2020

CHARACTERIZATION OF THE TSC/DYRK1A INTERACTION

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CHARACTERIZATION OF THE TSC/DYRK1A INTERACTION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Spring, 2020

Acknowledgements

I would like to thank my advisor Dr. Larisa Litovchick for her guidance and support through the past few years. This work would have not been possible without her constant advice and encouragement for improvement.

I would also like to thank both past and present lab members and all others for their friendship and support through the past few years. A special thanks to Dr. Vijay Menon, who started this project and guided me through some of the key experiments used in the study. Lots of gratitude to Sophia Gruszecki for all her help in the lab, and for being a very kind friend the past few years. I am especially thankful to my friend and lab mate Varsha Ananthapadmanabhan, for her continuous strong support and being a great friend through the entirety of my PhD.

A special thanks to my committee members Dr. Andrei Ivanov, Dr. Steven Grossman, Dr. Colleen Jackson-Cook and Dr. Frank Fang for their valuable advice that has guided me through my PhD.

I would like to thank my family, friends, as well as extended family for their unending love and support. Last but not the least, to my late father, to whom this entire endeavor is dedicated.

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ABBREVIATIONS

1. DMEM: Dulbeccos Minimal Essential Medium
2. TSC: Tuberous Sclerosis Complex
3. DYRK: Dual-Specificity Tyrosine Regulated Kinase
4. DS: Down Syndrome
5. mTORC1: Mammalian Target of Rapamycin Complex 1
6. MEF: Mouse Embryonic Fibroblasts
7. NFAT: Nuclear factor of activated T-cells
8. CDK: Cyclin Dependent Kinase
9. ID: Intellectual Disability
10. AD: Alzheimer's Disease
11. AMKL: Acute Megakaryoblastic Leukemia
12. ALL: Acute Lymphoblastic Leukemia
13. DEPTOR: DEP domain containing mTOR interacting protein
14. RAPTOR: Regulatory associated protein of mTOR
15. ATP: Adenosine Triphosphate
16. GAP: GTPase activating protein
17. ASD: Autism Spectrum Disorder
18. TAND: TSC-associated neuropsychiatric Disorders
19. RPPA: Reverse Phase Protein Array
20. GFP: Green Fluorescence Protein
21. LAM: Lymphangioleiomyomatosis
22. TBC1D7: TBC-domain family member 7

Abstract

CHARACTERIZATION OF THE TSC/DYRK1A INTERACTION

The Tuberous sclerosis complex (TSC) includes TSC1, TSC2 and the TBC1D7 subunits that together function as a principal inhibitor of the mTOR protein kinase complex 1 (mTORC1). mTORC1 is a master regulator of cell growth and proliferation that responds to signaling cues such as growth factors and nutrient availability. Proteomic studies in our lab revealed an interaction between the TSC subunits and DYRK1A, a ubiquitous protein kinase encoded by a gene located in the Down syndrome (DS) region on human chr21. In this study, we sought to validate the interaction of the TSC components with DYRK1A and to determine the functional significance of this interaction. Our analysis confirmed that DYRK1A interacts with TSC1 at the endogenous levels, and with TSC2 when overexpressed. Domain mapping of the DYRK1A-TSC interaction revealed that binding of TSC2 to DYRK1A requires TSC1. However, binding of TSC1 to DYRK1A does not require TSC2 as evidenced by the interaction observed in both WT and Null TSC2 Cells. We also observed that TSC1 is a substrate of DYRK1A kinase, and in this study, we report DYRK1A specific phosphorylation sites for the first time. Given that TSC is a major inhibitor of the mTORC1 pathway, we sought to assess the effect of DYRK1A loss on the mTORC1 activity. Interestingly, we did not observe any change in phosphorylation of mTORC1 substrates p70S6K and 4EBP1 under serum-starved conditions in both transient and CRISPR-Cas9 DYRK1A knockout (KO) cells. However, using FACS analysis, we noticed that cells lacking DYRK1A appeared smaller in size compared to controls in multiple cell lines. We also observed that global protein expression was significantly reduced in DYRK1A KO cell lines using the Puromycin-tagging Assay. Moreover, the decrease in global protein production was independent

of the TSC1 protein. We next hypothesized that interaction of DYRK1A with the TSC1 complex could have an effect on the function of DYRK1A kinase. Indeed, we observed a significant decrease in DYRK1A kinase activity as evidenced by the loss of Lin52 phosphorylation at its Serine28 residue in Tsc1-null MEFs. This effect however, was found to be limited to Tsc1-null MEFs and was not replicated in various other TSC1 knockdown cell lines. However, preliminary studies suggest that TSC1 knockdown cell lines are more resistant to DYRK1A mediated cell cycle arrest as compared to controls. Overall, our study introduces DYRK1A as a novel partner of the TSC components that could mediate its non-canonical functions including mTORC1-independent regulation of cell proliferation and protein synthesis.

I. BACKGROUND AND INTRODUCTION

Protein-Protein Interactions regulate key biological functions such as mediating signal transduction, enzyme regulation, nutrient sensing as well as maintenance of cellular integrity and cellular organization. Thus, one of the key questions that has to be answered during the study of a protein, is to figure out which other proteins it interacts with. In this dissertation, we describe a novel protein-protein interaction between a Dual-specificity Tyrosine Regulated Kinase (DYRK1A) and the components of the Tuberous Sclerosis complex (TSC1/TSC2). These proteins are involved in cellular functions such as cell cycle control, regulation of protein biosynthesis, autophagy and cell migration. The following sections will describe in detail the significance of both these proteins, and the molecular pathways that their interaction may regulate.

Chapter 1: THE DYRK FAMILY OF KINASES

Protein biosynthesis is often followed by post-translational modifications to give rise to a mature protein product. Out of the existing modifications, the phosphorylation of proteins has been regarded as the most common form of post-translation modification in the cell that greatly modifies the function of proteins. Phosphorylation is carried out by a family of enzymes known as the protein kinases. Eukaryotic protein kinases are clustered into a hierarchy of groups, families and sub-families, as per the taxonomic classification that was first devised by Hanks and Hunter in 1995 [1]. Out of these kinases, this study focuses on a single member of the DYRK family of kinases, DYRK1A.

1.1. THE EVOLUTIONARY CONSERVATION OF THE DYRK FAMILY

The DYRK family of kinases are proline directed dual specificity tyrosine regulated kinases that phosphorylate their substrates at serine/threonine (S/T) positions and auto phosphorylate at the tyrosine (Y) position [2]. Based on kinase domain homology, the DYRK family of proteins is divided into 3 groups - DYRK kinases, homeodomain-interacting protein kinases (HIPKs), and pre mRNA processing protein 4 kinases (PRP4s) [3]. The DYRK kinases are further sub-divided into 3 groups - the Yak group, which is not present in animals, and DYRK1 and DYRK2 [3]. The very first DYRK kinase, Yak1p, was discovered in budding yeast in 1989 [4]. Since this discovery, DYRK family members have been described in all eukaryotes including *Dictyostelium* (Dyrk1, Dyrk2), in nematodes like *C. elegans* (MBK1, MBK2), in fly *Drosophila melanogaster* (minibrain, dDyrk2, dDyrk3) and finally in mammals (DYRK1A, DYRK1B, DYRK2, DYRK3, DYRK4A and DYRK4B). (Fig.1).

1.1.a. DYRK kinases in lower eukaryotes and nematodes: Yak1p (*S. cerevisiae*), Pom1p (*S. pombe*) and YakA (*Dictyostelium*) are the three most well studied DYRK kinases in lower eukaryotes (Fig.1A). The DYRK kinase Yak1p found in fission yeast acts as a negative regulator of proliferation by regulating the Ras/cAMP pathway as well as the mammalian target of Rapamycin (TOR) Pathway [4, 5]. Inhibition of the mTOR pathway using rapamycin as well as glucose depletion causes Yak1p to localize to the nucleus where it can phosphorylate Msn2p and Hsf1p, two essential stress response transcription factors that are downstream of the RAS/cAMP pathway [5, 6]. Yet another substrate of this kinase is Crf1p, a transcriptional ribosomal protein co-repressor, whose localization is regulated by the mTOR pathway. The phosphorylation of Crf1p allows its nuclear retention which further leads to repression of ribosomal protein genes [5, 6].

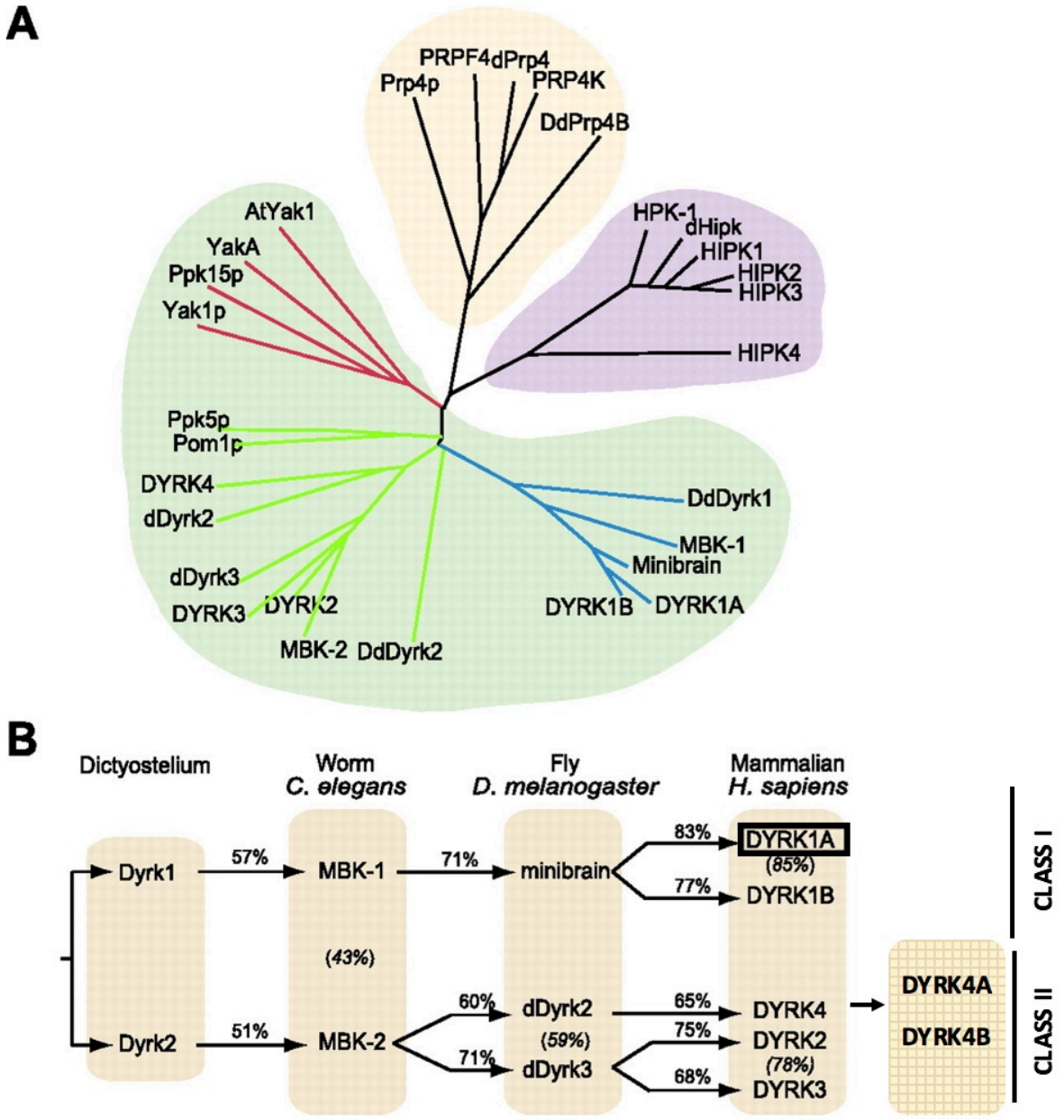


Fig.1. Evolutionary conservation of the DYRK1A kinase. Figure adopted and modified from- DYRK family of protein kinases: evolutionary relationships, biochemical properties and functional roles; Aranda, S; Laguna, A; de La Luna, S; Faseb Journal, 2011 Feb, Vol.25(2), pp.449-462. **A:** A phylogenetic tree representing the evolutionary diversion of kinase domain in different DYRK family members, dividing it into three families- DYRK (green), HIPK (Violet), PRP4K (yellow). **B:** The main classes of DYRK sub-family and their percentage of conservation at the protein level.

