upon DMEMminus treatment. Asterisk = $p$ -value<0.05. ....	142

## Summary

III.	Chapter III .....	121
I.	Introduction .....	126
II.	Aim and objectives .....	130
A.	Aim.....	130
B.	Objectives .....	130
III.	Material and Methods .....	131
A.	Cell lines.....	131
B.	Cell culture.....	131
C.	Protein isolation and Western Blotting.....	133
D.	Total RNA isolation and RNA sequencing.....	133
IV.	Results .....	136
A.	TSC1 and TSC2 protein analyses .....	136
B.	<i>TSC1</i> and <i>TSC2</i> mRNA analyses .....	140
C.	Global RNA analysis in the 1C2 cell line .....	143
V.	Discussion .....	149
A.	Cell line characterization.....	149
B.	<i>TSC1</i> and <i>TSC2</i> gene expression regulation.....	151
C.	Protein stress response in TSC1-negative cell line .....	152
VI.	Conclusions.....	154
VII.	References .....	155
VIII.	Supplementary Tables .....	168
IX.	Supplementary Figures .....	171



nucleotides and, similar to *TSC1*, is expressed in most tissues, with the highest mRNA amount in the cerebellum (Figure III-2B). Overall *TSC2* mRNA levels are nearly twice that observed for *TSC1* (Figure III-1B and Figure III-2B).

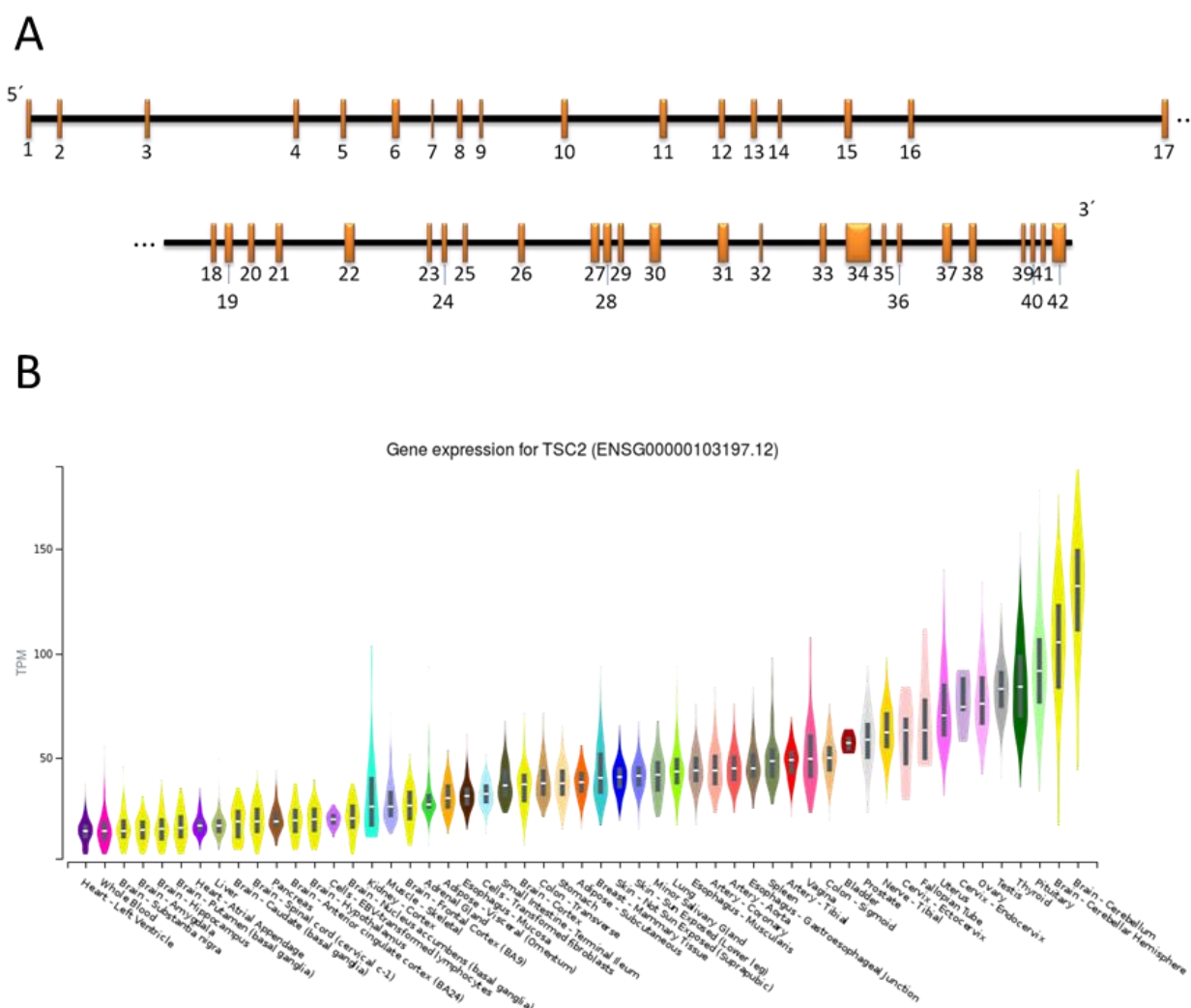


Figure III-2: *TSC2* locus and mRNA expression. (A) Schematic diagram of the *TSC2* gene exons and introns. Black lines represent introns, broad and narrow orange bars highlight coding exons and the UTR regions, respectively. (B) *TSC2* RNA-Seq expression data from the GTEx project (<https://www.gtexportal.org/home/gene/TSC2>). TPM: transcripts per million.

*TSC1* protein (NP\_000359.1) is a 130-kDa (1,164 amino acids) hydrophilic protein displaying a C-terminal domain required for interaction with *TSC2* (residues 762-848) (van Slegtenhorst, Nellist *et al.* 1998), predicted as a coiled-coil region. The 200 kDa *TSC2* protein (NP\_000539.2) has 1,807 amino acids, an N-terminal domain (residues 555-903) required for interaction with *TSC1*, and a C-terminal 163-residue GTPase activating (GAP) domain (GAP-Rheb residues 1562 to 1764) (Plank, Yeung *et al.* 1998, van Slegtenhorst, Nellist *et al.* 1998). Under conditions of amino acid depletion, Rag GTPase recruits *TSC2* to the lysosome, interacting with Rheb. mTORC1 is thus released from the lysosome and becomes inactivated. However, upon amino acid

withdrawal, cells with pathogenic variants on TSC2 cannot completely release TORC1 from the lysosome, hence failing to adjust to physiological conditions (Demetriades, Doumpas *et al.* 2014).

In cells lacking the TSC2 protein, TSC1 homodimers are prone to self-aggregation in detergent-resistant complexes, presenting a coarsely dotted cytoplasmic distribution by immunofluorescence analysis (Nellist, Sancak *et al.* 2005). If both genes are co-overexpressed, TSC1 protein becomes solubilized with a more homogeneous cytoplasmic distribution. Thus, it has been proposed that TSC2 may act as TSC1 chaperone (Nellist, van Slegtenhorst *et al.* 1999). On the other hand, Benvenuto, Li *et al.* (2000) suggested that TSC1 stabilizes TSC2.

TBC1D7, which has been reported as a protein from the TSC1/2 complex, stabilizes TSC1 dimers (Gai, Chu *et al.* 2016). The crystal structure of TBC1D7 in complex with the C-terminal part of TSC1 (TSC1-CC, residues 939–992, encoded by exon 22) revealed that two units of TSC1-CC form a parallel homodimer - two symmetric surfaces for interaction with TBC1D7 (Santiago Lima, Hoogeveen-Westerveld *et al.* 2014, Gai, Chu *et al.* 2016, Qin, Wang *et al.* 2016), stabilizing the TSC complex. Furthermore, the molecular chaperone heat-shock protein 90- $\alpha$  (HSP90, NP\_001017963.2), an essential component of the cellular homeostatic machinery in eukaryotes, may interact with TSC1 in the absence or presence of TSC2. The association between TSC1 and TSC2 decreases under heat shock, which does not affect the association between TSC1 and HSP90 (Inoue, Uyama *et al.* 2010). TSC1 has been proposed as HSP90 co-chaperone (Woodford, 2017; Sager 2018a, 2018b), as HSP90 binds *in vitro* and *in vivo* to denatured proteins and displays ATP-dependent anti-aggregation properties (Panaretou, Prodromou *et al.* 1998).

TSC2 had been identified as a target of AKT kinase regulation (Potter, Huang *et al.* 2001, Dan, Sun *et al.* 2002, Manning, Tee *et al.* 2002, Inoki, Li *et al.* 2003). The pathway of phosphatidylinositol-3 and AKT kinases was characterized as the main physiological regulation of TSC complex turnover, leading to a decrease in the affinity between TSC1 and TSC2, and degradation of both proteins mediated by their phosphorylation (Potter, Huang *et al.* 2001, Dan, Sun *et al.* 2002, Inoki, Li *et al.* 2003). The observed response to heat shock appears to be related to the phosphorylation of TSC2 by AKT, stimulating its degradation by the proteasome (Inoue, Uyama *et al.* 2010). The interaction of TSC1 with HSP90 is required for its localization in the outer membrane of the mitochondria, with or without TSC2, in a manner dependent on the phosphorylation of TSC1-Thr<sup>417</sup> (Inoue, Uyama *et al.* 2010). These data are corroborated by other observations that the knockout of *Tsc2* maintains Tsc1 at reasonable levels (Zhang, Gao *et al.* 2003, Pollizzi, Malinowska-Kolodziej *et al.* 2009). On the other hand, Western blotting of mouse *Tsc1*<sup>-/-</sup> embryonic fibroblast protein has shown reduced levels of the Tsc2 protein (Astrinidis, Senapedis *et al.* 2006), suggesting that lack of expression of both *Tsc1* alleles may subject Tsc2 to poly-ubiquitination and degradation by the proteasome. Overexpression of *TSC1* in Rat-2 cells or rat embryonic fibroblasts elevates the level of TSC2, and binding of TSC1 to TSC2 inhibits TSC2 ubiquitination. Finally, inhibition of the proteasome represses TSC2 degradation (Benvenuto, Li *et al.* 2000).

The stability of TSC1-TSC2 protein complex is central for the control of mTORC1 signaling. Further understanding of the complexity of *TSC1* and *TSC2* gene regulation is important as heterologous gene replacement can be a potential future therapy alternative for specific TSC lesions (Prabhakar, Zhang *et al.* 2015). However, it is as yet unknown whether in the absence of the TSC1 or TSC2 protein there may be transcriptional or post-transcriptional feedback control of *TSC1* and *TSC2* gene expression. Although it is clear that there should be inter-regulation of TSC1 and TSC2 protein stability, it is necessary to specifically understand the levels and mechanisms of regulation of endogenous gene expression. Hence, changes in mRNA amount may build a feedback control loop on TSC gene transcription, e. g., absence of *TSC1* gene expression may potentially affect the *TSC2* mRNA or TSC2 protein. On the other hand, similar amounts of *TSC1* mRNA would refute the hypothesis of transcriptional or mRNA half-life regulation. Moreover, the absence of *TSC1* should compromise the cell homeostasis under amino acid withdrawal due to lack of interaction with TSC2 or Hsp90. In this study, we intend to start understanding the regulatory relationship between *TSC1* and *TSC2* mRNA and protein.

## II. Aim and objectives

### A. Aim

To assess *TSC1* and *TSC2* gene product levels in search for possible transcriptional or translational feedback regulatory loops in cells lacking *TSC1* or *TSC2* proteins, and transcriptome response of *TSC1* knockout cell line on deprivation environment.

### B. Objectives

1. To compare *TSC1* and *TSC2* protein levels between the reference human cell line and human cell lines with bi-allelic inactivation of *TSC1* and/or *TSC2* genes;
2. To assess the activity of the mTORC1 pathway reported by the phosphorylation of S6 protein Ser<sup>235</sup> and Ser<sup>236</sup> residues; having nutrient starvation, insulin treatment and rapamycin supplementation as proof-of-concept controls;
3. To compare the transcriptome between cells with bi-allelic inactivation of *TSC1* the reference human cell; and
4. To compare the transcriptome of cells with bi-allelic inactivation of *TSC1* growing under complete medium and without supplementation.

### III. Material and Methods

Experiments were conducted in the Netherlands, mostly at Erasmus Medical Centre, Rotterdam, Amsterdam University or at the GenomeScan (Leiden, the Netherlands). Data analyses was performed at *Instituto de Biociências, Universidade de São Paulo* (São Paulo, Brazil) and *Hospital Sírio-Libanês, São Paulo, Brazil*.

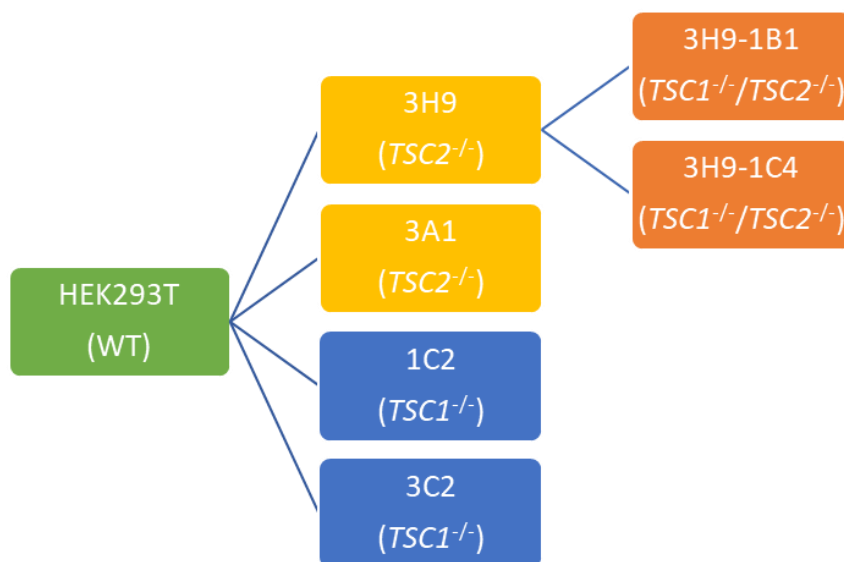
#### A. Cell lines

Seven cell lines employed had been generated before: HEK293T cell line, and six cell lines derived from HEK293T in which *TSC1* or *TSC2* gene genomic segments have been genetically edited by the CRISPR-Cas9 strategy (Dufner-Almeida et al., submitted). Figure III-3 depicts the outflow of genetic modifications, and Table III-1 presents cell line names and *TSC1* and *TSC2* genotypes.

