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Finding a cure for tuberous sclerosis complex: From genetics through to targeted drug therapies

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Abstract

Tuberous sclerosis complex (TSC) is a rare, autosomal dominant genetic condition caused by a mutation in either the TSC1 or TSC2 gene. Phenotypically, this leads to aberrant cell growth and the formation of benign tumors called hamartomas in multiple organs. Understanding the mechanisms of pathology that are caused through the presence of disease causing mutations is a real hurdle for many rare genetic disorders; a limiting factor that restricts knowledge of the disease and any hope of a future cure. Through the discovery of the TSC1 and TSC2 genes and the signaling pathways responsible for the pathology of TSC, a new drug target called mechanistic target of rapamycin complex 1 (mTORC1) was discovered. Rapamycin, an mTORC1 inhibitor, is now the only pharmacological therapy approved for the treatment of TSC. This chapter summarizes the success story of TSC and explores the future possibilities of finding a cure.

1 Introduction

1.1 Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is a rare, autosomal dominant genetic condition that currently affects approximately 2 million people globally (Henske, Jóźwiak, Kingswood, Sampson, & Thiele, 2016) and has an incidence of 1/6000–10,000 live births annually (Leung & Robson, 2007). TSC is caused by a mutation affecting either the TSC1 or TSC2 gene and is typically characterized by the formation of benign tumors, called hamartomas, in multiple organ systems (Crino, Katherine, & Henkse, 2006). Primarily, the organs affected by the disease include the brain, skin, kidney and heart, although this list is not exhaustive. Interestingly, TSC exhibits a large degree of phenotypic variability and patient morbidity is subsequently influenced by the site, penetrance, and severity of hamartoma formation. Furthermore, the quality of life TSC patients is often severely affected by epileptic seizures, and a proportion of patients are also affected by neurodevelopmental disorders which include autism and intellectual disability. Overall, the heterogeneous nature of the condition combined with the range of tissues it can affect makes TSC a particularly challenging disease to both diagnose and to treat.

Discovering the genetic defect(s) attributed to TSC has played an instrumental role in our knowledge of the disease pathology and has accelerated translational research within this field, as well as other related genetic disorders. Historically, the causative gene(s) of TSC were unknown, but were later found to be the tumor suppressor genes TSC1 and TSC2. TSC was first clinically described in the 1880s by the French physician, Bourneville (Gómez, 1995) and was eponymously named 'Bourneville's disease'.

However, it was not until much later, in the 1990s, that the responsible genes were finally discovered. For rare genetic disorders, the discovery of causative gene mutations and mechanism(s) of pathology is a bottleneck for potential cures. To successfully design therapies that abolish the disease state in patients, it is essential to first uncover the gene(s) responsible for the disease pathogenesis. Only then is it possible to begin unravelling the complex function of the gene in question, i.e., the "normal" or "homeostatic" function of the gene followed by determination of the underlying molecular pathology linked to loss of gene function.

Being categorized as a "rare disease" is a stigma that hinders progression of research, as mainstream funding tends to favor research areas that would benefit larger patient groups. Much of our knowledge of TSC and the therapy that is now in clinical practice is largely because of charitable funding that is dedicated to TSC research. The story of TSC has been a remarkable journey, involving clinical diagnosis, genetics, cell biology, drug therapy and future directions that are ever evolving based on the needs of the patients. Good communication between patients, patient-advocates, clinicians, researchers and politicians has also been a catalyst of success. Indeed, the TSC community is passionate about making forward progress with an aim to enhance the quality of life of patients. Furthermore, TSC research has also had a marked impact on our broader knowledge of human disease, as the TSC-pathway is implicated in many common diseases that affect the general population, for example, diabetes and cancer, as well as other age-related diseases. Therefore, research on genetic disorders, whilst rare, gives us the opportunity to explore disease mechanisms that also have a wider clinical impact on society.

1.1.1 Clinical features of TSC

The prognosis of TSC largely depends on the severity of the disease phenotype. Individuals with a low disease burden generally fare well and have a normal life-expectancy; however, for those severely affected, it can have a significant impact on both morbidity and mortality (Martin et al., 2017). As described, TSC primarily affects the brain, skin, eyes, kidney and lung (depicted in Fig. 1). Given the heterogeneity of disease severity, genetic testing now serves as a vital tool in diagnosis, especially in cases where clinical evidence is insufficient for a clear diagnosis. Furthermore, genetic testing also serves to determine whether there is a positive family history of TSC, i.e., whether the causative mutation was inherited from a parent in an autosomal dominant fashion. This is important, because approximately 60–70% of TSC cases occur de novo, due to a sporadic germline mutation in either TSC1 or TSC2. Prior to highly specific genetic testing, TSC was primarily diagnosed using a defined clinical criteria which is still used as an adjunct to diagnosis today. The current clinical criteria defines TSC diagnosis, as "definite" or as "possible." The TSC diagnosis is based on the presence of clinical evidence that fulfill the "major" and "minor" diagnostic criteria. The list of diagnostic criteria is shown below (Table 1) and is the current recommendation for attaining a diagnosis (please refer to Northrup & Krueger, 2013). In summary, Individuals presenting with two "major" features or one "major" feature with two "minor" features or positive genetic testing fulfill the diagnostic criteria for having TSC as being "definite." Individuals with a minimum of either one "major" feature, or two or more "minor" features meet criteria for having TSC as being "possible." When a "definite" diagnosis is determined, genetic testing is common practice to ascertain whether the disease causing mutation in either TSC1 or TSC2 is familial or sporadic, that is necessary for genetic counseling of the patients. Given the complexity of the disease, a large team of TSC clinical specialists are typically involved in the long-term clinical surveillance of the patients. Typically, mutations within TSC2 give rise to more severe disease manifestations when compared to mutations within TSC1 that cause a milder disease phenotype. Consequently, genetic evidence adds prognostic value as it helps predict the likely future disease burden for the patient.

Table 1 Clinical diagnostic criteria.

Table adapted from Northrup, H., & Krueger, D. A. (2013). Tuberous sclerosis complex diagnostic criteria update: Recommendations of the 2012 international tuberous sclerosis complex consensus conference. *Pediatric Neurology*, 49(4), 243–254.

- Angiofibromas or fibrous cephalic plaque
- Angiomyolipomas
- Ungual fibromas
- Shagreen patch
- Multiple retinal hamartomas
- Subependymal nodules
- Subependymal giant cell astrocytoma • Cardiac rhabdomyoma
- Cortical dysplasias
- Lymphangioleiomyomatosis

Minor features:

- Intraoral fibromas
- “Confetti” skin lesions • Dental enamel pits
- Retinal achromic patch • Multiple renal cysts
- Nonrenal hamartomas

Definite diagnosis: 2 “major” features, or 1 “major” feature with ≥ 2 “minor” features. Possible diagnosis: either 1 “major” or ≥ 2 “minor” features.

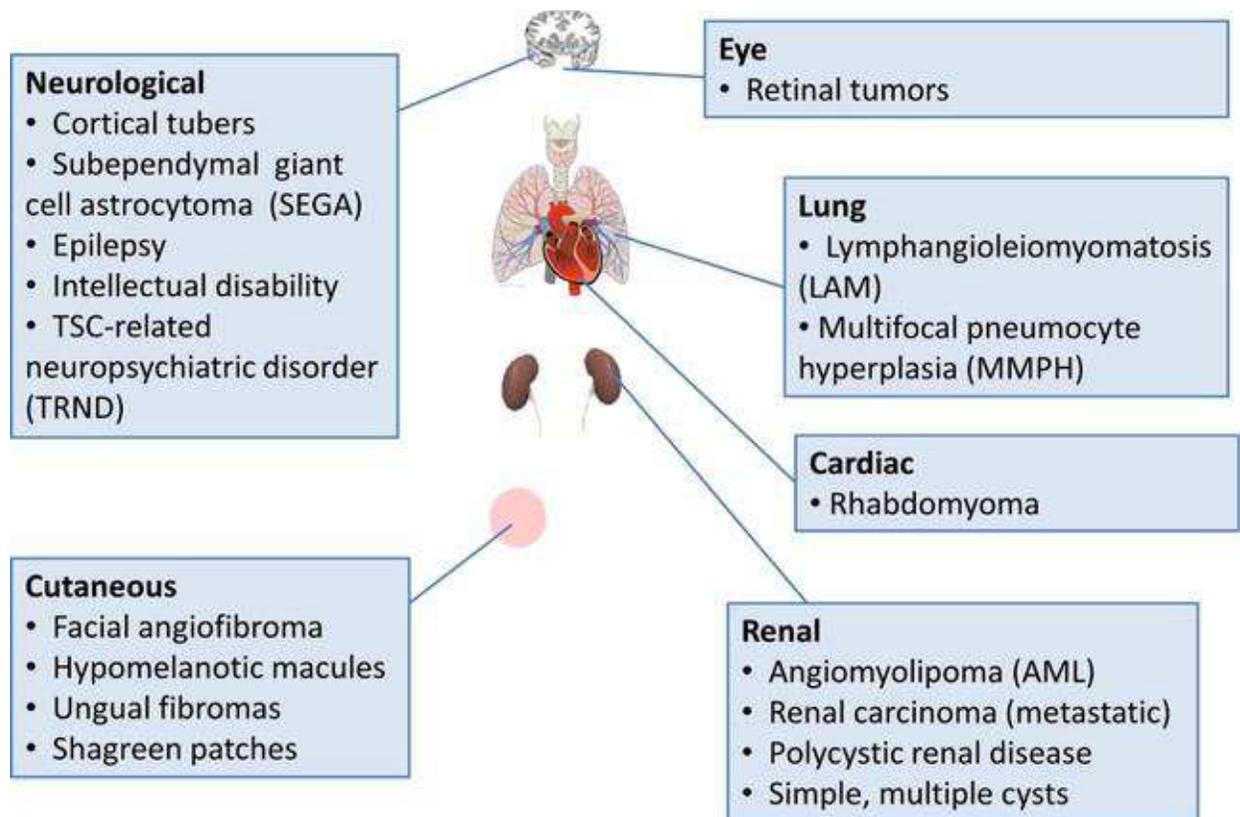


Fig. 1 Clinical features of TSC. Schematic summarizing the main clinical manifestations of TSC as categorized by organ system. For a more detailed description of the clinical aspects of TSC, see review by Crino et al. (2006).

