Molecular features of low-grade developmental brain tumours

Focusing on subependymal giant cell astrocytomas in tuberous sclerosis complex

Bongaarts, A.

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Chapter one

General introduction & outline of the thesis

New insights into a spectrum of developmental malformations related to mTOR dysregulations: challenges and perspectives

Mühlebner A[1], Bongaarts A[1], Sarnat HB[2], Scholl T[3] & Aronica E[1]

1.Department of Neuropathology, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands. 2.Departments of Paediatrics, Pathology (Neuropathology) and Clinical Neurosciences, University of Calgary Cumming School of Medicine and Alberta Children's Hospital Research Institute (Owerko Centre), Calgary, AB, Canada. 3.Department of Paediatric and Adolescent Medicine, Medical University of Vienna, Vienna, Austria

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Bongaarts A[1]*, Glowacka J[2]*, Wojdan K[3], Mühlebner A[1], Aronica E[1,4] & Mills JD[1]

1.Department of Neuropathology, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands. 2.Warsaw University of Technology, The Faculty of Electronics and Information Technology, Warsaw, Poland. 3.Warsaw University of Technology, Institute of Heat Engineering, Warsaw, Poland. 4.Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede, The Netherlands

submitted

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Low-grade gliomas

Clinically brain tumours can present with various symptoms, of which seizures are the most prominent [1]. Several factors are associated with the epileptogenicity of brain tumours, such as the type of tumour, grade, anatomical location, size, time interval before resection and the age of the patient. Particularly, slow-growing, low-grade glial and glioneuronal tumours are found to be highly epileptogenic. Most likely the slow growth rate gives enough time for reorganization of the adjacent cortex or other brain areas such as the hippocampus, leading to increased hypersensitivity and ultimately increasing the chance of epileptogenesis [2]. Low grade gliomas (LGGs) are considered grades I/II according to the WHO [3]. Low-grade astrocytic and oligodendroglial tumours include diffuse astrocytoma and oligodendrogliomas, whereas low-grade glioneuronal tumours include a large spectrum of entities such as desmoplastic infantile astrocytoma/ganglioglioma (DIA/DIG), papillary glioneuronal tumour, rosette-forming glioneuronal tumour, pilomyxoid astrocytoma, pleomorphic xanthoastrocytoma (PXA), pilocytic astrocytomas (PA), subependymal giant cell astrocytoma (SEGA), ganglioglioma (GG) and dysembryoplastic neuroepithelial tumour (DNT). According to the 2016 WHO classification, diagnosis of these tumours is based predominately on histological criteria, however interobserver variations are present and the histological characteristics overlap between entities, making classification based on their morphology alone challenging [4-6]. Therefore, the use of molecular markers based on genetic alterations and epigenetics is suggested [7].

While adult low-grade gliomas are capable of developing into higher-grade lesions, pLGGs seldomly transform into a malignant state, which most likely is a consequence of the molecular and genetic differences [8,9]. In adult patients with LGGs, a genetically based classification exists, where low-grade astrocytomas are defined by *Isocitrate dehydrogenase* (*IDH*) mutations, usually combined with *ATRX chromatin remodeler* (*ATRX*) and *tumour protein 53* (*TP53*) mutations, while oligodendrogliomas have whole arm 1p/19q co-deletion combined with *IDH* mutations [10,11]. Moreover, the presence of *IDH1/2* mutations can exclude the diagnosis of GG, DNT and gangliocytoma [3]. In paediatric LGGs (pLGGs) these genetic alterations are more rare. Genetic events involving the extracellular signal–regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway are most common in pLGGs, such as the *B-Raf proto-oncogene, serine/threonine kinase* (*BRAF*) c.1799T>A (p.V600E; *BRAFV600E*) mutation or the *KIAA1549-BRAF* fusion gene, which both result in constitutive activation of BRAF [12-19]. Other genetic alterations in *fibroblast growth factor receptor* (*FGFR*) *1/2/3*, *Raf-1 proto-oncogene, serine/threonine kinase* (*RAF1*), *ALK receptor tyrosine kinase* (*ALK*), *neurotrophic receptor tyrosine kinase 2* (*NTRK2*), *MET proto-oncogene, receptor tyrosine kinase* (*MET*), *ROS proto-oncogene 1, receptor tyrosine kinase* (*ROS1*), *MYB Proto-Oncogene, Transcription Factor* (*MYB*), *MYB proto-oncogene like 1* (*MYBL1*), *IDH1/2*, and *H3.3 histone A* (*H3F3A*) are less common [20-22]. Additionally, in contrast to most adult gliomas, pLGGs can also have a hereditary component. For example, SEGAs are low grade brain tumours that account for ~2% of pLGGs and occur almost exclusively in patients with tuberous sclerosis complex (TSC), a neurocutaneous disease caused by a germline mutation in either the *TSC1* or *TSC2* gene, which results in

constitutive activation of the mechanistic target of rapamycin complex (mTOR) pathway [3,23-26].

Although, some genetic alterations are enriched in certain tumour entities they are almost never exclusive to one entity alone. For example, the *BRAFV600E* mutation is a common genetic driver in GGs (18-56%) and can together with tyrosine kinase activating *FGFR1* gene mutations be helpful in distinguishing GGs from DNTs [13,15,16,27,28]. However, *BRAF* mutations also occur in other tumour entities including DNTs, making it difficult to distinguish between GGs and DNTs based only on the presence of these mutations alone [14,17,29]. Therefore, an integrated diagnosis combining both histological and molecular features based on genetic and genomic hallmarks is needed [3,30,31].

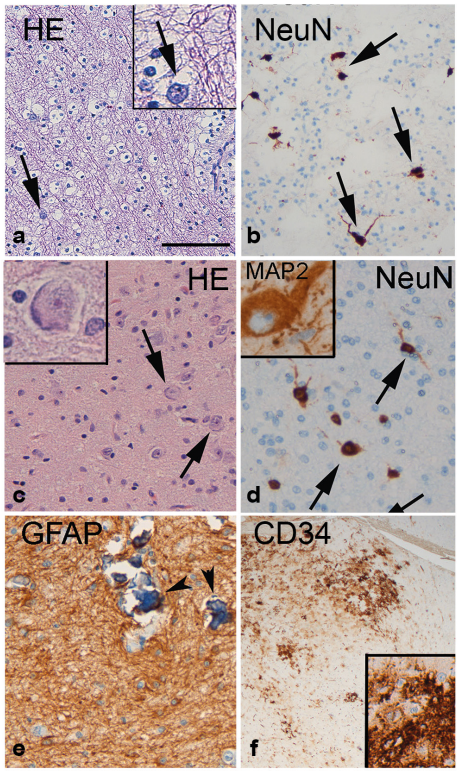

Figure 1. Histopathology of a *FGFR1* mutated DNT and a *BRAFV600E* mutated GG. a. HE staining of DNT showing a typical glioneuronal element, with floating neurons (arrows) surrounded by a prominent population of oligodendroglia-like cells (OLC) (insert: higher magnification showing floating neurons). **b.** NeuN staining showing the neuronal component of DNT. **c**. HE staining of GG showing the mixture of neuronal cells (dysmorphic neurons), lacking uniform orientation (arrows and insert) and glial cells. **d.** NeuN staining indicating the neuronal component (nuclear staining, arrows) of GG (insert: higher magnification showing MAP2 positivity indicating the neuronal component in dysmorphic neuron). **e**. GFAP staining indicating the astroglial tumour component in GG (arrowheads indicates micro-calcifications). **f.** CD34 staining showing positive cell aggregates infiltrated into adjacent neocortex in GG (insert: higher magnification showing cells with intense CD34 immunoreactivity). Scale bar in **a**: **a**: 160 µm; **b, c**: 80 µm; **d, e**: 40 µm; **f**: 3 mm.

Low-grade epilepsy-associated tumours

Although it is not unusual for brain tumours to clinically manifest with seizures, low-grade epilepsy-associated tumours (LEATs) are generally slowly growing tumours that most often arise in younger age groups with a mean age of seizure onset at 16.5 years [6,32]. LEATs were first introduced by Luyken et al. as tumours that were commonly encountered in patients that underwent surgery, who had been investigated and treated for drug-resistant seizure episodes for 2 years or longer [33]. The most commonly found tumours that are characterized as LEATs are DNTs and GGs. Additionally, several other low-grade tumours

have been classified as LEATs such as papillary glioneuronal tumours (PGNT), rosette-forming glioneuronal tumours (RGNT), polymorphous low-grade neuroepithelial tumours of the young (PLNTY), mutinodular and vacuolating neuronal tumour (MVNT) of the cerebrum, diffuse leptomeningeal glioneuronal tumour (DLGNT), PA, PXA, diffuse astrocytomas, oligodendrogliomas and angiocentric glioma.

Histologically DNTs can display a typical multinodular architecture with glial nodules and/or specific glioneuronal elements (characterized by columnar bundles of axons surrounded by oligodendrocyte-like cells oriented perpendicularly to the cortical surface and separated by a myxoid matrix that contains floating neurones) (Figure 1a-b) [3,34]. GGs consist of a mixture of neurons and glial tumour cells mainly represented by a large spectrum of astroglial cells (Figure 1c-e). Furthermore, CD34 expression in tumour satellites has been suggested to reflect offspring from dysplastic or developmentally compromised neural precursors (Figure 1f) [35]. The neuronal component, which varies in amount, is represented by dysplastic neurons with abnormal shape and size, lacking uniform orientation and often expression of the phosphorylated form of the downstream target ribosomal S6 protein (S6) of mTORC1. Interestingly, the *BRAFV600E* mutation, which is generally found in glioneuronal tumours (GNTs), has been shown to be significantly associated with the expression of pS6 in these tumours [17]. BRAF-induced phosphorylation of LKB1 may represent a possible mechanism contributing to mTOR activation in *BRAFV600E* mutated GNTs, possibly through uncoupling of the LKB1-AMPK-mTOR signalling. However, seizure activity itself and the inflammatory environment (i.e. via interleukin-1β) could also contribute to the activation of the PI3K-AKT3-mTOR signalling pathway [36,37]. Furthermore, the recently reported genetic alterations detected in LEATs provide evidence of a functional connection between two major signalling pathways: RAS–RAF–MAPK and PI3K–AKT–mTOR [6].

The ERK/MAPK pathway

The majority of LEATs and pLGGs are driven by a single genetic event that results in activation of the affected ERK/MAPK pathway (Figure 2) [21,22,38]. In total there are four conventional MAPK cascades defined based on the components ERK1/2, c-Jun N-terminal kinase (JNK), p38 MAPK and ERK5, which can all regulate basic cell processes such as proliferation, differentiation and motility [39-41]. The mammalian ERK/MAPK pathway includes the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK2, together forming the Ras-Raf-MEK-ERK pathway [42]. Most often extracellular agents such as growth factors, cytokines and hormones activate the ERK/MAPK pathway by binding and activating the transmembrane glycoproteins of the receptor tyrosine kinase (RTK) family. The phosphorylated residues of RTK can function as binding sites for proteins containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, such as growth factor receptor-binding protein 2 (GRB2) [43]. Together with GRB2, guanine nucleotide exchange factors (GEFs) such as Son of Sevenless (SOS) convert GDP to GTP resulting in RAS activity [44,45]. This process may be reversed by GTPase-activating proteins (GAPs), such as neurofibromin [46]. RAS activation can recruit RAF kinases to the plasma membrane for RAF activation [47]. The C-terminal catalytic

domain of RAF can interact with MEK resulting in phosphorylation of MEK1 and/or MEK2, which subsequently activates ERK1 and ERK2 [48,49]. Upon activation ERK1/2 translocate to the nucleus where they can regulate transcription of genes involved in cell proliferation, differentiation, survival and apoptosis [50]. Moreover, the ERK/MAPK pathway is known to be involved in brain development [51,52]. Therefore, it is not surprising that dysregulation of the ERK/MAPK pathway can lead to the occurrence and progression of LEATs and pLGGs.