#### B. Cell culture

Cell lines were seeded in round tissue culture dish (6 cm; Sigma-Aldrich, St. Louis, Missouri, USA) in Dulbecco's Modified Eagle's Medium (DMEM, BioWhittaker, Radnor, Pennsylvania, USA), supplemented with 5% fetal calf serum and penicillin-streptomycin, constituting the complete medium or DMEMplus, and maintained at 37°C and 10% CO<sub>2</sub>. After splitting to ten round dishes, cells were kept under the same culturing conditions until 80% confluence. Cells from three plates (group DMEMplus) were harvested and submitted to DNA, RNA and protein isolation. After washing the cells three times in PBS (Dulbecco's Phosphate Buffer Saline (0.0095M) – BioWhittaker - Radnor, Pennsylvania, USA), medium from the seven remaining plates was switched to DMEM minimum medium (without supplementation), considered as DMEMminus. The starvation period lasted for 16 hours (ON - overnight), after which RNA and protein were isolated from two plates (group DMEMminus). Rapamycin (Cell Signaling Technology, Danvers, Massachusetts, USA) suspended in DMSO was added to cells from the five remainder dishes to final concentration of 10 nM. After one hour, RNA and protein were isolated from two plates (group DMEMminus - RAPA-1h) while culture continued for further 15 hours in three plates. On the fourth day, RNA and protein were isolated from two plates (DMEMminus - RAPA O/N) and cells in the final plate were treated with insulin at 200 nM suspended in HEPES buffer (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 minutes (group DMEMminus – RAPA O/N – INS), washed and harvested for protein isolation (Figure II-2). All seven cell lines were simultaneously submitted to the same protocol, and the experiment was repeated at least three times.

A



B

Cell Line	TSC1 genotype	TSC2 genotype
HEK293T (WT)	c.965T>C (p.M322T) heterozygote	wild-type
1C2 (TSC1 <sup>-/-</sup> )	c.-13_5del homozygote c.965T>C (p.M322T) heterozygote c.3194_3206del (p.T1065Kfs*22) homozygote	wild-type
3C2 (TSC1 <sup>-/-</sup> )	c.989_3199del (p.S331_M1067del) heterozygote c.1002_3204del (p.T335Kfs*22) heterozygote c.1002dup (p.T335Dfs*6) homozygote c.3196insT (p.T1066Ifs*33) homozygote	wild-type
3A1 (TSC2 <sup>-/-</sup> )	c.965T>C (p.M322T) heterozygote	c.121dup (p.T41Nfs*26) homozygote c.116_4989+358del (p.I39Rfs*42) heterozygote
3H9 (TSC2 <sup>-/-</sup> )	c.965T>C (p.M322T) heterozygote	c.122_4990-284del38842 (p.T41Rfs*42) homozygote
3H9-1B1 (TSC1 <sup>-/-</sup> /TSC2 <sup>-/-</sup> )	c.-8_3196del (p.?) homozygote	c.122_4990-284del38842 (p.T41Rfs*42) homozygote
3H9-1C4 (TSC1 <sup>-/-</sup> /TSC2 <sup>-/-</sup> )	c.-8_3196del (p.?) homozygote	c.122_4990-284del38842 (p.T41Rfs*42) homozygote

Figure III-3: Cladogram and genotype of wildtype cell line and six edited cell lines by the CRISPR-Cas9. (A) Schematic representation of parental wild type HEK293T cell line and derived cell line. (B) Genotype of each seven cell lines.

## C. Protein isolation and Western Blotting

Cells were lysed in 50 mM Tris-HCl (pH 7.6), 100 mM NaCl, 50 mM NaF, 1% Triton-X-100, 1 mM ethylenediamine tetra-acetic acid (EDTA) and complete protease inhibitor cocktail (Roche, Basileia, Switzerland). Proteins lysate were submitted to Criterion™ TGX™ Precast Gel (4 -15%; Bio-Rad, Hercules, California, USA) electrophoresis at 100V for 10-15 minutes followed by one hour at 120V; and transferred to nitrocellulose filters in the Trans-blot® Turbo™ (Bio-Rad, Hercules, California, USA) using the pre-set program for high molecular weight (2.5 Amp, 25 V, 10min.). Finally, the membrane proteins were blocked in 5% powder milk in DPBS (Dulbecco's Phosphate Buffer Saline (0.0095M) – BioWhittaker - Radnor, Pennsylvania, USA) for 30 minutes, followed by specific primary and secondary antibody incubations, and fluorescence captured under the conditions described in chapter 2. The experiment was repeated at least three times and Student's *t* test was used for pairwise comparison.

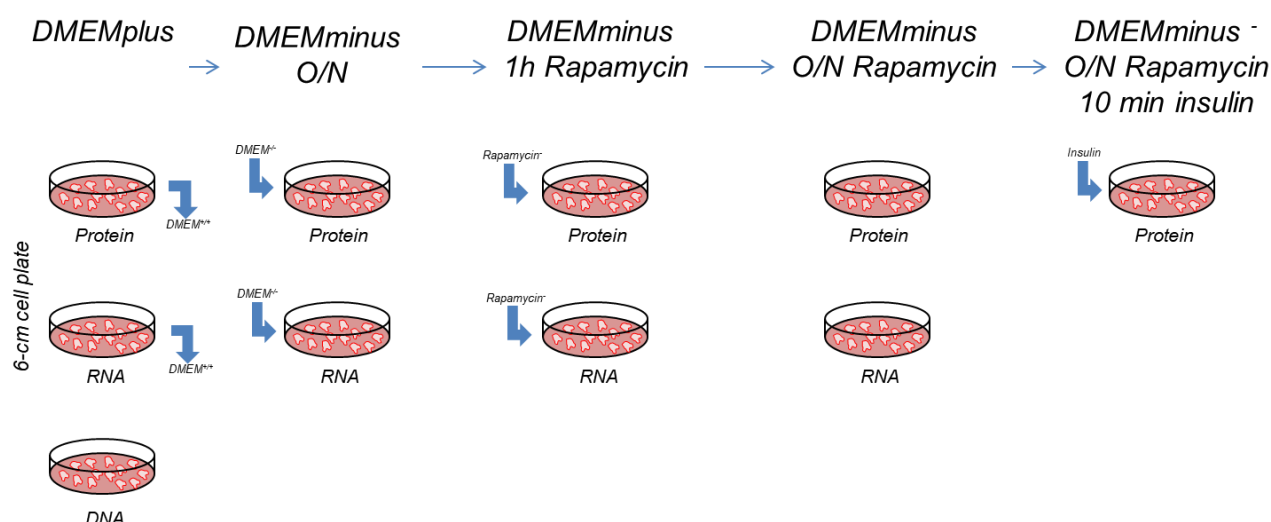


Figure III-4: Workflow of cell culture, DNA isolation, RNA Isolation and protein isolation from all seven cell lines. O/N: overnight.

## D. Total RNA isolation and RNA sequencing

Total RNA was isolated with RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer conditions. Briefly, cells were washed in DPBS, and then lysis buffer - 1% β-mercaptoethanol in RTL buffer (QIAGEN, Hilden, Germany) - was added. Cells were detached and transferred to a 1.5-mL tube. One volume of 70% ethanol was added, homogenized, and the solution was transferred to an RNeasy® mini-spin column, and centrifuged for 15 seconds at 8,000 X *g*. Column was washed with buffer RW1 and centrifuge for 15 sec at 12000*g*, and contents incubated with DNase I for 15 minutes at room temperature. Finally, the column was washed three times and RNA eluted in 50 µL of RNase-free water. RNA concentration and quality

were measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and BioAnalyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) ().

A total of 30 RNA samples (Table III-1) were used for library construction and Illumina HiSeq 4000 sequencing at GenomeScan (Leiden, The Netherlands). To assess the quality of the samples, concentration was determined using the Fragment Analyzer. Sample preparation was performed according to NEBNext Ultra Directional RNA Library Prep Kit for Illumina protocol (NEB #E7420S/L; New England Biolabs, Ipswich, Massachusetts, USA). Briefly, mRNA was isolated from total RNA using oligo-dT magnetic beads. After mRNA fragmentation on broad peak between 300-500 bp, cDNA was synthesized following kit specifications. Ligation with sequencing adapters followed, and the resulting product was submitted to PCR bridge amplification. Product quality and yield after sample preparation was measured with the Fragment Analyzer (Supplementary Figure III-2). The size of the resulting products was consistent with the expected size distribution (a broad peak between 300-500 nucleotides). A cDNA concentration of 3.0 nM was used (Supplementary Table III-2). Clustering and cDNA sequencing using the Illumina cBot and HiSeq 4000 were performed according to the manufacturer's protocols (Supplementary Table III-3).

Table III-1: Total of samples per cell line for each treatment - DMEMplus or DMEMminus.

Cell Line	Treatment	
	DMEMplus	DMEMminus
HEK293T (WT)	3	3
1C2 (TSC1 <sup>-/-</sup> )	2	2
3C2 (TSC1 <sup>-/-</sup> )	2	2
3A1 (TSC2 <sup>-/-</sup> )	2	2
3H9 (TSC2 <sup>-/-</sup> )	2	2
3H9-1B1 (TSC1 <sup>-/-</sup> /TSC2 <sup>-/-</sup> )	2	2
3H9-1C4 (TSC1 <sup>-/-</sup> /TSC2 <sup>-/-</sup> )	2	2

The mRNA-Seq pipeline starts with quality filtering and trimming of the sequence of optimized standard thresholds: Illumina sequencing adapters were removed, bases with read Q-score below 22 trimmed off, base reads with at least 36 nucleotide-long kept in the data set. Alignment was based on Burrows–Wheeler Transform to ensure maximum sensitivity for mapping reads to exons of genes. All the genes found in the reference annotation were included, even if they do not have any associated expression. Each expressed gene is listed separately per Ensembl Gene ID.

Statistical analysis and comparison between treatment and between samples were performed using DEseq2 script (Love, Huber *et al.* 2014) on R program (Version 3.5.1, ). Briefly, average of the normalized count values divided by size factors, taken over all samples (baseMean); expression changed due to treatment or cell lines (log2FoldChange), standard error estimate for the log2 fold change (lfcSE), *p*value and *padj* (Benjamini-Hochberg BH-adjusted *p*values for false positive results) were obtained. All *p*values in results' table assigned

as NA were excluded from analysis because it contained an extreme count outlier, or the expression was not detected. Only genes with  $p_{adj} < 0.1$  were considered differentially expressed.

Comparisons between gene sets identified from RNA-Seq analysis between 1C2 and HEK293T cell lines from DMEMplus or DMEMminus groups or from 1C2 (DMEMplus) and 1C2 (DMEMminus) groups yielded differentially expressed genes under significance of adjusted p value  $< 0.1$ . Differentially up- or down-regulated genes were submitted to gene ontology (GO) grouping for non-redundant biological processes, employing the over-representation enrichment analysis at WEB-based GENE SeT Analysis Toolkit (2019 Version; <http://webgestalt.org>), a set of computational tools developed and maintained at Dr. Bing Zhang laboratory at the Baylor College of Medicine (Houston, TX, USA). Gene ID was gene symbol, and the reference set for enrichment analysis was the human genome. Additionally, the following cut-off values were considered: at least five genes for a GO category, a maximum of 2,000 genes for a GO category, ten GO categories expected from set cover, and significance level for false discovery rate (FDR)  $< 0.05$  among the top 10 GO categories (Benjamini statistical test corrected by Hochberg).

## IV. Results

### A. TSC1 and TSC2 protein analyses

In the present study, we screened six HEK293T-derived cell lines with bi-allelic inactivation of the *TSC1* and/or *TSC2* genes (Figure III-3B) for the expression of these genes at both mRNA and protein levels. As presented in Figure III-5 (panels A-C), under DMEMplus conditions, the protein encoded by each CRISPR-Cas9-mediated knocked-out gene is not detected, as expected. Knocking-out *TSC1* or *TSC2* genes in homozygosity does not appear to affect the expression of AKT or S6 proteins (Figure III-5, D and E), but to relate to at least 50% reduction of the partnering protein, e.g., *TSC2* when *TSC1* is inactivated, or *TSC1* when *TSC2* is knocked out (Figure III-5, B and C).