1.1.1.1 Skin

Cutaneous manifestations occur in almost all patients with TSC and therefore form an integral part of the diagnostic criteria (Cardis & DeKlotz,

2017). Patients often have light colored spots on their skin called hypomelanotic macules (hyp that means less than normal, while melanotic refers to the skin pigmentation) as well as other cutaneous irregularities including angiofibromas, cephalic fibrous plaques, patches of thickened, raised skin called shagreen patches, and fibrous nail-bed growths called unguis fibromas. In some patients, skin features are present at birth, whilst in others cutaneous involvement develops later in childhood or even in early adulthood. The most common skin lesion is the facial angiofibroma, which are small nodular tumors that typically grow across the nose and cheeks (Hake, 2010). The term “angio” refers to the increase number of blood vessels within these benign tumors. Angiofibromas arise due to uncontrolled cellular proliferation, which is the underlying pathological mechanism of TSC. Although they do not pose a direct risk to health, facial angiofibromas often require treatment because their physical appearance is of great concern to patients. The development of skin features is unpredictable, however, most TSC patients (approximately 70%) eventually have at least one skin feature and many will have several.

1.1.1.2 Kidney

Renal angiomyolipomas (AMLs) affect approximately 80% of individuals with TSC and are a leading cause of morbidity in adulthood (Pirson, 2018). Renal AMLs are highly vascular, often bilateral lesions which are composed of adipose and smooth muscle tissue derived from the renal parenchyma. The vascular nature of these tumors makes them prone to aneurysmal formation and patients are at high risk spontaneous hemorrhage, especially when these tumors exceed 4 cm in diameter (Van Baal, Smits, Keeman, Lindhout, & Verhoef, 1994). Historically, surgery was the main treatment option for renal AMLs; however, surgery was not a definitive option as most patients experience some degree of recurrence. Since then, drug options have now been made available for the treatment of AMLs, these will be discussed later in the chapter. TSC patients are also at increased risk of other benign renal lesions such as simple cysts and adenomas, as well as malignant cancers such as renal carcinoma (Rakowski et al., 2006). Interestingly, TSC is genetically linked to development of polycystic kidney disease (PKD). This is because the PKD1 locus is proximal to the TSC2 gene on chromosome 16, which can give rise to the TSC/PKD contiguous gene syndrome with severe renal manifestations. There is also signaling cross talk between the proteins that are encoded from the PKD and TSC genes that involves the normal formation of cilia. Dysfunction of PKD-TSC, which is under studied, can give rise to renal cysts, hypertension and eventually renal failure (discussed in Dere, Wilson, Sandford, & Walker, 2010).

1.1.1.3 Lung

Pulmonary lymphangiomyomatosis (LAM) is a severe complication of TSC which mainly affects female patients, usually between the onset of puberty and menopause (Moss et al., 2012). It is estimated that 300,000 women have LAM worldwide, which includes patients that have either TSC-LAM or those with sporadic LAM. LAM is hormonally driven by oestrogen and progesterone and is characterized by progressive infiltration of abnormal smooth muscle tissue into the pulmonary parenchyma (Sun et al., 2014); the formation of lung cysts and parenchymal lung destruction leads to a decline in lung function and subsequently respiratory failure. Histologically, an unusual type of muscle-like cell metastasizes to the lungs and airways, in addition to the blood and lymph vessels. The origin of these invasive lung directed muscle-like cells is unknown; however, the uterus and renal angiomyolipomas are possible sources. These muscle-like cells progressively destroy the lungs, making it difficult for oxygen exchange between the alveoli and the blood cells. Clinically, as the disease progresses, women become short of breath and complain of chest pain. Respiratory insufficiency and/or pulmonary hypertension develop in individuals with extensive cystic lesions. LAM cells express oestrogen and progesterone receptors, which account for how oestrogen and progesterone enhance LAM-disease progression. It is advised that all women with TSC that are 18 years and older should have a computerized tomography (CT) scan of the chest and undergo pulmonary function testing (Krueger, 2013).

1.1.1.4 Brain

The name “tuberous sclerosis” was coined from the characteristic cortical tubers (or potato-like nodules) affecting the brain, these are a type of focal cortical dysplasia, which can disrupt the integrity of the cerebral cortex and calcify with age to become hard and sclerotic. Other examples of brain lesions include subependymal nodules and subependymal giant cell astrocytomas (SEGAs) that grow within the brain’s ventricular system, leading to hydrocephalus (Roth et al., 2013). Space occupying lesions are associated with development of epilepsy and are the most prevalent clinical manifestation of TSC. Approximately 90% of individuals with TSC will experience a seizure during their lifetime, the most common of which include infantile spasms, tonic-clonic seizures and absence seizures. Epilepsy is particularly challenging to treat in TSC

patients. A proportion of seizures are resistant to anti-epileptic medications (Wang & Fallah, 2014), making them extremely debilitating for patient and difficult to control. Furthermore, in approximately half of all patients, TSC is associated with some degree of intellectual disability (Joinson et al., 2003). Autism spectrum disorder and intellectual disability occur in about 40% of TSC patients with differing degrees of severity; some individuals exhibit severe intellectual disability while others only have mild impairment.

1.1.1.5 Heart

Cardiac rhabdomyomas occur spontaneously in > 50% of TSC patients but often resolve themselves in early childhood and typically cause no major clinical complications. However, in neonates and infants, cardiac rhabdomyomas may cause congestive heart failure and/or arrhythmias. The presence of cardiac rhabdomyomas detected during ultra-sound scans of an unborn foetus often functions as an early indication of TSC that is then later confirmed with diagnostic genetic testing.

1.1.1.6 Clear knowledge gaps TSC and treatment options

TSC is a “complex” disease that requires careful management and clinical surveillance of many organ systems. Historically, brain and kidney tumor were managed through surgical intervention if they were considered a risk to the health of the patient. Skin angiofibromas were also treated with abrasive or laser therapy, whilst a cocktail of anti-seizure drugs was routinely used to control seizures with varying degrees of success. Treatment options were extremely limited for TSC patients and were more focused on disease management rather than a cure. In order to advance treatment options for patients and improve their quality of life, greater understanding of disease mechanisms was required. The real breakthrough in TSC research was the discovery of the causative genes. This work then progressed to the sequential delineation of the cell signaling pathways central to TSC and the tumor suppressor functions of both TSC1 and TSC2. Fortunately, new therapeutic options were made available as a consequence of these landmark discoveries and will be discussed below. This advancing knowledge has certainly raised the profile of this “rare” genetic disease, accelerating the research on TSC and related diseases. Undoubtedly, the larger body of researchers that are now working on TSC will deepen our understanding of this complex disease with the intent to find a cure or to prevent disease onset. There are certainly many questions which remain unanswered, for instance what drives tumor growth? Why do muscle-like cells migrate and invade into the lung? What causes epilepsy and how do we prevent this in TSC? TSC has lost its “rare disease” stigma to a certain extent, as its relationship to the pathogenesis of other related diseases is becoming more widely recognised.

1.1.2 Genetics of TSC

TSC is inherited in an autosomal dominant fashion, or can occur de novo due to a sporadic germline mutation (Northrup, Koenig, Pearson, et al., 1999). As described, a mutation can affect either the TSC1 gene, which encodes for a protein called harmartin or, more commonly, the TSC2 gene which codes for the protein tuberin. The proteins are now more commonly referred to as TSC1 and TSC2, respectively. The TSC1 gene was discovered in 1997 and is found on chromosome 9q34 (van Slechtenhorst et al., 1997), while the TSC2 gene was discovered in 1993 and is found on chromosome 16p13.3 (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). TSC1 spans 53 kb of genomic DNA with 23 exons (encoding for TSC1: 1164 amino acids and is 130 kDa in size). TSC1 is a hydrophilic protein and is ubiquitously expressed. TSC2 spans approximately 43 kb of genomic DNA with 41 exons (encoding for TSC2: 1807 amino acids and is 198 kDa in size). Notable features of TSC2 are that this protein contains an N-terminal hydrophilic domain that interacts with TSC1 and a conserved 163 amino acid region within the C-terminus called a GTPase activating protein (GAP) domain. Extensive studies of TSC1 and TSC2 have revealed a wide spectrum of disease causing mutations. Typically, TSC1 mutations are more likely to be familial whereas TSC2 mutations tend to be germline mutations and are associated with a greater disease severity. TSC exhibits a large degree of allelic variation; there are over 200 different mutations described in TSC1 and almost 800 in TSC2.