Treatment and diagnosis of LEATs and pLGGs

Currently the treatment of choice for LEATs and pLGGs is gross total surgical resection [53]. However, in cases where total resection is not possible, or cases with recurrence or malignant transformation, additional radiation or chemotherapy is needed, which can negatively affect survival rates and the quality of life of patients [33,54-59]. Therefore, a better understanding of the molecular profile of these tumours could help to improve the postsurgical treatment regimen [33,58,59]. Over the past decades, the development of sequencing techniques has transformed the landscape of molecular biology especially at the levels of transcriptomics and genomics. Molecular stratification of pLGGs and LEATs has led to novel diagnostic strategies and trials with more specific inhibitors, such as MEK and mTOR inhibitors [60-62]. Clinical trials with such inhibitors seem promising for the treatment of pLGGs and LEATs with *BRAF* mutation or other alterations of the ERK/MAPK pathway including GGs (for review see [63]). However, despite the advances, response to treatment with these inhibitors can be variable and the clinical implications of these molecular markers are not always clear [64-66]. Moreover, response to treatment with these inhibitors can be variable depending on the genetic mutations emphasizing the risk of trials without a good molecular characterization. Furthermore, although some histological entities are enriched for molecular events, they are not always mutually exclusive. Therefore, more comprehensive analyses of low grade gliomas, including LEATs and pLGGs, using novel sequencing techniques in combination with conventional methods are needed to identify novel targets for therapy and to further improve treatment and diagnosis.

Tuberous sclerosis complex

TSC is a neurocutaneous disorder caused by mutations in either the *TSC1* or *TSC2* gene, resulting in dysregulation of the mTOR pathway, which demonstrates a direct link between a genetic mutation and brain pathology [67-71]. Clinically, TSC is associated with epilepsy, autism and intellectual disability. About 90% of individuals with TSC exhibit TSC-Associated Neuropsychiatric Disorders (TANDs) which includes behavioral, psychiatric, intellectual, neuropsychological, and psychosocial issues [72-75]. In addition to the neurological manifestations, TSC can also cause dermatologic manifestations (facial angiofibromas), renal angiomyolipomas, pulmonary lymphangioleiomyomatosis and cardiac rhabdomyoma [71,76,77].

Neuropathological examination of TSC brain specimens reveals three major lesions: subependymal nodules (SENs), SEGAs and cortical tubers (Figure 3) [26,78-82]. Cortical tubers are lesions that are predominantly found in frontal and temporal regions and can extend into the white matter. They show cortical dyslamination and consist of dysmorphic neurons

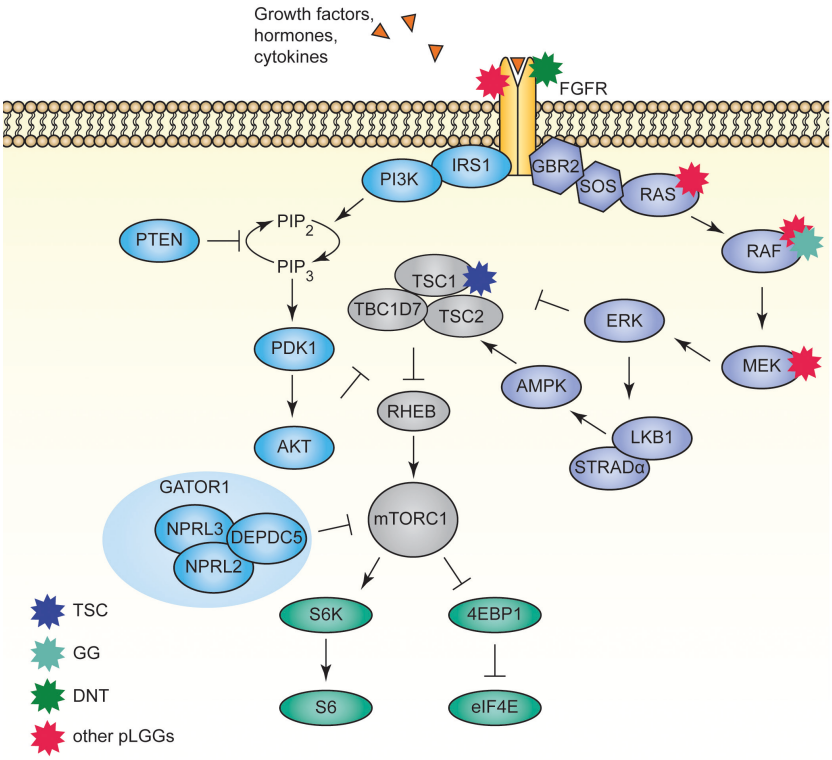

Figure 2. A schematic overview of the MAPK and mTOR pathways showing the proteins that are affected by mutations in TSC, GG, DNT and other pLGGs adapted from [224]. IRS1, insulin receptor substrate 1; PI3K, PI3kinase; PDK1, phosphoinositide-dependent kinase-1; PTEN, phosphatase and tensin homologue; AKT, protein kinase B; GBR2, Growth factor receptor-bound protein 2; SOS, Son of Sevenless; RAF, RAF proto-oncogene serine/threonine-protein kinase; MEK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; LKB1, tumour suppressor liver kinase B1; STRADα, STE20-related kinase adaptor alpha; AMPK, AMP-activated protein kinase; TBC1D5, TBC1 Domain Family Member 5; RHEB, ras homolog enriched in brain; mTORC1, mammalian target of rapamycin complex 1; DEPDC5, DEP Domain Containing 5; NPRL2, NPR2 Like, GATOR1 Complex Subunit; NPRL3, NPR3 Like, GATOR1 Complex Subunit; GATOR1, Gap Activity Toward Rags 1; S6K1, p70S6kinase; S6, ribosomal S6 protein; 4EBP1, eIF4E-binding protein 1; eIF4E, binding of eukaryotic translation.

(characterised by abnormal morphology and orientation, an enlarged cell body and cytoplasmic accumulation of neurofilaments within the nucleus) and giant cells (characterised by an enlarged cell body, often multiple nuclei and eosinophilic cytoplasm) (Figure 3G-J) [74,78,79,81,83-86]. Giant cells express both neuronal and glial markers, suggesting aberrant differentiation before cell migrate into the cortex [87-90] (for review see [70,91]). Dysmorphic neurons are characterised by the expression of multiple cortical layer markers, independent of their laminar location and similar to that of cortical projection neurons, suggesting specific changes in a subgroup of intermediate progenitor cells [70,81,92]. Moreover, the morphological features of dysmorphic neurons and giant cells can be explained by the enhanced activation

of the mTOR pathway (indicated by increased phosphorylation of S6K1 and S6) (figure 3G) [69,70,91]. Recently, three distinct histological cortical tuber subtypes (designated as types A, B and C) have been described based on the proportion of calcifications, dysmorphic neurons and giant cells [80].

Cortical tubers can already develop during embryonic brain development and can therefore be detected prenatally [70,93-97]. Additionally, elevated mTORC1 activation has been shown in prenatal cortical tubers, before seizure development [70,97,98]. Seizures in the fetus are not physiologically possible before 24–25 weeks of gestation because synaptogenesis in the cortical plate is not initiated until 22 weeks of gestation [99]. These findings indicate the importance of the mTOR pathway during brain development and highlight the role of mTOR dysregulation in pathological changes of the brain [69,70].

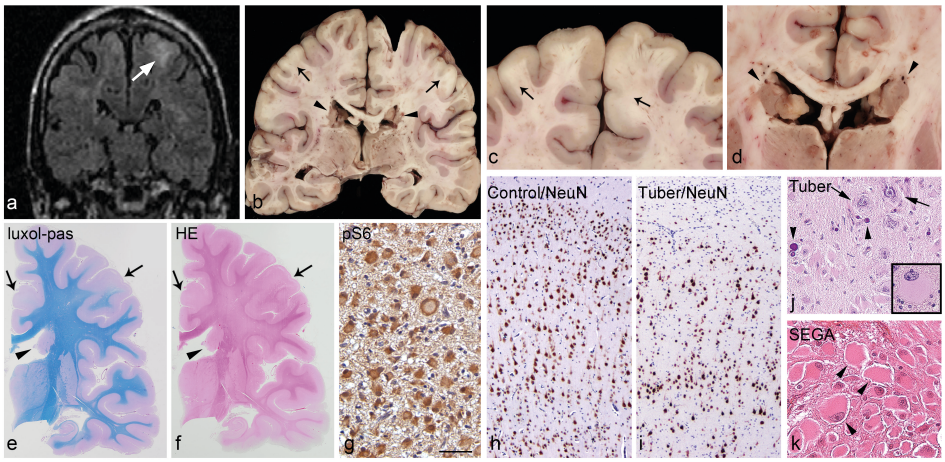

Figure 3. Tuberous sclerosis complex (TSC) adapted from [224]. a. MRI showing a TSC lesion (histologically proven; arrow). **b-d.** Coronal sections of the brain (32-year-old patient with a germline mutation in the *TSC2* gene; [83,86]) showing several regions with blurring of the cortex/white matter junction (arrows in **b** and **c**) and subependymal nodules (arrowheads in **b** and arrows in **d**). **b,c.** A high magnification of abnormal brain regions with loss of a distinct cortex/white matter junction and mushroom-like appearance of the gyri, indicating the presence of tubers. **d.** A high magnification of the SENs which appear as firm oval-shaped structures projecting into the ventricles. **e,f.** Histological stains: Luxol fast blue-PAS-staining (**e**) and haematoxylin and eosin (H&E; panel **f**), showing cortical tubers (arrows) and subependymal nodules (arrowheads; [83,86]). **g.** phospho-S6 ribosomal protein (pS6) staining positive in giant cells of a tuber [97]. **h,i.** NeuN staining showing difference in the architecture between the perituberal cortex (**h**) and the tuberal cortex (**i**) with dyslamination within the cortical tuber. **j,k.** H&E staining of a tuber (**j**) and SEGA (**k**). Tuber showing large dysmorphic neurones (arrows), calcification (arrowheads) and a giant cell in insert [83,86]. SEGA showing giant cells (arrowheads) with a mixed glial background and blood vessels. Scale bars in **g**: **g**: 60 µm; **h,i**: 250 µm; **j,k**: 40 µm.

The mTOR pathway and TSC

The mTOR pathway is another signaling cascade that plays an important role in brain development and neurological diseases such as TSC [23,100-102]. Although this pathway is generally thought to be the driver of dysplasia's, enhanced mTOR activation has also

been seen in low grade gliomas including, GGs, DNTs and SEGAs (Figure 2). Similarly, to the ERK/MAPK pathway, the mTOR pathway is activated by growth factors, cytokines and hormones. As a member of the phosphatidylinositol 3-kinase related kinase (PIKK) family, mTOR functions as catalytic subunit of two distinct multi-protein complexes called mTORC1 and mTORC2, which have distinct molecular compositions [103]. Specific to mTORC1 are regulatory-associated protein of mTOR (RAPTOR) and proline-rich AKT1 substrate 40 kDa (PRAS40), whereas mTORC2 contains rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated protein kinase-interacting protein 1 (mSIN1), and protein observed with RICTOR (PROTOR). Dysregulation of mTOR may affect proliferation and migration of neural progenitor cells, neuronal soma size, dendritogenesis and formation of dendritic spines, axon outgrowth, astrocyte proliferation and cortical lamination [103]. The role of mTORC1 in neurodevelopment is well established. Via phosphoinositide-3' kinase (PI3K) and phosphoinositide-dependent kinase-1 (PDK1), AKT is activated and can inhibit the TSC complex formed by hamartin (encoded by *TSC1*), tuberin (encoded by *TSC2*), together with TBC1 domain family member 7 (TBC1D7). This TSC complex functions as a GAP for small G-protein Ras homology enriched in brain (RHEB), acting as an upstream regulator of mTORC1 [104-106]. Downstream of mTORC1, the ribosomal protein S6 kinase beta-1 (S6K1)/S6 and eukaryotic initiation factor 4E-binding protein 1(4E-BP1)/ Eukaryotic translation initiation factor 4E (eIF4E) can initiate translation [107].