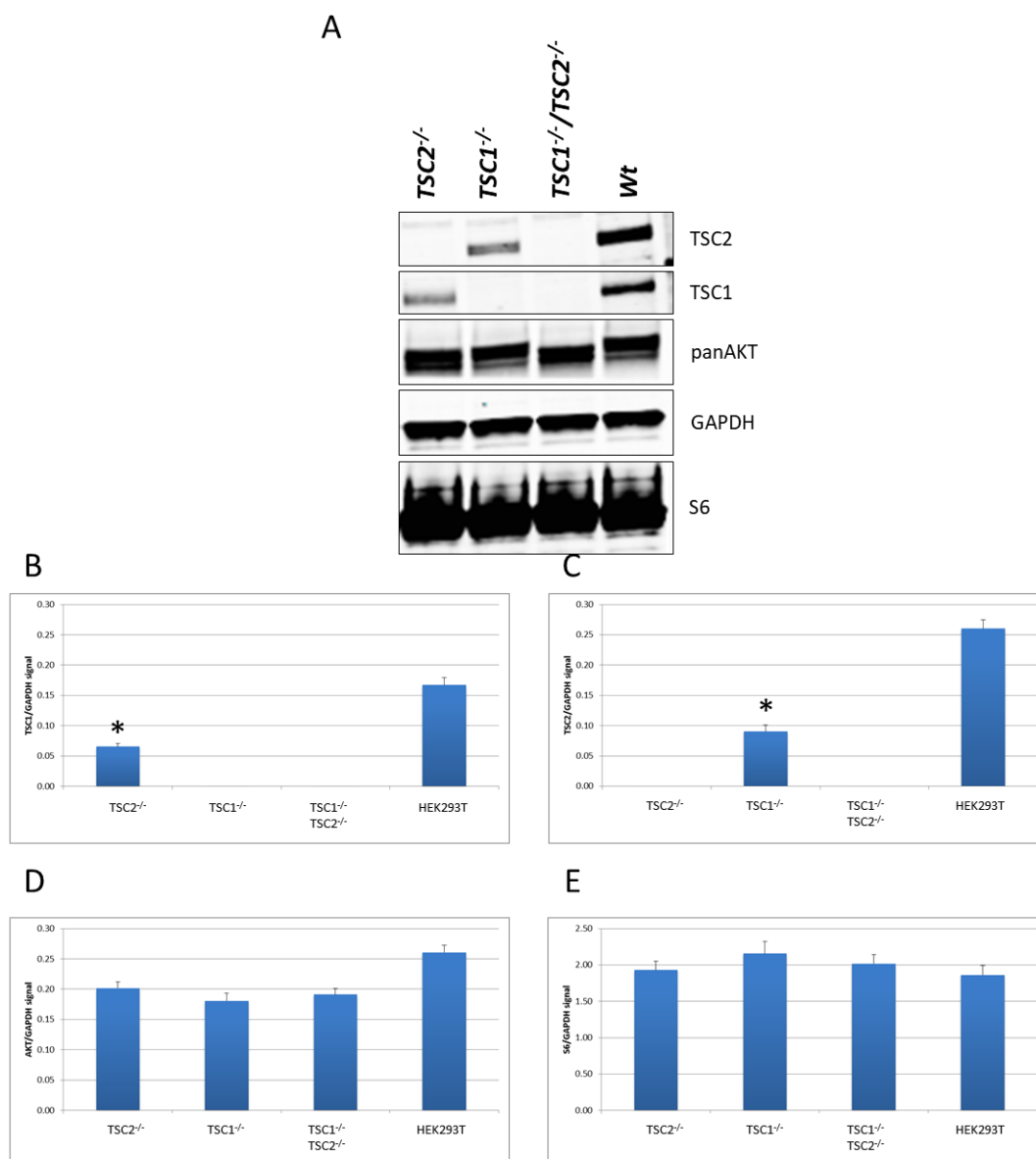


Figure III-5: TSC1, TSC2, AKT and S6 protein levels assessed by Western blotting. (A) Western blots showing protein bands for TSC2, TSC1, panAKT, GAPDH and S6 protein, as shown on the right side, respectively from HEK293T-derived cell lines *TSC2*<sup>-/-</sup>, *TSC1*<sup>-/-</sup>, *TSC2*<sup>-/-</sup>/*TSC1*<sup>-/-</sup> and HEK293T reference. (B-E) Signal relative to GAPDH signal respectively for TSC1 (B), TSC2 (C), AKT (D) and S6 (E) for HEK293T-derived cell lines *TSC2*<sup>-/-</sup>, *TSC1*<sup>-/-</sup>, *TSC2*<sup>-/-</sup>/*TSC1*<sup>-/-</sup> and HEK293T reference. All values were normalized to GAPDH. Asterisk represents a statistically significant difference between *TSC1*<sup>-/-</sup> or *TSC2*<sup>-/-</sup> under the same culturing condition to HEK293T (*p*-value < 0.05).

TSC1 and TSC2 proteins were reassessed on blots in all seven cell lines in a single electrophoresis for each culturing condition. Lack of TSC1 and TSC2 proteins was reconfirmed for the respective knock-out genotypes (Figure III-6). As expected, lack of TSC1 or TSC2 protein directly related to the levels of S6/Ser<sup>235</sup>/Ser<sup>236</sup> phosphorylation, reporting mTORC1 hyperactivity (Figure III-6). For instance, all cells from DMEMplus group and the knocked-out cells from DMEMminus group had S6/Ser<sup>235</sup>/Ser<sup>236</sup> hyperphosphorylated (Figure III-6, first and second panels from second bottom row). On the contrary, DMEMminus reference HEK293T cells did not (Figure III-6, second panel from second bottom row). Addition

of rapamycin, for one or 16 hours, down-regulated mTORC1 activity as shown by hypophosphorylation of the reporter S6/Ser<sup>235</sup>/Ser<sup>236</sup> (Figure III-6, third to fifth panels from second bottom row).

The PI3 kinase -Akt pathway is activated under growth conditions. Akt activity depends on two phosphorylated residues; one located in the catalytic domain (threonine 308 – Akt-Thr<sup>308</sup>), and the second, serine 473 (Akt-Ser<sup>473</sup>), in the C-terminal regulatory domain (Nicholson and Anderson 2002). Thus, Akt residues Thr<sup>308</sup> and Ser<sup>473</sup> are phosphorylated in response to insulin-dependent PI3K activity. As observed in figure 6, complete medium (left panel) and ten-minute insulin treatment (far right panel) of cells associate with positive blot signals for both Akt phosphorylated residues. Serum withdrawal did not change Akt activity, as reported by the blot signals observed for antibodies specific for the phosphorylated residues (Figure 6, second panel). Signals for both phosphorylated residues reporting AKT activity decreased when cells were treated overnight with rapamycin for 16 hours (Figure III-6, second right panel), though they appeared unchanged when rapamycin treatment lasted for only one hour (Figure III-6, middle panel). As Akt is upstream to the TSC1/2 complex, its residues Thr<sup>308</sup> and Ser<sup>473</sup> were expected to be phosphorylated in all cell lines. However, 3H9-1B1 cell line showed unphosphorylated Akt under all conditions, except for insulin treatment.

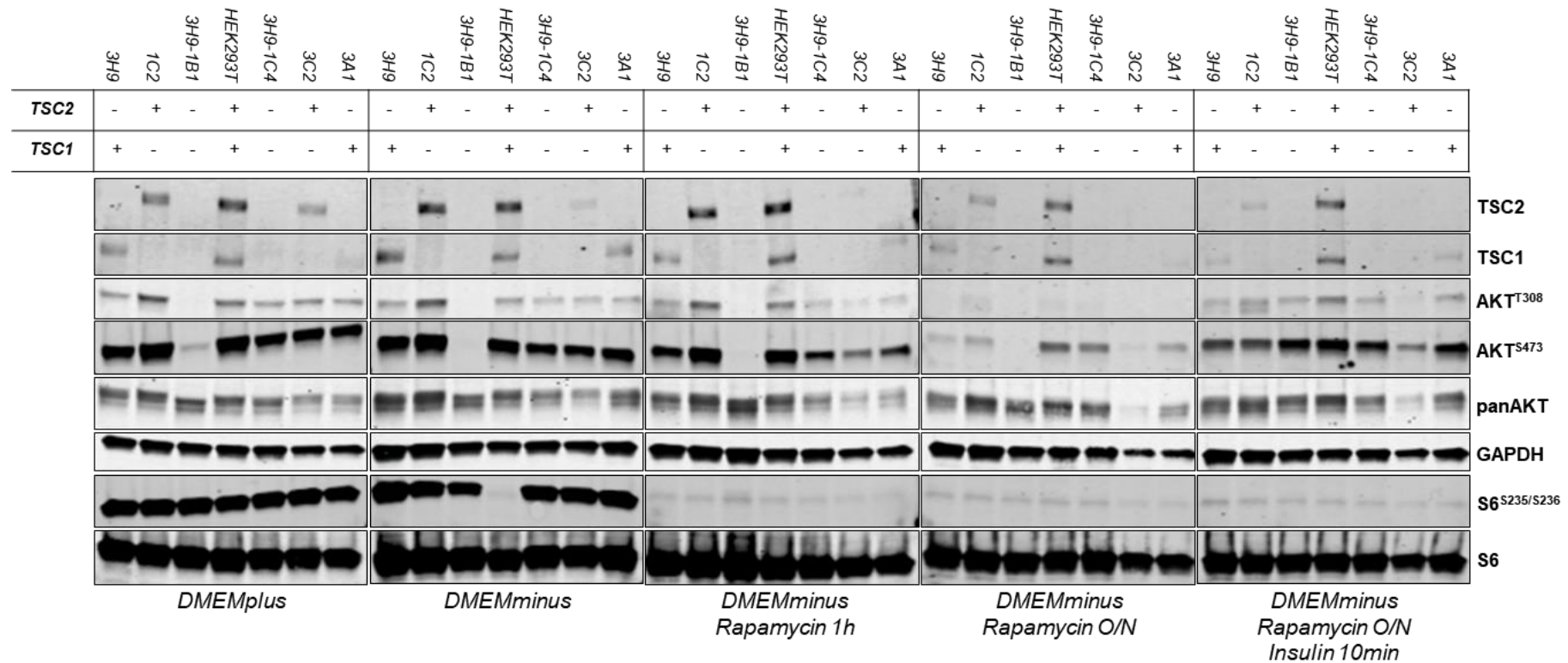


Figure III-6: Specific protein profile from genetically modified cell lines. (A) Table of cell lines showing if there is expression (+) or knock-out (-) of HEK293T *TSC1* and *TSC2* genes. (B) Western Blot of proteins isolated from cell lines according to table (A) expressing (+) *TSC1* and *TSC2* or not (-). Antibodies employed on blots detect proteins identified on the right end side (*TSC2*, *TSC1*, phosphorylated Thr<sup>308</sup> from AKT, phosphorylated Ser<sup>473</sup> from AKT, panAKT, GAPDH, both phosphorylated Ser<sup>235</sup> and Ser<sup>236</sup> from S6; and S6. The five blot panels represent distinct cell growth media and treatments, as labeled from left to right: DMEMplus, DMEMminus, DMEMminus and rapamycin for 1 hour, DMEMminus and rapamycin for 16 hours (O/N), and DMEMminus, rapamycin for 16 hours (O/N) and 10-minute treatment with insulin.

An apparent decrease was observed for TSC1 protein amount in serum-deprived cells treated with rapamycin, in the lack of TSC2 expression (Figure III-6, cell lines 3H9 and 3A1, three right panels). Moreover, treatment with rapamycin for 16 hours also apparently reduced TSC2 protein levels in the two cell lines without TSC1 protein (1C2 and 3C2). However, gel lanes seem to have different amount of lysate protein, causing an apparent reduction of total protein levels.

## B. *TSC1* and *TSC2* mRNA analyses

Reference HEK293T cells, under complete (DMEMplus) or minimum (DMEMminus) medium, disclosed *TSC2* mRNA RPKM values respectively 2.13 and 2.70 times higher than *TSC1* transcript RPKM (Figure III-7). These data show that independently on the presence of calf serum *TSC2* mRNA levels are at least twice that of *TSC1* mRNA in the reference HEK293T cell line, as previously reported in databases for other cell lines and tissue (Figure III-1 and Figure III-2).

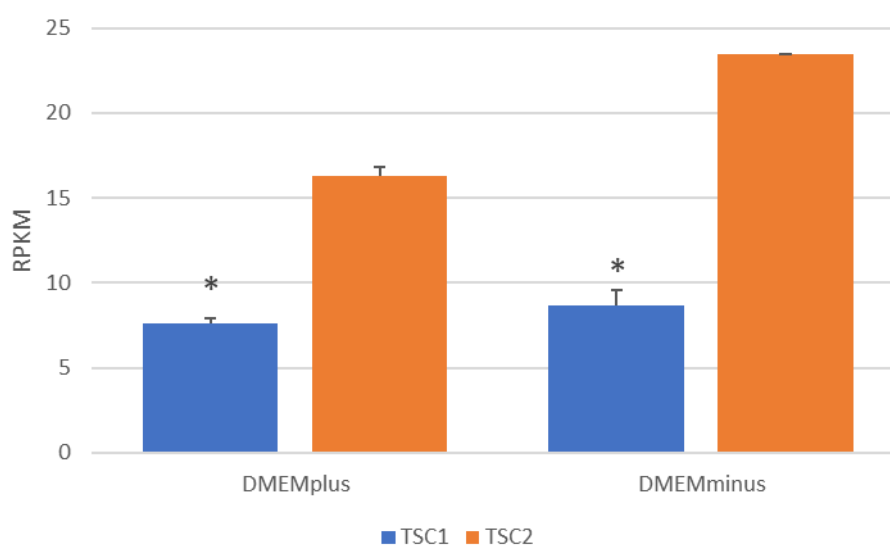


Figure III-7: *TSC1* and *TSC2* mRNA RPKM upon DMEMplus and DMEMminus treatments. Student *t*-test was calculated, and asterisk represents a statistically significant difference between *TSC1* and *TSC2* RPKM, under the same culturing condition ( $p$ -value < 0.05).

As *TSC2*-to-*TSC1* mRNA ratio appeared similar to RNA-Seq reports on other tissues and cell lines, we subsequently analyzed their mRNA amount in HEK293T-derived cell lines for two groups, DMEMplus and DMEMminus. For that, we employed DESeq2 pairwise comparisons to the reference HEK293T cell line, a test based on binary logarithmic fold change of the gene total mRNA read count. In DMEMplus group, in the lack of the TSC1 protein (cell lines 1C2 and 3C2, and 3H9-1B1 and 3H9-1C4), there is a significant reduction of *TSC1* mRNA (Figure III-8A). The fold change of those reductions was in average 39% (34% to 44%) as the binary logarithm varied between -0.60 and -0.83 (Figure III-8A).

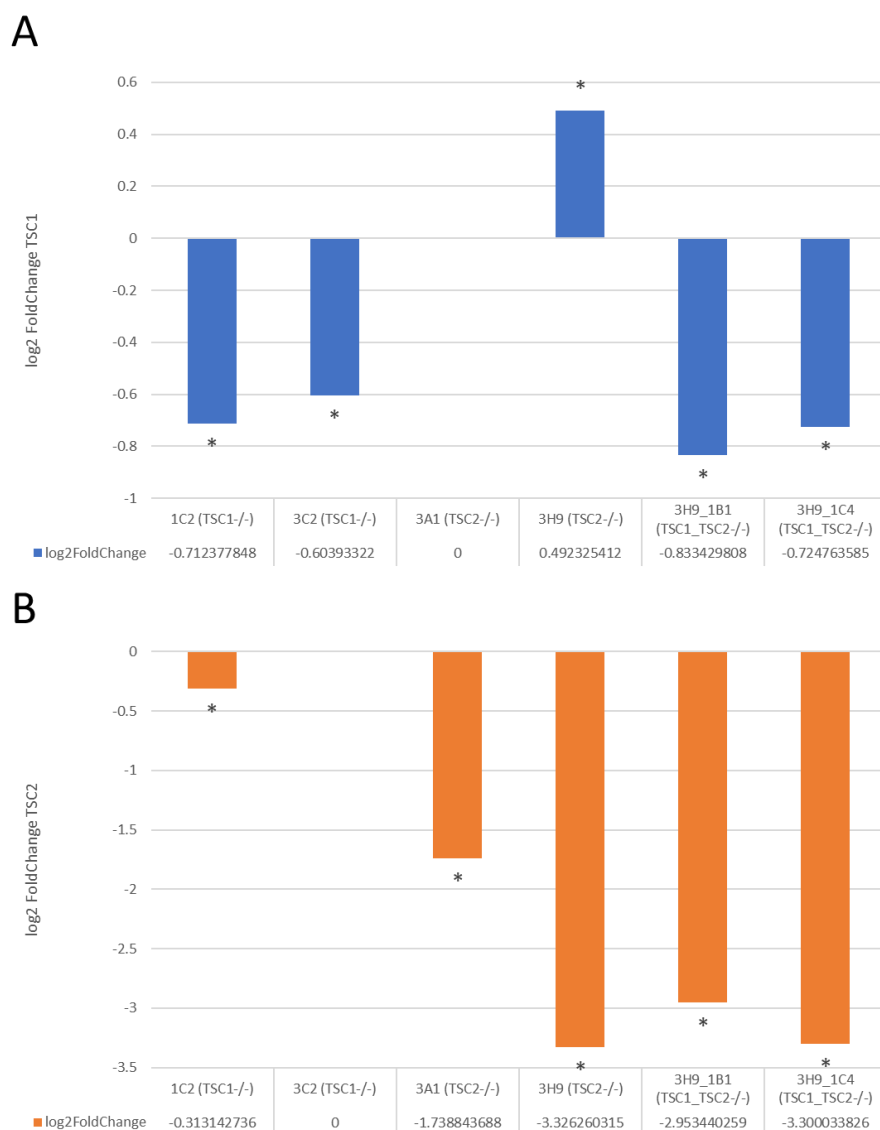


Figure III-8: Log2FoldChange of *TSC1* (A) and *TSC2* (B) transcript amount upon DMEMplus treatment. Asterisk =  $p$ -value<0.05.