TSC has a high penetrance but variable expressivity, meaning that almost all patients carrying the mutation will develop the disease but will be affected to different degrees. This disease pattern can be attributed and described by the Knudson’s “two-hit” model of tumor-suppressor genes (Hino & Kobayashi, 2017) (Fig. 2). Of interest, Alfred G. Knudson used a dominantly inherited tumor model for his genetic studies that happened to be the Eker rat. In these Eker rats, it is now known that tumor development is predisposed by a germline mutation in Tsc2. The Eker rat is routinely used as a TSC model to study this familial tumor predisposing syndrome. In line with Knudson’s research, patients with TSC are characterized by loss of heterozygosity due to the acquisition of a somatic mutation in the remaining functional copy of either TSC1 or TSC2

(Green, Smith, & Yates, 1994). As an example of the “two-hit” model, the distribution of facial angiofibromas can be attributed to UV-induced mutations in the second copy of TSC1 or TSC2 which then results in aberrant growth of these skin tumors (Tyburczy et al., 2013). These small benign tumors are usually scattered on the central face, especially on the nose and cheeks, which are areas of the face that tend to be more sun exposed. Angiofibromas are found in most individuals with TSC over 5 years of age and indicate a real need to protect against UV-damage from an early age. Consequently, reduced sun exposure is advised for TS patients to reduce the number and severity of angiofibromas (Tyburczy et al., 2013).

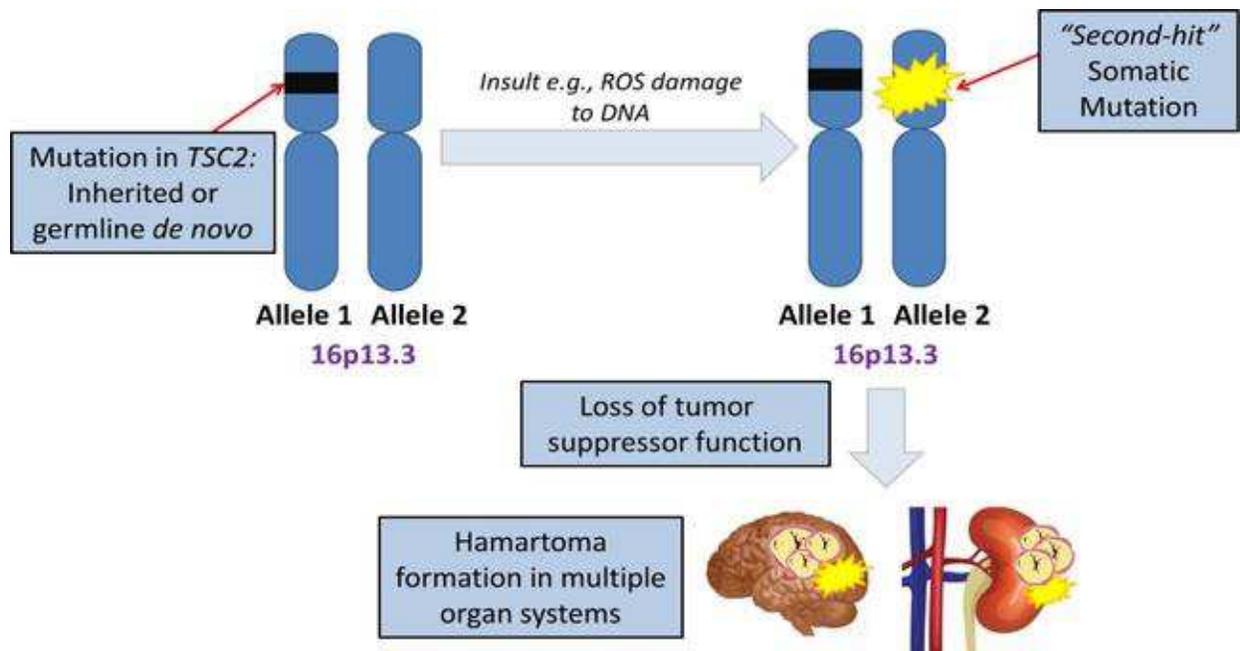


Fig. 2 Knudson’s “two-hit” model of tumor-suppressor genes. A schematic illustration of how an inherited mutation in TSC2 can progress to tumor formation by acquisition of a somatic mutation in the unaffected allele.

Understanding the genetics of TSC was instrumental in the delineation of the functional activity of the TSC1 and TSC2 proteins in the cell. The longstanding question was: “How do TSC1 and TSC2 inhibit tumor growth?” The high degree of C-terminal truncating mutations of TSC2 that removed the GAP domain, as well as a cluster of pathogenic TS-patient derived TSC2 mutations within the GAP domain, suggested that the GAP domain of TSC2 was functionally important (Maheshwar et al., 1997).

GAP domains of proteins function to switch small G proteins from an active GTP-bound state to an inactive GDP-bound state. The spread of TSC2 mutations implicated that TSC2 was likely to regulate a small G protein involved in cellular growth. Indeed, it was later discovered that the small G protein, Ras homologue enriched in brain (Rheb), was negatively regulated by the GAP domain of TSC2, when TSC2 formed a functional complex with TSC1 (Tee, Manning, Roux, Cantley, & Blenis, 2003). Rheb, when in an active GTP-bound state, was found to potently activate mechanistic target of rapamycin complex 1 (mTORC1) involved in cell growth control. It is now known that TSC1 and TSC2 bind together to form a tumor suppressor complex to inhibit cellular growth through mTORC1 (Plank, Yeung, & Henske, 1998). Consequently, loss of function mutations within TSC1 and TSC2 leads to aberrant mTORC1 activity that drives cell proliferation and tumor growth within TSC. Since the discovery of the TSC1 and TSC2 genes, research on the signaling pathway central to TSC has accelerated our understanding and has positioned mTORC1 a new drug target for the treatment of TSC.

It should be highlighted that TSC pathology is unlikely to be fully accountable by just mTORC1 hyperactivity alone. It is possible that Rheb-GTP can modulate other downstream functions in addition to mTORC1 activation. It is also possible that TSC1/TSC2 has additional tumor suppressor functions. In addition to the TSC2-GAP domain, TSC2 has other potential functional domains that are under studied. One such under studied

domain of TSC2 is called the transcriptional activation (TA) domain, which is C-terminal and is postulated to interact with the oestrogen-receptor as well as Ca^{2+} -calmodulin. This TA domain has been shown to be required for the nuclear translocation of TSC2 (York, Lou, & Noonan, 2006). It is feasible that this TA domain is linked to the pathology of LAM, where disease progression in LAM is driven by oestrogen. Also, the potential for Ca^{2+} -calmodulin binding to the TA domain is suggestive of Ca^{2+} signaling in the normal function of TSC2. It is possible that the involvement of TSC2 in Ca^{2+} -signaling is linked to the normal function of cilia. This is because PKD is a Ca^{2+} -channel in cilia and TSC2 interacts with PKD (Dere et al., 2010). The TA domain is proximal to the GAP domain, so both these functional domains are commonly lost with disease causing C-terminal truncating mutations of TSC2. Clearly, research is still required to fully understand these multi-functional TSC1 and TSC2 proteins and how they regulate cell processes. Furthermore, it is vital that we understand how TSC disease-causing mutations of both TSC1 and TSC2 can cause cellular pathology.

1.1.3 Molecular pathology and mechanistic target of rapamycin (mTOR)

Whilst there are still clear knowledge gaps regarding the tumor suppressor role of TSC1/TSC2, the most well-described role to date is the inhibition of mTORC1. mTORC1 is a serine-threonine protein kinase complex that is considered as the master-regulator of protein synthesis, metabolism, cell growth and drives angiogenesis and metastasis (Benjamin, Colombi, Moroni, & Hall, 2011). Generally, mTORC1 upregulates anabolic processes to increase cellular biomass via lipogenesis, protein synthesis and gluconeogenesis (Laplante & Sabatini, 2009), whilst also inhibiting catabolic processes, such as autophagy. mTORC1 consists of the core components; mTOR, rapamycin-associated protein of TOR (Raptor) and mLST8. mTOR is the catalytic subunit of the complex that phosphorylates downstream proteins on serine/threonine residues. Raptor serves as a scaffold protein, which interacts with downstream mTORC1 substrates and is essential for directing mTORC1-mediated phosphorylation and activation of downstream proteins to drive cell growth. mTORC1 is negatively regulated by proline-rich Akt substrate of 40 kDa (PRAS40) and DEP domain containing mTOR-interacting protein (DEPTOR) (Yang et al., 2013). Less is known about PRAS40 and DEPTOR, but are important negative regulators that ensure the proper control of mTORC1 activation in cells.