Loss of function mutations in *TSC1* or *TSC2* result in constitutive activation of the mTORC1 pathway [108]. In TSC, germline mutations in *TSC1* or *TSC2* can be familial inherited in an autosomal dominant fashion, but more often are sporadic in nature. Approximately, 60% of patients have a *TSC2* mutation, while 30% of patients have a *TSC1* mutation [109]. In 10-15% of TSC patients, a TSC mutation cannot be identified, suggesting a potential low-level somatic mosaicism [110]. Interestingly, *TSC2* mutations generally result in more severe clinical manifestations than *TSC1* mutations in TSC[111]. Lesions in TSC are thought to be caused by biallelic inactivation of either *TSC1* or *TSC2*, also known as loss of heterozygosity (LOH), reflecting mosaicism that originates from loss of the corresponding wild-type allele. However, second-hit mutations are not always detected in cortical tubers and SEGAs [70,112,113]. Potentially, only a small number of cells within the tuber/SEGA have LOH in either *TSC1* or *TSC2*, suggesting limitations in the detection of second-hit mutations in these lesions [70]. Alternatively, the mono-allelic TSC mutation, perhaps together with a destructive non-autonomous phenotype of mutated cells is sufficient for tuber/SEGA development [70,114]. Furthermore, other pathways might also be at play in the development of these lesions.

Interestingly, ERK/MAPK activation can result in downstream activation of mTORC1 by inhibiting the TSC complex, whereas mTORC1 inhibition can lead to ERK/MAPK activation in turn, indicating that these pathways are intrinsically linked [115-118]. Moreover, it has been shown that both the mTORC1 and the ERK/MAPK pathway can be activated by the lysosomal Ragulator complex consisting of late endosomal/lysosomal adaptor, MAPK and mTOR activator 1–5 (LAMTOR1/p18, LAMTOR2/p14, LAMTOR3/MP1, LAMTOR4/C7orf59 and LAMTOR5/HBXIP) [119-122]. Dysregulation of the ERK/MAPK and mTOR pathway has been

implicated in SEGAs and targeting both pathways in pLGGs has been suggested [123].

Subependymal giant cell astrocytoma

SEGAs are low-grade glioneuronal tumours classified as WHO grade I and represent 1%-2% of all pediatric brain tumours [3,23]. Since 2012, The International Tuberous Sclerosis Complex Consensus Conference has defined SEGA as either a lesion that is located at the caudothalamic groove with a size >1 cm or a subependymal lesion of any size with serial growth based on consecutive imaging [77]. Although SEGAs are benign and slow growing tumours, extensive growth can cause obstruction of the cerebrospinal fluid tract leading to hydrocephalus and in some cases even sudden death [124,125].The prevalence of SEGAs in patients with TSC ranges from 5% to 25% [124,126-129]. SEGAs can already be detected in fetal and neonatal periods, but generally develop during the first two decades of life [97,127,130-134]. The occurrence of de novo SEGAs after the age of 18 years is relatively low, however tumour growth can still be observed in ~20% of adult patients [134-136]. SEGAs are thought to arise from SENs along the ependymal lining of the lateral ventricles at the height of the foramen of Monro [138-140].

Histologically, SEGAs consist of spindle cells, gemistocytic-like cells and giant cells and are indistinguishable from SEN (Figure 3K). They can also present vascular stroma and parenchymal or vascular calcifications. Initially, it was thought that SEGAs have an astrocytic character, however more recent studies have demonstrated a mixed glio-neuronal phenotype. SEGAs are usually strongly positive for glial fibrillary acid protein (GFAP) and S-100 protein as well as for neuronal markers, i.e. neurofilament proteins (NF) and synaptophysin [141-143]. Due to this mixed phenotype, it has been suggested that their origin lies in neural progenitors that normally reside in the subependymal zone [144]. The percentage of KI67 positive cells in SEGAs is low at 1-2%, which is in accordance with their benign phenotype [145]. However, rare cases with anaplastic features and increased KI67 positivity have been reported [146,147]. Positive staining for mTOR activation has been found predominately in giant cells but not in spindle cells of SEGA [108,143,145,148]. HLA-DR positive microglial cells localize around giant cells in SEGA, which is interesting considering that the mTOR pathway can activate immune responses [149,150].

At the molecular level little is known about establishment and progression of SEGAs. Evidence of second-hit inactivation of *TSC1* or *TSC2* has been reported in SEGAs [108,114]. However, second hit mutations are not always seen in SEGAs and might already be present in SEN, suggesting that additional molecular mechanisms may play a role in their progression and growth. Recently, it was shown that that the overall mutation burden is low in SEGA, which is consistent with their slow growing character [114]. In the same study it was shown that gene ontologies related to cell systems such as inflammation, extracellular matrix organization and synaptic transmission were mainly affected in SEGAs [114]. However, it is still unknown how the deregulation of these cell systems could contribute to SEGA pathology and needs to be further investigated.

Treatment options in TSC

The goal of epilepsy treatment in TSC is to prevent and control seizures as soon as possible.

Antiepileptic drugs (AEDs) are available for seizure treatment, with vigabatrin the AED of choice for infantile spasms and/or focal seizures in TSC [151-153]. Treatment with vigabatrin prior to seizure development improves long-term epilepsy and neurodevelopment outcomes. Other AEDs such as, ACTH (natural or synthetic), prednisolone or GABAergic AEDs other than vigabatrin such as topiramate, carbamazepine and oxcarbazepine can be used as second line therapy [154-157].

Surgery can be an appropriate treatment option in TSC-associated epilepsy that is inadequately controlled after trials of two AEDs, provided that the lesions are well-defined [151]. Cortical tubers are thought to represent the neuropathological substrate for epilepsy in TSC patients and are targeted for surgical resection. Epilepsy surgery where the epileptogenic focus is easily recognizable can improve the quality of life and IQ, especially in postoperative patients who remained seizure-free. Although epilepsy surgery often results in freedom from seizures, increasing evidence supports the importance of the perituberal cortex in TSC [158-165].

Currently, surgical resection is the treatment of choice for symptomatic SEGA (i.e. tumour growth, hydrocephalus, hemorrhage or cystization, and clinical deterioration) [124,125,166-174]. Complete and safe removal of the tumour is curative in almost all cases, whereas subtotal removal increases the risk of recurrence [124,175]. Tumour size correlates negatively with the probability of total resection [124,174]. Therefore, it seems reasonable to resect the tumour as early as possible when no other risk factors are at play. When the probability of total resection seems low or when other potential complications with surgery are foreseen treatment with mTOR inhibitors is preferred. Moreover, treatment with mTOR inhibitors can reduce tumour size to improve the success rate of surgical resection or can even prevent the need for surgery altogether [176,177].

The first report of mTOR inhibitors as a TSC treatment occurred in 2006, where patients with SEGA treated with sirolimus demonstrated on average a 55% reduction in tumour volume, which is supported by other independent studies [61,178-180]. However, to date the only mTOR inhibitor that is Food and Drug Administration (FDA) approved is everolimus. The EXIST-1 study showed that everolimus can reduce volume size of SEGA with at least a 50% reduction in 35% of the patients [181]. The follow up open-label studies from EXIST-1 showed that this amount increased further to 58% of patients with a tumour reduction of more than 50%, indicating that even after 2.5 years patients can still benefit from treatment with everolimus [182,183]. In accordance, a 5 year study showed that the majority of patients could maintain the tumour reduction that was achieved in the first 6 months [184]. In a small cohort of congenital SEGA a good response was also seen [185,186]. In addition to treatment of SEGA, mTOR inhibitors may have additional benefit for treatment of other TSC related manifestations, including seizure control, cognitive development, and volume reduction of other TSC tumours [187].

Although mTOR inhibitors have been shown to be effective in patients with TSC, the response to mTOR inhibitors can be variable and cessation of treatment may result in tumour regrowth [61,182,188-191]. Furthermore, treatment with mTOR inhibitors can produce

side effects, including stomatitis, diarrhea, nasopharyngitis, pyrexia, and upper respiratory tract infections[181,192,193]. Therefore, identifying other targets for therapy are of the utmost importance and can be established with new techniques such as next-generation sequencing.

Unraveling the transcriptome using RNA-sequencing

Over the past two decades, the development of various next-generation sequencing have transformed the landscape of research in molecular biology [194]. The use of microarrays, which uses a predesigned set of probes, have been replaced by next generation RNA-sequencing (RNA-seq), which allows for the entire gene expression profile of a sample to be assessed in a high-throughput manner [194,195]. Giving a snapshot of the gene-expression profile of a tissue or cell at a specific moment in time. Additionally, alternative splicing events, novel transcripts, gene fusion events, map transcription start sites, sequence variation in transcripts and circular RNAs (circRNAs) can be detected with RNA-seq [194-197]. Furthermore, modifications of the standard RNA-seq workflow has given rise to several different RNA-seq based technologies, including small RNA-seq, single-cell RNA-seq (scRNA-seq), single-nuclei RNA-seq (snRNA-seq) and spatial transcriptomics.

A next-generation RNA-seq experiment can be divided into three distinct phases; sample or library preparation, sequencing, and data analysis. During library preparation, RNA is isolated from a sample and reversed transcribed into cDNA, which is followed by ligation of adapters to the end of the ensuing molecules resulting in the generation of cDNA library [196]. The ligation of adapters introduces a unique barcode to each sample allowing for multiplexing of samples during sequencing. Throughout the library preparation steps there are a number of options which can be chosen that will impact on the data produced [195,198]. RNA for RNA-seq can be poly-A selected or selected via ribosomal(ribo)-depletion; poly-A selection enriches for mRNAs and the polyadenylated fraction of non-coding RNAs (ncRNAs), while ribo-depletion enriches for mRNA, pre-mRNA and ncRNA and also allows for the identification of circRNAs [199]. The generated cDNA library is then subject to sequencing, utilising the sequencing-by-synthesis strategy. While sequencing itself is a rather trivial process there are a number of parameters or sequencing conformations that must be considered, including read-length, single-end (SE) or paired-end (PE), and read depth [200]. Regardless of the type of RNA-seq carried out the data-analysis workflow is made up of the following steps; quality control (QC), mapping of reads, quantification of expression of genes or transcripts to generate a count (or expression) matrix. Once a gene or transcript count matrix has been constructed differentially expressed genes can be identified, followed by a pathway or gene ontology enrichment analysis. More advanced analysis techniques can also be used, including weighted gene co-expression network analysis (WGCNA) or various machine learning techniques.

For pLGGs RNA-seq has provided evidence that supports the role of the ERK/MAPK pathway in these tumours and has helped in identifying genetic alteration in these tumours [21,22,38]. Recently, the transcriptional profile of LEATs identified four clusters each with distinct signature genes that do not fully resemble the histopathological classification of LEATs [201].