The absence of the *TSC2* protein (cell lines 3A1, 3H9, 3H9-1B1 and 3H9-1C4) related to a significant decrease in *TSC2* mRNA amount (Figure III-8B). Notably, the fold change of these *TSC2* mRNA reductions were considerably higher, in average 84% (70% to 90%) as the binary logarithm varied between -1.74 and -3.33 (Figure III-8B). The limited length of the *TSC2* mRNA from the cell lines 3A1, 3H9, 3H9-1B1 and 3H9-1C4 (85% the size of the full-length wild-type *TSC2* mRNA) generates a relatively lower number of reads. Thus, when adjusted to the wild-type transcript length and data size (RKPM) a drastic reduction is expected. Remarkably, 3A1 cell line, which is heterozygous for the gross *TSC2* deletion, presented a less severe reduction of *TSC2* mRNA (70%; Figure III-8B).

When the effect of one protein absence was evaluated on the mRNA levels from the partnering gene, cell lines with the same knock-out genotype were discordant. Cells without the *TSC1* protein had no effect or

discrete reduction of *TSC2* mRNA (1C2 and 3C2; Figure III-8B). On the other hand, cells lacking the TSC2 protein had equal amounts or significantly more *TSC1* mRNA than the reference HEK293T cell line (Figure III-8A).

When the same mRNA analysis was conducted for the DMEMminus group, we observed fold-change reductions of *TSC2* mRNA in the absence of TSC2 protein similar to the comparisons from the DMEMplus group, in average 88% reduction (78% to 93%). The lowest reduction (78%) was from the cell line with one allele with *TSC2* gross deletion. We additionally disclosed a concordant, significant, more discrete reduction of *TSC2* mRNA in both cell lines (1C2 and 3C2) that have no TSC1 protein (Figure III-9B). In the DMEMminus group, four cell lines had undetectable change in *TSC1* mRNA levels in the binary logarithmic scale. One cell line without TSC1 protein (1C2) had a significant reduction of *TSC1* mRNA (39% decrease), as observed for the DMEMplus group (Figure III-9A). In another cell line, which lacks TSC2 protein (3H9), *TSC1* mRNA increased nearly 70% when compared to the reference HEK293T cell line (Figure III-9A).

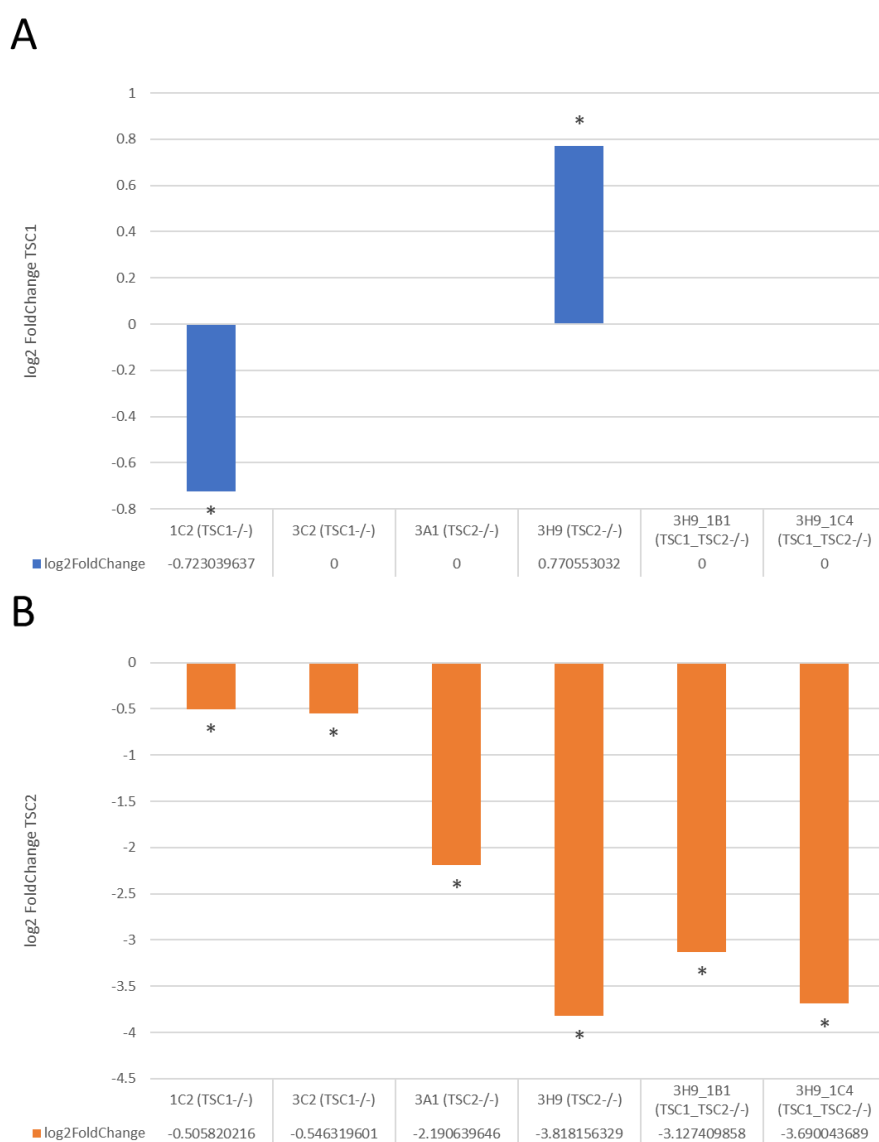


Figure III-9: Log2FoldChange of *TSC1* (A) and *TSC2* (B) transcript amount upon DMEMminus treatment. Asterisk =  $p$ -value<0.05.

Table III-2: Observed *TSC1* and *TSC2* mRNA and protein levels for each knock-out cell line comparatively to HEK293T.

Knocked-out gene(s)		<i>TSC1</i>		<i>TSC2</i>		<i>TSC1 and TSC2</i>	
Cell line		1C2	3C2	3A1	3H9	3H9-1B1	3H9-1C4
Protein	<i>TSC1</i>	-	-	↓	↓	-	-
	<i>TSC2</i>	↓	↓	-	-	-	-
mRNA (DMEMplus)	<i>TSC1</i>	↓	↓	-	↑	↓	↓
	<i>TSC2</i>	↓	-	↓	↓	↓	↓
mRNA (DMEMminus)	<i>TSC1</i>	↓	-	-	↑	-	-
	<i>TSC2</i>	↓	↓	↓	↓	↓	↓

(-) unchanged or not observed; (↓) reduced; (↑) increased.

As observed in Table III-2, 1C2 was the only cell line that independently on the medium consistently decreased *TSC1* and *TSC2* mRNA levels. This cell line expresses no *TSC1* protein. It presented decreased *TSC2* protein as well as *TSC1* and *TSC2* mRNA amounts. The two CRISPR/Cas9-generated deletions in 1C2 are fairly small, in homozygosity. One deletion abolishes the translational start codon, and the other generates a frameshift, with no significant impact on the *TSC1* mRNA length. Therefore, we selected the 1C2 cell line for further experiments, aiming at global analyses of the effects of the lack of *TSC1* protein on the cell transcriptome.

### C. Global RNA analysis in the 1C2 cell line

The number of differentially expressed genes in the 1C2 cell line compared to HEK293T (reference) gene set is indicated in Table III-3 for both DMEMminus and DMEMplus groups. 1C2 genes with less mRNA or more mRNA than the reference cell line were considered respectively down- or up-regulated. In all comparisons, Benjamini-Hochberg test was employed with FDR <0.05.

Table III-3: Number of genes down- or up-regulated in 1C2 cell line in regard to HEK293T in DMEMplus or DMEMminus groups.

Groups compared	Number of down-regulated genes and labeled group ID*	Number of up-regulated genes and labeled group ID*
1C2 X HEK293T (DMEMminus)	645 (B2)	474 (C2)
1C2 X HEK293T (DMEMplus)	1,411 (B3)	1,746 (C3)

\*Group ID in parenthesis

Gene ontology (GO) analysis of 645 genes down-regulated in 1C2 in minimum medium (DMEMminus) significantly grouped them in ten sets, as presented in Table III-4. The gene sets at this table is similar to the gene sets identified for down-regulated genes from DMEMplus group (N=1,411; Table III-5), and relate to post-transcription steps of gene expression.

Table III-4: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMminus group.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0006401	RNA catabolic process	341	10.66	43.15	0	0
GO:0090150	Establishment of protein localization to membrane	313	97.86	48.03	0	0
GO:0006413	Translational initiation	192	60.03	84.96	0	0
GO:0070972	Protein localization to endoplasmic reticulum	137	42.83	95.72	0	0
GO:0006605	Protein targeting	412	12.88	38.04	5,55E-12	9,44E-10
GO:0002181	Cytoplasmic translation	92	28.76	73.01	5,84E-09	8,28E-07
GO:0022613	Ribonucleoprotein complex biogenesis	440	13.76	28.35	4,54E-05	5,51E-03
GO:0071826	Ribonucleoprotein complex subunit organization	245	76.60	32.64	2,10E-03	2,22E-5
GO:0034248	Regulation of cellular amide metabolic process	385	12.04	21.60	1,99E-4	1,88E-2
GO:0006284	Base-excision repair	43	13.44	52.07	3,39E-4	2,88E-2

Table III-5: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMplus group.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0022613	Ribonucleoprotein complex biogenesis	440	40.71	27.51	0	0
GO:0006401	RNA catabolic process	341	31.55	29.80	0	0
GO:0006413	Translational initiation	192	17.76	37.72	0	0
GO:0070972	Protein localization to endoplasmic reticulum	137	12.67	37.08	4,44E-12	9,44E-10
GO:0071826	Ribonucleoprotein complex subunit organization	245	22.66	26.91	3,63E-09	6,00E-07
GO:0002181	Cytoplasmic translation	92	85.11	39.95	4,23E-09	6,00E-07
GO:0016072	rRNA metabolic process	236	21.83	24.73	2,58E-06	3,13E-04
GO:0090150	Establishment of protein localization to membrane	313	28.96	21.76	2,25E-05	2,39E-03
GO:0008380	RNA splicing	417	38.58	19.70	5,59E-05	5,28E-03
GO:0006605	Protein targeting	412	38.11	19.68	7,44E-05	6,33E-03

Up-regulated genes were significantly enriched in two gene sets for the DMEMminus group (Table III-6), and in at least ten GO sets for the DMEMplus group (Table III-7). Those gene sets relate to response to topologically incorrect protein and autophagic mechanism in DMEMminus group. The DMEMplus group of up-regulated genes was enriched in gene sets that relate to cell growth and morphogenesis.

Table III-6 Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMminus group.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0035966	Response to topologically incorrect protein	188	43.417	36.85	7,63E-6	6,48E-3
GO:0061919	Process utilizing autophagic mechanism	473	10.923	22.89	1,07E-4	4,56E-2

Table III-7: Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, in DMEMplus group.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO: 0034330	Cell junction organization	285	19.73	23.32	5,77E-04	4,90E-5
GO: 0022604	Regulation of cell morphogenesis	473	32.74	18.94	7,86E-03	3,34E-4
GO: 2000147	Positive regulation of cell motility	493	34.12	18.17	3,17E-6	8,45E-4
GO: 0010975	Regulation of neuron projection development	475	32.88	18.25	3,97E-6	8,45E-4
GO: 0060560	Developmental growth involved in morphogenesis	225	15.57	22.47	5,16E-6	8,78E-4
GO: 0090066	Regulation of anatomical structure size	487	33.71	17.80	8,74E-6	1,12E-4
GO: 0045785	Positive regulation of cell adhesion	392	27.132	18.797	9,25E-6	1,12E-3
GO: 0048638	Regulation of developmental growth	314	21.733	19.325	2,91E-5	3,09E-3
GO: 0048732	Gland development	434	30.039	17.644	3,70E-5	3,49E-3
GO: 0045926	Negative regulation of growth	250	17.304	20.227	5,17E-5	4,39E-3

As shown in Table III-3, we labeled as B2, B3, C2, and C3 the sets of differentially expressed genes from comparisons between 1C2 and HEK293T cell lines, if they were respectively down-regulated in DMEMminus or DMEMplus groups, or up-regulated in DMEMminus or DMEMplus groups. These gene group identifications were used to show in Table III-8 the intersections that were generated between gene sets from two experimental groups. The number of retrieved genes is indicated in the same table.

Table III-8: Number of genes observed when groups assigned in Table III-3 were intersected.