In the presence of growth factor ligands such as insulin or epidermal growth factor (EGF), the mTORC1 pathway is turned on via upstream activation of receptor tyrosine kinases, such as the insulin receptor and EGF receptor, respectively (Tee, Anjum, & Blenis, 2003). These growth regulating signaling inputs activate the PI3K-Akt pathways and Ras-Raf-ERK that both converge on the TSC1/TSC2 complex (Fig. 3). Inhibition of TSC1/TSC2 through phosphorylation of TSC1 and TSC2 results in impairment of the GTPase activating protein (GAP) function of TSC2. Inhibition of TSC1/TSC2 results in the switching of Rheb to the active GTP-bound form that serves to potently activate mTORC1 (Tee, Manning, et al., 2003). In resting conditions when cellular growth is repressed, Rheb remains in an inactive, GDP-bound state and prevents mTORC1 activation (to tell the cell to stop growing). Consequently, a loss-of-function mutation in either TSC1 or TSC2, as seen in TSC, leads to heightened levels of GTP-bound Rheb and aberrant activation of mTORC1, which drives the pathophysiology of TSC (Fig. 4). For instance, mTORC1 hyperactivity causes enhanced cellular proliferation, tumorigenesis and ultimately the hamartomatous phenotype observed in TSC.

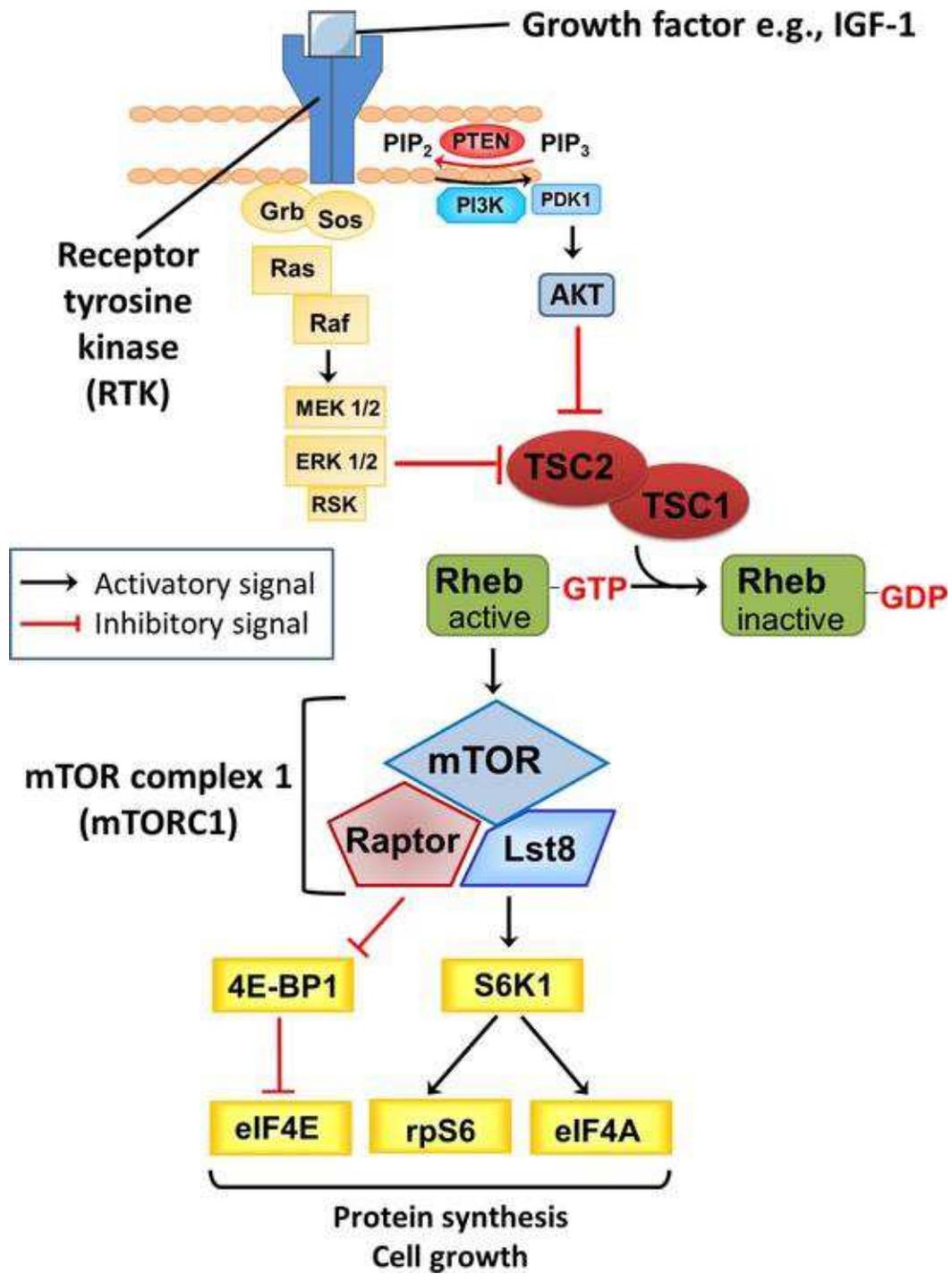


Fig. 3 mTORC1 signaling pathway. Schematic summarizing the key upstream and downstream signaling pathways of mTORC1. Ligand binds to a receptor tyrosine kinase and induces autophosphorylation. Phosphorylation subsequently activates the Ras/ERK and PI3K/Akt signaling pathways, both of which result in a cascade of phosphorylation reactions that leads to the inactivation of the TSC1/TSC2 complex. Rheb-GTP is favored when TSC1/TSC2 is negatively inhibited through activation of the Ras/ERK and PI3K/Akt signaling pathways. Active GTP-bound Rheb binds to and activates mTORC1 to drive cellular growth through 4E-BP1 and S6K1 (and other downstream targets).

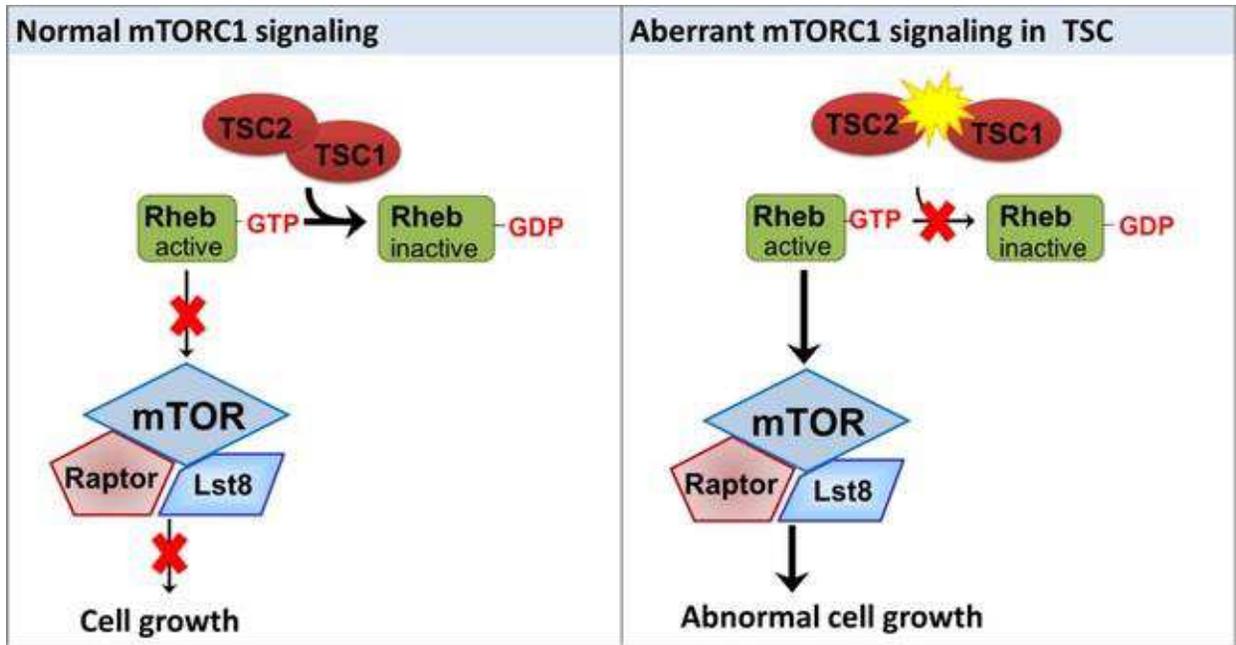


Fig. 4 mTORC1 signaling in the absence of activatory ligand. In normal cells, in the absence of an activatory ligand, TSC1/TSC2 forms an active tumor suppressor complex. The GTPase activation domain of TSC2 subsequently converts Rheb-GTP to Rheb-GDP, which inactivates Rheb. Consequently, mTORC1 remains inactive and cell growth is restricted. In contrast, a patient-derived mutation in either TSC1 or TSC2 prevents inactivation of Rheb. This causes Rheb to be constitutively GTP-bound, stimulating mTORC1 to drive abnormal cell growth.