Another lower eukaryotic DYRK kinase, Pom1p, is essential for providing positional information for cell growth and cell division via regulation of the G2/M phase-transition of the cell cycle [7-9]. One of the three *Dictyostelium* DYRK kinases, YakA, is also involved in the regulation of the cell cycle in a similar manner as its homolog Yak1p in the fission yeast [10]. Interestingly, YakA-null cells were found to be smaller than wild type cells and were also found to progress faster through the cell cycle. Correspondingly, over-expression of YakA under the control of a conditional promoter causes cell cycle arrest [10].

In the nematode *Caenorhabditis elegans* (*C.elegans*), two DYRK genes have been identified - Minibrain Kinase homologue-1 (mbk-1) and mbk-2. While mbk-1 loss-of-function mutants are viable and morphologically similar to wild types, mbk-2 loss-of-function mutants are embryonically lethal [11]. Interestingly, extra copies of mbk-1 cause behavioral defects in the chemotaxis of nematodes towards volatile chemoattractants [11].

1.1.b. DYRK kinases in *Drosophila:* *Drosophila melanogaster,* the fruit fly, has been found to have three DYRK sub-family members – *Minibrain (mnb),* dDYRK2 and dDYRK3 [12, 13]. The study of these *Drosophila* kinases is largely responsible for the existing functional model of DYRK kinases [14, 15]. The most widely studied of these kinases is the *Minibrain*, so named due to the smaller brain size of hypomorphic mutant flies. Specific morphologoical alterations include reduction in the size of the optic lobes as well as the central brain hemispheres. These morphological changes are accompanied by corresponding behavioral defects in memory, learning, visual as well as olfactory components [12]. dDYRK2, the homolog of mammalian DYRK4 is involved in embryogenesis while dDYRK3 may be involved in regulation of the transcription factor NFAT (Nuclear Factor of Activated T-cells) [13, 16].

1.1.c DYRK kinases in mammals: The mammalian DYRK kinase subfamily is made up of at least 7 members including DYRK1A, DYRK1B, DYRK1C, DYRK2, DYRK3, DYRK4A and DYRK4B [17]. Structurally, members of this subfamily are characterized by the presence of a kinase domain and a DYRK homology (DH) Box domain that is common to all members [17]. Additionally, DYRK1A and DYRK1B are thought to be paralogs, and are further chracterized by the presence of a nuclear localization signal (NLS) at the N-terminal region and a PEST motif at the C-terminal region. The rest of the mammalian DYRK family members are differentiated from DYRK1A/B by the presence of the NAPA-domain that is also observed in DYRK kinases from other species. (Fig. 2). Out of these mammalian DYRK kinases, since DYRK1A is our protein of interest, the next few sections will be highlighting the specific functions and phenotypes associated with this kinase.

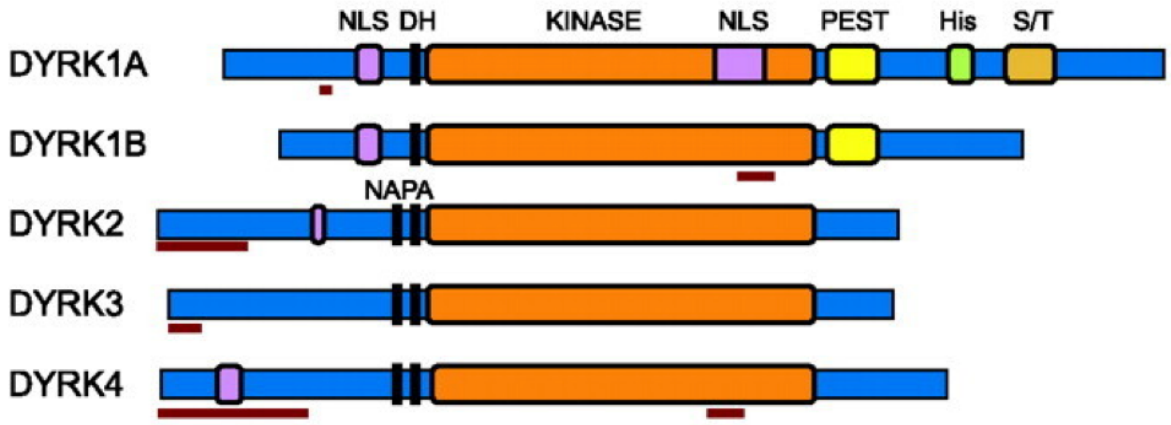


Fig.2. The mammalian DYRK kinases. Adopted from- DYRK family of protein kinases: evolutionary relationships, biochemical properties and functional roles; Aranda, S; Laguna, A; de La Luna, S; Faseb Journal, 2011 Feb, Vol.25(2), pp.449-462. Identified domains include: NLS, Nuclear localization signal; DH, DYRK homology; NAPA, N-terminal autophosphorylation accessory region; PEST, motif rich in proline, glutamic acid, serine and threonine residues; HIS, poly-histidine stretch; S/T, serine/threonine enriched residues; Red lines indicate the regions modulated by splicing events.

1.2. THE MAMMALIAN DYRK1A

The mammalian DYRK1A kinase is an emerging tumor supressor protein that is encoded by a gene located in the Down Syndrome Critical Region (DSCR) on chromosome 21. It is the most ubiquitously expressed kinase of the DYRK family and plays an important role in G0/G1 cell cycle arrest by promoting the assembly of a transcriptional repressor complex known as the DREAM (DP, RB-like, E2F4 and MuvB) [18, 19]. Moreover, DYRK1A has been found to be overexpressed in the brain of patients with Down Syndrome (DS), and its substrates have been implicated in the neuropathological phenotypes oberved in DS patients [20-23]. Additionally, a unique characteristic of DYRK1A is that it is thought to be a constitutively active kinase, with its kinase activity directly proportional to the amount of protein produced. This feature makes it highly dosage sensitive because both upregulation and down regulation of DYRK1A can have physiological implications [24]. Like the other members of its sub-family, DYRK1A is a dual-specificity serine/threonine (S/T) kinase that autophosphorylates a conserved tyrosine (Y) residue in its own activation loop. The optimal substrate sequence for DYRK1A has been recognized as **RPX(S/T)P**, with a preference for proline at the +1 position in its consensus sequence, making it a proline directed kinase [2]. However, not all DYRK1A specific phosphorylation sites that have been catalogued match the consensus sequence, and hence other mechanisms may also be involved in the direction of DYRK1A towards specific substrates. This section highlights in further detail the role of DYRK1A in cell cycle regulation and its role in neuropathological diseases such as DS and Alzheimer's disease (AD) as well as in tumorigenesis.

1.2.a. The role of DYRK1A in DREAM-mediated cell cycle control.

DREAM (DP, RB-like, E2F4 and MuvB) complex promotes G0/G1 arrest by repression of cell cycle dependent genes [18]. The DREAM complex consists of retinoblastoma-like protein p130, E2F4, DP1 and a core complex of 5 MuvB-like proteins that include LIN9, LIN37, LIN52, LIN54 and RBBP4. The assembly of DREAM complex requires DYRK1A-mediated phosphorylation of LIN52 at its Serine28 (S28) position, which further leads to repression of cell cycle regulated genes. Upon entry into the cell cycle, p130 dissociates from the MuvB core, which can then interact with the B-MYB transcription factor and transactivate G2/M phase late cell cycle genes [19]. Inhibition of DYRK1A or mutation in the S28 site of LIN52 disrupts DREAM complex assembly and reduces the ability of cells to enter quiescence.

1.2.b. The role of DYRK1A in cyclin-mediated cell cycle control.

Apart from its role in inhibition of the cell cycle through the DREAM complex, DYRK1A also regulates the cell cycle by phosphorylating cyclin D1, a positive regulator of the cell cycle, at Thr286 which causes its proteosomal degradation, as well as by phosphorylating $p27^{Kip}$, a cyclin dependent kinase (CDK) inhibitor at the Ser10 position and enhancing its stability during the G0/G1 phase of the cell cycle [25]. Keeping with this observation, a study using live cell microscopy and single cell imaging, has shown that Dyrk1a mediates a dose-dependent increase in the duration of the G1 phase through direct phosphorylation and degradation of cyclin D1. Moreover, inhibition of Dyrk1a activity or reduction in Dyrk1a levels stabilized cyclin D1 and propelled the cells into 2 different fates - one with accelerated proliferation and the other with the proliferation arrest due to increased p21 protein levels [26]. These results indicate that the upregulation as well as downregulation of DYRK1A could lead to transient or reversible cell cycle arrest and/or progression and have similar consequences on tissue growth as well.

1.2.c. Role of DYRK1A kinase in additional signaling pathways

It has been reported that overexpression of *mnb,* the Drosophila homolog of DYRK1A, inhibits the Hippo pathway, a known inhibitor of proliferation and inducer of apoptosis in fly models, and promotes organ growth [27]. Correspondingly, overepression of DYRK1A in tumor cell lines can inhibit proliferation and colony formation. The variability in the effects observed with DYRK1A loss and overexpression may be cell type or species dependent. This variability, however, underline the dosage sensitivity of the DYRK1A gene. Yet another effect of DYRK1A imbalance is seen with DYRK1A modulation of the Rei-dependent transcription factor (REST), that is found to be downregulated in the brain of Down syndrome patients [28]. REST is a master regulator of neuronal cell differentiation. Recent studies have indicated that REST can activate DYRK1A transcription through a negative feedback loop. The imbalance of DYRK1A can destabilize REST protein expression and transcription activity through ubiquitination and subsequent degradation. These results imply that DYRK1A can act both as a positive regulator or a negative regulator of the REST protein. In summary, existing literature on DYRK1A functions in the regulation of cell cycle and tumorigenesis indicate that, based on the context, DYRK1A can function both as a tumor supressor and an oncogene (Fig. 3).