Intersected groups	Number of genes from intersection or intersection subtracted
$B2 \cap B3$	513
$C2 \cap C3$	326
$B2 - (B2 \cap B3)$	133
$B3 - (B2 \cap B3)$	1,234
$C2 - (C2 \cap C3)$	149
$C3 - (C2 \cap C3)$	1,086
B2: down-regulated genes in DMEMminus group from 1C2 X HEK293T comparison	
B3: down-regulated genes in DMEMplus group from 1C2 X HEK293T comparison	
C2: up-regulated genes in DMEMminus group from 1C2 X HEK293T comparison	
C3: up-regulated genes in DMEMplus group from 1C2 X HEK293T comparison	

The intersections between down-regulated gene sets ( $B2 \cap B3$ ) represent significantly down-regulated genes independently on the presence of serum. As shown in Table III-9, the twelve gene sets enriched in this intersection group ( $N = 513$ ) relates to post-transcriptional steps of gene expression, notably mRNA translation, and ATP generation. Thus, these biological processes appear down-regulated in the lack of the TSC1 protein, independently on culture serum (trophic factors). Differentially expressed genes uniquely detected in the presence of serum were enriched in gene sets also directly related to gene expression,

however, mostly to the nuclear steps of gene expression (Table III-10). Down-regulated genes detected independently on serum in DMEMminus but not in DMEMplus samples ( $B2 - (B2 \cap B3)$ ,  $N = 133$ ) were not significantly enriched in gene sets.

Table III-9: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, independently on the serum presence.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0090150	Establishment of protein localization to membrane	313	79.68	55.22	0	0
GO:0006413	Translational initiation	192	48.88	98.20	0	0
GO:0070972	Protein localization to endoplasmic reticulum	137	34.88	10.90	0	0
GO:0006401	RNA catabolic process	341	86.81	47.23	1,11E-12	2,36E-10
GO:0006605	Protein targeting	412	10.49	41.95	5,55E-12	9,44E-10
GO:0002181	Cytoplasmic translation	92	23.42	85.39	1,24E-09	1,76E-07
GO:0022613	Ribonucleoprotein complex biogenesis	440	11.20	32.14	6,45E-06	7,84E-04
GO:0071826	Ribonucleoprotein complex subunit organization	245	62.37	36.88	7,53E-04	8,00E-6
GO:0009141	Nucleoside triphosphate metabolic process	304	77.39	24.55	3,02E-4	2,85E-2
GO:0009123	Nucleoside monophosphate metabolic process	321	81.72	23.25	5,91E-4	5,02E-2
GO:0006091	Generation of precursor metabolites and energy	469	11.940	20.101	9,64E-4	7,44E-2
GO:0016072	rRNA metabolic process	236	60.080	24.967	1,07E-3	7,63E-2

Table III-10: Gene sets significantly enriched in down-regulated genes in 1C2 cell line in regard to HEK293T, detected in DMEMplus but not in DMEMminus samples

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0022613	Ribonucleoprotein complex biogenesis	440	29.50	25.76	1,33E-10	1,13E-07
GO:0006397	mRNA processing	487	32.65	21.74	3,64E-06	1,55E-03
GO:0008380	RNA splicing	417	27.96	22.53	8,74E-06	2,48E-03
GO:0006403	RNA localization	228	15.29	27.47	1,70E-05	3,61E-03
GO:0006401	RNA catabolic process	341	22.86	23.18	7,26E-05	1,23E-6
GO:1903311	Regulation of mRNA metabolic process	266	17.84	24.11	6,12E-04	8,66E-6
GO:0016072	rRNA metabolic process	236	15.82	24.64	1,39E-03	1,68E-5
GO:0010608	Posttranscriptional regulation of gene expression	486	32.59	19.33	3,15E-03	3,34E-5
GO:0051169	Nuclear transport	356	23.87	20.95	4,94E-03	4,66E-5
GO:0071826	Ribonucleoprotein complex subunit organization	245	16.43	23.13	1,03E-6	8,76E-5

There was no significant enrichment in gene sets for up-regulated genes in 1C2 cell line when compared to HEK293, independently on the serum presence ( $C2 \cap C3$ ,  $N = 326$ ). Gene sets such as response to topologically incorrect protein and regulation of protein stability were enriched in up-regulated genes in

DMEMminus but not in DMEMplus (Table III-11). Enriched gene sets for genes up-regulated in DMEMplus but not DMEMminus samples relate to cell morphogenesis (Table III-12).

Table III-11: Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, detected in DMEMminus but not in DMEMplus samples.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0035966	Response to topologically incorrect protein	188	14.47	62.19	1,45E-5	1,23E-2
GO:0031647	Regulation of protein stability	267	20.55	48.65	4,02E-5	1,70E-2

Table III-12: Gene sets significantly enriched in up-regulated genes in 1C2 cell line in regard to HEK293T, detected in DMEMplus but not in DMEMminus samples

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0034330	Cell junction organization	285	15.34	26.08	2,43E-04	2,06E-5
GO:0022604	Regulation of cell morphogenesis	473	25.46	19.64	3,70E-6	1,57E-3
GO:0060560	Developmental growth involved in morphogenesis	225	12.11	23.95	1,12E-5	3,18E-3
GO:0048638	Regulation of developmental growth	314	16.90	21.30	1,50E-5	3,18E-3
GO:0043087	Regulation of GTPase activity	472	25.40	18.50	3,40E-5	5,79E-3
GO:2000147	Positive regulation of cell motility	493	26.53	18.09	4,97E-5	7,04E-3
GO:0045785	Positive regulation of cell adhesion	392	21.10	18.96	7,60E-5	8,59E-3
GO:0010975	Regulation of neuron projection development	475	25.56	17.99	8,08E-5	8,59E-3
GO:0031589	Cell-substrate adhesion	332	17.87	19.59	1,11E-4	1,04E-2
GO:0090066	Regulation of anatomical structure size	487	26.21	17.55	1,45E-4	1,23E-2

When only the 1C2 cell line was considered for global RNA analyses, and the groups DMEMminus and DMEMplus (reference) were compared, we retrieved 72 and 51 genes that were respectively down - and up-regulated in 1C2 DMEMminus group. Gene sets that revealed a significant enrichment are presented in Table III-13 and Table III-14. We observed that response to topologically incorrect protein is both down- and up-regulated. Response to endoplasmic reticulum stress is down-regulated, whereas protein folding and response to temperature stress are up-regulated, consistently with the role of TSC1 protein as heat-shock protein co-chaperone.

Table III-13: Gene sets significantly enriched in down-regulated genes in DMEMminus group in regard to DMEMplus, in the 1C2 cell line.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0034976	Response to endoplasmic reticulum stress	268	10.47	76.22	9,07E-6	7,71E-3
GO:0006730	One-carbon metabolic process	25	0.10	30.64	1,23E-4	3,49E-2
GO:0035966	Response to topologically incorrect protein	188	0.73	81.49	9,04E-5	3,49E-2
GO:0070972	Protein localization to endoplasmic reticulum	137	0.54	93.18	1,94E-4	4,13E-2

Table III-14: Gene sets significantly enriched in up-regulated genes in DMEMminus group in regard to DMEMplus, in the 1C2 cell line.

Gene Set	Description	Size	Expect	Ratio	pValue	FDR
GO:0035966	Response to topologically incorrect protein	188	0.49510	18.178	1,08E-05	6,22E-03
GO:0051131	Chaperone-mediated protein complex assembly	20	0.052671	94.929	1,46E-05	6,22E-03
GO:0006457	Protein folding	210	0.55304	12.657	1,09E-6	3,09E-4
GO:0009266	Response to temperature stimulus	201	0.52934	11.335	1,30E-5	2,76E-3
GO:0070841	Inclusion body assembly	22	0.057938	51.780	2,51E-5	4,27E-3
GO:0046677	Response to antibiotic	316	0.83220	72.098	1,62E-4	2,29E-2
GO:0044839	Cell cycle G2/M phase transition	213	0.56094	89.136	2,27E-4	2,75E-2
GO:0071559	Response to transforming growth factor beta	238	0.62678	79.773	3,78E-4	4,02E-2

## V. Discussion

### A. Cell line characterization

In this study, we employed six cell lines derived from HEK293T cell line by CRISPR-Cas9 editing of the *TSC1* (chromosome 9q34.1) and/or *TSC2* (chromosome 16p13.3) genes. HEK293 cell line has been first obtained after immortalization by adenovirus transduction of primarily cultured human embryonic kidney (HEK) cells (Graham, Smiley *et al.* 1977). Although the identity of the cell that originally derived the HEK293 line is unknown, later analyses disclosed that HEK293 cells exhibit some adrenal cell properties, including neuronal-like morphophysiology (Lin, Boone *et al.* 2014). Therefore, the original HEK293 line should not be considered as a kidney-derived cell line, but a complex, heterogeneous cell line, likely derived from cells of the adrenal glands, which are anatomically related to the kidneys (Lin, Boone *et al.* 2014). Among further genomic modifications these cells have undergone, HEK293T cell line, employed in our study, originated upon SV40 transfection of the HEK293 cell line (Rio, Clark *et al.* 1985). HEK293T presents a combination of karyotypes with some chromosomes in three copies, leading to the classification of the cell line as hypotriploid or pseudotriploid (Lin, Boone *et al.* 2014). In 19 HEK293T chromosome metaphases examined by Lin, Boone *et al.* (2014), six cells presented t(9,17) translocations, five had duplicated chromosome 9 short arm, two cells had deleted 9q, one cell had a 9q duplication, and two cells had a translocation involving chromosome 16 (t(16,21) and t(16,22)). Although Lin, Boone *et al.* (2014) did not demonstrate the translocation breakpoints nor 9q34.1 and 16p13.3 trisomy in HEK293T cells, their data tend to suggest a situation of eventual di- or monosomy for the *TSC1* and *TSC2* genes. As *TSC1* and *TSC2* genes are considered haplosufficient in non-neuronal cells, we believe that HEK293T chromosome 9 and 16 ploidies are not a concern for the present study.

CRISPR-Cas9-edited HEK293T cell lines analyzed in the present study have been previously produced in the Nellist laboratory (Erasmus Medical Centre, Rotterdam, The Netherlands), and have *TSC1* and *TSC2* genotypes indicated in Figure III-3. Our Western blot analyses have shown that all cell lines that had either gene inactivated did not express the respective protein (Figure III-5 and Figure III-6), and had mTORC1 hyperactivated, as presented in the functional assay by S6-phosphorylation reporter (Figure III-6). Hyperactivated mTOR was observed under both DMEMplus and DMEMminus conditions, similarly to reports from the literature (Tee, Fingar *et al.* 2002, Garami, Zwartkruis *et al.* 2003). Amino acids, such as leucine and arginine, activate mTORC1 independently on growth factors from serum in cells where *TSC1* or *TSC2* is knocked out (Byfield, Murray *et al.* 2005, Nobukuni, Joaquin *et al.* 2005, Smith, Finn *et al.* 2005). As DMEM minimum medium contains 15 out of the 20 amino acids, it is believed that the growth factor-independent, amino-acid-dependent mTORC1 activation pathway should be undertaken. This pathway is regulated by the Rag GTPases, which allow mTORC1 to interact with the lysosome membrane. Therefore, in DMEMminus conditions, cells

negative for TSC1 or TSC2 present hyperphosphorylated S6-Ser<sup>235</sup> and Ser<sup>236</sup> (Figure III-6, second panel). Under conditions of amino acid depletion, Rag GTPases recruit TSC2 to the lysosome, interacting with Rheb. mTORC1 is thus released from the lysosome and becomes inactivated (Bar-Peled, Schweitzer *et al.* 2012). As seen in Figure III-6 (third and fourth panels), mTORC1 activity was inhibited upon rapamycin treatment for one or 16 hours, as shown by hypophosphorylation of the reporter S6-Ser<sup>235</sup>/Ser<sup>236</sup>.

Upon insulin or insulin-like growth factor (IGF) binding, the insulin/IGF receptor becomes activated by auto-phosphorylation, starting a signalling cascade that activates the PI3 kinase (PI3K)-Akt pathway. PI3K activity on membrane lipids is essential for Akt and PDK1 (phosphoinositide-dependent kinase 1) translocation to the cell membrane. PDK1 has been demonstrated to phosphorylate Akt-Thr<sup>308</sup>, thus inhibiting the TSC1/2 complex (Kwiatkowski and Manning 2005). On the other hand, considerable efforts have been made to understand the roles of different kinases shown to phosphorylate Akt-Ser<sup>473</sup>. For instance, mTORC2 (mechanistic target of rapamycin complex 2) has been reported to phosphorylate Akt-Ser<sup>473</sup> under a variety of physiological conditions, and to facilitate phosphorylation of AKT-Thr<sup>308</sup> by PDK1 (Sarbasov, Guertin *et al.* 2005). Similarly to mTORC1, the large molecular complex mTORC2 contains mTOR and mLST8 (mammalian lethal with Sec13 protein 8), but differs from the former complex as they present different additional partnering proteins, subcellular localization, functions and sensitivity to rapalogs. mTORC2 phosphorylates several members of protein kinase families A, B (Akt), C and G, affecting cytoskeleton remodelling, ion transport and cell survival, growth, proliferation (Reviewed by Menon, 2018).

We observed that serum starvation for 16 hours did not apparently affect the phosphorylation status of Akt residues Thr<sup>308</sup> and Ser<sup>473</sup> not even when followed by one-hour treatment with rapamycin (Figure 6). It is as yet difficult to explain the lack of effect of serum deprivation on Akt dephosphorylation. However, as there should be a reciprocal relation between Akt and the two mTOR complexes, different possibilities could be raised, such as inhibition of specific phosphatases, a delayed response to repress mTORC2 or susceptibility of Akt Thr<sup>308</sup> and Ser<sup>473</sup> to additional kinases.

The *TSC1*<sup>-/-</sup>, *TSC2*<sup>-/-</sup> 3H9-1B1 cell line had AKT hypophosphorylated in all treatments, a situation restored upon insulin treatment. A marked reduction in Akt activation was reported in *Tsc2*<sup>-/-</sup>/*Tp53*<sup>-/-</sup> and *Tsc1*<sup>-/-</sup> cells in response to serum (Zhang, Cicchetti *et al.* 2003). The combination of double knock-out 3H9-1B1 cell line and the intrinsic inhibition of p53 in HEK293T (Liljestrom, Klein *et al.* 2006) could explain why this cell line did not respond to Akt kinases (Figure III-6). However, 3H9-1B1 cell line did not considerably differ from 3H9-1C4, except for Akt phosphorylation status.