Interestingly, one of the groups, containing LEATs enriched for astrocytic differentiation, *BRAF* mutation and activation of MAPK/FGFR/EGFR pathways was at higher risk of tumour recurrence than the other three groups. This highlights how RNA-seq can provide novel insights that can be translated to the clinic. Furthermore, in TSC, RNA-seq has identified novel pathways and small RNAs that might play a role in TSC pathogenesis of cortical tubers [114,201]. With the constant development of novel RNA-seq methods we are just at the beginning of fully understanding the transcriptome and the molecular features of such neurological diseases and tumours.

microRNAs

Amongst all small RNA species, microRNAs (miRNAs) have received the most interest as a molecule for study. These short RNAs are crucial post-transcriptional regulators of gene expression/ miRNAs work in concert with the RNA-inducing silencing complex (RISC) to direct post-transcriptional repression of target mature mRNA transcripts by binding to complementary 3' untranslated regions (UTR) [203]. Furthermore, each miRNA can regulate the expression of multiple target genes, and each transcript can be targeted by multiple miRNAs [204]. Thus, miRNAs can potentially regulate entire pathways or networks of genes and as such are immensely important regulators of the transcriptome; so far miRNAs appear to play pivotal roles in regulation of cellular proliferation, differentiation and apoptosis [205]. Interestingly, approximately 70% of all known miRNAs are expressed in the brain and the role of miRNAs in various neurodevelopmental processes, such as neurogenesis, cell fate determination, migration and synapse development has been shown [206,207].

In TSC, dysregulation of miRNAs has been shown, identifying the mir34 family among the most significantly overexpressed miRNAs [202]. Alterations in the expression of numerous miRNAs have also been described in pLGGs, including PA and GGs [208-213]. Moreover, a decision tree has been proposed based on the expression of miRNAs for pediatric neuronal and mixed neuronal-glial tumours suggesting that miRNAs can be useful for classifying tumours that are difficult to distinguish by classical histopathological examination [214].

Methylation profiling of low grade gliomas

Epigenetics refers to the process by which specific epigenetic marks can influence gene regulation and transposon activity without altering the actual sequence of the DNA. The most studied epigenetic marker is DNA-methylation, which is characterized by the addition of a methyl or hydroxymethyl by DNA methyltransferases (DNMTs) to cytosine residues in CG, CXG and CXX DNA sequences (CpG-sites; where X corresponds to A, T, or C), producing 5-methylcytosines (5-mC). When located in a gene promoter region it is generally associated with silencing of gene expression [215]. DNA-methylation is thought to play a role in several key processes including genomic imprinting, X-chromosome inactivation, repression of transposable elements, aging and carcinogenesis. One of the most widely utilized methylation analysis method utilizes specialized microarrays developed by Illumina known as the Infinium 450k microarray or the more recently developed Infinium 850k microarray [216]. First, DNA is bisulfite-treated and the specific CpG sites are detected through hybridization

to complementary probes. For each potential methylation site of interest there are two probes, one that hybridizes with the converted CpG site and one which hybridizes with the original sequence. By calculating the ratio between the intensity of these two probes it is possible to calculate the methylation level of at each site and compare across conditions. The 450K microarray contains 1.5% of the CpGs in the human genome, whereas the 850K microarray contains 90% of the CpGs on the 450K microarray plus an additional 350K CpGs, including CpGs in the promotor regions as well as gene body regions [216]. The array output data can be normalized using various packages in R, including minfi and methylumi, which include options for normalizing for probe intensity as well as background corrections using the control probes [217,218]. After normalization either the β-values (which are equivalent to the absolute DNA methylation levels) or the M-Value (a Logit transformation of the β-values) can be used for further analysis [219]. However, despite all these normalization tools bias can occur due to batch effects, the presence of SNPs and unspecific binding of probes [217,218].

Recently, a machine learning approach for classification of CNS tumours based on the analysis of genome-wide DNA methylation patterns has been developed [220]. Using this classifier, SEGAs were classified as low-grade glioma. Furthermore, no differences were found in *TSC1* and *TSC2* mutated SEGAs and no epigenetic silencing of *TSC1* or *TSC2* has been seen in TSC related tumours [114-220]. Further, for LEATs methylation profiling using the 450K methylation array has been shown to be useful in classifying subtypes [221]. The study by Stone et al. showed that *BRAF* and *FGFR1* altered tumours have different DNA methylation profiles. In accordance with this, another study showed that DNT and GG have distinct methylation signatures and that tumours with diffuse growth patterns and immunopositivity for CD34, are more similar to GGs than DNT [222]. Furthermore, methylation profiling has also been used to identify two subtypes of DLGNT, with one group appearing to be less aggressive in clinical outcome [223]. Taken together, these studies highlight the importance of DNA methylation in integrating molecular diagnostics and classification.

Scope and outline of this thesis

In this thesis, we aimed to investigate the molecular mechanisms involved in the pathology of SEGAs in TSC and GNTs, in order to find potential targets for the development of novel treatments. We therefore examined the molecular pathways and miRNAs in SEGAs and GNTs, by investigating the (epi)genomic, transcriptomic and proteomic profile of SEGAs and the genetic abnormalities in GNTs.

In **chapter 2**, the prevalence of the *BRAFV600E* mutation in a large cohort of TSC related SEGAs was investigated. Additionally, massively parallel sequencing of *TSC1*/*TSC2* was performed to confirm that SEGAs fit the classic model of two hit *TSC1* or *TSC2* inactivation.

In **chapter 3**, the methylation profile of SEGAs was investigated using the Illumina Infinium HumanMethylation450 BeadChip, where we attempted to identify subgroups and pathways that could play a role in SEGA pathogenesis. Additionally, we tried to link certain methylation changes to the expression of inflammation, mTOR activation, glial and neuronal markers in SEGAs.

In **chapter 4**, the coding and non-coding transcriptome of SEGAs was analysed. The expression of the ERK/MAPK pathway as well as the Ragulator complex (LAMTOR1-LAMTOR5) were investigated in SEGAs. Subsequently, the effect of an ERK inhibitor and one miRNA were investigated in patient-derived SEGA cultures or foetal astrocyte-enriched cell cultures, respectively.

In **chapter 5**, we go in more detail on the role of the extracellular matrix in SEGAs, which was identified in chapter 4. As such, the expression of the MMPs and TIMPs were investigated in SEGAs. Additionally, the regulation of MMPs by one miRNA was evaluated using foetal astrocyte-enriched cell cultures.

In **chapter 6**, we aimed to identify miRNAs that could help distinguish GGs from DNTs and other low and high grade gliomas, including SEGAs. The expression of two selected miRNAs were analysed in GGs, DNTs, peritumoural tissue and several astrocytoma entities. Moreover, the role of these miRNAs were investigated in the cell cycle in a pediatric low grade astrocytoma cell line.

In **chapter 7** the content of this thesis is summarized and discussed.

References

1. van Breemen, M. S., Wilms, E. B. & Vecht, C. J. Epilepsy in patients with brain tumours: epidemiology, mechanisms, and management. *Lancet Neurol* 6, 421-430, doi:10.1016/S1474-4422(07)70103-5 (2007).
2. Wolf, H. K., Roos, D., Blumcke, I., Pietsch, T. & Wiestler, O. D. Perilesional neurochemical changes in focal epilepsies. *Acta Neuropathol* 91, 376-384, doi:10.1007/s004010050439 (1996).
3. Louis, D. N. *et al.* The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol* 131, 803-820, doi:10.1007/s00401-016-1545-1 (2016).
4. van den Bent, M. J. Interobserver variation of the histopathological diagnosis in clinical trials on glioma: a clinician's perspective. *Acta Neuropathol* 120, 297-304, doi:10.1007/s00401-010-0725-7 (2010).
5. Bruner, J. M., Inouye, L., Fuller, G. N. & Langford, L. A. Diagnostic discrepancies and their clinical impact in a neuropathology referral practice. *Cancer* 79, 796-803, doi:10.1002/(sici)1097-0142(19970215)79:4<796::aid-cncr17>3.0.co;2-v (1997).
6. Blumcke, I. *et al.* Low-grade epilepsy-associated neuroepithelial tumours - the 2016 WHO classification. *Nat Rev Neurol* 12, 732-740, doi:10.1038/nrneurol.2016.173 (2016).
7. Fukuoka, K. *et al.* Clinical impact of combined epigenetic and molecular analysis of pediatric low grade gliomas. *Neuro Oncol*, doi:10.1093/neuonc/noaa077 (2020).
8. Hartmann, C. *et al.* Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. *Acta Neuropathol* 118, 469-474, doi:10.1007/s00401-009-0561-9 (2009).
9. Sturm, D., Pfister, S. M. & Jones, D. T. W. Pediatric Gliomas: Current Concepts on Diagnosis, Biology, and Clinical Management. *J Clin Oncol* 35, 2370-2377, doi:10.1200/JCO.2017.73.0242 (2017).
10. Horbinski, C. *et al.* Isocitrate dehydrogenase 1 analysis differentiates gangliogliomas from infiltrative gliomas. *Brain Pathol* 21, 564-574, doi:10.1111/j.1750-3639.2011.00480.x (2011).
11. Yan, H. *et al.* IDH1 and IDH2 mutations in gliomas. *N Engl J Med* 360, 765-773, doi:10.1056/NEJMoa0808710 (2009).
12. Dougherty, M. J. *et al.* Activating mutations in BRAF characterize a spectrum of pediatric low-grade gliomas. *Neuro Oncol* 12, 621-630, doi:10.1093/neuonc/noq007 (2010).
13. Schindler, G. *et al.* Analysis of BRAF V600E mutation in 1,320 nervous system tumors reveals high mutation frequencies in pleomorphic xanthoastrocytoma, ganglioglioma and extra-cerebellar pilocytic astrocytoma. *Acta Neuropathol* 121, 397-405, doi:10.1007/s00401-011-0802-6 (2011).
14. Chappe, C. *et al.* Dysembryoplastic neuroepithelial tumors share with pleomorphic xanthoastrocytomas and gangliogliomas BRAF(V600E) mutation and expression. *Brain Pathol* 23, 574-583, doi:10.1111/bpa.12048 (2013).
15. Dahiya, S. *et al.* BRAF(V600E) mutation is a negative prognosticator in pediatric ganglioglioma. *Acta Neuropathol* 125, 901-910, doi:10.1007/s00401-013-1120-y (2013).
16. Koelsche, C. *et al.* Mutant BRAF V600E protein in ganglioglioma is predominantly expressed by neuronal tumor cells. *Acta Neuropathol* 125, 891-900, doi:10.1007/s00401-013-1100-2 (2013).
17. Prabowo, A. S. *et al.* BRAF V600E mutation is associated with mTOR signaling activation in glioneuronal tumors. *Brain Pathol* 24, 52-66, doi:10.1111/bpa.12081 (2014).
18. Jones, D. T. *et al.* Tandem duplication producing a novel oncogenic BRAF fusion gene defines the majority of pilocytic astrocytomas. *Cancer Res* 68, 8673-8677, doi:10.1158/0008-5472.CAN-08-2097 (2008).