Both Rheb and mTORC1 are ubiquitously expressed in all cells in the human body, which provides an explanation for the multi-system nature of TSC. However, not all organs in the body are affected in TSC. Tumor distribution in TSC patients to the kidneys, brain and skin is suggestive of a dominant role that the TSC1/2 → Rheb/mTORC1 plays in the development and maintenance of these organs. These organs are considered as highly sensitive to blood oxygen levels and are highly metabolic. The metabolically driven environment of the kidney is presumably why tumors occur in the kidney. mTORC1 is known to drive metabolic transformation, and is a drug target in the treatment of kidney cancer (mTOR in the cancer setting is reviewed in Rad, Murray, & Tee, 2018). mTORC1 is also linked to oxygen sensing pathways that drive blood vessel growth through angiogenesis. A big player in this is hypoxia inducible factor (HIF), which is a common driver of kidney cancer. The connection of TSC1/TSC2 with mTORC1 and HIF can be related to enhanced blood vessel formation in renal angiomyolipomas, as well as in facial angiofibromas.

mTORC1 has two well described downstream substrates: ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiating factor 4E-binding protein 1 (4E-BP1), both of which are translation modulators (reviewed in Guertin & Sabatini, 2007) (depicted in Fig. 3). In resting conditions, hypophosphorylated 4E-BP1 binds to and represses eIF4E with high affinity, out-competing eIF4G to prevent translation initiation (Fig. 5). During a cell growth input (from insulin or EGF, as examples), mTORC1 subsequently phosphorylates 4E-BP1, causing it to dissociate from eIF4E, which then allows eIF4E to interact with eIF4G. The interaction of eIF4G with eIF4E then allows the recruitment of other translation initiation factors to enhance the efficiency of translation of mRNAs. Through enhancement of eIF4E, mTORC1 drives mRNA translation of a specific set of mRNAs that are involved in cell growth control (for further reading, refer to a review on mTOR and cell growth control in cancer Rad et al., 2018).

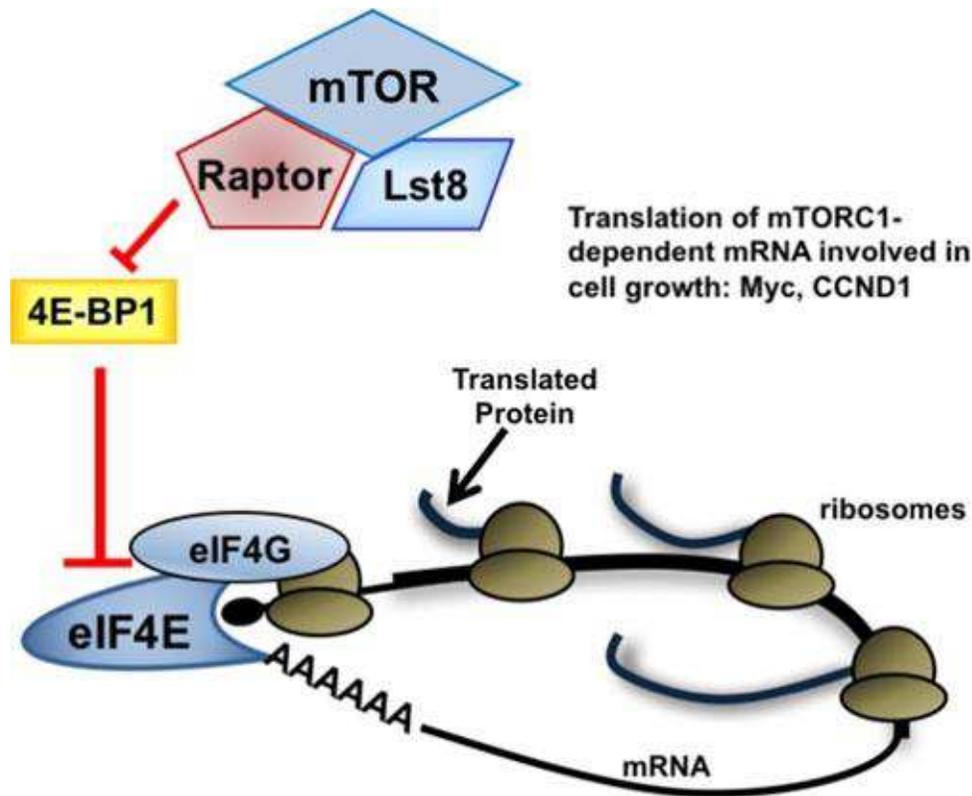


Fig. 5 Translation initiation through mTORC1-4E-BP1 and eIF4E/eIF4G. In a resting state, 4E-BP1 is bound to eIF4E which prevents association with eIF4G. To promote protein translation, mTORC1 phosphorylates 4E-BP1, causing its dissociation from eIF4E. eIF4E then associates with eIF4G to allow recruitment of ribosomes to the 5'-end of the mRNA, which then scan to the start codon to translate the mRNA. Proteins whose translation is enhanced by mTORC1 include: Myc and CCND1, involved in cell growth control.

mTORC1 also phosphorylates S6K1 on Thr389, which is required for S6K1 activation. S6K1 is a Ser/Thr protein kinase and enhances translation efficiency through phosphorylation of multiple downstream targets that are responsible for the recruitment of mRNA encoding for components of the translation machinery, e.g., ribosomal proteins and protein translation factors, thus increasing the capacity for protein synthesis to build cellular biomass (reviewed in Rad et al., 2018). Other downstream actions of mTORC1 include inhibition of autophagy, a catabolic process characterized by the formation of endocytic structures called autophagolysosomes that serve to initiate the degradation of intracellular organelles, such as mitochondria. Less well-characterized pathways involving mTORC1 include the generation of lipids necessary for membrane expansion of a growing cell. The central role of mTORC1 is to control the progressive growth of a cell, where mTORC1 drives the capacity of a cell to effectively build cellular biomass. Consequently, high activity of mTORC1 will significantly shorten the time a proliferating cell has in the first growth (G1) phase of the cell cycle. Cancer cells typically activate mTORC1 to enhance proliferative drive to quickly by-pass the rate-limiting G1-phase of the cell cycle. Based on sporadic mutations found in cancer, it is roughly estimated that > 50% of cancers possess genetic mutations in upstream signaling components that would lead to constitutive hyperactivation of mTORC1. Interestingly, many discoveries relating to the mTORC1 pathway have been made using TSC-model systems in research. As a result, TSC research has had a clear beneficial influence on our current knowledge of sporadic cancers that are driven by mTORC1.

In addition to inputs via growth factors and upstream growth factor receptors, the energy and nutrient status of the cell is also an important regulatory signaling input of mTORC1 (Bond, 2016). In the absence of either energy or nutrients (amino acids), mTORC1 is effectively turned off. This makes physiological sense for a cell, as the building of cellular biomass has a high demand on both energy and amino acids. Pathways regulated by mTORC1 (such as protein translation) are highly energy consuming, as well as also requiring an abundance of free amino acids. Therefore, it is critically important that the cell can quickly adapt when energy or nutrients become limited so that energy and nutrient homeostasis can be restored. Consequently, by halting cell growth when nutrients and energy are low, cellular stress is also kept to a minimum.

When energy levels are low, i.e., when glucose and ATP become depleted, the enzyme 5'AMP-activated protein kinase (AMPK) becomes activated. AMPK is a serine/threonine protein kinase that allosterically interacts with both ATP and AMP, thus it can directly sense the ratio of ATP:AMP in the cellular environment (Kemp et al., 1999). Consequently, when AMP levels are elevated in energy-deprived cells, AMPK becomes activated. AMPK functions to conserve energy by inhibiting an array of anabolic processes, which also includes inhibition of mTORC1 through phosphorylation of TSC2 (Kim, Yang, Kim, & Ha, 2016). Furthermore, AMPK inhibits lipogenesis by phosphorylation of acetyl CoA carboxylase (ACC), an enzyme that usually enhances fatty acid synthesis. AMPK also enhances catabolic processes that serve to increase the availability of energy for the cell, an example of which is autophagy. Whilst mTORC1 inhibits autophagy, AMPK stimulates autophagy, which exists to recycle protein and lipid components, regenerate ATP, and to free up amino acids. AMPK also enhances the breakdown of fatty acids so that they can be used as an energy source for the cell (Mihaylova & Shaw, 2011). By switching on processes that generate energy, while restricting energy-consuming processes (such as protein translation), the cells can quickly restore their energy levels to homeostasis.

Nutrient sensing and the regulation of mTORC1 are complex and will only be briefly described in this chapter (for a detailed review, see Dunlop & Tee, 2014). When amino acids become limiting, mTORC1 is cytoplasmically localized and is not able to interact with GTP-Rheb, which is lysosomally tethered by a fatty prenylation moiety. This means that mTORC1 is kept away from Rheb in an inactive state when nutrient levels are insufficient, irrespective of a growth signaling input via upstream growth factor receptors. When nutrients become replenished, mTORC1 is actively translocated to the surface of the lysosome by Rag small G protein heterodimers (Sancak et al., 2008). Once at the membrane surface of the lysosome, mTORC1 is then susceptible to a growth signal input that switches Rheb to an active Rheb-GTP bound state (via TSC1/TSC2, as described above). mTORC1 binding to Rheb-GTP then drives mTORC1 signal transduction. A recent study showed that TSC1/TSC2 is directly regulated by the amino acid arginine, as arginine can displace Rheb away from the TSC1/TSC2 complex, leading to another level of nutrient regulation (Carroll et al., 2016). Nutrient signaling toward mTORC1 ensures that a cell will only grow when there is a plentiful supply of amino acids.