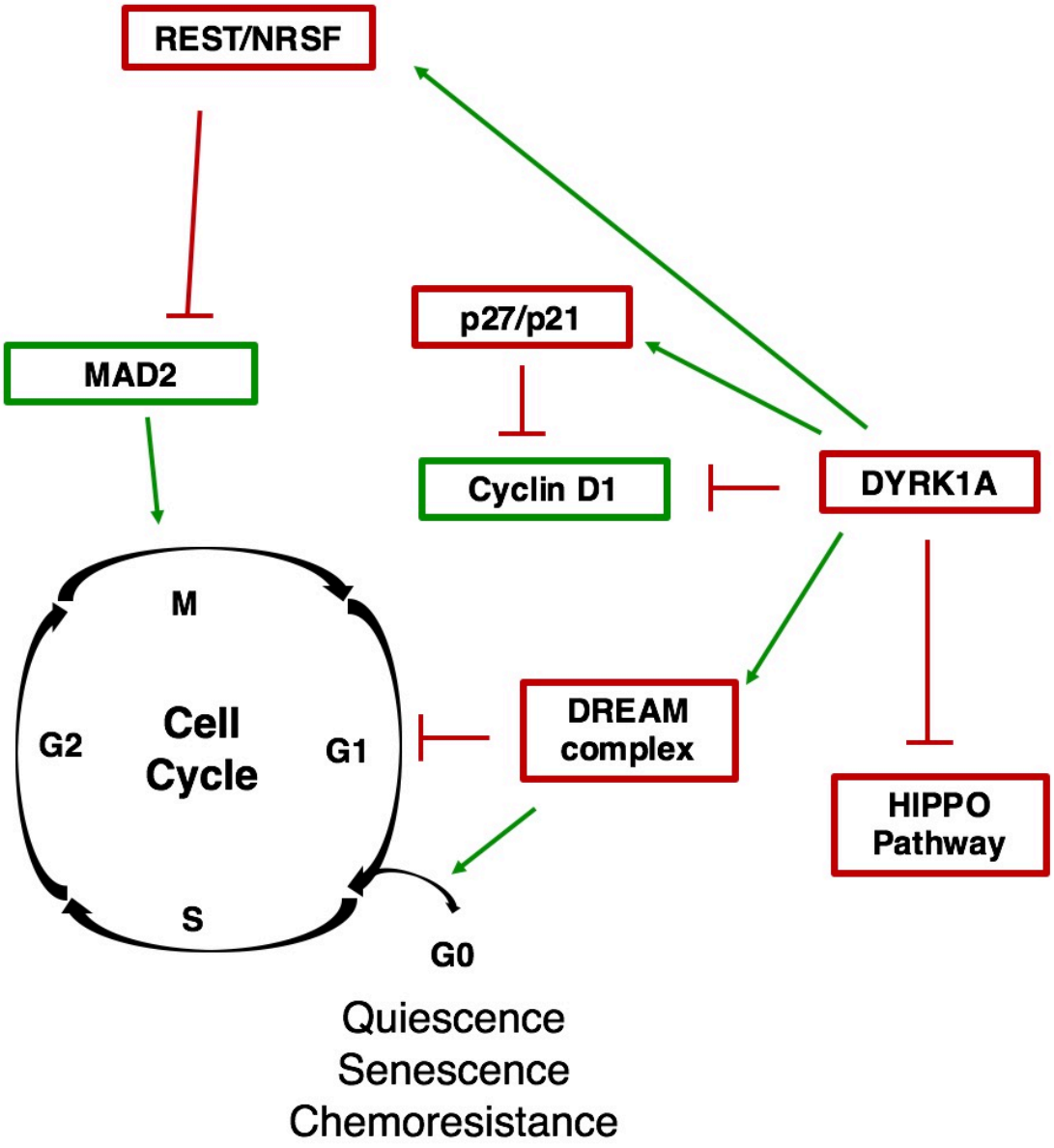


Fig.3. The role of DYRK1A in cell cycle control. Figure adapted from- P Fernández-Martínez, C Zahonero & P Sánchez-Gómez (2015) DYRK1A: the double-edged kinase as a protagonist in cell growth and tumorigenesis, Molecular & Cellular Oncology, 2:1, e970048. The figure shows activating (Green lines, green box) and inhibitory pathways (Red line, Red Box).

1.2.d. Pathological effects of DYRK1A dysfunction: Vertebrate Dyrk1a kinase is ubiquituosly expressed in different stages of development, and its absence is embryonically lethal. In mice,

heterozygous animals are viable but are considerably smaller in size than wild type mice throughout their lifetime. Moreover, heterozygous mice are characterized by changes in motor development, dopaminergic deficiency, reduced neonatal viability, decrease in the number of neurons and impaired spatial development [29, 30]. Moreover, haploinsufficiency of DYRK1A in humans causes a recognizable syndrome characterized by seizures, intellectual disability, congenital microcephaly, skeletal defects and global developmental delay [31]. Conversely, overexpression of Dyrk1a in transgenic mice causes motor and cognitive defects, hyperactivity as well as neurodevelopmental delay [32]. Down syndrome, caused by partial or complete trisomy of chromosome 21, is characterized by abnormal brain development, reduced brain size and neuronal number as well as reduced dendritic branches [33, 34]. DYRK1A plays an important role in the regulation of neuronal functions and neurodevelopment, and has been mapped to the Down syndrome critical region on chromosome 21, further reinforcing the idea that overexpression of this protein is linked to intellectual disability (ID) observed in Down syndrome patients. DYRK1A also hyperphosphorylates the *tau* protein at Ser202, Ser404 and Thr212 positions which leads to the formation of amyloid plaques that are characteristic of patients with Alzheimer's disease (AD). It is not surprising, therefore, that most individuals with DS show early onset AD [35]. The prevalence of AD in DS is thought to be <5% under the ages of 40 and is thought to roughly double at each 5-year interval, although this might not be true for 100% of patients [36, 37]. The importance of DYRK1A in regulating the neuropathological symptoms in AD and DS has made it a very promising therapeutic target. For instance, a recent study has shown that chronic Dyrk1a inhibition in a transgenic model for AD (3xTg-AD) ameliorated the cognitive defects seen in AD mice [38]. Moreover, similar improvement was seen in a model for DS (Ts65Dn) with competitive Dyrk1a inhibition [39].

Recent reports have shown heterozygous mutations in DYRK1A in patients that show a vast array of phenotypes including intellectual disability with autism spectrum disorder, microcephacy, seizures, impaired speech and cardiac defects, thereby defining a new syndromic disorder [40-42]. Such mutations are generally found in the kinase domain of the DYRK1A molecule close to the ATP or peptide binding sites [43]. *De novo* mutations in the DYRK1A gene have also been shown to cause the autosomal dominant mental retardation-7 (MRD7) syndrome [44]

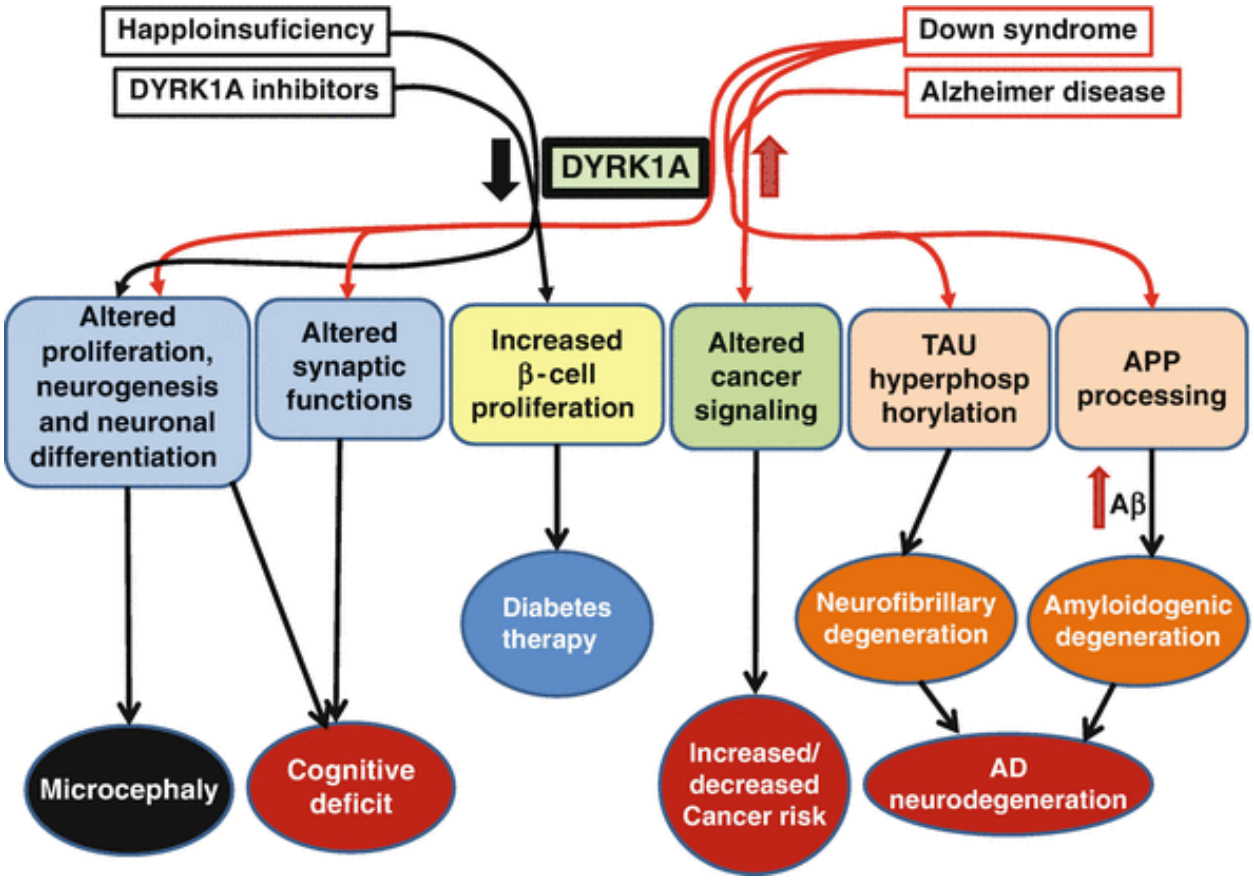


Fig.4. The role of DYRK1A in disease pathology. Figure adopted from- Tejedor F.J. (2018) Dyrk1a. In: Choi S. (eds) Encyclopedia of Signaling Molecules. Springer, Cham. The pathological effects of DYRK1A overexpression (Red arrows) and haploinsufficiency/inhibition (black arrows) are illustrated.

An additional importance of DYRK1A dosage is seen in the incidence of acute megakaryoblastic leukemia (AMKL), acute lymphoblastic leukemia (ALL) and different solid tumors in DS patients. Epidemiological studies have shown that individuals with DS have an increased risk of AMKL and ALL in combination with acquired mutations in the global transcription factor-1 (GATA-1) gene [45]. Similar mutations in non-DS patients have no such predisposition to leukemia. Contrary to this, individuals with DS show a decreased risk for most solid tumors [46, 47].

Apart from its role in neurological disorders, DYRK1A has also been shown to have a role in the regulation of β-cell proliferation. β-cells are present in the islets of pancreas and loss of these cells is the characteristic feature of both Type I and Type II diabetes. In mice, haploinsufficiency of DYRK1A induces diabetes through decreased β-cell proliferation and reduced β-cell mass along with severe glucose intolerance [48]. Alternately, inhibition of DYRK1A kinase activity causes an increase in the proliferation of human β-cells [49].

The variability in these results further underline the importance of DYRK1A dosage, and emphasize the importance of trying to achieve "*normal*" levels of DYRK1A while devising a therapeutic strategy for any disease model (Fig.4).

CHAPTER 2: THE TUBEROUS SCLEROSIS COMPLEX (TSC)

Prokaryotic and eukaryotic cells are required to sense diverse environmental signals in order to mount approriate physiological responses that are essential for cell survival. Eukaryotic cells in multisystem organisms recognize a variety of signaling cues including nutrient, cytokine and growth factor availability as well as stress recognition. One of the major protein complexes that has been known to integrate these signaling cues is the Tuberous Sclerosis Complex.