19. Penman, C. L., Faulkner, C., Lowis, S. P. & Kurian, K. M. Current Understanding of BRAF Alterations in Diagnosis, Prognosis, and Therapeutic Targeting in Pediatric Low-Grade Gliomas. *Front Oncol* 5, 54, doi:10.3389/fonc.2015.00054 (2015).
20. Guerreiro Stucklin, A. S. *et al.* Alterations in ALK/ROS1/NTRK/MET drive a group of infantile hemispheric gliomas. *Nat Commun* 10, 4343, doi:10.1038/s41467-019-12187-5 (2019).
21. Zhang, J. *et al.* Whole-genome sequencing identifies genetic alterations in pediatric low-grade gliomas. *Nat Genet* 45, 602-612, doi:10.1038/ng.2611 (2013).
22. Ryall, S. *et al.* Integrated Molecular and Clinical Analysis of 1,000 Pediatric Low-Grade Gliomas. *Cancer Cell* 37, 569-583 e565, doi:10.1016/j.ccell.2020.03.011 (2020).
23. Jozwiak, S., Mandera, M. & Mlynarski, W. Natural History and Current Treatment Options for Subependymal Giant Cell Astrocytoma in Tuberous Sclerosis Complex. *Semin Pediatr Neurol* 22, 274-281, doi:10.1016/j.spen.2015.10.003 (2015).
24. European Chromosome 16 Tuberous Sclerosis, C. Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell* 75, 1305-1315, doi:10.1016/0092-8674(93)90618-z (1993).
25. van Slegtenhorst, M. *et al.* Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* 277, 805-808, doi:10.1126/science.277.5327.805 (1997).
26. DiMario, F. J., Jr. Brain abnormalities in tuberous sclerosis complex. *J Child Neurol* 19, 650-657, doi:10.1177/08830738040190090401 (2004).
27. Rivera, B. *et al.* Germline and somatic FGFR1 abnormalities in dysembryoplastic neuroepithelial tumors. *Acta Neuropathol* 131, 847-863, doi:10.1007/s00401-016-1549-x (2016).
28. Lee, D. *et al.* BRAF V600E mutations are frequent in dysembryoplastic neuroepithelial tumors and subependymal giant cell astrocytomas. *J Surg Oncol* 111, 359-364, doi:10.1002/jso.23822 (2015).
29. Qaddoumi, I. *et al.* Genetic alterations in uncommon low-grade neuroepithelial tumors: BRAF, FGFR1, and MYB mutations occur at high frequency and align with morphology. *Acta Neuropathol* 131, 833-845, doi:10.1007/s00401-016-1539-z (2016).
30. Ellison, D. W. *et al.* cIMPACT-NOW update 4: diffuse gliomas characterized by MYB, MYBL1, or FGFR1 alterations or BRAF(V600E) mutation. *Acta Neuropathol* 137, 683-687, doi:10.1007/s00401-019-01987-0 (2019).
31. Louis, D. N. *et al.* cIMPACT-NOW update 6: new entity and diagnostic principle recommendations of the cIMPACT-Utrecht meeting on future CNS tumor classification and grading. *Brain Pathol* 30, 844-856, doi:10.1111/bpa.12832 (2020).
32. Blumcke, I., Aronica, E., Urbach, H., Alexopoulos, A. & Gonzalez-Martinez, J. A. A neuropathology-based approach to epilepsy surgery in brain tumors and proposal for a new terminology use for long-term epilepsy-associated brain tumors. *Acta Neuropathol* 128, 39-54, doi:10.1007/s00401-014-1288-9 (2014).
33. Luyken, C. *et al.* The spectrum of long-term epilepsy-associated tumors: long-term seizure and tumor outcome and neurosurgical aspects. *Epilepsia* 44, 822-830, doi:10.1046/j.1528-1157.2003.56102.x (2003).
34. Daumas-Duport, C. Dysembryoplastic neuroepithelial tumours. *Brain Pathol* 3, 283-295, doi:10.1111/j.1750-3639.1993.tb00755.x (1993).
35. Blumcke, I. *et al.* The CD34 epitope is expressed in neoplastic and malformative lesions associated with chronic, focal epilepsies. *Acta Neuropathol* 97, 481-490, doi:10.1007/s004010051017 (1999).
36. Liu, J. *et al.* Evidence for mTOR pathway activation in a spectrum of epilepsy-associated pathologies. *Acta Neuropathol Commun* 2, 71, doi:10.1186/2051-5960-2-71 (2014).
37. Rossini, L. *et al.* FCD Type II and mTOR pathway: Evidence for different mechanisms involved

in the pathogenesis of dysmorphic neurons. *Epilepsy Res* 129, 146-156, doi:10.1016/j.eplepsyres.2016.12.002 (2017).

38. Johnson, A. *et al.* Comprehensive Genomic Profiling of 282 Pediatric Low- and High-Grade Gliomas Reveals Genomic Drivers, Tumor Mutational Burden, and Hypermutation Signatures. *Oncologist* 22, 1478-1490, doi:10.1634/theoncologist.2017-0242 (2017).
39. Keshet, Y. & Seger, R. The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Mol Biol* 661, 3-38, doi:10.1007/978-1-60761-795-2_1 (2010).
40. Cargnello, M. & Roux, P. P. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* 75, 50-83, doi:10.1128/MMBR.00031-10 (2011).
41. Lawrence, M. C. *et al.* The roles of MAPKs in disease. *Cell Res* 18, 436-442, doi:10.1038/cr.2008.37 (2008).
42. Guo, Y. J. *et al.* ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med* 19, 1997-2007, doi:10.3892/etm.2020.8454 (2020).
43. Bos, J. L., Rehmann, H. & Wittinghofer, A. GEFs and GAPs: critical elements in the control of small G proteins. *Cell* 129, 865-877, doi:10.1016/j.cell.2007.05.018 (2007).
44. Margarit, S. M. *et al.* Structural evidence for feedback activation by Ras.GTP of the Ras-specific nucleotide exchange factor SOS. *Cell* 112, 685-695, doi:10.1016/s0092-8674(03)00149-1 (2003).
45. Bandaru, P., Kondo, Y. & Kuriyan, J. The Interdependent Activation of Son-of-Sevenless and Ras. *Cold Spring Harb Perspect Med* 9, doi:10.1101/cshperspect.a031534 (2019).
46. Mitin, N., Rossman, K. L. & Der, C. J. Signaling interplay in Ras superfamily function. *Curr Biol* 15, R563-574, doi:10.1016/j.cub.2005.07.010 (2005).
47. Marais, R., Light, Y., Paterson, H. F. & Marshall, C. J. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J* 14, 3136-3145 (1995).
48. Crews, C. M. & Erikson, R. L. Purification of a murine protein-tyrosine/threonine kinase that phosphorylates and activates the Erk-1 gene product: relationship to the fission yeast byr1 gene product. *Proc Natl Acad Sci U S A* 89, 8205-8209, doi:10.1073/pnas.89.17.8205 (1992).
49. Lavoie, H. & Therrien, M. Regulation of RAF protein kinases in ERK signalling. *Nat Rev Mol Cell Biol* 16, 281-298, doi:10.1038/nrm3979 (2015).
50. Chambard, J. C., Lefloch, R., Pouyssegur, J. & Lenormand, P. ERK implication in cell cycle regulation. *Biochim Biophys Acta* 1773, 1299-1310, doi:10.1016/j.bbamcr.2006.11.010 (2007).
51. Zsarnovszky, A. & Belcher, S. M. Spatial, temporal, and cellular distribution of the activated extracellular signal regulated kinases 1 and 2 in the developing and mature rat cerebellum. *Brain Res Dev Brain Res* 150, 199-209, doi:10.1016/j.devbrainres.2004.03.012 (2004).
52. Cheng, P., Alberts, I. & Li, X. The role of ERK1/2 in the regulation of proliferation and differentiation of astrocytes in developing brain. *Int J Dev Neurosci* 31, 783-789, doi:10.1016/j.ijdevneu.2013.09.008 (2013).
53. Wisoff, J. H. *et al.* Primary neurosurgery for pediatric low-grade gliomas: a prospective multi-institutional study from the Children's Oncology Group. *Neurosurgery* 68, 1548-1554; discussion 1554-1545, doi:10.1227/NEU.0b013e318214a66e (2011).
54. Packer, R. J. *et al.* Carboplatin and vincristine chemotherapy for children with newly diagnosed progressive low-grade gliomas. *J Neurosurg* 86, 747-754, doi:10.3171/jns.1997.86.5.0747 (1997).
55. Marcus, K. J. *et al.* Stereotactic radiotherapy for localized low-grade gliomas in children: final results of a prospective trial. *Int J Radiat Oncol Biol Phys* 61, 374-379, doi:10.1016/j.ijrobp.2004.06.012 (2005).

56. Merchant, T. E., Conklin, H. M., Wu, S., Lustig, R. H. & Xiong, X. Late effects of conformal radiation therapy for pediatric patients with low-grade glioma: prospective evaluation of cognitive, endocrine, and hearing deficits. *J Clin Oncol* 27, 3691-3697, doi:10.1200/JCO.2008.21.2738 (2009).
57. Armstrong, G. T. *et al.* Survival and long-term health and cognitive outcomes after low-grade glioma. *Neuro Oncol* 13, 223-234, doi:10.1093/neuonc/noq178 (2011).
58. Majores, M. *et al.* Tumor recurrence and malignant progression of gangliogliomas. *Cancer* 113, 3355-3363, doi:10.1002/cncr.23965 (2008).
59. Ray, W. Z. *et al.* Clinicopathologic features of recurrent dysembryoplastic neuroepithelial tumor and rare malignant transformation: a report of 5 cases and review of the literature. *J Neurooncol* 94, 283-292, doi:10.1007/s11060-009-9849-9 (2009).
60. Banerjee, A. *et al.* A phase I trial of the MEK inhibitor selumetinib (AZD6244) in pediatric patients with recurrent or refractory low-grade glioma: a Pediatric Brain Tumor Consortium (PBTC) study. *Neuro Oncol* 19, 1135-1144, doi:10.1093/neuonc/now282 (2017).
61. Franz, D. N. *et al.* Rapamycin causes regression of astrocytomas in tuberous sclerosis complex. *Ann Neurol* 59, 490-498, doi:10.1002/ana.20784 (2006).
62. Slegers, R. J. & Blumcke, I. Low-grade developmental and epilepsy associated brain tumors: a critical update 2020. *Acta Neuropathol Commun* 8, 27, doi:10.1186/s40478-020-00904-x (2020).
63. Ryall, S., Tabori, U. & Hawkins, C. Pediatric low-grade glioma in the era of molecular diagnostics. *Acta Neuropathol Commun* 8, 30, doi:10.1186/s40478-020-00902-z (2020).
64. Martins, F. *et al.* A review of oral toxicity associated with mTOR inhibitor therapy in cancer patients. *Oral Oncol* 49, 293-298, doi:10.1016/j.oraloncology.2012.11.008 (2013).
65. Kondyli, M. *et al.* Trametinib for progressive pediatric low-grade gliomas. *J Neurooncol* 140, 435-444, doi:10.1007/s11060-018-2971-9 (2018).
66. Sievert, A. J. *et al.* Paradoxical activation and RAF inhibitor resistance of BRAF protein kinase fusions characterizing pediatric astrocytomas. *Proc Natl Acad Sci U S A* 110, 5957-5962, doi:10.1073/pnas.1219232110 (2013).
67. Sapir, T. *et al.* Building Bridges Between the Clinic and the Laboratory: A Meeting Review - Brain Malformations: A Roadmap for Future Research. *Front Cell Neurosci* 13, 434, doi:10.3389/fncel.2019.00434 (2019).
68. Curatolo, P. Mechanistic target of rapamycin (mTOR) in tuberous sclerosis complex-associated epilepsy. *Pediatr Neurol* 52, 281-289, doi:10.1016/j.pediatrneurol.2014.10.028 (2015).
69. Curatolo, P. *et al.* Early onset epileptic encephalopathy or genetically determined encephalopathy with early onset epilepsy? Lessons learned from TSC. *Eur J Paediatr Neurol* 20, 203-211, doi:10.1016/j.ejpn.2015.12.005 (2016).
70. Curatolo, P., Moavero, R., van Scheppingen, J. & Aronica, E. mTOR dysregulation and tuberous sclerosis-related epilepsy. *Expert Rev Neurother* 18, 185-201, doi:10.1080/14737175.2018.1428562 (2018).
71. Northrup, H., Krueger, D. A. & International Tuberous Sclerosis Complex Consensus, G. Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 Iinternational Tuberous Sclerosis Complex Consensus Conference. *Pediatr Neurol* 49, 243-254, doi:10.1016/j.pediatrneurol.2013.08.001 (2013).
72. de Vries, P. J. *et al.* TSC-associated neuropsychiatric disorders (TAND): findings from the TOSCA natural history study. *Orphanet J Rare Dis* 13, 157, doi:10.1186/s13023-018-0901-8 (2018).
73. de Vries, P. J. *et al.* Tuberous sclerosis associated neuropsychiatric disorders (TAND) and the TAND Checklist. *Pediatr Neurol* 52, 25-35, doi:10.1016/j.pediatrneurol.2014.10.004 (2015).