Energy and nutrient homeostasis is markedly compromised in TSC-tumor cells. In normal cell physiology, mTORC1 would not remain active for long periods of time, which grants a cell an ample period for the cell to rest and effectively recover. Signaling pathways typically fluctuate in activity, where rhythmic cycles of active and inactive states exist to ensure that the growth of a cell (or maintenance of a cell) is carefully monitored. For instance, it would be detrimental for a cell to grow when energy or nutrients become rate limiting. In the case of TSC-tumor cells, it appears that these mTORC1 hyperactive cells are particularly sensitive to energy starvation (Inoki, Zhu, & Guan, 2003). Thus, it is possible that energy starvation could be a potential vulnerability to exploit therapeutically. Persistently elevated mTORC1 activity makes cells less flexible at retaining homeostasis under conditions of cellular stress (in this case, energy stress). In order to design future drug therapies, more work examining TSC-tumour vulnerabilities is required.

1.1.4 Current therapeutics in TSC

Delineation of the TSC1/TSC2 signaling pathway through mTORC1 was a major advance in our understanding of the pathophysiology of TSC. These earlier basic studies implicated that mTORC1 could be a drug target for TSC that was later confirmed in pre-clinical and then clinical studies using mTORC1 drug inhibitors. It was fortunate that there was a clinically approved mTORC1 inhibitor that could be repositioned to TSC patients, which certainly sped up the translational process from bench to bedside. The mTORC1 inhibitors are analogues of rapamycin, which was discovered in the early 1970s, secreted from the bacteria *Streptomyces hygroscopicus* in Easter Island (also known as "Rapa Nui"). Rapamycin (later renamed as Sirolimus) and analogues of rapamycin (such as Everolimus) are classed as first generation mTOR inhibitors. Whilst rapamycin was originally classified as an anti-fungal compound, it was subsequently found to possess immunosuppressant and anti-proliferative properties. Later, the drug target of rapamycin was discovered in yeast and was historically named as the target of rapamycin (TOR), which was much later renamed as mTOR, prior to discovery of what we now understand to be mTOR complex 1 (mTORC1). The immunosuppressive effect of rapamycin is caused via a reduced rate of T-lymphocyte proliferation. Thus, it is not surprising that side-effects of rapamycin include an increased risk of infection. Rapamycin can also reduce the wound heal response in patients, which is in part through the reduced proliferative drive of fibroblasts and keratinocytes that are needed to efficiently close the wound through multiple rounds of rapid cell replication. Rapamycin allosterically inhibits mTORC1 by forming a drug:protein complex with FK502-binding protein 12 (FKBP12) (Van Duyne, Standaert, Karplus, Schreiber, & Clardy, 1993). The rapamycin:FKBP12 (drug:protein) complex allosterically interacts with mTOR on

the FKBP12:rapamycin binding (FRB) domain, which inhibits the ability of mTORC1 to phosphorylate downstream substrates, such as 4E-BP1 and S6K1. Rapalogues are now clinically approved for the treatment of AMLs and SEGAs in TSC patients as it reduces tumor burden and provides symptomatic relief for patients (Bissler et al., 2008). There has been much interest in rapamycin by the cancer research community due to the anti-proliferative properties of the drug. However, the use of rapamycin as a single-agent therapy in both TSC and sporadic cancer is subject to several significant limitations. First, activation of downstream components of the mTORC1 pathway, such as S6K1, initiates a negative feedback loop which antagonizes upstream signaling pathways that feed onto mTORC1 (Li, Kim, & Blenis, 2014). In addition, not all mTORC1 downstream substrates are fully sensitive to rapamycin inhibition, which is because rapamycin can only partially inhibit mTORC1. As an example, 4E-BP1 has been shown to be re-phosphorylated to enhance eIF4E-mediated protein translation after just 12 h of rapamycin therapy, exhibiting resistance to rapamycin treatment (Choo, Yoon, Kim, Roux, & Blenis, 2008). It should be noted that the low-level toxicity profile of rapamycin is probably a reflection of its incomplete allosteric inhibition of mTORC1, and whilst a more potent inhibitor might have better outcomes at blocking mTORC1, toxicity might become an issue. The low level of toxicity presented upon treatment with rapamycin is likely a reflection that some mTORC1-mediated cell processes are not completely turned off upon longer periods of rapamycin treatment.

Another issue with the use of rapalogues is that both data from in vitro and clinical studies provide evidence that rapamycin is a cytostatic agent, and that cessation of therapy results in rebound and regrowth of TSC-tumours (Bissler et al., 2008). mTORC1 inhibition can also lead to enhanced cell survival via the induction of autophagy, and this exists as another potential reason as to why rapamycin therapy is limited. Despite these limitations, rapalogues enhance the quality of life for TSC patients, through reducing tumour growth of AMLs, SEGAs and facial angiofibromas. While mTORC1 inhibitors are successful at controlling tumor growth, the tumors do not completely regress with treatment, which means that patients with TSC will be taking mTOR inhibitors for extended periods of time (maybe for the rest of their lives).

Whilst an overall success, it is important to examine the shortfalls and ask the question: “Is there anything more we can do to improve therapy?” Future mTORC1 inhibitors that are more potent might be one avenue of approach and could show greater efficacy in the treatment of TSC. Second generation inhibitors of mTOR are ATP-competitive inhibitors that interact to the ATP-binding pocket of the kinase domain to prevent the docking of ATP required for kinase activity. Such ATP-competitive inhibitors, whilst more potent in repressing mTORC1, may be impractical when considered as a long-term therapy for TSC in addition to a greater risk of toxicity. mTOR complex 2 (mTORC2) would also be inhibited by these second-generation inhibitors, a rapamycin-insensitive mTOR protein kinase complex involved in the regulation of the actin cytoskeleton and other less well characterized downstream pathways.

One possible solution is a third generation mTORC1 inhibitor that has enhanced potency. This third generation mTORC1 inhibitor would keep the specificity to inhibit mTORC1 but would not inhibit mTORC2. The third generation mTORC1 inhibitor is called RapaLink-1 and looks very promising. RapaLink-1 has greater specificity to inhibit mTORC1 when compared to rapalogues. RapaLink-1 could be classified as a chimeric or a dual-hybrid drug as it possesses elements of both the first and second generation mTORC1 inhibitors, i.e., has the property of a rapalogue linked to an ATP-competitive inhibitor. This dual-acting drug can simultaneously associate with and allosterically inhibit mTORC1 (via the FRB domain of mTOR) while also binding within the ATP-binding pocket of mTOR (Fan et al., 2017). Importantly, RapaLink-1 shows a higher efficacy to specifically target mTORC1, whilst not inhibiting mTORC2. This third generation mTORC1 inhibitor has been shown to reduce acquired drug resistance that can occur when using rapalogues in the cancer setting. While RapaLink-1 is still in early pre-clinical stages, this third-generation inhibitor holds much promise in the treatment of TSC as well as other disease settings, such as cancer.

1.2 Molecular pathways for therapeutic exploitation

While inhibiting mTORC1 reduces the disease state in TSC, this line of therapy does not remove the diseased cells. Consequently, TSC patients would be taking an mTORC1 inhibitor therapy indefinitely, which raises issues of long-term toxicity as well as creating a financial burden on the national health service for supply of the commercial drug. Furthermore, it is not clear what the consequences of long-term therapy with mTORC1 inhibitors would be for young children with TSC. mTORC1 is necessary for brain development, therefore the use of mTORC1 inhibitors may, theoretically, have an impact on cognitive and learning outcomes in younger patients. Ethically, this means it would be unlikely that these drug therapies could be tested in

clinical trials on young children. Due to the limitations of rapamycin and mTORC1 inhibitors, there is a clear unmet need for novel therapeutics in TSC, which is the primary focus of TSC research. Drug development often follows the identification of a molecular target, or pathway, with the potential for therapeutic exploitation. Ideally, TSC therapeutics should be targeted at pathways that invoke a cytotoxic response within the tumour cells, as opposed to temporarily inducing growth arrest.

To give an example of a therapy in the cancer setting that exploits a vulnerability, we can consider the concept of genotoxic drugs, which are cytotoxic to cancer cells by inducing DNA-damage. Why would you want to damage the DNA within a patient with cancer? The concept of this therapy is that the normal cells within the body can tolerate the DNA damaging agent, and respond to the DNA damage appropriately. Normal cells would stop proliferating; allowing them time to effectively repair the DNA damage. Once the DNA damage is repaired and the cellular stress dissipates, the normal cells can resume their normal activities. In contrast to the normal cells, cancer cells are unable to turn off proliferative drive in the presence of DNA damage. This inflexibility is a major conflict within a cancer cell; cancer cells will continue to proliferate in the presence of DNA damage, this would act as a trigger to induce a cell death response. While DNA damage has been a powerful treatment for cancer through chemotherapy agents, such treatments for TSC patients would be unfeasible because they give rise to second-hit mutations that could potentially affect healthy cells and give rise to a new tumour. Conceptually, TSC patients require a therapy that is non-genotoxic but can still elevate cell stress in tumor cells.