2.1. The Characteristics and Functions of the Tuberous Sclerosis Complex

The Tuberous Sclerosis Complex (TSC) includes TSC1 (Hamartin), TSC2 (tuberin) and TBC1D7 subunits that regulate important cellular processes such as cell growth and protein synthesis by integrating growth factor, nutrient and stress signaling pathways [50]. Mutations in the *TSC1* and *TSC2* tumor suppressor genes cause an autosomal dominant tumor syndrome called tuberous sclerosis (TS). Tuberous sclerosis is a multi-system autosomal dominant genetic disorder that is characterized by formation of benign tumors called hamartomas in various organs including brain, kidneys, skin, lungs and heart. Additionally, serious clinical manifestations of TS are characterized by severe neurological defects that include, but are not limited to epilepsy, autism spectral disorders (ASD) and intellectual disability. The hamartomas caused by mutations in the TSC genes rarely cause malignant tumors and have been associated with defects in various cellular functions such as adhesion, cellular migration and proliferation [51]. Functionally, the TSC serves as a principal inhibitor of the mechanistic target of rapamycin-1 (mTORC1) protein kinase complex [52]. The mTORC1 complex is a master regulator of cell growth and proliferation that integrates and responds to signaling cues such as growth factors and nutrient availability, *via* pathways that converge at the TSC. Additionally, like DYRK1A, the TSC is also highly conserved in evolution with homologs in *S. pombe* (yeast), *Drosophila* and mice. In this section we will describe the

structure of the TSC proteins and detail the mechanisms by which they regulate mTORC1 and its downstream pathways as well as the mTORC1-independent functions of the TSC components.

2.1.a The structure of the Tuberous Sclerosis Complex

TSC1 and TSC2 form the two main components of the TSC. The TSC2 gene was discvered in 1993 and codes for a 200 KDa protein tuberin. Structurally, tuberin is composed of a hydrophobic N-terminal region and a conserved 163 amino acid stretch at the C-terminus that is homologous to GTPase activating proteins (GAP) domain of Rap1 Gap [53]. The GAP domain of TSC2 regulates the activity of Rheb, a Ras homologue enriched in brain, that lies upstream of the mTORC1 complex and promotes its activity in response to specific growth signals. The GAP activity of TSC2 switches Rheb from its mTORC1-activating GTP-bound active state to GDP-bound inactive state [54, 55] (Fig.5).

The TSC1 protein (hamartin) is a 130 KDa protein that does not show a significant homology to any other known mammalian protein [56]. The TSC1/TSC2 complex mainly functions as a heterodimer. Structurally, the N-terminal domain of TSC2 binds to TSC1 at multiple regions, including a coiled-coil region close to the C-terminal region of TSC1 [57-59]. The TSC1 and TSC2 proteins with their various functional domains are shown in Fig.6. The next sections will describe in greater detail the canonical and non-canonical functions of the TSC proteins as well as the pathological significance of mutations in these genes.

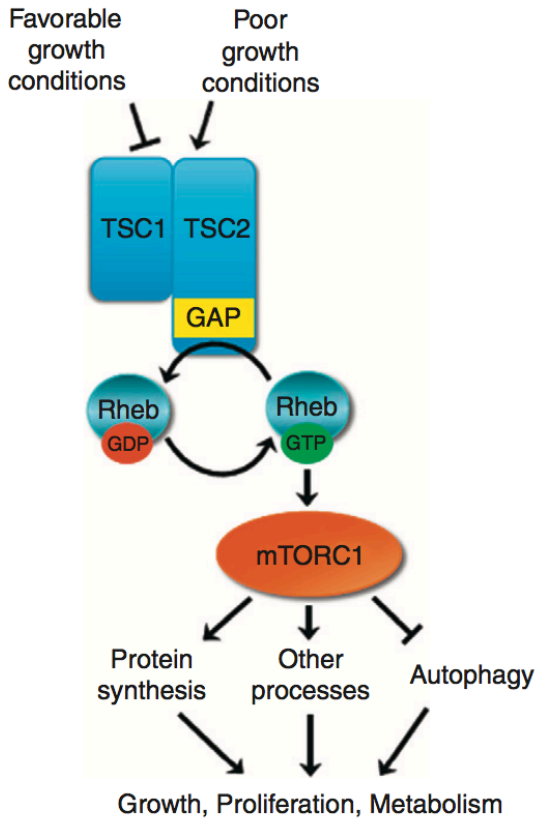


Fig.5. TSC inhibits the mTORC1 pathway through the activity of the Rheb protein. Figure adopted from- *Dibble, C.C. and B.D. Manning,* The TSC1–TSC2 Complex. *2010.* ***28****: p. 21-48.* GAP activity of TSC2 switches Rheb from its mTORC1 activating GTP-bound active state to GDP-bound inactive state, thus inhibiting mTORC1 activation.

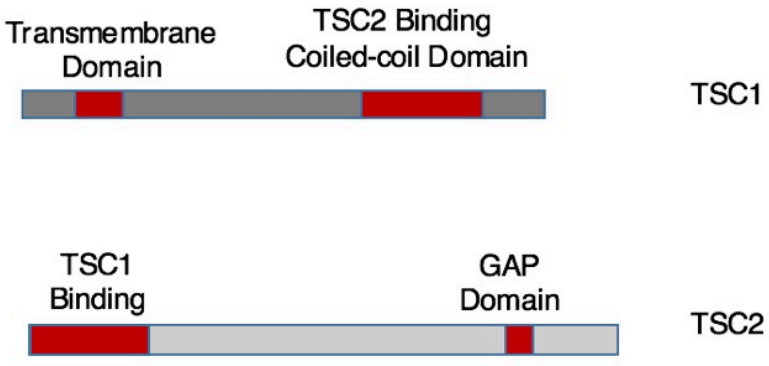


Fig.6. The components of the Tuberous Sclerosis Complex (TSC). The N-terminal domain of TSC2 binds to the C-terminal coiled-coil domain of TSC1.

2.1.b. The tumor supressive function of the TSC

As noted earlier, TSC is a principal inhibitor of the mechanistic target of rapamycin (mTOR). mTOR is an atypical serine threonine kinase that interacts with several different proteins to form two distinct multi-subunit complexes - mTORC1 and mTORC2. Besides mTOR, both these complexes include mammalian lethal sec13 protein 8, DEP domain containing mTOR-interacting protein (DEPTOR) and the Tti2/Tel2 complex. The regulatory associated protein of mTOR (RAPTOR) and proline rich Akt substrate 40KDa (PRAS40) are specific to mTORC1 while the rapamycin insensitive companion of mTOR (RICTOR), mammalian stress activated map kinase interacting protein-1 (mSin1) and protein observed with RICTOR 1/2 (PROCTOR 1/2) are exclusive to the mTORC2 complex [60]. The mTORC1 complex directly phosphorylates two important translational regulators - the eukaryotic translational initiation factor 4E (eIF4E) binding protein-1 (4EBP1) and the 70KDa ribosomal protein S6 Kinase (p70S6K), at multiple sites [61]. The phosphorylation of 4EBP1 prevents it from binding to eIF4E, the cap binding protein, which further forms the eIF4F complex required for cap-dependent translation initiation. According to the current model, cellular stress causes the translocation of TSC2 from the cytoplasm to the lysosomal membrane, where mTORC1 is also located. Upon lysosomal recruitment, TSC2 inhibits mTORC1 through its GAP activity on Rheb, which is also localized in the lysosomes [62].

Apart from its role in protein biosynthesis through the substrates mentioned above, mTORC1 also controls lipid synthesis, cellular metabolism, ATP production and negatively regulates autophagy as well as lysosome biogenesis. It is not surprising, therefore, that mTOR has been found to be deregulated in an array of human diseases including metabolic disorders and cancer.

2.1.c. Upstream regulators of the TSC/mTORC1 pathway

Five major intracellular and extracellular cues integrate on the mTORC1 pathway. These include growth factors, stress, oxygen, energy status and amino acids. Among the signaling cues that impinge on mTORC1 are insulin and insulin like growth factor (IGF1), that stimulate the PI3K/Ras pathway that includes the effector kinases protein kinase B (PKB), also known as AKT, extracellular signal regulated kinase 1/2 (ERK1/2) and ribosomal S6 kinase (RSK1). These kinases directly phosphorylate and inactivate the TSC components, thereby activating the mTORC1 complex and promoting protein synthesis [63, 64]. Additionally, mTORC1 can also be activated by proinflammatory cytokines such as tumor necrosis factor-α (TNFα). Finally, the canonical Wnt pathway, which is a major regulator of cell growth and differentiation, can also activate mTORC1 by inactivating glycogen synthase kinase 3β (GSK3β). GSK3β normally phosphorylates and promotes the functions of TSC [65]. In addition to signaling cues that activate the mTORC1 pathway, cellular stressors such as hypoxia and low energy state, result in adenosine monophosphate-activated protein kinase (AMPK) dependent phosphorylation of TSC2, thus increasing TSC2 mediated mTORC1 inhibition [66]. In summary, the upstream regulation of the TSC/mTORC1 pathway is highly complex and regulated at multiple levels (Fig.7). Specifically, this pathway is differentially regulated by phosphorylation of the TSC components by multiple protein kinases, which are conserved across different species. These protein kinases have been reported to both activate and inhibit TSC based on specific phosphorylation sites. Table 1 shows several examples of these activating and inhibitory phosphorylation sites, along with the respective upstream kinases [54] (PhosphositePlus®).

Site	Kinase	Function	Conservation
TSC1			
S487	IKKβ	Inhibit	Mammals
S584	CDK1	Inhibit	Vertebrate
TSC2			
S540	ERK	Inhibit	Vertebrate
S1371	GSK3	Activate	Human
T1271	AMPK	Activate	Vertebrate

Table 1. Regulation of TSC by upstream kinases.

While some of these upstream kinases and their respective target sites have been studied in great detail, the functions of a large number of phosphorylated sites on the TSC components remain elusive and need to be explored further.