74. Curatolo, P., Moavero, R. & de Vries, P. J. Neurological and neuropsychiatric aspects of tuberous sclerosis complex. *Lancet Neurol* 14, 733-745, doi:10.1016/S1474-4422(15)00069-1 (2015).
75. Leclezio, L., Jansen, A., Whittemore, V. H. & de Vries, P. J. Pilot validation of the tuberous sclerosis-associated neuropsychiatric disorders (TAND) checklist. *Pediatr Neurol* 52, 16-24, doi:10.1016/j.pediatrneurol.2014.10.006 (2015).
76. Crino, P. B., Nathanson, K. L. & Henske, E. P. The tuberous sclerosis complex. *N Engl J Med* 355, 1345-1356, doi:10.1056/NEJMra055323 (2006).
77. Roth, J. *et al.* Subependymal giant cell astrocytoma: diagnosis, screening, and treatment. Recommendations from the International Tuberous Sclerosis Complex Consensus Conference 2012. *Pediatr Neurol* 49, 439-444, doi:10.1016/j.pediatrneurol.2013.08.017 (2013).
78. Mizuguchi, M. & Takashima, S. Neuropathology of tuberous sclerosis. *Brain Dev* 23, 508-515, doi:10.1016/s0387-7604(01)00304-7 (2001).
79. Aronica, E. & Crino, P. B. Epilepsy related to developmental tumors and malformations of cortical development. *Neurotherapeutics* 11, 251-268, doi:10.1007/s13311-013-0251-0 (2014).
80. Muhlebner, A. *et al.* Novel Histopathological Patterns in Cortical Tubers of Epilepsy Surgery Patients with Tuberous Sclerosis Complex. *PLoS One* 11, e0157396, doi:10.1371/journal.pone.0157396 (2016).
81. Aronica, E. & Muhlebner, A. Neuropathology of epilepsy. *Handb Clin Neurol* 145, 193-216, doi:10.1016/B978-0-12-802395-2.00015-8 (2017).
82. Cotter, J. A. An update on the central nervous system manifestations of tuberous sclerosis complex. *Acta Neuropathol* 139, 613-624, doi:10.1007/s00401-019-02003-1 (2020).
83. Boer, K. *et al.* Clinicopathological and immunohistochemical findings in an autopsy case of tuberous sclerosis complex. *Neuropathology* 28, 577-590, doi:10.1111/j.1440-1789.2008.00920.x (2008).
84. Grajkowska, W., Kotulska, K., Jurkiewicz, E. & Matyja, E. Brain lesions in tuberous sclerosis complex. Review. *Folia Neuropathol* 48, 139-149 (2010).
85. Zurolo, E. *et al.* Activation of Toll-like receptor, RAGE and HMGB1 signalling in malformations of cortical development. *Brain* 134, 1015-1032, doi:10.1093/brain/awr032 (2011).
86. Aronica, E., Becker, A. J. & Spreafico, R. Malformations of cortical development. *Brain Pathol* 22, 380-401, doi:10.1111/j.1750-3639.2012.00581.x (2012).
87. Crino, P. B., Trojanowski, J. Q., Dichter, M. A. & Eberwine, J. Embryonic neuronal markers in tuberous sclerosis: single-cell molecular pathology. *Proc Natl Acad Sci U S A* 93, 14152-14157, doi:10.1073/pnas.93.24.14152 (1996).
88. Lee, A. *et al.* Markers of cellular proliferation are expressed in cortical tubers. *Ann Neurol* 53, 668-673, doi:10.1002/ana.10579 (2003).
89. Talos, D. M., Kwiatkowski, D. J., Cordero, K., Black, P. M. & Jensen, F. E. Cell-specific alterations of glutamate receptor expression in tuberous sclerosis complex cortical tubers. *Ann Neurol* 63, 454-465, doi:10.1002/ana.21342 (2008).
90. Boer, K. *et al.* Doublecortin-like (DCL) expression in focal cortical dysplasia and cortical tubers. *Epilepsia* 50, 2629-2637, doi:10.1111/j.1528-1167.2009.02191.x (2009).
91. Orlova, K. A. & Crino, P. B. The tuberous sclerosis complex. *Ann N Y Acad Sci* 1184, 87-105, doi:10.1111/j.1749-6632.2009.05117.x (2010).
92. Muhlebner, A. *et al.* Specific pattern of maturation and differentiation in the formation of cortical tubers in tuberous sclerosis omplex (TSC): evidence from layer-specific marker expression. *J Neurodev Disord* 8, 9, doi:10.1186/s11689-016-9142-0 (2016).
93. Park, S. H. *et al.* Tuberous sclerosis in a 20-week gestation fetus: immunohistochemical study. *Acta*

Neuropathol 94, 180-186, doi:10.1007/s004010050691 (1997).
94. Chen, C. P., Su, Y. N., Hung, C. C., Shih, J. C. & Wang, W. Novel mutation in the TSC2 gene associated with prenatally diagnosed cardiac rhabdomyomas and cerebral tuberous sclerosis. *J Formos Med Assoc* 105, 599-603, doi:10.1016/S0929-6646(09)60157-1 (2006).
95. Glenn, O. A. & Barkovich, A. J. Magnetic resonance imaging of the fetal brain and spine: an increasingly important tool in prenatal diagnosis, part 1. *AJNR Am J Neuroradiol* 27, 1604-1611 (2006).
96. Wortmann, S. B., Reimer, A., Creemers, J. W. & Mullaart, R. A. Prenatal diagnosis of cerebral lesions in Tuberous sclerosis complex (TSC). Case report and review of the literature. *Eur J Paediatr Neurol* 12, 123-126, doi:10.1016/j.ejpn.2007.06.006 (2008).
97. Prabowo, A. S. *et al.* Fetal brain lesions in tuberous sclerosis complex: TORC1 activation and inflammation. *Brain Pathol* 23, 45-59, doi:10.1111/j.1750-3639.2012.00616.x (2013).
98. Tsai, V. *et al.* Fetal brain mTOR signaling activation in tuberous sclerosis complex. *Cereb Cortex* 24, 315-327, doi:10.1093/cercor/bhs310 (2014).
99. Sarnat, H. B., Flores-Sarnat, L. & Trevenen, C. L. Synaptophysin immunoreactivity in the human hippocampus and neocortex from 6 to 41 weeks of gestation. *J Neuropathol Exp Neurol* 69, 234-245, doi:10.1097/NEN.0b013e3181d0151f (2010).
100. Poore, B. *et al.* Inhibition of mTORC1 in pediatric low-grade glioma depletes glutathione and therapeutically synergizes with carboplatin. *Neuro Oncol* 21, 252-263, doi:10.1093/neuonc/noy150 (2019).
101. Rodriguez, E. F. *et al.* PI3K/AKT pathway alterations are associated with clinically aggressive and histologically anaplastic subsets of pilocytic astrocytoma. *Acta Neuropathol* 121, 407-420, doi:10.1007/s00401-010-0784-9 (2011).
102. Hutt-Cabezas, M. *et al.* Activation of mTORC1/mTORC2 signaling in pediatric low-grade glioma and pilocytic astrocytoma reveals mTOR as a therapeutic target. *Neuro Oncol* 15, 1604-1614, doi:10.1093/neuonc/not132 (2013).
103. Switon, K., Kotulska, K., Janusz-Kaminska, A., Zmorzynska, J. & Jaworski, J. Molecular neurobiology of mTOR. *Neuroscience* 341, 112-153, doi:10.1016/j.neuroscience.2016.11.017 (2017).
104. Inoki, K., Li, Y., Xu, T. & Guan, K. L. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* 17, 1829-1834, doi:10.1101/gad.1110003 (2003).
105. Sancak, Y. *et al.* The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320, 1496-1501, doi:10.1126/science.1157535 (2008).
106. Dibble, C. C. *et al.* TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. *Mol Cell* 47, 535-546, doi:10.1016/j.molcel.2012.06.009 (2012).
107. Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H. & Sabatini, D. M. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A* 95, 1432-1437, doi:10.1073/pnas.95.4.1432 (1998).
108. Chan, J. A. *et al.* Pathogenesis of tuberous sclerosis subependymal giant cell astrocytomas: biallelic inactivation of TSC1 or TSC2 leads to mTOR activation. *J Neuropathol Exp Neurol* 63, 1236-1242, doi:10.1093/jnen/63.12.1236 (2004).
109. Qin, W. *et al.* Ultra deep sequencing detects a low rate of mosaic mutations in tuberous sclerosis complex. *Hum Genet* 127, 573-582, doi:10.1007/s00439-010-0801-z (2010).
110. Peron, A., Au, K. S. & Northrup, H. Genetics, genomics, and genotype-phenotype correlations of TSC: Insights for clinical practice. *Am J Med Genet C Semin Med Genet* 178, 281-290, doi:10.1002/ajmg.c.31651 (2018).
111. Ogorek, B. *et al.* TSC2 pathogenic variants are predictive of severe clinical manifestations in TSC

infants: results of the EPISTOP study. *Genet Med*, doi:10.1038/s41436-020-0823-4 (2020).
112. Henske, E. P. *et al.* Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. *Am J Hum Genet* 59, 400-406 (1996).
113. Caban, C., Khan, N., Hasbani, D. M. & Crino, P. B. Genetics of tuberous sclerosis complex: implications for clinical practice. *Appl Clin Genet* 10, 1-8, doi:10.2147/TACG.S90262 (2017).
114. Martin, K. R. *et al.* The genomic landscape of tuberous sclerosis complex. *Nat Commun* 8, 15816, doi:10.1038/ncomms15816 (2017).
115. Carracedo, A. *et al.* Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J Clin Invest* 118, 3065-3074, doi:10.1172/JCI34739 (2008).
116. Han, S. *et al.* Phosphorylation of tuberin as a novel mechanism for somatic inactivation of the tuberous sclerosis complex proteins in brain lesions. *Cancer Res* 64, 812-816, doi:10.1158/0008-5472.can-03-3277 (2004).
117. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. & Pandolfi, P. P. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* 121, 179-193, doi:10.1016/j.cell.2005.02.031 (2005).
118. Ma, L. *et al.* Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer. *Cancer Res* 67, 7106-7112, doi:10.1158/0008-5472.CAN-06-4798 (2007).
119. Teis, D., Wunderlich, W. & Huber, L. A. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. *Dev Cell* 3, 803-814, doi:10.1016/s1534-5807(02)00364-7 (2002).
120. Bar-Peled, L., Schweitzer, L. D., Zoncu, R. & Sabatini, D. M. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. *Cell* 150, 1196-1208, doi:10.1016/j.cell.2012.07.032 (2012).
121. Nada, S., Mori, S., Takahashi, Y. & Okada, M. p18/LAMTOR1: a late endosome/lysosome-specific anchor protein for the mTORC1/MAPK signaling pathway. *Methods Enzymol* 535, 249-263, doi:10.1016/B978-0-12-397925-4.00015-8 (2014).
122. de Araujo, M. E. G. *et al.* Crystal structure of the human lysosomal mTORC1 scaffold complex and its impact on signaling. *Science* 358, 377-381, doi:10.1126/science.aao1583 (2017).
123. Arnold, A. *et al.* Synergistic activity of mTORC1/2 kinase and MEK inhibitors suppresses pediatric low-grade glioma tumorigenicity and vascularity. *Neuro Oncol* 22, 563-574, doi:10.1093/neuonc/noz230 (2020).
124. Cuccia, V. *et al.* Subependymal giant cell astrocytoma in children with tuberous sclerosis. *Childs Nerv Syst* 19, 232-243, doi:10.1007/s00381-002-0700-2 (2003).
125. Amin, S. *et al.* The outcome of surgical management of subependymal giant cell astrocytoma in tuberous sclerosis complex. *Eur J Paediatr Neurol* 17, 36-44, doi:10.1016/j.ejpn.2012.10.005 (2013).
126. Goh, S., Butler, W. & Thiele, E. A. Subependymal giant cell tumors in tuberous sclerosis complex. *Neurology* 63, 1457-1461, doi:10.1212/01.wnl.0000142039.14522.1a (2004).
127. Adriaensen, M. E. *et al.* Prevalence of subependymal giant cell tumors in patients with tuberous sclerosis and a review of the literature. *Eur J Neurol* 16, 691-696, doi:10.1111/j.1468-1331.2009.02567.x (2009).
128. Kothare, S. V. *et al.* Severity of manifestations in tuberous sclerosis complex in relation to genotype. *Epilepsia* 55, 1025-1029, doi:10.1111/epi.12680 (2014).
129. Kingswood, J. C. *et al.* TuberOus SClerosis registry to increase disease Awareness (TOSCA) - baseline data on 2093 patients. *Orphanet J Rare Dis* 12, 2, doi:10.1186/s13023-016-0553-5 (2017).