To find a new therapy, a suitable pathway or a cell process must be ascertained as a potential vulnerability. The idea is to find a pathway that we can target with a drug that is not tolerated by the TSC diseased cells and consequently triggers a cell death response. One such molecular pathway with potential therapeutic exploitation in TSC is the endoplasmic reticulum (ER) stress pathway. Previously, this chapter touched on the role of energy stress in mTORC1 regulation; energy stress should also be considered as a possible therapeutic option.

1.2.1 ER stress

The ER is a complex network of folded membrane tubules that perform a variety of cellular and metabolic processes involved in protein folding and trafficking between membrane organelles. The ER also has an important role in many anabolic processes including gluconeogenesis and lipogenesis, as well as protein synthesis, modification and maturation (Hetz, 2012). The ER receives several environmental and cellular inputs that function to maintain a homeostatic environment. Any disruption to the integrity of the ER, including energy starvation, calcium depletion and impaired protein transport, can lead to the induction of ER stress. ER stress is also implicated in the pathogenesis of a number of diseases including Alzheimer's disease, inflammatory bowel disease (IBD), diabetes mellitus and cancer (Lin, Walter, & Yen, 2008). In the context of mTORC1 hyperactive cells, ER stress is induced by the increased production of de novo proteins through mRNA translation, which leads to the accumulation of disorganized, unfolded protein in the ER. In response to this accumulation of unfolded protein within the ER, the ER employs an adaptive response termed the unfolded protein response (UPR) to restore homeostasis (Schroder & Kaufman, 2005). The UPR is essential for cell survival and is initiated by three distinct ER stress-sensor receptors: inositol-requiring enzyme 1 (IRE1 α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Oyadomari & Mori, 2004) (Fig. 6).

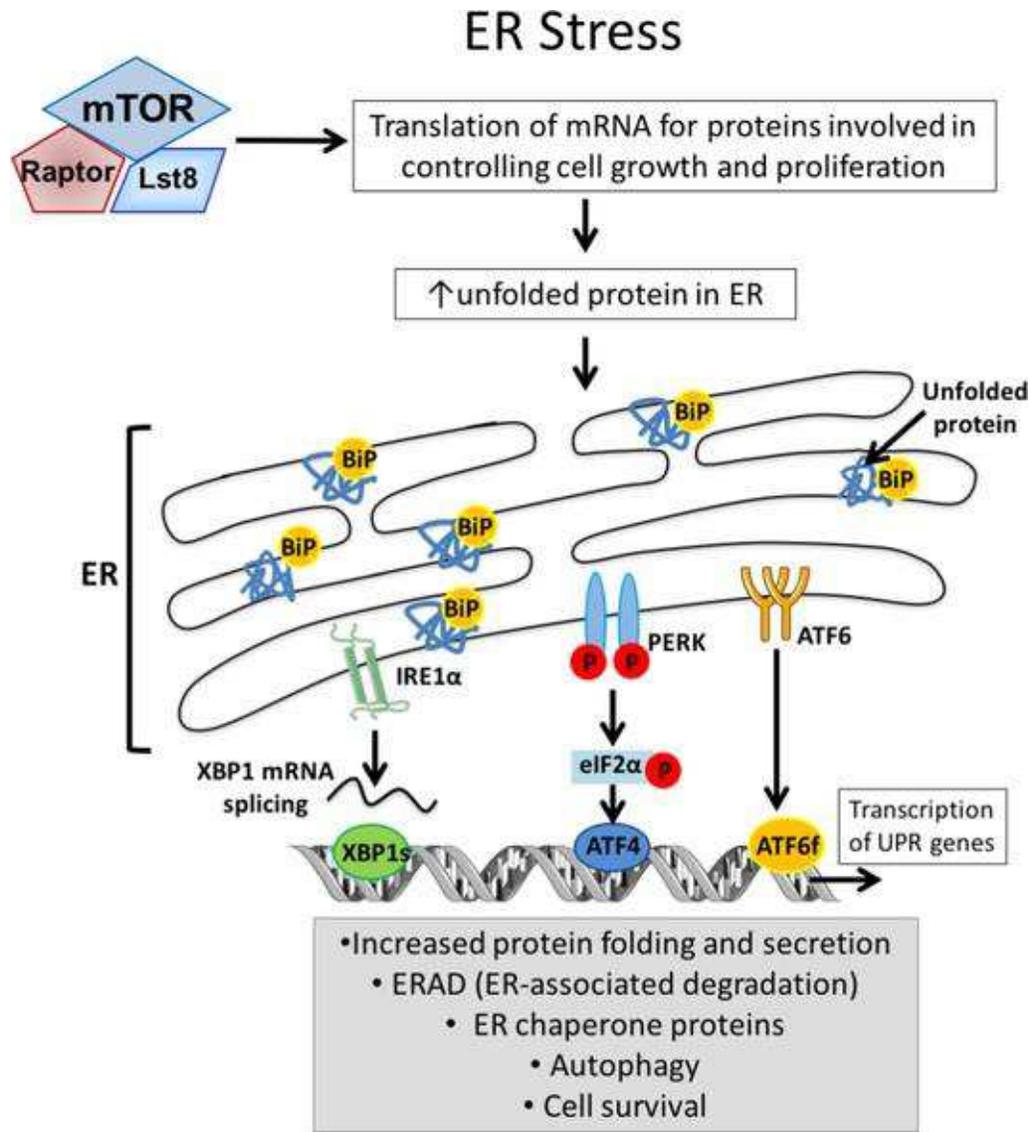


Fig. 6 Initiation of the ER stress response. Hyperactive mTORC1 leads to increased levels of mRNA translation and the accumulation of unfolded protein in the ER. Unfolded protein binds BiP which causes it to dissociate from IRE1 α , PERK and ATF6. Subsequent activation of each receptor results in production of transcription factors XBP1s, ATF4 and ATF6, respectively. These modulate gene transcription to restore ER homeostasis.

Under normal conditions, all three receptors exist in an inactive conformation when bound to a protein called binding immunoglobulin peptide (BiP). During ER stress, unfolded protein binds and reversibly dissociates BiP, leading to receptor activation and initiation of the UPR (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000). Each of the ER-stress sensor receptors employs a unique mechanism by which it modulates gene transcription. Under high stress conditions, IRE1 α forms large clusters which activates the cytosolic RNase domain; this then splices X-box binding protein 1 (XBP1). XBP1 spliced (XBP1s) is a stable transcription factor that enters the nucleus and induces transcription ER-associated degradation (ERAD) genes. Activation of PERK results in phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) which subsequently causes expression of ATF4 (activating transcription factor 4). ATF4 is responsible for modulating the transcription of genes involved in protein synthesis attenuation, autophagy and amino acid synthesis (Fusakio et al., 2016).

Cell viability is dependent on the balance between increased protein requirements and the ability of the UPR to resolve ER stress and initiate of self-repair mechanisms. However, if ER stress is prolonged and intensified, the cell reaches a critical threshold of stress in which it cannot survive. This is a vulnerability of TSC-tumour cells, as they are less efficient at restoring ER stress. During induction of the UPR, the cell

expresses a transcription factor called CCAAT-enhancer-binding protein homologous protein (CHOP) which is the key initiator of cell death when CHOP is expressed for a long duration of ER stress (Oyadomari & Mori, 2004). CHOP induction is predominantly mediated by ATF4 and studies have shown that CHOP-deficient cells are resistant to ER-stress induced apoptosis (Oyadomari & Mori, 2004). Binding of CHOP to its dimerization partner, CCAAT-enhancer binding protein (C/EBP), enables interaction with a collection of proteins termed downward-stream of CHOP (DOCs) (Wang et al., 1998). Subsequent effects include down-regulation of pro-survival proteins such as B-cell lymphoma 2 (Bcl-2) and induction of pro-apoptotic proteins such as the caspases that trigger apoptosis (Endo, Mori, Akira, & Gotoh, 2006). TS-tumor cells as well as many cancer cells with an elevated mTORC1 signaling pathway have an elevated levels of ER stress due to the higher levels of protein translation. Consequently, these cells have a higher basal level of ER stress and their UPR is elevated as a pro-survival mechanism (Schonthal, 2012). However, this persistent stimulation of the ER stress response could be considered as a potential vulnerability of the cell, where the balance might be shifted from a pro-survival, to a pro-apoptotic state (see Fig. 7). Specifically, angiomyolipoma cells (AMLs) taken from TSC patients have previously been shown to have elevated levels of ER stress which can be exacerbated further by ER stress inducers such as thapsigargin (Siroky et al., 2012).