Knocking-out *TSC1* or *TSC2* genes by CRISPR-Cas9 genome edition did not affect the overall protein levels of upstream AKT protein or downstream S6 protein (Figure III-6). The TSC2 levels in *TSC1*<sup>-/-</sup> cells were reduced relative to the wild-type cell line HEK293T; likewise, the protein levels of TSC1 in *TSC2*<sup>-/-</sup> cells (Table

III-2). Our data do not corroborate observations that the knockout of *Tsc2* maintains levels of *Tsc1* (Zhang, Gao *et al.* 2003, Pollizzi, Malinowska-Kolodziej *et al.* 2009). However, embryonic fibroblasts obtained from murine *Tsc1* knockout model (MEF *Tsc1*<sup>-/-</sup>) showed reduced levels of *Tsc2* (Astrinidis, Senapedis *et al.* 2006). *TSC1* transient transfection increased endogenous levels of *TSC2* in wild-type cells, and transient co-transfection of *TSC1* with *TSC2* in the same cell type increased *TSC2* levels (Benvenuto, Li *et al.* 2000). These observations can be attributed to the *Tsc1* property as a co-chaperone of Hsp90 and properly folding of *Tsc2* (Woodford, Sager *et al.* 2017). The absence of *TSC1* in human cell line might have the same effects as in mouse cells, what could inhibit the folding of *TSC2*, thus leading to its ubiquitination and degradation. In the same way, low levels of *TSC2* in knocked-out cell lines should lead to ubiquitination and degradation of *TSC1*. Part of non-degraded *TSC1* could accumulate in the cell as partially unfolded, self-aggregated protein, as suggested by Nellist, van Slegtenhorst *et al.* (1999). As *TSC1* protein has been considered a chaperone-allied protein, this scenario considering both protein degradation and aggregation could be closely examined to answer as well if other protein species are also unfolded and present in the *TSC1* aggregates.

## B. *TSC1* and *TSC2* gene expression regulation

The expression of *TSC2* has been observed to be higher than that of the *TSC1* gene at the mRNA level (Figure III-1 and 2), independently whether cells are growing under complete or minimum medium (Figure III-7). However, the half-life of mRNA depends on transcription activity as well as decay mechanisms. Thus it is recommended to refer to as alteration in mRNA amount or levels, as the responsible mechanisms cannot be inferred without further experiments. Furthermore, one cannot infer a quantitative correlation between mRNA and the respective protein taking into account the mRNA amount, as there are various post-transcriptional steps regulating gene expression.

Regardless of medium conditions, the reduction of *TSC2* mRNA expression has been more pronounced than those in *TSC1* mRNA levels. This higher decrease in expression of *TSC2* may be due to the genomic edition, which subtracted nearly 4.8 Kb of *TSC2* coding sequence (Figure III-3B). Consequently, the *TSC2*<sup>-/-</sup> cell lines should provide significantly lower RNA-Seq *TSC2* read count, and thus they do not constitute an appropriate model for *TSC1/2* gene regulation studies. Furthermore, the unaffected *TSC1* mRNA levels in the DMEMminus group (similar to HEK293T *TSC1* mRNA levels) suggest that the *TSC1* mRNA down-regulation should be sensitive to the presence of growth factors, *TSC1* and *TSC2* proteins. As mTORC1 has been functionally involved in transcriptional control as well translational regulation, it should be interesting to study if rapamycin treatment affects *TSC1* mRNA in the 1C2 cell line. We also disclosed a discrete reduction of *TSC2* mRNA in both cell lines that have no *TSC1* protein (Figure 9B) under minimum medium condition.

The extension of the mRNA 5'-untranslated regions (5'-UTR) is similar for human *TSC1* (234 bp) and *TSC2* (200 bp) mRNA. The 230-bp 5'UTR of mouse *Tsc1* is 88% (202/230) similar to the human orthologous

5'UTR. The 119-bp 5'UTR of mouse *Tsc2* is 70% (83/119) similar to the 5'UTR of the human orthologue. However, human *TSC1* (4,887 bp) and *TSC2* (109 bp) 3'- untranslated region (3'-UTR) lengths are significantly different. Human and mouse *Tsc1* 3'-UTR length and sequence are conserved, the latter containing 3,999 bp. On the other hand, although human and mouse *Tsc2* 3'-UTRs have similar lengths, the latter with 131 bp, the sequences display lower similarity (51%, 56/109). The longer *TSC1* 3'-UTR may infer that this mRNA is more responsive to translational regulation and more stable than *TSC2* mRNA, as it may harbor *cis* regulatory motifs in that transcribed segment. Furthermore, changes in mRNA amount may build a feedback control loop on TSC gene transcription. Studies based on quantitative RT-PCR of the *TSC1* and *TSC2* cDNA, combined with the use of chemical transcriptional, translational or proteasomal inhibitors are likely to clarify the type of regulation to which TSC mRNA and proteins should be submitted. The data presented here, along with the anatomy of the *TSC1* gene, suggest that *cis* elements in the mRNA 3'-UTR region could regulate the stability of the transcript. Finally, if RNA decay and translation appear to regulate the steady state of *TSC1* protein and mRNA, it would be worth screening *TSC1* mRNA for *cis*-regulating motifs.

### C. Protein stress response in TSC1-negative cell line

*TSC1*<sup>-/-</sup> 1C2 cell line has one deletion encompassing the translational start codon and another causing frameshift, basically with no impact on *TSC1* RNA length. Considering that more than 80% of TSC patients have point or small indel pathogenic variants (Niida, Lawrence-Smith *et al.* 1999, van Slegtenhorst, Verhoef *et al.* 1999, Dabora, Jozwiak *et al.* 2001, Au, Williams *et al.* 2007), the 1C2 cell line should be a good model study loss-of-function *TSC1* mutations. Although it was not an objective of the present work, we were provided with RNA-Seq from the seven cell lines under different growth conditions. As the 1C2 cell line appeared to consistently affect *TSC1* and *TSC2* mRNA and protein, its RNA-Seq data were analyzed under complete or minimum medium in comparison to HEK293T reference cell line.

Under growth conditions, 1C2 down-regulated mRNAs related to posttranscriptional steps of gene expression, notably RNA processes and translation (Table III-10), and was not considerably different from gene sets enriched in down-regulated genes for starvation conditions (Table III-4). However, the latter group was more represented by translation-associated GOs. It is important to note that the differentially expressed genes have not been identified or studied on an individual basis. Therefore, it is possible that translation-associated GOs be enriched in translation repression genes, which are expected to be down-regulated when mTOR is hyperactivated (Goncharova, Goncharov *et al.* 2002).

Likewise, it is important to put in evidence that the GO response to unfolded proteins has been identified in both down-regulated and up-regulated gene sets, inferring that, if truly positive evidences, a detailed analysis of each gene function is advised, as positive and negative regulatory functions could be represented. These data, as well as the gene sets for the ER stress response are consistent with the role of

TSC1 as HSP90 co-chaperone (Woodford, Sager *et al.* 2017, Sager, Woodford *et al.* 2018, Sager, Woodford *et al.* 2018).

The DMEMplus group presented up-regulated genes significantly enriched in cell growth and morphogenesis sets while, in DMEMminus set, topologically incorrect protein and autophagic mechanisms were enriched. Cell size phenotypes have been observed in *Tsc1*<sup>-/-</sup> cells from *Drosophila melanogaster* that dramatically increased in size, yet differentiated normally (Potter, Huang *et al.* 2001). Autophagic mechanisms are related to the TSC1/2 proteins, which up-regulate this pathway, which becomes inhibited upon mTORC1 hyperactivation (Martina, Chen *et al.* 2012, Efeyan, Zoncu *et al.* 2013).

We did not find significant enrichment in gene sets for up-regulated genes independently on the presence of serum. However, up-regulated gene sets in DMEMplus group did relate to cell morphogenesis. This transcriptional trait for cell morphology of 1C2 *TSC1*<sup>-/-</sup> are in agreement with previously reports, in which overexpression of the *TSC1* gene product hamartin results in changes of cell morphology (Benvenuto, Li *et al.* 2000).

Finally, when only the 1C2 cell line was considered for global RNA analyses between DMEMminus and DMEMplus groups, more than 50 down-regulated genes have been detected. Similarly to up-regulated genes on DMEMminus group, response to topologically incorrect protein is both down- and up-regulated, which means that incorrect protein folding is an intrinsic characteristic of the 1C2 *TSC1*<sup>-/-</sup> cell line. Interestingly, response to endoplasmic reticulum (ER) stress is down-regulated. The ER is the cellular compartment where synthesis of proteins for the secretory pathway takes place. The ER stress response has been observed in mTORC1 activation in TSC2-null Elt3 cells via c-MYC translation (Babcock, Nguyen *et al.* 2013). mTORC1 hyperactivation increases the basal level of ER stress via an accumulation of unfolded proteins (Clarke, Chambers *et al.* 2014, Johnson, Dunlop *et al.* 2018). Taking together, a stress on this cellular compartment due to mTORC1 hyperactivation may cause an accumulation of unfolded proteins leading to a negative feedback and down regulate genes related to post-transcriptional steps, as observed for 1C2 cell line. It is worth to note that ER stress can develop in TSC-associated cells due to mTORC1-driven protein translation. mTORC1 inhibition of ER stress by RAD001 suppressed angiomyolipoma cell proliferation in a cytostatic manner (Siroky, Yin *et al.* 2012). Finally, protein folding and response to temperature stress are up-regulated in the 1C2 cell line, consistent with the role of TSC1 protein as a heat-shock co-chaperone protein (Woodford, Sager *et al.* 2017).

## VI. Conclusions

In this study, we employed six cell lines derived from HEK293T cell line by CRISPR-Cas9 editing of the *TSC1* and/or *TSC2* genes. Western blotting analyses have shown no protein expressed by the respective inactivated gene, and mTORC1 hyperactivation. Serum starvation for 16 hours did not affect the phosphorylation status of Akt residues Thr<sup>308</sup> and Ser<sup>473</sup>, as well as the overall protein levels of upstream AKT protein or downstream S6 protein. TSC2 levels were reduced in *TSC1*<sup>-/-</sup> cells relative to the wild-type cell line HEK293T; likewise, the protein levels of TSC1 decreased in *TSC2*<sup>-/-</sup> cells. The *TSC1*<sup>-/-</sup> cell line 1C2 was selected for RNA-Seq analyses as it harbored limited *TSC1* indel variants produced by CRISPR/Cas editing, thus not impacting its mRNA length. It also consistently presented reduction of *TSC2* mRNA and protein amount. Upon serum withdrawal, 1C2 cells had significant alterations of mRNA amount of genes involved in protein folding, which clustered in two distinct groups of either up- or down-regulated mRNA levels. The gene IDs in each group are consistent with the role of TSC1 as co-chaperone of heat-shock proteins.

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## VIII. Supplementary Tables

Supplementary Table III-1: RNA sample information. RQN: RNA quality number. QC: Quality control. All samples passed on RQN and QC.

Customer ID	Material Type	RQN	Entry QC (ng/ul)
293T (DMEM +/+)	total RNA	10	1551.86
1C2 (DMEM +/+)	total RNA	10	1262.16
3H9 (DMEM +/+)	total RNA	10	1582.19
3H9-1B1 (DMEM +/+)	total RNA	10	1197.36
293T (DMEM -/-)	total RNA	10	1138.53
1C2 (DMEM -/-)	total RNA	10	885.78
3H9 (DMEM -/-)	total RNA	10	501.64
3H9-1B1 (DMEM -/-)	total RNA	10	754.1
293T (DMEM +/+)	total RNA	10	981.95
3C2 (DMEM +/+)	total RNA	10	911.37
3A1 (DMEM +/+)	total RNA	10	1016.13
3H9-1C4 (DMEM +/+)	total RNA	10	773.92
293T (DMEM -/-)	total RNA	10	858.66
3C2 (DMEM -/-)	total RNA	10	621.08
3A1 (DMEM -/-)	total RNA	10	816.27
3H9-1C4 (DMEM -/-)	total RNA	10	960.41
293T (DMEM +/+)	total RNA	10	867.13
3C2 (DMEM +/+)	total RNA	10	768.35
1C2 (DMEM +/+)	total RNA	10	1023.06
3A1 (DMEM +/+)	total RNA	10	547.62
3H9 (DMEM +/+)	total RNA	10	1357.19
3H9-1C4 (DMEM +/+)	total RNA	10	466.22
3H9-1B1 (DMEM +/+)	total RNA	10	774.4
293T (DMEM -/-)	total RNA	10	911.17
3C2 (DMEM -/-)	total RNA	10	887.26
1C2 (DMEM -/-)	total RNA	10	871.46
3A1 (DMEM -/-)	total RNA	10	1108.38
3H9 (DMEM -/-)	total RNA	10	1198.26
3H9-1C4 (DMEM -/-)	total RNA	10	1134.05
3H9-1B1 (DMEM -/-)	total RNA	10	825.42

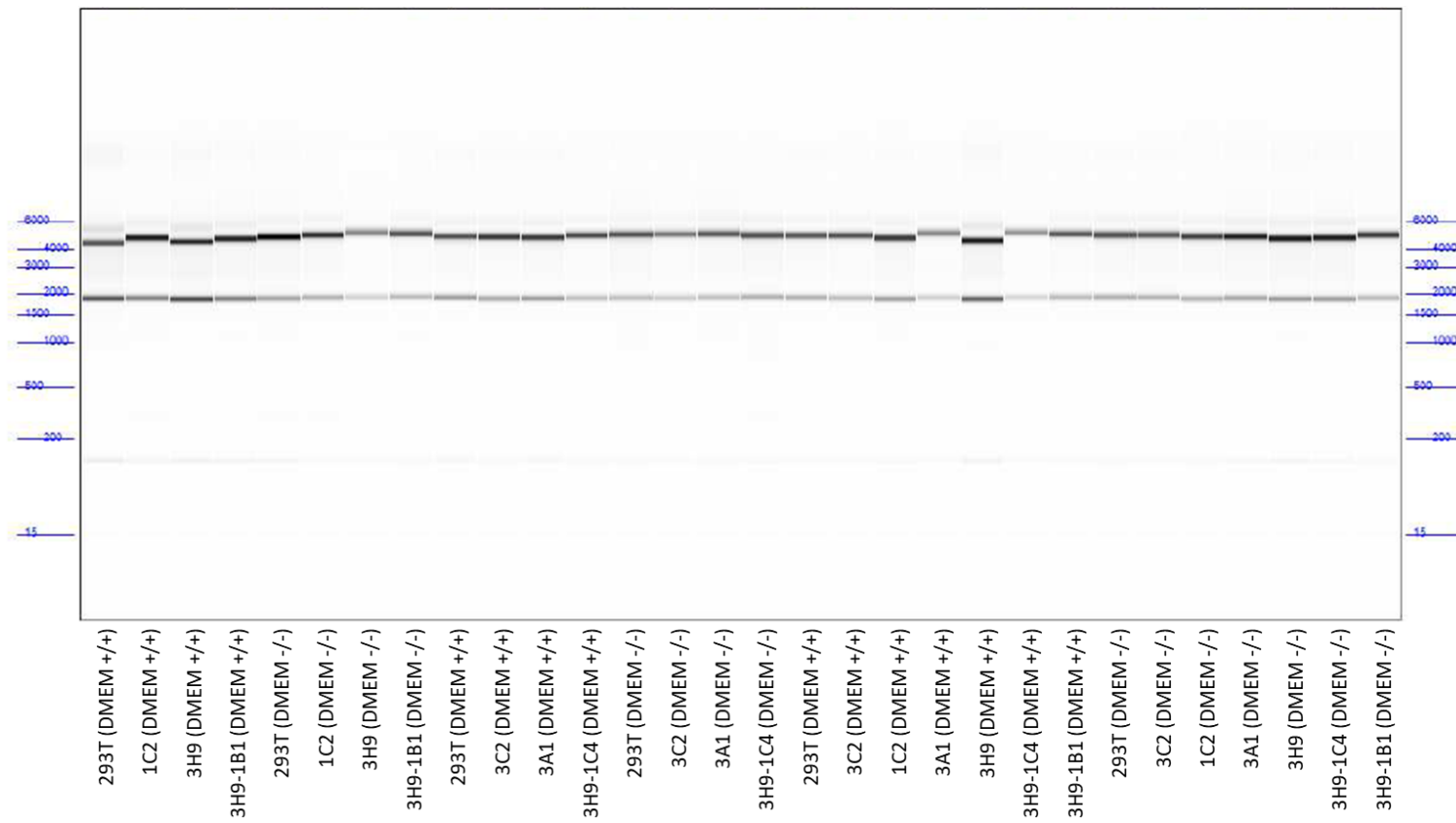
Supplementary Table III-2: Library construction and Molarity.