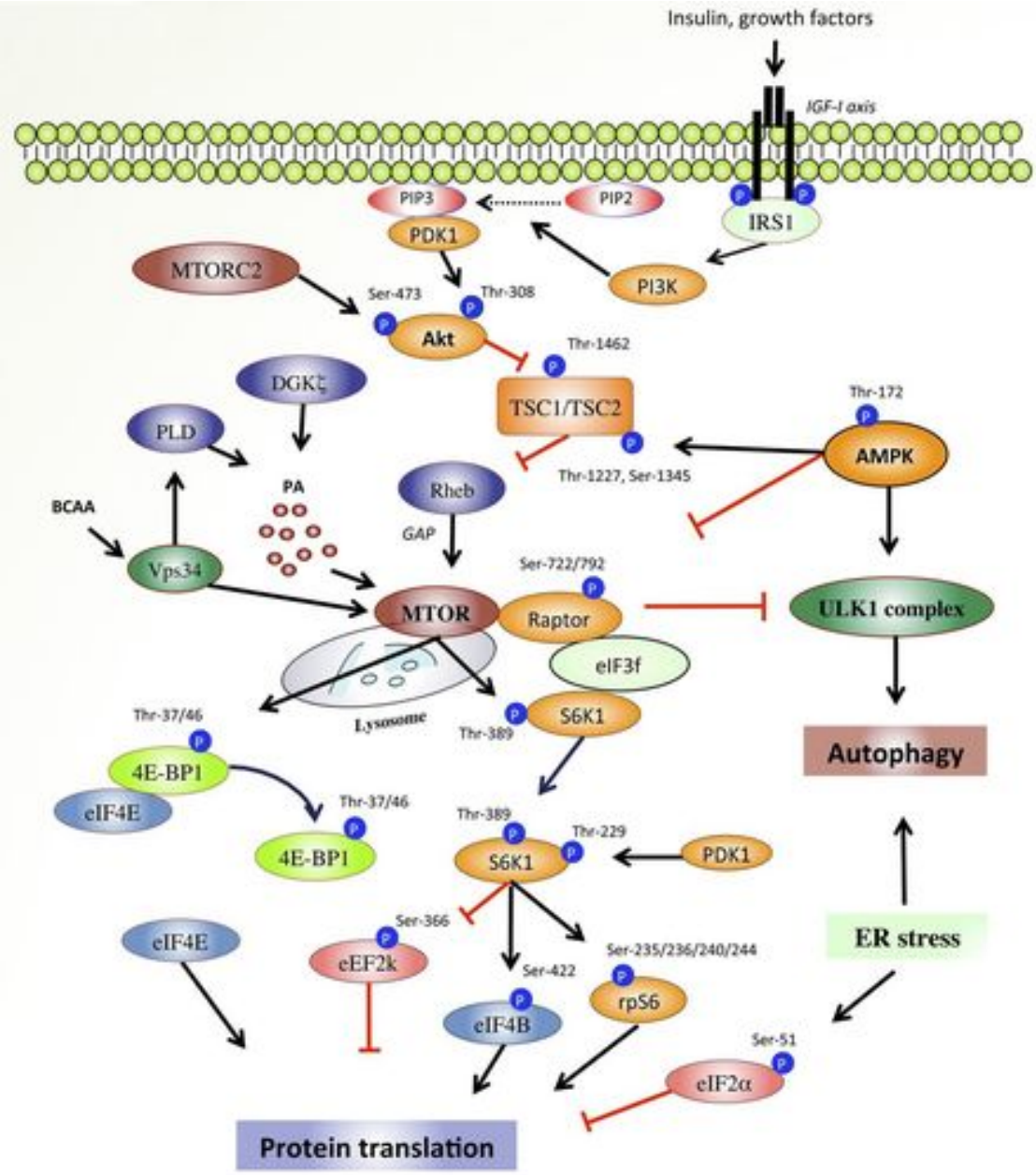


Fig.7. The complexity of the TSC/mTORC1 pathway. Figure adopted from: Anthony M. J. Sanchez; Henri Bernardi; Guillaume Py; Robin B. Candau; American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **2014,** 307, R956-R969.

2.1.d. Clinical presentation and pathophysiology of the tuberous sclerosis complex.

TS is characterized by multi-organ benign hamartomas, that very rarely turn malignant. Clinically, most patients exhibit dermatological, renal and neurological manifestations. These manifestations

are highly variable, ranging from asymptomatic to severe, even with the same mutations. The morbidity in TS patients is usually associated with pulmonary lymphangioleiomyomatosis (LAM), which is characterized by smooth muscle cell proliferation and cystic destruction of the lungs, and is primarily seen in young women. The most common dermatological manifestations include hypomelanotic macules (ash leaf spots), found in 90% of patients and facial angiofibromas that are present in about 75% of the patients [67]. Additionally, 20-30% patients develop an accumulation of collagen in the lumbosacral region, which as known as shagreen "leather" patches [68]. Another manifestation seen in about 30% patients, is the appearance of the "café au lait" spots - light brown macules that vary in size [69]. The most common renal manifestation in TSC occuring in about 50-80% patients is angiomyolipoma, benign tumors composed of blood vessels and thickened walls. Other renal manifestations include renal cysts, renal cell carcinoma and oncocytoma [70]. Interestingly, it has also been seen that while mutations in both TSC1 and TSC2 show similar phenotypic and clinical effects in patients, as well as mice models of Tuberous Sclerosis, the severerity of the phenotype of patients with TSC1 mutations are often milder as compared to those seen with TSC2 mutations.

2.1.e. Tuberous sclerosis associated neuropsychiatric disorders

Neuorological manifestations that are included in the broad umbrella collectively known as tuberous sclerosis associated neuropsychiatric disorders (TAND) are highly variable (Fig. 8). The most common neurological complication seen in 75% to 90% of patients is seizures. Behavioral manifestations range from mild effects, such as poor eye contact, sleep problems and hyperactivity, to more severe disorders on the autism spectrum including self injury and aggressive behavior. About half of the patients with TS are thought to have some form of intellectual disability. Other manifestations include anxiety, depression, and autism spectrum disorder (ASD). Some patients

are also found to have academic difficulties and an impaired working memory [71]. The exact reason for the neurological synmptoms observed in TS patients is not clearly understood. Interestingly, many of the neurological symptoms seen in TS patients are similar to those, in Down syndrome or haploinsufficiency of DYRK1A, including autism, seizures and intellectual disability.

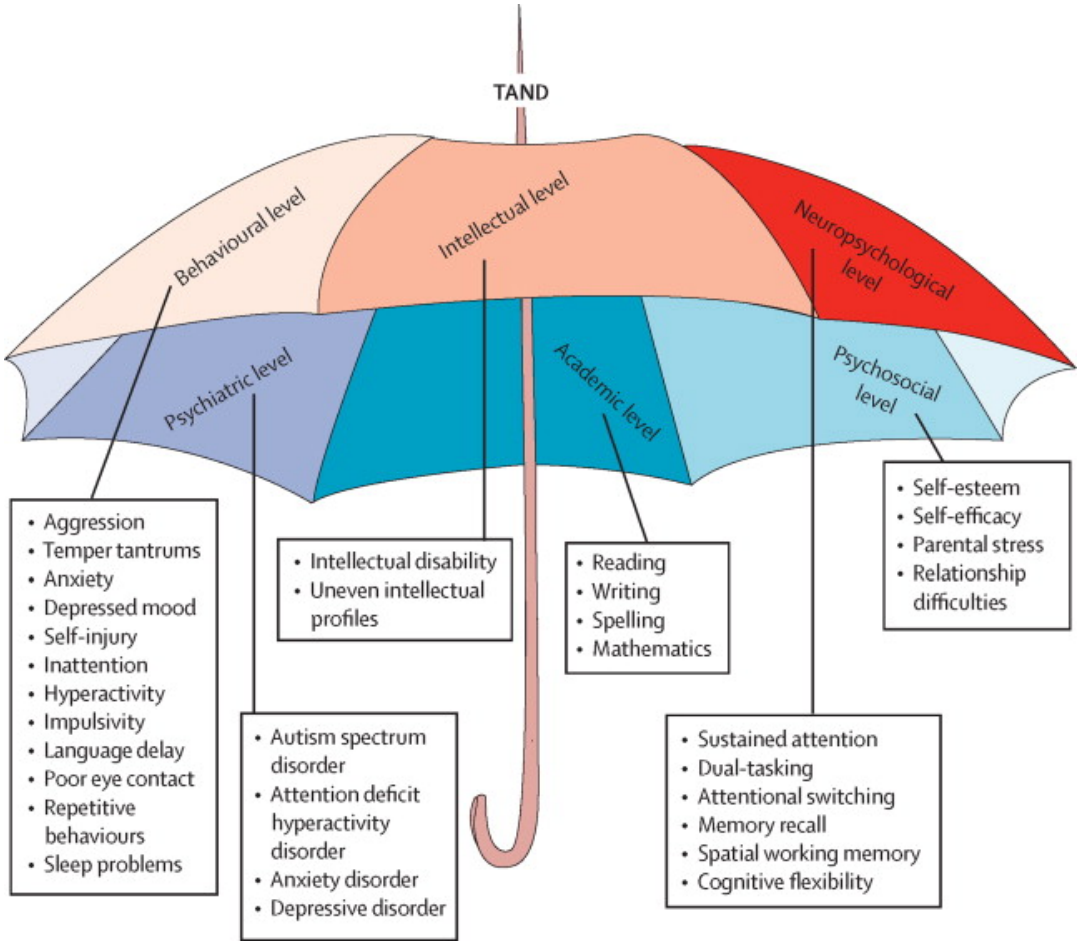


Fig.8. The clinical complexity of TSC-associated neuropsychiatric disorders. Figure adopted from: Curatolo P, Moavero R, de Vries PJ: Neurological and neuropsychiatric aspects of tuberous sclerosis complex. Lancet Neurol. 2015; 14(7): 733–45.

2.1.f. The non-canonical functions of the TSC

The most extensively studied functions of TSC are the ones associated with the canonical mTORC1 axis. Recent reports, however, have suggested that the TSC and the TSC/Rheb axis also act on mTORC1 independent non-canonical pathways. Studies with Tsc1 and Tsc2 null mouse embryonic fibroblasts have shown that these cells have longer cilia and a greater percentage of cells are multi-ciliated in these cells as compared to the wild type cells. [72, 73]. TSC may also regulate amino-acid uptake, vascular endothelial growth factor-A (VEGF-A) secretion, and the NOTCH signalling pathway partially in an mTORC1 independent manner through Rheb [74].

One of the more widely reported non-canonical functions of TSC is rheb-dependent as well as rheb-independent regulation of the actin cytoskeleton. TSC can also regulate the actin cytoskeleton by directly regulating the Rho family of GTPases. The Rho family of GTPases consist of three key proteins- RhoA, Rac1 and Cdc42. RhoA promotes the formation of stress fibers and focal adhesions while Rac induces the formation of lamellopodia and membrane ruffles, and Cdc42 stimulates the formation of filopodia [75]. TSC2 regulates the actin cytoskeleton through its N-terminal domain that also overlaps with TSC1 binding, which is important for regulation [76]. In summary, there is evidence that TSC may have important functions that are independent of its role as an inhibitor of the mTORC1 complex. This is emphasized by the fact the TSC proteins are reported to have multiple binding partners, and the functions of all of these interactions have not yet been fully elucidated [77].

In this study, we describe in detail one such novel partner of TSC - the DYRK1A kinase. Our studies demonsrate that TSC1 plays a more crucial role in this interaction as compared to TSC2. Therefore, the next section will describe the functions of TSC1 (hamartin) in greater detail.

2.2. THE FUNCTIONAL SIGNIFICANCE OF HAMARTIN

While numerous studies have been carried out elucidating the functions of the TSC2 protein, relatively little is known about the significance of the TSC1 protein. One of the earliest and best studied functions of TSC1 is its role in stabilizing the TSC2 protein. Specifically, TSC2 interacts with HERC1, a HECT domain containing ubiquitin E3 ligase, which ubiquitinates TSC2 and targets it for proteosomal degradation. Moreover, TSC2 interacts with HERC1 at its N-terminal region, the same region required for interaction with the TSC1 protein. TSC1 thus acts as a competetive binding partner for TSC2 and prevents its destabilization by the HERC1 ligase. Consistent with these findings, the free fraction of TSC2 (tuberin) is found to be highly ubiquinated in cells, while the fraction bound to hamartin is not ubiquinated [78, 79]. These reports are also consistent with observations by other groups as well.

Recent reports have suggested additional functions for TSC1 that are independent of its role in TSC2 stabilization. Hamartin is expressed throughout the cell cycle. High levels of TSC1 negatively regulate cell proliferation by regulating the G1 phase of the cell cycle possibly through increasing the p27 expression [80].