130. Oikawa, S., Sakamoto, K. & Kobayashi, N. A neonatal huge subependymal giant cell astrocytoma: case report. *Neurosurgery* 35, 748-750, doi:10.1227/00006123-199410000-00025 (1994).
131. Torres, O. A. *et al.* Early diagnosis of subependymal giant cell astrocytoma in patients with tuberous sclerosis. *J Child Neurol* 13, 173-177, doi:10.1177/088307389801300405 (1998).
132. Raju, G. P., Urion, D. K. & Sahin, M. Neonatal subependymal giant cell astrocytoma: new case and review of literature. *Pediatr Neurol* 36, 128-131, doi:10.1016/j.pediatrneurol.2006.08.009 (2007).
133. Phi, J. H. *et al.* Congenital subependymal giant cell astrocytoma: clinical considerations and expression of radial glial cell markers in giant cells. *Childs Nerv Syst* 24, 1499-1503, doi:10.1007/s00381-008-0681-x (2008).
134. Adriaensen, M. E., Zonnenberg, B. A. & de Jong, P. A. Natural history and CT scan follow-up of subependymal giant cell tumors in tuberous sclerosis complex patients. *J Clin Neurosci* 21, 939-941, doi:10.1016/j.jocn.2013.08.022 (2014).
135. Tsai, J. D. *et al.* Association between the growth rate of subependymal giant cell astrocytoma and age in patients with tuberous sclerosis complex. *Childs Nerv Syst* 32, 89-95, doi:10.1007/s00381-015-2947-4 (2016).
136. Jansen, A. C. *et al.* Newly Diagnosed and Growing Subependymal Giant Cell Astrocytoma in Adults With Tuberous Sclerosis Complex: Results From the International TOSCA Study. *Front Neurol* 10, 821, doi:10.3389/fneur.2019.00821 (2019).
137. Jansen, A. C. *et al.* Clinical Characteristics of Subependymal Giant Cell Astrocytoma in Tuberous Sclerosis Complex. *Front Neurol* 10, 705, doi:10.3389/fneur.2019.00705 (2019).
138. Nabbout, R. *et al.* Early diagnosis of subependymal giant cell astrocytoma in children with tuberous sclerosis. *J Neurol Neurosurg Psychiatry* 66, 370-375, doi:10.1136/jnnp.66.3.370 (1999).
139. Morimoto, K. & Mogami, H. Sequential CT study of subependymal giant-cell astrocytoma associated with tuberous sclerosis. Case report. *J Neurosurg* 65, 874-877, doi:10.3171/jns.1986.65.6.0874 (1986).
140. Fujiwara, S., Takaki, T., Hikita, T. & Nishio, S. Subependymal giant-cell astrocytoma associated with tuberous sclerosis. Do subependymal nodules grow? *Childs Nerv Syst* 5, 43-44, doi:10.1007/BF00706748 (1989).
141. Bonnin, J. M., Rubinstein, L. J., Papasozomenos, S. C. & Marangos, P. J. Subependymal giant cell astrocytoma. Significance and possible cytogenetic implications of an immunohistochemical study. *Acta Neuropathol* 62, 185-193, doi:10.1007/BF00691851 (1984).
142. Sharma, M. C. *et al.* Subependymal giant cell astrocytoma--a clinicopathological study of 23 cases with special emphasis on histogenesis. *Pathol Oncol Res* 10, 219-224, doi:10.1007/BF03033764 (2004).
143. Buccoliero, A. M. *et al.* Subependymal giant cell astrocytoma: a lesion with activated mTOR pathway and constant expression of glutamine synthetase. *Clin Neuropathol* 35, 295-301, doi:10.5414/NP300936 (2016).
144. Ess, K. C., Kamp, C. A., Tu, B. P. & Gutmann, D. H. Developmental origin of subependymal giant cell astrocytoma in tuberous sclerosis complex. *Neurology* 64, 1446-1449, doi:10.1212/01.WNL.0000158653.81008.49 (2005).
145. Barrows, B. D., Rutkowski, M. J., Gultekin, S. H., Parsa, A. T. & Tihan, T. Evidence of ambiguous differentiation and mTOR pathway dysregulation in subependymal giant cell astrocytoma. *Turk Patoloji Derg* 28, 95-103, doi:10.5146/tjpath.2012.01107 (2012).
146. Telfeian, A. E., Judkins, A., Younkin, D., Pollock, A. N. & Crino, P. Subependymal giant cell astrocytoma with cranial and spinal metastases in a patient with tuberous sclerosis. Case report. *J Neurosurg*

100, 498-500, doi:10.3171/ped.2004.100.5.0498 (2004).
147. Grajkowska, W. *et al.* Subependymal giant cell astrocytomas with atypical histological features mimicking malignant gliomas. *Folia Neuropathol* 49, 39-46 (2011).
148. Buccoliero, A. M. *et al.* Subependymal giant cell astrocytoma (SEGA): Is it an astrocytoma? Morphological, immunohistochemical and ultrastructural study. *Neuropathology* 29, 25-30, doi:10.1111/j.1440-1789.2008.00934.x (2009).
149. Boer, K. *et al.* Inflammatory processes in cortical tubers and subependymal giant cell tumors of tuberous sclerosis complex. *Epilepsy Res* 78, 7-21, doi:10.1016/j.eplepsyres.2007.10.002 (2008).
150. Lim, H. K. *et al.* Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. *J Biol Chem* 278, 45117-45127, doi:10.1074/jbc.M303789200 (2003).
151. Curatolo, P. *et al.* Management of epilepsy associated with tuberous sclerosis complex: Updated clinical recommendations. *Eur J Paediatr Neurol* 22, 738-748, doi:10.1016/j.ejpn.2018.05.006 (2018).
152. Chiron, C., Dumas, C., Jambaque, I., Mumford, J. & Dulac, O. Randomized trial comparing vigabatrin and hydrocortisone in infantile spasms due to tuberous sclerosis. *Epilepsy Res* 26, 389-395, doi:10.1016/s0920-1211(96)01006-6 (1997).
153. Elterman, R. D., Shields, W. D., Mansfield, K. A., Nakagawa, J. & Group, U. S. I. S. V. S. Randomized trial of vigabatrin in patients with infantile spasms. *Neurology* 57, 1416-1421, doi:10.1212/wnl.57.8.1416 (2001).
154. Cusmai, R., Moavero, R., Bombardieri, R., Vigevano, F. & Curatolo, P. Long-term neurological outcome in children with early-onset epilepsy associated with tuberous sclerosis. *Epilepsy Behav* 22, 735-739, doi:10.1016/j.yebeh.2011.08.037 (2011).
155. Jozwiak, S. *et al.* Antiepileptic treatment before the onset of seizures reduces epilepsy severity and risk of mental retardation in infants with tuberous sclerosis complex. *Eur J Paediatr Neurol* 15, 424-431, doi:10.1016/j.ejpn.2011.03.010 (2011).
156. Bombardieri, R., Pinci, M., Moavero, R., Cerminara, C. & Curatolo, P. Early control of seizures improves long-term outcome in children with tuberous sclerosis complex. *Eur J Paediatr Neurol* 14, 146-149, doi:10.1016/j.ejpn.2009.03.003 (2010).
157. Overwater, I. E. *et al.* Epilepsy in children with tuberous sclerosis complex: Chance of remission and response to antiepileptic drugs. *Epilepsia* 56, 1239-1245, doi:10.1111/epi.13050 (2015).
158. Liang, S. *et al.* Long-term outcomes of epilepsy surgery in tuberous sclerosis complex. *J Neurol* 264, 1146-1154, doi:10.1007/s00415-017-8507-y (2017).
159. Fujiwara, H. *et al.* Resection of ictal high frequency oscillations is associated with favorable surgical outcome in pediatric drug resistant epilepsy secondary to tuberous sclerosis complex. *Epilepsy Res* 126, 90-97, doi:10.1016/j.eplepsyres.2016.07.005 (2016).
160. Fallah, A. *et al.* Resective Epilepsy Surgery for Tuberous Sclerosis in Children: Determining Predictors of Seizure Outcomes in a Multicenter Retrospective Cohort Study. *Neurosurgery* 77, 517-524; discussion 524, doi:10.1227/NEU.0000000000000875 (2015).
161. Mohamed, A. R. *et al.* Intrinsic epileptogenicity of cortical tubers revealed by intracranial EEG monitoring. *Neurology* 79, 2249-2257, doi:10.1212/WNL.0b013e3182768923 (2012).
162. Moshel, Y. A. *et al.* Do tubers contain function? Resection of epileptogenic foci in perirolandic cortex in children with tuberous sclerosis complex. *Epilepsia* 51, 1242-1251, doi:10.1111/j.1528-1167.2009.02493.x (2010).
163. Major, P. *et al.* Are cortical tubers epileptogenic? Evidence from electrocorticography. *Epilepsia* 50, 147-154, doi:10.1111/j.1528-1167.2008.01814.x (2009).