Customer ID	Molarity (nM)
293T (DMEM +/+)	5.33
1C2 (DMEM +/+)	3.75
3H9 (DMEM +/+)	4.40
3H9-1B1 (DMEM +/+)	5.00
293T (DMEM -/-)	7.30
1C2 (DMEM -/-)	7.57
3H9 (DMEM -/-)	8.10
3H9-1B1 (DMEM -/-)	4.28
293T (DMEM +/+)	6.82
3C2 (DMEM +/+)	7.05
3A1 (DMEM +/+)	10.40
3H9-1C4 (DMEM +/+)	4.44
293T (DMEM -/-)	6.89
3C2 (DMEM -/-)	6.56
3A1 (DMEM -/-)	7.13
3H9-1C4 (DMEM -/-)	9.10
293T (DMEM +/+)	6.62
3C2 (DMEM +/+)	5.05
1C2 (DMEM +/+)	7.48
3A1 (DMEM +/+)	6.44
3H9 (DMEM +/+)	7.08
3H9-1C4 (DMEM +/+)	9.79
3H9-1B1 (DMEM +/+)	4.23
293T (DMEM -/-)	5.32
3C2 (DMEM -/-)	4.71
1C2 (DMEM -/-)	5.01
3A1 (DMEM -/-)	6.72
3H9 (DMEM -/-)	3.68
3H9-1C4 (DMEM -/-)	3.56
3H9-1B1 (DMEM -/-)	3.13

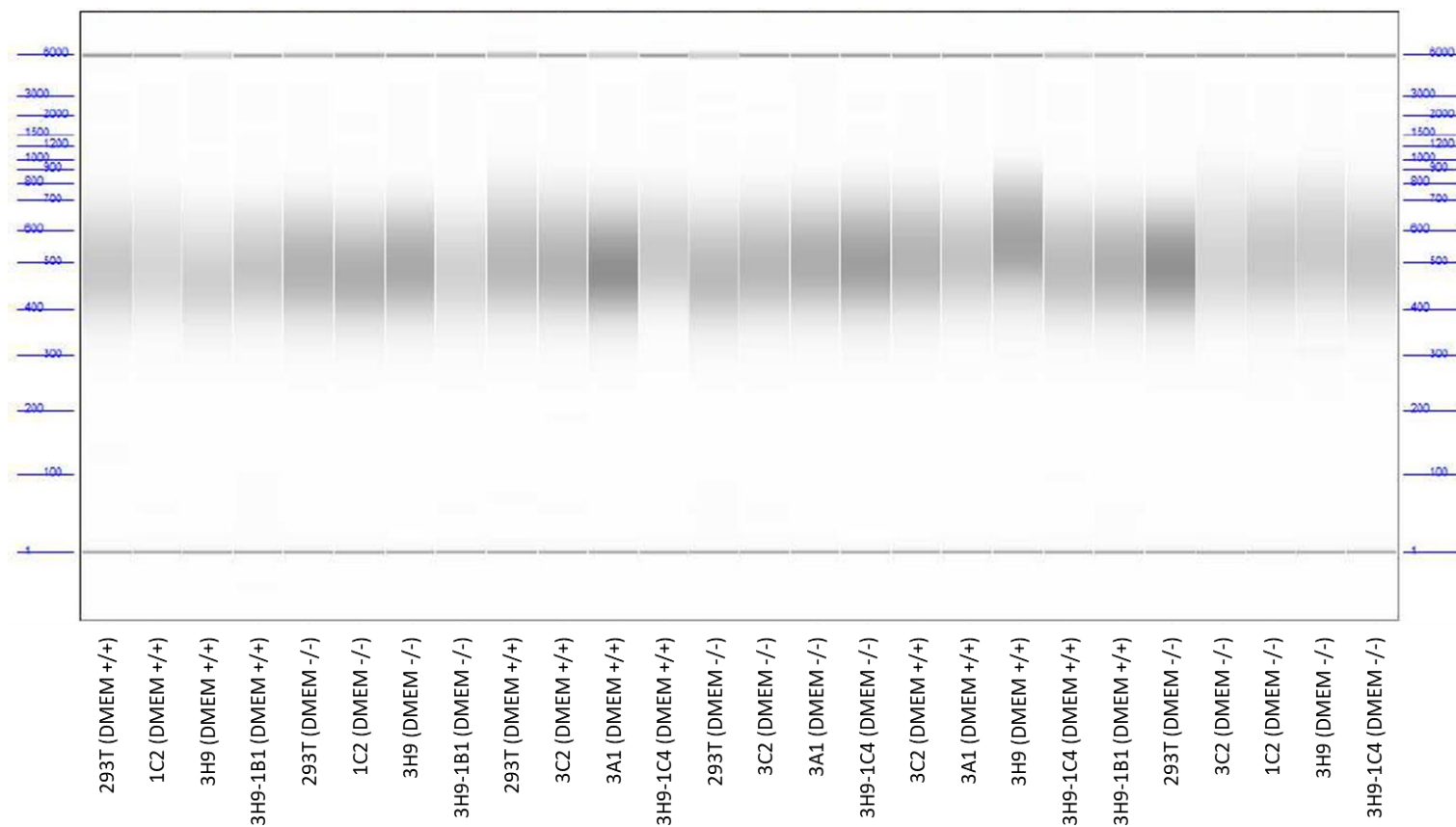
Supplementary Table III-3: Run yields, clusters and percentage of Q30 per sample.

<b>Sample ID</b>	<b>Yield (Mb)</b>	<b>Clusters</b>	<b>% &gt;=Q30</b>
293T (DMEM +/+)	4,359	14,435,335	90.68
1C2 (DMEM +/+)	3,543	11,731,379	90.14
3H9 (DMEM +/+)	4,518	14,957,866	91.35
3H9-1B1 (DMEM +/+)	4,192	13,881,889	90.68
293T (DMEM -/-)	4,357	14,428,154	90.83
1C2 (DMEM -/-)	4,204	13,918,424	90.96
3H9 (DMEM -/-)	4,281	14,175,446	90.64
3H9-1B1 (DMEM -/-)	3,578	11,847,647	90.72
293T (DMEM +/+)	3,835	12,698,726	90.25
3C2 (DMEM +/+)	3,611	11,954,942	90.54
3A1 (DMEM +/+)	3,984	13,191,862	90.69
3H9-1C4 (DMEM +/+)	3,970	13,146,674	89.93
293T (DMEM -/-)	4,052	13,416,176	90.31
3C2 (DMEM -/-)	3,840	12,715,334	90.37
3A1 (DMEM -/-)	3,554	11,770,421	90.06
3H9-1C4 (DMEM -/-)	5,331	17,651,436	90.11
293T (DMEM +/+)	6,269	20,757,887	91.08
3C2 (DMEM +/+)	4,114	13,620,545	90.06
1C2 (DMEM +/+)	6,498	21,514,943	90.54
3A1 (DMEM +/+)	4,255	14,088,555	90.05
3H9 (DMEM +/+)	3,561	11,791,372	89.18
3H9-1C4 (DMEM +/+)	3,894	12,895,789	90.23
3H9-1B1 (DMEM +/+)	3,529	11,685,430	89.36
293T (DMEM -/-)	3,711	12,287,753	90.18
3C2 (DMEM -/-)	7,429	24,598,652	89.78
1C2 (DMEM -/-)	3,477	11,513,170	89.65
3A1 (DMEM -/-)	5,368	17,775,044	89.78
3H9 (DMEM -/-)	4,043	13,384,615	88.99
3H9-1C4 (DMEM -/-)	3,959	13,110,984	89.72
3H9-1B1 (DMEM -/-)	3,940	13,045,184	89.55

## IX. Supplementary Figures



Supplementary Figure III-1: Gel image of total RNA fragment analysis. Separation at 8.0 kV, for 40.0 min.



Supplementary Figure III-2: Gel image of NGS Fragment 1-6000 bp analysis. Separation at 6.0 kV, for 50.0 min.

# Portfolio

## Complementary Studies

1. 2017: Short course in NGS in DNA Diagnostics Course. (Course load: 24h). Erasmus Medical Center, ERASMUS MC, Rotterdam, Holand
2. 2016: Short course in New Resources at the UCSC Genome Browser for the Display and Interpretation. (Course load: 2h). American Society Of Human Genetics, Bethesda, United States of America
3. 2015: Short course in Genome Editing. (Course load: 6h). Latin American School of Human and Medical Genetics, ELAG, Brazil
4. 2015: Short course in Next Generation Sequencing. (Course load: 6h). Latin American School of Human and Medical Genetics, ELAG, Brazil
5. 2014: Short course in iSeqTools to demistify the cloud and genomics. (Course load: 4h). American Society of Human Genetics, ASHG, United States of America
6. 2014: Short course in Principio da Técnica de interferências por RNA. (Course load: 4h). Sociedade Brasileira de Genética, SBG, Ribeirao Preto, Brazil
7. 2014: Short course in Ensembl Highlights: Intermediate/Advanced Workshop. (Course load: 4h). American Society of Human Genetics, ASHG, United States of America

## Chapters of published books

1. **Dufner-Almeida, Luiz Gustavo**; do Carmo, Ramon Torreglosa; Masotti, Cibele; Haddad, L A, *Understanding human DNA variants affecting pre-mRNA splicing in the NGS era* In: Advances in Genetics.103 ed.: Elsevier, 2019, p. 39-90. ISBN: 9780128171592, Home page: <https://linkinghub.elsevier.com/retrieve/pii/S0065266018300348> (Appendix 3)

## Complete articles published in journals

1. **Almeida, Luiz Gustavo Dufner**; Cruz, Dayane Bernardino da; Mingroni Netto, Regina Célia; Batissocho, Ana Carla; Ferraz, Jeane Oiticica Ramalho; Silva, Rodrigo Salazar, *Stem-cell therapy for hearing loss: are we there yet?*. Brazilian Journal of Otorhinolaryngology, v.85, p.520 - 529, 2019. [doi:10.1016/j.bjorl.2019.04.006] (Appendix 4)

## Papers published in event annals

1. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Masulk, L.; Richards, M.; Miranda, N.; Silva, I. M. W.; Chiavegatto, S.; Passos-Bueno, M. R.; Antoniuki, S. A.; Rosemberg, S.; Haddad, L A, *Segmental genomic deletions and splicing mutations causing tuberous sclerosis complex (TSC)* In: American Society of Human Genetics 66th Annual Meeting, 2016, Vancouver, BC, Canada. American Society of Human Genetics 66th Annual Meeting. , 2016. p.1 – 1996
2. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Rosemberg, S.; Haddad, Luciana A H, *A relatively high rate of TSC1 mutations causing tuberous sclerosis complex (TSC) in a cohort of 21 patients* In: XI Course of the Latin American School of Human and Medical Genetics, 2015, Caxias do Sul, RS., XI Course of the Latin American School of Human and Medical Genetics2015.

3. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Masulk, L.; Richardz, M.; Miranda, N.; Antoniuk, S. A.; Rosemberg, S.; HADDAD, L. A. *Tuberous sclerosis complex-causing mutations analyzed in a cohort of 41 Brazilian patients* In: ASHG 65th Annual Meeting, 2015, Baltimore, MD, USA. ASHG 65th Annual Meeting, 2015.
4. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Masulk, L.; Richardz, M.; Miranda, N.; Antoniuk, S. A.; Rosemberg, S.; HADDAD, L. A., *Analyses of TSC genes among Brazilian tuberous sclerosis complex (TSC) patients* In: ASHG 64th Annual Meeting, 2014, San Diego, Ca, USA. ASHG 64th Annual Meeting. 2014.