A recent report suggests that both TSC1 and TSC2 interact with chaperone proteins Hsp90 and Hsp70. Hsp90 and Hsp70 are molecular chaperones that are involved in regulation of client protein folding and stability. While inhibition of Hsp90 and Hsp70 leads to ubiquitination and subsequent degradation of TSC2, the same effects are not observed with TSC1. Additionally, TSC1 binds to Hsp90 at its C-terminal domain, which harbors the MEEVID motif, a highly conserved tetratricopeptide repeat binding domain (TPR), that is known to mediate interaction with other co-chaperone proteins. Additionally, TSC1 inhibits Hsp90 ATPase activity and increases the binding

of Hsp90 to ATP and inhibitors. In summary, TSC1 functions as a co-chaperone of Hsp90 and can facilitate the folding of kinase and non-kinase clients [81].

Yet another function reported for TSC1 is its role in regulation of protein synthesis through regulation of nucleophosmin, a 38KDa protein that is specifically localized to the the nucleolus. Nucleophosmin has been implicated in cellular processes, such as cell growth and proliferation, embryonic development, protein chaperoning, regulation of p53, as well as centrosome duplication and ribosomal biogenesis [82, 83]. Specifically, loss of TSC1 causes a significant increase in the levels of the nucleophosmin protein, which in turn leads to an increase in the pool of actively transcribing ribosomes and increased protein synthesis [84].

It is well understood that TSC1 and TSC2 are regulated by phosphorylation by multiple protein kinases on specific sites. Many of these sites have not yet been characteried. Moreover, while TSC1 is known to interact with multple other proteins, the functional importance of these interactions have not been well characterized. Hence, in this study we hypothesized that DYRK1A phosphorylates TSC1 at specific positions, and regulates its downstream functions including the regulation of the mTORC1 pathway. DYRK1A can thus act as a novel regulator of the mTORC1 pathway through the interaction with TSC. Alternately, we hypothesized that the interaction between TSC/DYRK1A could also have an effect on the functions of the DYRK1A kinase itself.

II. MATERIALS AND METHODS

1. Cell culture

Human and mouse cell lines were obtained from ATCC and maintained in T-75 flasks. Dulbecco's Minimum Essential Medium (DMEM) (Hyclone #SH30243.01) was used for all cell lines except HCT116 cells that were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium. All growth media was supplemented with 20 mM GlutaMax [ThermoFisher #35050061] and 500 units/ml Penicillin/Streptomycin antibiotics [ThermoFisher #15070063]. Additionally, the media was supplemented with 10% fetal bovine serum (FBS), as per ATCC standards. The table below lists the cell lines used in the study. All cell cultures were tested for mycoplasma contamination before use.

Name	Source	Nutrient Medium
T98G	Glioblastoma cells; adherent fibroblasts (human brain) ATCC# CRL-1690	DMEM; High glucose, with 4mM L-glutamine and Sodium pyruvate
U2OS	Osteosarcoma cells; adherent epithelial (human bone) ATCC# HTB-96	DMEM; High glucose, with 4mM L-glutamine and Sodium pyruvate
Mouse Embryonic Fibroblasts (MEFs)	Spontaneously immortalized fibroblasts (mouse) Gift from Dr. R. Moran	DMEM; High glucose, with 4mM L-glutamine and Sodium pyruvate
HCT116	Colorectal carcinoma; adherent epithelial ATCC# CCL-247	RPMI

Table 2. Cell lines used in the study.

2. Cell lysates and protein estimation

Cells were plated and grown according to appropriate protocols as needed for each experiment. Before collection, cells were first washed with phosphate buffered saline (PBS). The cells were then scraped and collected in 100-500 µl of PBS containing protease inhibitory cocktail [EMD Millipore #539131-1vl] and phosphatase inhibitors [EMD Millipore # 524625-1ml] to protect protein integrity and phosphorylation. The cells were then spun down at 1000 g for 5 mins, at 4^{o}C to collect cell pellets. These cell pellets were then stored for further use at -80^{o}C, or utilized for further analysis. After centrifugation, cell pellets were resuspended in approximately 10 times their estimated volume of either ice cold EBC lysis buffer [Boston Bioproducts #C-1410] or RIPA lysis buffer [Boston Bioproducts #BP-115X] supplemented with protease and phosphatase inhibitors as well as β-mercaptoethanol (β-ME) [1:10000] [BioRad #1610710]. The samples were then incubated in the lysis buffer for at least 10 min (for EBC) or about 2-5 min (for RIPA). The lysates were then clarified by centrifugation at 14,000 g for 15 min. After centrifugation, the lysates were collected in a separate tube for protein estimation. Protein estimation was performed using the Biorad DC protein assay kit [BioRad #5000112], as per the manufacturers protocol. The absorbance was read using a spectrophotometer at 750 nm. The BSA standard curve was also plotted and used to calculate the concentration of the samples and for normalization.

3. Immunoprecipitation (IP)

Cells were collected and lysed as described above. The lysates were then normalized for protein concentration and 10% of the total sample was removed and stored as the "input". To the rest of the lysate, a mixture of Protein A sepharose beads (20 µl of a 50% suspension) along with 1 µg of the bait antibody was added. Wherever necessary, a normal IgG antibody was used as a control. Next, all tubes were placed onto a rocker or a rotator in the cold room and allowed to incubate

overnight. Next day, the beads were collected by centrifugation at 10,000 g for 15 seconds at 4°C. The supernatant was aspirated. The beads were then washed 5 times with high salt (200 mM) EBC buffer to eliminate any non-specific binding. Next, 30 µl of 2X sample buffer [BioRad #1610737] was added to the beads and the samples were boiled for 5 minutes at 95°C. The samples were then chilled on ice and analyzed by polyacrylamide gel electrophoresis (PAGE) or stored in -80°C until ready to use.

4. Western Blots

Samples were prepared by mixing the protein sample with premade SDS-PAGE loading buffer. Biorad gradient gels were used for separation of proteins. The samples were loaded onto a polyacrylamide gel, and run at 200V for the required period of time. Protein ladders from Biorad [#1610394] were used as a control for protein size. The gels were then transferred onto nitrocellulose membranes for 45 mins to 1 hr at 15 V, using the semi-dry transfer technique and 1X transfer buffer containing 20% methanol. The nitrocellulose membrane containing the transferred proteins was then stained using Ponceau S staining solution to confirm proper transfer. The membrane was then blocked with 3% non-fat dry milk in TBS-T [BostonBioproducts #BM-260] (Tris-buffered saline + tween) containing 0.01% sodium azide (Blocking solution). After blocking, the membrane was incubated for 1hr at room temperature with the primary antibody (diluted in blocking buffer), or overnight at 4°C. The membrane was then washed thrice using TBS-T for 5 mins each and then incubated with the HRP-conjugated secondary antibody for 1 hr at RT. The membrane was then washed thrice with TBS-T for 10 mins each. The membranes were soaked with the substrate solution [Forte - EMD Millipore #WBLUF0500; Classico - EMD Millipore #WBLUC0500] and developed using the Biorad imager or X-ray films. After development, the membranes were stripped for reprobing or wrapped and stored at -20°C until

further use. For membrane reprobing, the membrane was incubated with stripping buffer for 10-15 minutes. Next, the membranes were washed thrice with TBS-T and then placed into a new container for blocking. Then the same steps were repeated as mentioned above for probing with a new antibody.

5. siRNA transfections using RNAImax reagent.

Cells were plated on 6-well plates at a density of ~ 150,000/well and were allowed to attach overnight. The next day, cells were transfected with 10 nM of either siControl [Ambion #AM4613], siDYRK1A [Ambion #AM16104] or siTSC1 [Ambion #AM16708] silencer RNA. The opti-MEM medium [ThermoFisher #31985-070] was used as the transfection medium. Transfections were performed with RNAiMAX as per the manufacturer's protocol. The cells were incubated at 37^0C for 48hrs and then collected and lysed as per the protocols described above.

6. Plasmid transfections using PEI, Mirus2020 reagent, or BioT reagent

Cells were plated in p100 dishes at ~ 1.5 million per plate. DYRK1A, TSC1 or TSC2 DNA plasmid constructs were added to 250 µl of opti-MEM at the required concentration. PEI or Mirus2020 [MIR5404] was added at a 1:3 ratio (3 µl reagent to every 1 µg of DNA) to a separate tube containing 250 µl of opti-MEM. For BioT reagent [Bioland Scientific LLC #B01-01], the reaction mixture was prepared in PBS in a single tube. The DNA to reagent ratio for BioT reagent was titrated for each cell line as per manufacturers protocol. The reagent containing medium was then added to the tube containing the DNA and mixed well. The mixture was incubated for 25 minutes. The DNA/reagent mixture was then added dropwise to the plates. DMEM containing no antibiotics was used as the transfection medium. The transfection medium was changed 24 hrs after transfection. Cells were collected 48 hrs after transfection.

7. Propidium Iodide staining for cell cycle analysis.

Cells were plated at a confluency of around 70% in p100 plates. Untreated cells were collected 24 hrs after plating by trypsinization and centrifugation at 1000 rpm for 4 minutes. The cells were then resuspended in ice-cold freshly prepared 70% ethanol. Transfected cells were collected at specific time-points in a similar manner. Cells were vortexed well before fixing overnight at 4^{o}C in 70% ethanol. The fixed cells can be stored at 4^{o}C until they are stained. After fixation, the cells were collected by centrifugation for 5 mins at 4000 rpm. The ethanol supernatants were discarded. The cells were then resuspended with PBS, transferred to 5 ml round bottom tubes and centrifuged at 4000 rpm for 5 mins. This PBS wash was performed twice. After the final wash, the cells were resuspended in the cell cycle staining solution (PBS+ RNAase+ Propidium iodide). The cells were then stained in the dark at room temperature for 1-2 hrs or stained overnight at 4^{o}C. After completion of staining, the cells were analyzed by flow cytometry at the VCU Flow cytometry core using the FACS Canto instrument.

8. Preparing cells for RPPA analysis.

T98G glioblastoma control and DYRK1A KO CRISPR cell lines were plated at a density of 150,000 cells per well of a 6-well plate. Each condition utilized three wells. The cells were then allowed to attach overnight and were lysed using the lysis buffer obtained from the RPPA core at MD Anderson center. The lysates were normalized using the BioRad assay described above. Sample buffer without Bromophenol Blue (also obtained from the MD Anderson center) was added to the samples and the samples were boiled for 5 mins at 95^{o}C. The lysates were then stored at -80^{o}C until they were ready to be shipped to the MD Anderson Center for RPPA analysis. The experiment was performed in three biological repeats.

9. Production of lentiviral particles and preparing stable TSC1 knockdown cell lines using lentiviral transfections

Mission shRNA bacterial glycerol stocks specific for TSC1 knockdown were obtained from Millipore Sigma [SHCLNG-NM_000368]. Purified shRNA vectors were prepared using the bacteria glycerol stocks as per the manufacturer's protocol. For viral production, HEK293T packaging cells are plated in 6-well plates such that they are 50-80% confluent. A transfection cocktail was prepared for each shRNA by mixing with the pCMV-ΔR8.2 packaging plasmid and pCMV-VSVG envelope plasmid. Transfections were performed using PEI transfection reagent and cells were incubated with the transfection components for 12-16 hrs. Fresh medium was added 16 hr post-transfection. Virus conditioned medium (VCM) collections were made at days 3 and 4 post-transfection. The VCM was centrifuged at 2000 rpm for 10min and filtered through a 0.45 μm filter, aliquoted and stored at -80^{0}C for future use. Puromycin sensitivity was determined for each host cell line before viral infection. Target cells were first grown on 6-well plates at a confluency of about 60%. An additional well was plated as a positive control for puromycin selection. The next day, the medium was changed to 1 ml of fresh medium with 10 μg/ml polybrene. For lentiviral transfection, 1 ml of VCM was added to each well such that the final concentration of polybrene was 5 μg/ml. The cells were then incubated overnight at 37^0 C, 5% CO2. After 24 hrs of infection, fresh culture medium was added to the cells. For selection, puromycin was added to the cells at the appropriate concentration identified for each cell line. The cells were selected with puromycin for 3-5 days or until all the cells in the positive control well are dead. The selected cells were then analyzed for TSC1 knockdown using western blots to select the clones that showed the highest knockdown.