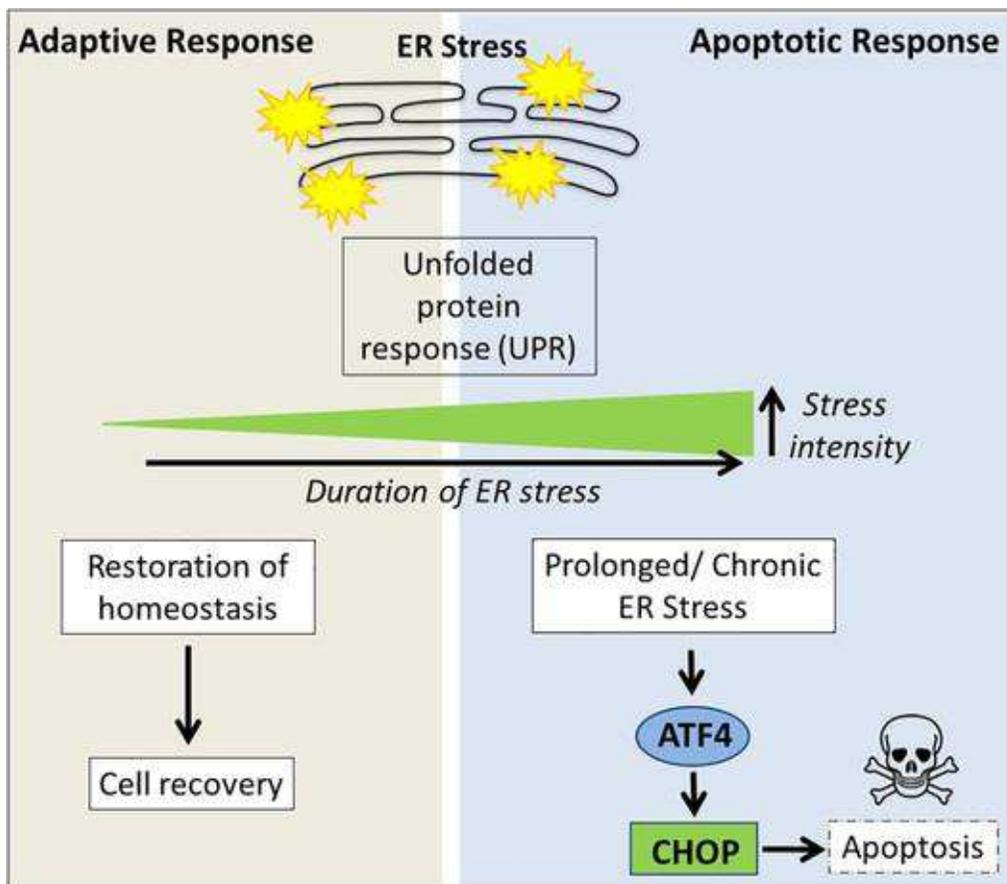


Fig. 7 Outcomes of the ER stress response: cell survival versus cell death. Lower intensity ER stress leads to induction of the UPR, restoration of homeostasis and ultimately cell recovery/survival. Prolonged ER stress at higher intensities leads to induction of the pro-apoptotic protei CHOP, predominantly by the transcription factor, ATF4. CHOP leads to induction of apoptosis. ER stress is a potential vulnerability in TSC-diseased cells that could be therapeutically exploited to kill the tumor cells.

Forward research progress has been made regarding testing ER stress inducers as a potential therapy for TSC. Recent studies showed that combinatory therapy with an ER stress inducer, Nelfinavir, in the presence of an autophagy inhibitor, Chloroquine (Johnson et al., 2015), or a proteosomal inhibitor, Bortezomib (Johnson et al., 2018), showed promise in both in vitro and in vivo models of TSC to reduce tumor size and to selectively kill the TSC-diseased cells. There was a marked difference when comparing rapamycin to these drug combinations. For instance, rapamycin was very efficient at shrinking the tumor volume, but after removal of rapamycin, cells within the tumor quickly recovered. In

contrast, Nelfinavir and Bortezomib were effective at killing the tumors in vitro, where there was not cell recovery after removal of this drug combination (Johnson et al., 2018). These studies demonstrate a potent cytotoxic response to TSC-diseased cells with ER stress inducers, which are tolerated by the normal cells with an intact TSC1/TSC2 signaling pathway (Fig. 8). The cytotoxic response within the TSC-diseased cells is presumably because they are heavily compromised in their ability to recover from an acute ER stress insult.

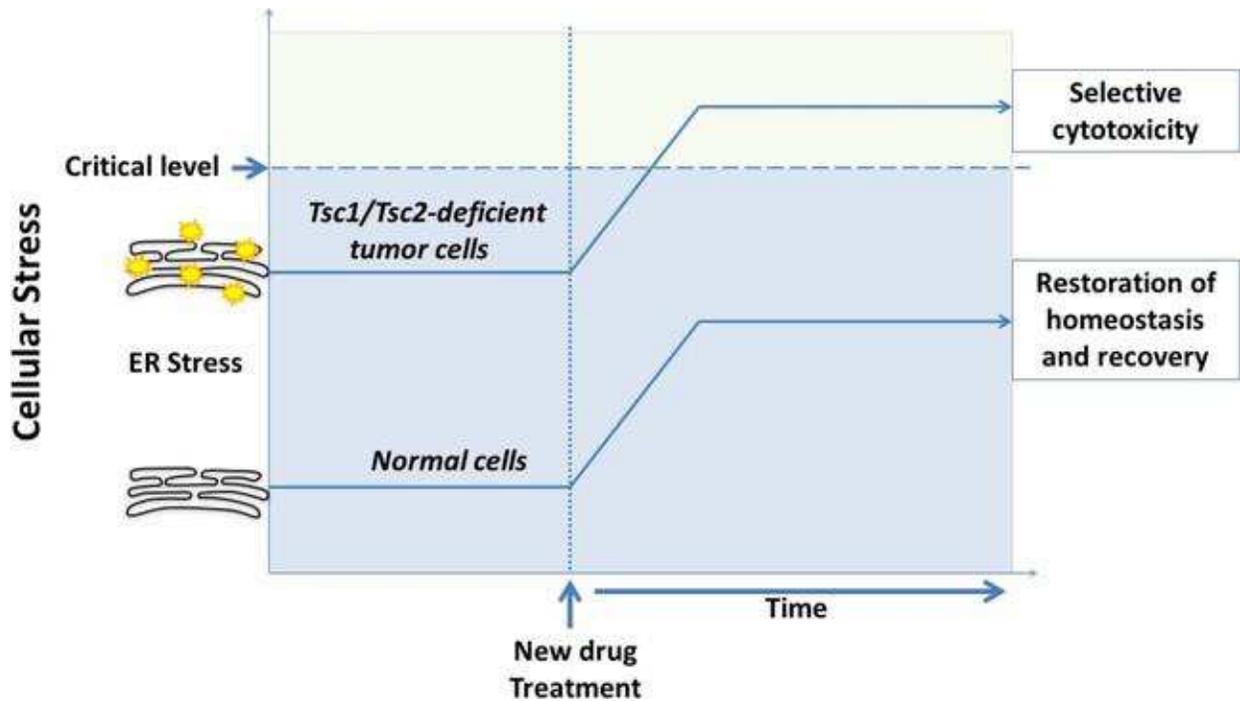


Fig. 8 ER stress response as a tumor vulnerability. *Tsc2*-deficient tumor cells have a higher basal level of ER stress that could be enhanced by therapeutic drug treatment to evoke a cytotoxic response. In contrast, the normal cells containing functional TSC1 and TSC2 would tolerate ER stress as they still have the capacity to recover from ER stress.

1.3 Future directions for TSC therapy

A significant challenge in TSC research is to identify a drug, or drug combination, that is superior to conventional cytostatic agents, such as mTOR inhibitors. Unfortunately, drug development is a lengthy process and, because of this, patients may not infer benefit for many years. The idea of exploring tumor vulnerability using already clinically approved drug combinations is not a new concept; however, the repositioning of these drugs could revolutionize the treatment of conditions with limited therapeutic options, such as TSC. In addition, utilizing TSC as a model system to screen for future anti-cancer drugs also holds much promise.

While chemotherapy agents are not suitable for TSC patients, the principle behind their success when treating cancer could be followed. Rather than using a genotoxic drug, non-genotoxic drugs would instead be employed that target key vulnerabilities of the TSC diseased cells. Potential pathways for future therapeutic exploitation include homeostatic pathways involving energy, nutrients and the ER. Autophagy and the proteasome are also considered as potential cell survival pathways. Furthermore, pathways involved in cellular growth and metabolism are starting to show potential for the treatment of TSC, where blocking of nucleotide precursors is also cytotoxic to TSC-diseased cells (Valvezan et al., 2017).

In summary, a significant amount of knowledge has been gained regarding the genetics and the cellular pathology of TSC. This

knowledge has been instrumental in the current therapy of TSC using mTORC1 inhibitors, which is a relative clinical success but not without limitations. However, it is very possible that preventative therapy with mTORC1 inhibitors for life-long treatment is sufficient to increase the quality of life of TSC patients. The idea of curing the disease is still an ongoing challenge, a challenge that is supported by the TSC community. Our hope is that our combined efforts will lead to new and better drug therapies, and possibly a cure, for TSC patients in the near future.

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Conflict of Interest

The authors declare no conflict of interest.

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