## Presentation of work and lecture

1. Nanhoe, S.; **Dufner-Almeida, L.G.**; Elfferich, P.; Hoogeveen-Westerveld, M.; Swenker, R.; Hossinzadeh, M.; Kom-Gortat, R.; Brouwer, R.; Ijcken, W. V.; Ekong, R.; Povey, S.; Nellist, M., *Improving molecular diagnostics for tuberous sclerosis complex*, 2017, International TSC symposium
2. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Masulk, L.; Richardz, M.; Miranda, N.; Silvia, I. M. W.; Chiavegatto, S.; Passos-Bueno, M. R.; Antoniuk, S. A.; Rosemberg, S.; Haddad, L. A., *Segmental genomic deletions and splicing mutations causing tuberous sclerosis complex (TSC)*, 2016, Vancouver, BC, Canada; American Society of Human Genetics 66th Annual Meeting
3. Carvalho, D. S.; Antoniuk, S. A.; Cardozo, L. F. M.; Bruck, I.; Haddad, L. A.; **Dufner-Almeida, L.G.**, *Tuberous Sclerosis Complex: Clinical Evaluation in 47 patients*, 2016, Lisboa.
4. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Rosemberg, S.; Haddad, L. A., *A relatively high rate of TSC1 mutation causing tuberous sclerosis complex (TSC) in a cohort of 21 patients*, 2015, Brazil; Evento: XI Course of the Latin American School of Human and Medical Genetics
5. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Masulk, L.; Richardz, M.; Miranda, N.; Antoniuk, S. A.; Rosemberg, S.; Haddad, L. A., *Tuberous sclerosis complex-causing mutations analyzed in a cohort of 41 Brazilian patients*, 2015, United States, ASHG 65th Annual Meeting
6. **Dufner-Almeida, L.G.**; Almeida, J. P. G.; Masulk, L.; Richardz, M.; Miranda, N.; Antoniuk, S. A.; Rosemberg, S.; Haddad, L. A., *Analyses of TSC genes among Brazilian tuberous sclerosis complex (TSC) patients*, 2014., United States, ASHG 64th Annual Meeting.

## Participation in events

1. Lecture at ABET 2015, *Mão na massa: entendendo porque a Esclerose Tuberosa se manifesta*.
2. Poster / Panel Presentation in ASHG 65th Annual Meeting, 2015, *Tuberous sclerosis complex-causing mutations analyzed in a cohort of 41 Brazilian patients*.
3. Poster / Panel Presentation in XI Course of the Latin American School of Human and Medical Genetics, 2015, *A relatively high rate of TSC1 mutations causing tuberous sclerosis complex (TSC) in a cohort of 21 patients*.
4. Poster / Panel Presentation in 60° Congresso Brasileiro de Genética, 2014, *TSC1 gene DNA variant analysis in tuberous sclerosis complex (TSC) patients*.
5. Poster / Panel Presentation in ASHG 64th Annual Meeting, 2014, *Analyses of TSC genes among Brazilian tuberous sclerosis complex (TSC) patients*.

# Appendix

## Appendix 1



Número de Identificação: LGF \_\_\_\_\_

**Projeto de pesquisa: Análise dos genes causadores do complexo da esclerose tuberosa****TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO  
(Maiores de 18 anos)  
COLETA DE SANGUE**

Você está sendo convidado(a) a participar do projeto de pesquisa acima citado. Este documento contém todas as informações necessárias sobre a pesquisa que estamos fazendo. Sua colaboração neste estudo será muito importante para entendermos um pouco mais sobre o complexo da esclerose tuberosa.

Eu, \_\_\_\_\_,  
residente e domiciliado(a) na \_\_\_\_\_,  
inscrito(a) no CPF \_\_\_\_\_, portador(a) da cédula de identidade,  
RG \_\_\_\_\_ e nascido(a) em \_\_\_\_/\_\_\_\_/\_\_\_\_, declaro que fui esclarecido(a) pelo  
pesquisador, entendi o que me foi explicado e tive oportunidade para lhe fazer perguntas  
sobre o projeto "Análise dos genes causadores do complexo da esclerose tuberosa".  
**Consinto de livre e espontânea vontade em participar do estudo "Análise dos genes  
causadores do complexo da esclerose tuberosa" e estou ciente que:**

- (a) O objetivo do estudo é identificar mutações associadas ao complexo da esclerose tuberosa e entender como a esclerose tuberosa se manifesta.
- (b) Será feita coleta de sangue (cerca de 10 mililitros), o que poderá causar pequeno incômodo ou dor no momento da coleta (introdução da agulha na veia).
- (c) O sangue se destinará à extração de DNA, que deverá ser armazenado em laboratório pelo tempo necessário e utilizado em pesquisa de mutações associadas ao complexo da esclerose tuberosa.
- (d) Uma aliquota codificada (sem identificação) do DNA em quantidade suficiente para esta análise poderá ser enviada a um laboratório especializado no exterior.
- (e) A análise do sequenciamento do DNA será realizada somente para mutações causadoras e modificadoras do complexo da esclerose tuberosa.
- (f) Resultados dos meus exames clínico e laboratoriais poderão ser utilizados para fins de publicações científicas, aulas ou seminários científicos. No entanto, a publicação de imagens fotográficas só será possível com meu consentimento abaixo.
- (g) Mutações em qualquer segmento dos genes *TSC1* ou *TSC2* podem causar a esclerose tuberosa, o que faz a análise laboriosa e demorada. Por este motivo e por se tratar de um novo projeto de pesquisa, os resultados poderão ser obtidos nos próximos anos.



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- (h) Nas condições atuais, cerca de 10% dos pacientes com o complexo da esclerose tuberosa não têm a mutação identificada no laboratório. Isso se deve à limitação técnica.
- (i) Ao final desta pesquisa, poderei tomar conhecimento dos resultados, em consulta com a responsável pelo projeto, quando terei a oportunidade de esclarecimentos e aconselhamento genético. Em relação a esta alternativa, minha opção é:
- ☐ Desejo conhecer os resultados desta pesquisa.
- ☐ Não desejo conhecer os resultados desta pesquisa.
- (j) Tenho a liberdade de desistir e retirar minha participação deste estudo a qualquer momento, sem necessidade de explicação.
- (k) Minha participação ou minha desistência não resultará em ônus ou compensação financeira, não implicará em interrupção ou recusa de atendimento médico nem me trará benefício terapêutico ou assistencial médico.
- (l) O DNA ficará sob a responsabilidade do Instituto de Biociências da Universidade de São Paulo e sob a guarda da pesquisadora responsável pela pesquisa, Profa. Dra. Luciana Amaral Haddad, pelo tempo necessário para a identificação e caracterização das mutações relacionadas ao complexo da esclerose tuberosa.

**Assinale com um X uma das duas alternativas:**

☐ **Concordo** que imagens fotográficas obtidas ao meu exame médico ou de exames de imagem sejam utilizadas para fins de publicações científicas, aulas ou seminários científicos sobre o complexo da esclerose tuberosa, mantendo-se sempre a confidencialidade sobre minha identidade.

☐ **Não concordo** que imagens fotográficas obtidas ao meu exame médico ou de exames de imagem sejam utilizadas para fins de publicações científicas, aulas ou seminários científicos sobre o complexo da esclerose tuberosa.

\_\_\_\_\_, \_\_\_\_ de \_\_\_\_\_ de 20 \_\_\_\_

Assinatura do sujeito da pesquisa ou responsável legal  
Nome legível: .....

Assinatura do pesquisador  
Profa. Dra. Luciana Amaral Haddad  
Médica, CRM 22803  
Telefone: 11-3091-0971

Termo de consentimento livre e esclarecido — Análise dos genes causadores do complexo da esclerose tuberosa — Página 2/2



## Appendix 2



Número de Identificação: LGF \_\_\_\_\_

**Projeto de pesquisa: Análise dos genes causadores do complexo da esclerose tuberosa****TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

(Menores de 18 anos ou indivíduos que não podem assumir a responsabilidade de assinar)

**COLETA DE SANGUE**

Seu/sua filho(a) ou o(a) paciente sob sua responsabilidade está sendo convidado(a) a participar do projeto de pesquisa acima citado. Este documento contém todas as informações necessárias sobre a pesquisa que estamos fazendo. Sua colaboração neste estudo será muito importante para entendermos um pouco mais sobre o complexo da esclerose tuberosa.

Eu, \_\_\_\_\_,  
residente e domiciliado(a) na \_\_\_\_\_,  
inscrito(a) no CPF \_\_\_\_\_ e portador(a) da cédula de identidade, RG \_\_\_\_\_, declaro que fui esclarecido(a) pelo pesquisador, entendi o que me foi explicado e tive oportunidade para lhe fazer perguntas sobre o projeto "Análise dos genes causadores do complexo da esclerose tuberosa".

Consinto de livre e espontânea vontade que \_\_\_\_\_,  
sob minha responsabilidade,  
nascido(a) em \_\_\_\_/\_\_\_\_/\_\_\_\_ participe do projeto de pesquisa "Análise dos genes causadores do complexo da esclerose tuberosa".

**Estou ciente que:**

- (a) O objetivo do estudo é identificar mutações associadas ao complexo da esclerose tuberosa e entender como a esclerose tuberosa se manifesta.
- (b) Será feita coleta de sangue (cerca de 10 mililitros) de veia do(a) paciente, o que poderá causar pequeno incômodo ou dor no momento da coleta (introdução da agulha na veia).
- (c) O sangue se destinará à extração de DNA, que deverá ser armazenado em laboratório pelo tempo necessário e utilizado em pesquisa de mutações associadas ao complexo da esclerose tuberosa.
- (d) Uma alíquota codificada (sem identificação) do DNA em quantidade suficiente para esta análise poderá ser enviada a um laboratório especializado no exterior.
- (e) A análise do sequenciamento do DNA será realizada somente para mutações causadoras e modificadoras do complexo da esclerose tuberosa.
- (f) Resultados dos exames clínico e laboratoriais do(a) paciente poderão ser utilizados para fins de publicações científicas, aulas ou seminários científicos. No entanto, a publicação de imagens fotográficas só será possível com meu consentimento abaixo.



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- (g) Mutações em qualquer segmento dos genes *TSC1* ou *TSC2* podem causar a esclerose tuberosa, o que faz a análise laboriosa e demorada. Por este motivo e por se tratar de um novo projeto de pesquisa, os resultados poderão ser obtidos nos próximos anos.
- (h) Nas condições atuais, cerca de 10% dos pacientes com o complexo da esclerose tuberosa não têm a mutação identificada no laboratório. Isso se deve à limitação técnica.
- (i) Ao final desta pesquisa, poderei tomar conhecimento dos resultados, em consulta com a responsável pelo projeto, quando terei a oportunidade de esclarecimentos e aconselhamento genético. Em relação a esta alternativa, minha opção é:
- ( ) Desejo conhecer os resultados desta pesquisa.
- ( ) Não desejo conhecer os resultados desta pesquisa.
- (j) Tenho a liberdade de desistir e retirar a participação deste estudo a qualquer momento, sem necessidade de explicação.
- (k) A participação do(a) paciente ou desistência não resultará em ônus ou compensação financeira, não implicará em interrupção ou recusa de atendimento médico nem lhe trará benefício terapêutico ou assistencial médico.
- (l) O DNA ficará sob a responsabilidade do Instituto de Biociências da Universidade de São Paulo e sob a guarda da pesquisadora responsável pela pesquisa, Profa. Dra. Luciana Amaral Haddad, pelo tempo necessário para a identificação e caracterização das mutações relacionadas ao complexo da esclerose tuberosa.

**Assinale com um X uma das duas alternativas:**

- ( ) **Concordo** que imagens fotográficas obtidas ao exame médico ou de exames de imagem do(a) paciente sob minha responsabilidade sejam utilizadas para fins de publicações científicas, aulas ou seminários científicos sobre o complexo da esclerose tuberosa, mantendo-se sempre a confidencialidade sobre sua identidade.
- ( ) **Não concordo** que imagens fotográficas obtidas ao exame médico ou de exames de imagem do(a) paciente sob minha responsabilidade sejam utilizadas para fins de publicações científicas, aulas ou seminários científicos sobre o complexo da esclerose tuberosa.

\_\_\_\_\_, \_\_\_\_ de \_\_\_\_\_ de 20\_\_\_\_

Assinatura do sujeito da pesquisa ou responsável legal  
Nome legível: .....

Assinatura do pesquisador  
Profa. Dra Luciana Amaral Haddad  
Médica, CRM 22803  
Telefone: 11-3091-0971

Termo de consentimento livre e esclarecido – Análise dos genes causadores do complexo da esclerose tuberosa – Página 2/2



## Appendix 3



### CHAPTER TWO

# Understanding human DNA variants affecting pre-mRNA splicing in the NGS era

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## Contents

1. Introduction	40
2. Pre-mRNA splicing	40
2.1 Co-transcriptional exon definition and pre-mRNA splicing	46
2.2 The extended intron definition	48
2.3 Cajal bodies and nuclear speckles: snRNP biogenesis, maturation and storage	49
3. DNA variants affecting pre-mRNA splicing	51
3.1 Classical SV: DNA variants in canonical splicing sites	51
3.2 The minigene approach and gene expression analyses for functional assessment of SV at the mRNA level	56
3.3 "Miscalled" missense, nonsense and synonymous DNA variants?	59
3.4 Deep intronic SV	64
3.5 Interfering in exon skipping by antisense oligonucleotide	69
4. Pre-mRNA splicing alterations and cancer	70
4.1 Alternative splicing and the hallmarks of cancer	70
4.2 Somatic mutations affecting <i>cis</i> - and <i>trans</i> -regulatory factors in cancer	71
4.3 Genetic basis of alternative splicing in cancer	74
5. Concluding remarks	76
Acknowledgments	77
References	77

## Abstract

Pre-mRNA splicing, an essential step in eukaryotic gene expression, relies on recognition of short sequences on the primary transcript intron ends and takes place along transcription by RNA polymerase II. Exonic and intronic auxiliary elements may modify the strength of exon definition and intron recognition. Splicing DNA variants (SV) have been associated with human genetic diseases at canonical intron sites, as well as exonic

## Appendix 4

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## REVIEW ARTICLE

Stem-cell therapy for hearing loss: are we there yet?<sup>☆</sup>

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## KEYWORDS

Cellular therapy;  
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## Abstract

**Introduction:** Mammalian hair cells and auditory neurons do not show regenerative capacity. Hence, damage to these cell types is permanent and leads to hearing loss. However, there is no treatment that re-establishes auditory function. Regenerative therapies using stem cells represent a promising alternative.

**Objective:** This article aims to review the current literature about the main types of stem cells with potential for application in cell therapy for sensorineural hearing loss, the most relevant experiments already performed in animals, as well as the advances that have been recently made in the field.

**Methods:** Research included the databases PubMed/MEDLINE, Web of Science, Science Direct and Scielo, as well as gray literature. Search strategy included the following main terms: "stem cells", "hair cells" and "auditory neurons". Additionally, the main terms were combined with the following secondary terms: "mesenchymal", "iPS", "inner ear", "auditory". The research was conducted independently by three researchers.

**Results:** Differentiation of stem cells into hair cells and auditory neurons has a high success rate, reaching up to 82% for the first and 100% for the latter. Remarkably, these differentiated cells are able to interact with hair cells and auditory neurons of cochlear explants through formation of new synapses. When transplanted into the cochlea of animals with hearing loss, auditory restoration has been documented to date only in deafferented animals.

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