164. Wang, Y. *et al.* Neocortical hyperexcitability in a human case of tuberous sclerosis complex and mice lacking neuronal expression of TSC1. *Ann Neurol* 61, 139-152, doi:10.1002/ana.21058 (2007).
165. Madhavan, D. *et al.* Surgical outcome in tuberous sclerosis complex: a multicenter survey. *Epilepsia* 48, 1625-1628, doi:10.1111/j.1528-1167.2007.01112.x (2007).
166. Krueger, D. A., Northrup, H. & International Tuberous Sclerosis Complex Consensus, G. Tuberous sclerosis complex surveillance and management: recommendations of the 2012 International Tuberous Sclerosis Complex Consensus Conference. *Pediatr Neurol* 49, 255-265, doi:10.1016/j.pediatrneurol.2013.08.002 (2013).
167. Holanda, F. J. & Holanda, G. M. Tuberous sclerosis - neurosurgical indications in intraventricular tumors. *Neurosurg Rev* 3, 139-150, doi:10.1007/BF01644066 (1980).
168. de Ribaupierre, S. *et al.* Subependymal giant-cell astrocytomas in pediatric tuberous sclerosis disease: when should we operate? *Neurosurgery* 60, 83-89; discussion 89-90, doi:10.1227/01.NEU.0000249216.19591.5D (2007).
169. Cai, R. & Di, X. Combined intra- and extra-endoscopic techniques for aggressive resection of subependymal giant cell astrocytomas. *World Neurosurg* 73, 713-718, doi:10.1016/j.wneu.2010.02.068 (2010).
170. Roszkowski, M., Drabik, K., Barszcz, S. & Jozwiak, S. Surgical treatment of intraventricular tumors associated with tuberous sclerosis. *Childs Nerv Syst* 11, 335-339, doi:10.1007/BF00301665 (1995).
171. Pascual-Castroviejo, I. Neurosurgical treatment of tuberous sclerosis complex lesions. *Childs Nerv Syst* 27, 1211-1219, doi:10.1007/s00381-011-1488-8 (2011).
172. Rodgers, S. D., Bassani, L., Weiner, H. L. & Harter, D. H. Stereotactic endoscopic resection and surgical management of a subependymal giant cell astrocytoma: case report. *J Neurosurg Pediatr* 9, 417-420, doi:10.3171/2011.12.PEDS11349 (2012).
173. Nishio, S. *et al.* Subependymal giant cell astrocytoma: clinical and neuroimaging features of four cases. *J Clin Neurosci* 8, 31-34, doi:10.1054/jocn.2000.0767 (2001).
174. Kotulska, K. *et al.* Surgical treatment of subependymal giant cell astrocytoma in tuberous sclerosis complex patients. *Pediatr Neurol* 50, 307-312, doi:10.1016/j.pediatrneurol.2013.12.004 (2014).
175. Giordano, F. *et al.* Neurosurgical treatment of subependymal giant cell astrocytomas in tuberous sclerosis complex: a series of 44 surgical procedures in 31 patients. *Childs Nerv Syst* 36, 951-960, doi:10.1007/s00381-019-04449-w (2020).
176. Arroyo, M. S., Krueger, D. A., Broomall, E., Stevenson, C. B. & Franz, D. N. Acute Management of Symptomatic Subependymal Giant Cell Astrocytoma With Everolimus. *Pediatr Neurol* 72, 81-85, doi:10.1016/j.pediatrneurol.2017.04.008 (2017).
177. Moavero, R. *et al.* Everolimus Alleviates Obstructive Hydrocephalus due to Subependymal Giant Cell Astrocytomas. *Pediatr Neurol* 68, 59-63, doi:10.1016/j.pediatrneurol.2016.11.003 (2017).
178. Lam, C. *et al.* Rapamycin (sirolimus) in tuberous sclerosis associated pediatric central nervous system tumors. *Pediatr Blood Cancer* 54, 476-479, doi:10.1002/pbc.22298 (2010).
179. Cardamone, M. *et al.* Mammalian target of rapamycin inhibitors for intractable epilepsy and subependymal giant cell astrocytomas in tuberous sclerosis complex. *J Pediatr* 164, 1195-1200, doi:10.1016/j.jpeds.2013.12.053 (2014).
180. Koenig, M. K., Butler, I. J. & Northrup, H. Regression of subependymal giant cell astrocytoma with rapamycin in tuberous sclerosis complex. *J Child Neurol* 23, 1238-1239, doi:10.1177/0883073808321764 (2008).
181. Franz, D. N. *et al.* Efficacy and safety of everolimus for subependymal giant cell astrocytomas associated with tuberous sclerosis complex (EXIST-1): a multicentre, randomised, placebo-

controlled phase 3 trial. *Lancet* 381, 125-132, doi:10.1016/S0140-6736(12)61134-9 (2013).
182. Franz, D. N. *et al.* Everolimus for subependymal giant cell astrocytoma in patients with tuberous sclerosis complex: 2-year open-label extension of the randomised EXIST-1 study. *Lancet Oncol* 15, 1513-1520, doi:10.1016/S1470-2045(14)70489-9 (2014).
183. Franz, D. N. *et al.* Long-Term Use of Everolimus in Patients with Tuberous Sclerosis Complex: Final Results from the EXIST-1 Study. *PLoS One* 11, e0158476, doi:10.1371/journal.pone.0158476 (2016).
184. Franz, D. N. *et al.* Everolimus for subependymal giant cell astrocytoma: 5-year final analysis. *Ann Neurol* 78, 929-938, doi:10.1002/ana.24523 (2015).
185. Kuki, I. *et al.* Efficacy and safety of everolimus in patients younger than 12months with congenital subependymal giant cell astrocytoma. *Brain Dev* 40, 415-420, doi:10.1016/j.braindev.2018.01.001 (2018).
186. Fukumura, S., Watanabe, T., Takayama, R., Minagawa, K. & Tsutsumi, H. Everolimus Treatment for an Early Infantile Subependymal Giant Cell Astrocytoma With Tuberous Sclerosis Complex. *J Child Neurol* 30, 1192-1195, doi:10.1177/0883073814544703 (2015).
187. Moavero, R. & Curatolo, P. Long-term use of mTORC1 inhibitors in tuberous sclerosis complex associated neurological aspects. *Expert Opinion on Orphan Drugs* 8, 215-225, doi:10.1080/21678707.2020.1789862 (2020).
188. Bissler, J. J. *et al.* Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. *N Engl J Med* 358, 140-151, doi:10.1056/NEJMoa063564 (2008).
189. Krueger, D. A. *et al.* Long-term treatment of epilepsy with everolimus in tuberous sclerosis. *Neurology* 87, 2408-2415, doi:10.1212/WNL.0000000000003400 (2016).
190. Krueger, D. A. *et al.* Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. *N Engl J Med* 363, 1801-1811, doi:10.1056/NEJMoa1001671 (2010).
191. McCormack, F. X. *et al.* Efficacy and safety of sirolimus in lymphangioleiomyomatosis. *N Engl J Med* 364, 1595-1606, doi:10.1056/NEJMoa1100391 (2011).
192. Kaplan, B., Qazi, Y. & Wellen, J. R. Strategies for the management of adverse events associated with mTOR inhibitors. *Transplant Rev (Orlando)* 28, 126-133, doi:10.1016/j.trre.2014.03.002 (2014).
193. Soefje, S. A., Karnad, A. & Brenner, A. J. Common toxicities of mammalian target of rapamycin inhibitors. *Target Oncol* 6, 125-129, doi:10.1007/s11523-011-0174-9 (2011).
194. van Vliet, E. A. *et al.* Standardization procedure for plasma biomarker analysis in rat models of epileptogenesis: Focus on circulating microRNAs. *Epilepsia* 58, 2013-2024, doi:10.1111/epi.13915 (2017).
195. Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 10, 57-63, doi:10.1038/nrg2484 (2009).
196. Mills, J. D. & Janitz, M. Alternative splicing of mRNA in the molecular pathology of neurodegenerative diseases. *Neurobiol Aging* 33, 1012 e1011-1024, doi:10.1016/j.neurobiolaging.2011.10.030 (2012).
197. Jeck, W. R. & Sharpless, N. E. Detecting and characterizing circular RNAs. *Nat Biotechnol* 32, 453-461, doi:10.1038/nbt.2890 (2014).
198. Kukurba, K. R. & Montgomery, S. B. RNA Sequencing and Analysis. *Cold Spring Harb Protoc* 2015, 951-969, doi:10.1101/pdb.top084970 (2015).
199. Szabo, L. & Salzman, J. Detecting circular RNAs: bioinformatic and experimental challenges. *Nat Rev Genet* 17, 679-692, doi:10.1038/nrg.2016.114 (2016).
200. Conesa, A. *et al.* A survey of best practices for RNA-seq data analysis. *Genome Biol* 17, 13, doi:10.1186/s13059-016-0881-8 (2016).
201. Delev, D. *et al.* Long-term epilepsy-associated tumors: transcriptional signatures reflect clinical

course. *Sci Rep* 10, 96, doi:10.1038/s41598-019-56146-y (2020).
202. Mills, J. D. *et al.* Coding and small non-coding transcriptional landscape of tuberous sclerosis complex cortical tubers: implications for pathophysiology and treatment. *Sci Rep* 7, 8089, doi:10.1038/s41598-017-06145-8 (2017).
203. Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215-233, doi:10.1016/j.cell.2009.01.002 (2009).
204. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15-20, doi:10.1016/j.cell.2004.12.035 (2005).
205. Zhao, Y. & Srivastava, D. A developmental view of microRNA function. *Trends Biochem Sci* 32, 189-197, doi:10.1016/j.tibs.2007.02.006 (2007).
206. Cao, X., Yeo, G., Muotri, A. R., Kuwabara, T. & Gage, F. H. Noncoding RNAs in the mammalian central nervous system. *Annu Rev Neurosci* 29, 77-103, doi:10.1146/annurev.neuro.29.051605.112839 (2006).
207. Rajman, M. & Schratt, G. MicroRNAs in neural development: from master regulators to fine-tuners. *Development* 144, 2310-2322, doi:10.1242/dev.144337 (2017).
208. Prabowo, A. S. *et al.* Differential expression and clinical significance of three inflammation-related microRNAs in gangliogliomas. *J Neuroinflammation* 12, 97, doi:10.1186/s12974-015-0315-7 (2015).
209. Birks, D. K. *et al.* Survey of MicroRNA expression in pediatric brain tumors. *Pediatr Blood Cancer* 56, 211-216, doi:10.1002/pbc.22723 (2011).
210. Jones, T. A. *et al.* Molecular analysis of pediatric brain tumors identifies microRNAs in pilocytic astrocytomas that target the MAPK and NF-kappaB pathways. *Acta Neuropathol Commun* 3, 86, doi:10.1186/s40478-015-0266-3 (2015).
211. Ames, H. M., Yuan, M., Vizcaino, M. A., Yu, W. & Rodriguez, F. J. MicroRNA profiling of low-grade glial and glioneuronal tumors shows an independent role for cluster 14q32.31 member miR-487b. *Mod Pathol* 30, 204-216, doi:10.1038/modpathol.2016.177 (2017).
212. Ho, C. Y. *et al.* MicroRNA profiling in pediatric pilocytic astrocytoma reveals biologically relevant targets, including PBX3, NFIB, and METAP2. *Neuro Oncol* 15, 69-82, doi:10.1093/neuonc/nos269 (2013).
213. Yuan, M. *et al.* MicroRNA (miR) 125b regulates cell growth and invasion in pediatric low grade glioma. *Sci Rep* 8, 12506, doi:10.1038/s41598-018-30942-4 (2018).
214. Zakrzewska, M. *et al.* Expression-based decision tree model reveals distinct microRNA expression pattern in pediatric neuronal and mixed neuronal-glial tumors. *Bmc Cancer* 19, 544, doi:10.1186/s12885-019-5739-5 (2019).
215. Law, J. A. & Jacobsen, S. E. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* 11, 204-220, doi:10.1038/nrg2719 (2010).
216. Moran, S., Arribas, C. & Esteller, M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenomics* 8, 389-399, doi:10.2217/epi.15.114 (2016).
217. Bock, C. Analysing and interpreting DNA methylation data. *Nat Rev Genet* 13, 705-719, doi:10.1038/nrg3273 (2012).
218. Morris, T. J. & Beck, S. Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data. *Methods* 72, 3-8, doi:10.1016/j.ymeth.2014.08.011 (2015).
219. Du, P. *et al.* Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics* 11, 587, doi:10.1186/1471-2105-11-587 (2010).

220. Capper, D. *et al.* DNA methylation-based classification of central nervous system tumours. *Nature* 555, 469-474, doi:10.1038/nature26000 (2018).
221. Stone, T. J. *et al.* Comprehensive molecular characterisation of epilepsy-associated glioneuronal tumours. *Acta Neuropathol* 135, 115-129, doi:10.1007/s00401-017-1773-z (2018).
222. Huse, J. T. *et al.* Polymorphous low-grade neuroepithelial tumor of the young (PLNTY): an epileptogenic neoplasm with oligodendroglioma-like components, aberrant CD34 expression, and genetic alterations involving the MAP kinase pathway. *Acta Neuropathol* 133, 417-429, doi:10.1007/s00401-016-1639-9 (2017).
223. Deng, M. Y. *et al.* Molecularly defined diffuse leptomeningeal glioneuronal tumor (DLGNT) comprises two subgroups with distinct clinical and genetic features. *Acta Neuropathol* 136, 239-253, doi:10.1007/s00401-018-1865-4 (2018).
224. Muhlebner, A., Bongaarts, A., Sarnat, H. B., Scholl, T. & Aronica, E. New insights into a spectrum of developmental malformations related to mTOR dysregulations: challenges and perspectives. *J Anat* 235, 521-542, doi:10.1111/joa.12956 (2019).