10. Detection of mTORC1 activity

Cells were plated on 6-well plates at a density of ~ 150,000/well and were allowed to attach overnight. The cells were then starved for 6, 12 or 24 hours, with DMEM containing 0% FBS. Control and starved cells were collected at specific time-points and lysed as per the protocols described in the previous sections. The lysates were normalized for protein concentration and prepared for western blot analysis. The cells were probed for mTORC1 downstream target proteins p-4EBP1 and p-p70S6K.

11. Puromycin tagging assay for protein synthesis

T98G and NIH3T3 cells were plated on 6-well plates at a density of ~ 150,000/well and were allowed to attach overnight. The cells were pulsed with 10 µg/ml puromycin for 10 mins and then collected and processed as described above. A control group was included that was pre-treated with Cyclohexamide (CHX) for 1 hr before puromycin pulse, to inhibit protein translation. The cells were then analyzed by western blots and probed with anti-puromycin antibody. The experiments were performed in three biological repeats. Puromycin signal in the entire lane was quantitated using ImageJ software and were normalized to the loading control.

12. EdU staining

Cells were plated on coverslips in 6-well plates at a density of ~ 150,000/well and were allowed to attach overnight. Cycling cells stained for the 5'ethynyl-2'deoxyuridine (EdU) reagent as per the manufacturer's protocol [ABP Biosciences iClick EdU Andy Fluor 488 imaging kit #A003]. The stained and fixed cells were imaged at 10X using fluorescent microscopy.

13. Wound Healing

Cells were plated on 6-well plates at a density of ~ 250,000/well and were allowed to attach overnight. The cells were allowed to grow until they were confluent. A 20 µl pipette tip was then

used to make a vertical scratch in the most confluent area of the monolayer. Any debris created due to the wound was washed away using media. The wells were then marked for analysis and a 0 h image was immediately taken. The wound was then imaged at 6, 12 and 24 hours and the percent of wound healing was calculated. The extent of wound closure at 0h and 24h was calculated using the ImageJ software.

14. Site-directed mutagenesis

For site directed mutagenesis, primers for specific mutations were designed using the SnapGene Software. Site-directed mutagenesis was then performed using the QuikChange II XL Site-Directed Mutagenesis Kit [Agilent #200521] as per the manufacturer's protocol. Confirmation of mutagenesis was obtained by sequencing using mutation-specific sequencing primers.

15. Immunofluorescence and confocal microscopy

Cells were plated on coverslips in 6-well plates at a density of 150,000/well and were allowed to attach overnight. For Phalloidin staining, coverslips were first coated with collagen and then plated. The cells were then fixed in 10% Formaldehyde for 15 mins. After fixation, the cells were permeabilized with PBS containing 0.5% Triton X-100. The cells were then washed with PBS thrice, and blocked for 1hr at RT with 3% bovine serum albumin (BSA). The cells were then stained with the appropriate primary antibody diluted in the blocking buffer for 1 hr at room temperature. This was followed by three washes with PBS for 5 min each. The cells were then stained with appropriate secondary antibody (diluted at 1:1000) for 1 hr at RT. This was followed by three washes with PBS for 5 min each. The coverslips were then dried and mounted on slides using mounting medium with or without DAPI as per requirement. Microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy facility, supported in part with

funding from the NIH-NCI cancer center support grant-P30 CA016059. Images were taken on Zeiss LSM 700 Laser scanning microscope.

16. Trypan blue cell viability staining

Cells were plated at a density of ~5000 in 12 well plates. Each cell line was plated in triplicates. Cell number was measured on days 1, 3 and 5. Cells were stained with trypan blue at a ratio of 1:1 before counting. Number of cells on day 5 was normalized to the number of cells on day 1 to create a quantitative graph.

17. Antibodies and plasmids

The antibodies used in the study were mainly obtained from Cell signaling technology (CST) or Sigma-aldrich. The antibodies used include TSC2 [CST#4308S], TSC1 [CST#4906S], p70S6K-total protein [CST#9202S], p-p70S6K (Thr389)[CST#4205S], 4EBP1 (total) [CST#9644S], p-4EBP1 (Thr37/46) [CST#2855S], p-4EBP1 (Thr70)[CST#9455S], GFP [CST#2956S], DYRK1A [CST#8765S], DYRK1A [Sigma-aldrich #ST1650], Myc-tag [CST#2278S], V5-tag [CST13202S], GFP-trap [Cromotek#gta-20], V5-tag [BioRad #MCA1360GA], Puromycin [Kerafest #EQ0001], IgG [Sigma-Aldrich #NI01]. The TSC1 and TSC2 constructs (in a pcDNA3.1 backbone) used in this study were a gift from Dr. Mark Nellist.

III. RESULTS

PART I: BIOCHEMICAL CHARACTERIZATION OF THE TSC/DYRK1A INTERACTION

1. Validating and mapping the interaction between the TSC components (TSC1/TSC2) and DYRK1A

Introduction: Protein interactions regulate a wide variety of cellular processes, making it imperative to study the interacting partners of a protein of interest. MudPIT (Multidimentional Protein Identification Technology) proteomic analysis carried out in the lab revealed TSC as a potential interactor of the DYRK1A kinase protein (Fig.9) [85]. The observation of this interaction was interesting since both TSC and DYRK1A are known for their tumor supressive functions. Moreover, disregulation of both the TSC components and DYRK1A have been implicated in neurological and behavioral diseases.

Previously, it has been shown that the N-terminal region of TSC1 is required for its stability and expression in the cell while the C-terminal region is important for binding to TSC2. Also, The C-terminal region of TSC1 binds to the N-terminal region of TSC2 [57]. Interestingly, it has also been shown that binding of TSC2 to various truncated TSC1 proteins is sufficient to promote the mTORC1 inhibition activities of TSC1 [86]. Hence, it is important to characterize the biochemical association between two interacting proteins in order to shed light on their functions. Therefore, this part of the study describes in detail the biochemical characterization of the interaction between the TSC1, TSC2 and DYRK1A proteins. Both wild type and truncated mutants of these three proteins that have been used in this study have been extensively used for mapping experiments in previous studies [57, 86].

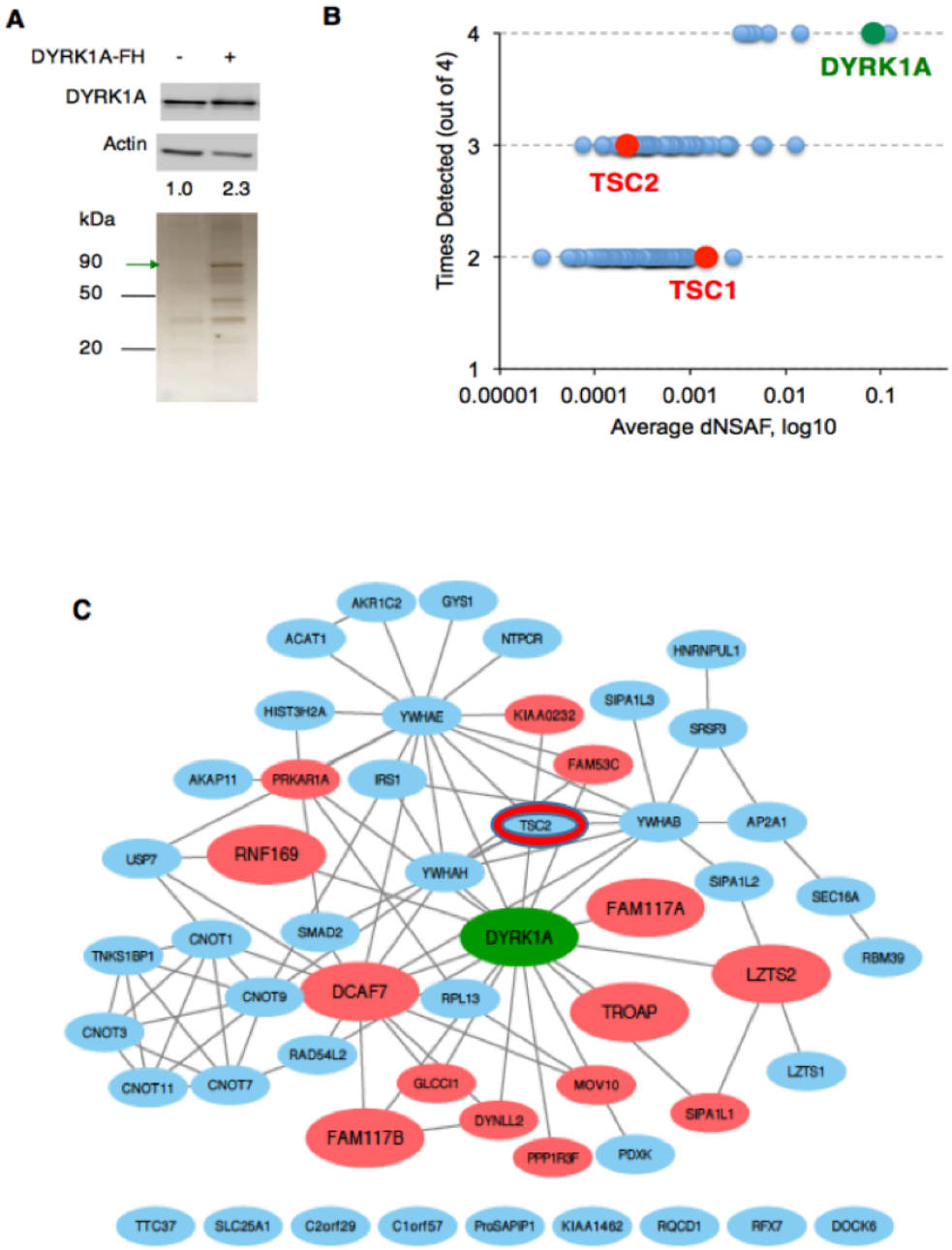


Fig.9. Proteomic analysis reveals the interaction between DYRK1A kinase and TSC. (Figure adapted from the paper by V. Menon, V. Ananthapadmanabhan et al., Cell Cycle, 2019) MudPIT (Multidimensional Protein Identification technology) analysis was performed using control or Flag-HA(FH)-DYRK1A-expressing T98G glioblastoma cells. Four biological replicates were analyzed to identify proteins that specifically and reproducibly co-precipitated with HA-DYRK1A. **A:** Scheme of DYRK1A MudPIT analysis and a representative silver stained gel showing 10% of the sample analyzed by MudPIT. **B:** Graph showing relative enrichment (dNSAF) of HA-DYRK1A interacting proteins detected in 2, 3 or all 4 replicate MudPIT experiments. **C:** Representation of the DYRK1A interactome. DYRK1A is shown as a

green circle, red and blue circles correspond to genes either listed in the BioGrid database or new DYRK1A-binding proteins, respectively.

Since DYRK1A is a serine/threonine kinase, in this section we also investigated whether TSC1 and TSC2 are substrates for DYRK1A. DYRK1A is known to phosphorylate its substrates on the consensus site **RPX(S/T) P.** Careful examination of both TSC1 and TSC2 recognized two potential DYRK1A consensus sites in the TSC2 protein but none in the TSC1 protein. In this study, we report for the first time the identification of potential DYRK1A specific non-consensus sites in the TSC1 protein.

1.1 Characterization of DYRK1A interaction with TSC1 and TSC2

The first step after the observation of the DYRK1A/TSC interaction in the MudPIT proteomic data was to confirm this interaction in human cell lines. For this purpose, Myc-tagged TSC1 and HA-tagged TSC2 were co-expressed with GFP-tagged wild type (WT) DYRK1A or GFP-tagged DYRK1A with a kinase activity inactivating mutation (Y321F) in T98G glioblastoma cells, followed by immunoprecipitation of HA-TSC2. TSC1 and TSC2 were found to bind both WT and kinase mutant DYRK1A, suggesting that kinase activity of DYRK1A is not essential for binding to the TSC components (experiment by Dr. Vijay Menon; the data not shown). The next step was to confirm the interaction between the TSC subunits and DYRK1A under endogenous conditions and to assess whether the interaction is mediated by either of these proteins. For this purpose, reciprocal immunoprecipitation was performed for endogenous TSC1 and DYRK1A as well as for endogenous TSC2 and DYRK1A, using T98G glioblastoma cell lines. TSC1 and DYRK1A were found to interact under endogenous conditions (Fig.10A). However, we were unable to detect the interaction between the endogenous DYRK1A and TSC2 under similar conditions in T98G cells.

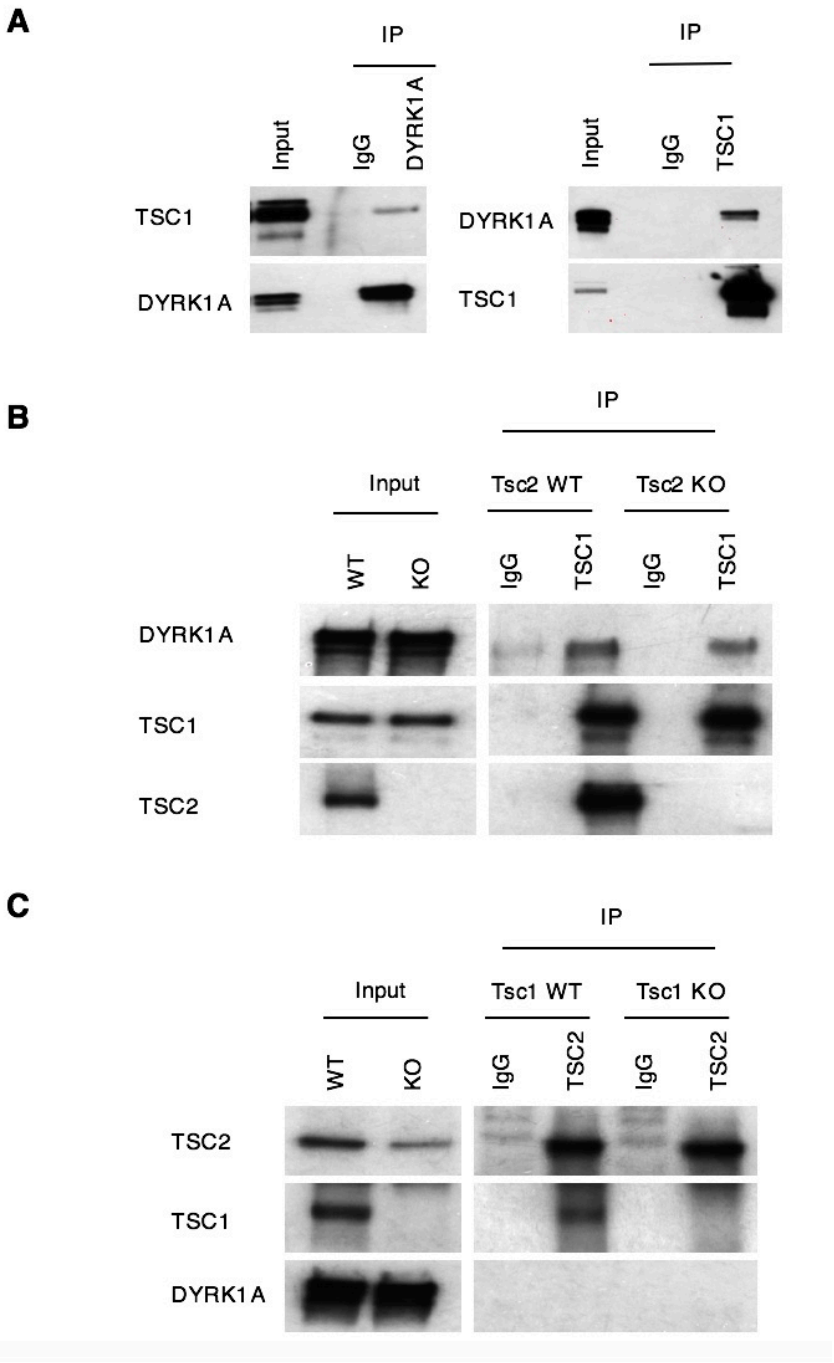


Fig.10. TSC1 binds to DYRK1A in a TSC2 independent manner. A: Immunoprecipitation/Western Blot shows the co-precipitation between TSC1 and DYRK1A in T98G cells at the endogenous level. **B:** Immunoprecipitation/Western Blot shows the co-precipitation between TSC1 and DYRK1A at the endogenous level in both the Tsc2 WT and the Tsc2 KO mouse embryonic fibroblasts. This indicates that TSC1 interacts with DYRK1A in the absence of TSC2. **C:** Immunoprecipitation/Western Blot shows that TSC2 and DYRK1A do not co-precipitate in both the Tsc1 WT the Tsc1 KO mouse embryonic fibroblasts.

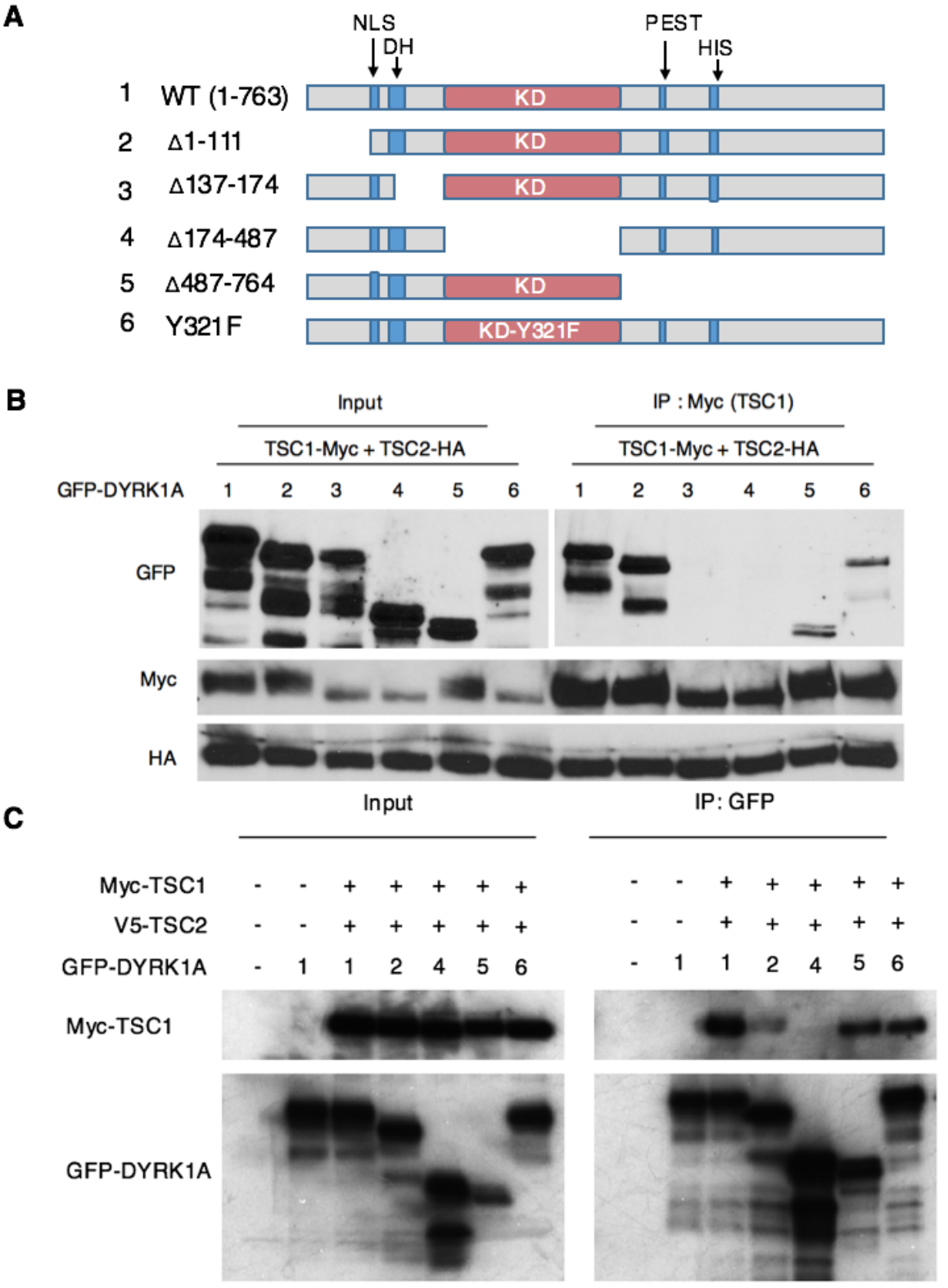
Next, to verify whether TSC2 is required for the interaction of TSC1 with DYRK1A, endogenous mouse Tsc1 was pulled down from Tsc2 WT and Tsc2 KO MEFs and probed for DYRK1A. Tsc1 was found to interact with DYRK1A both in the presence and absence of Tsc2 (Fig. 10B). As seen in T98G cells, we could not detect DYRK1A and Tsc2 interaction in MEFs, both in the presence and absence of Tsc1 (Fig. 10C). TSC2 interaction with DYRK1A can however be observed under overexpressed conditions for both these proteins. These results indicate that TSC complex binds to DYRK1A primarily through TSC1.

1.2. The kinase domain is required for DYRK1A binding to TSC1.

Once it was confirmed that DYRK1A binds to TSC1 (both endogenous and overexpressed conditions) and TSC2 (overexpressed) conditions, the next step was to characterize the specific domains involved in these interactions. First, various deletion mutants of GFP-tagged DYRK1A were co-expressed with both HA-tagged TSC2 and Myc-tagged TSC1 (Fig. 11A, 11C). This was followed by immunoprecipitation using anti-Myc antibody. The deletion mutants of DYRK1A included both an N-terminal and C-terminal deletion construct (Δ1-111, Δ487-764), a construct lacking the section just before the kinase domain (Δ137-174), a construct lacking the kinase domain (Δ174-487) and a kinase dead mutant (Y321F). TSC1 and TSC2 were found to bind all DYRK1A fragments except for those lacking the kinase domain or a section just before the kinase domain (*Experiment 11B performed by Dr. Vijay Menon; S. Joshi, V. Menon et al, manuscript in preparation*). Interestingly, TSC1 and TSC2 were also found to bind to the kinase dead mutant, emphasizing that the kinase activity itself is not required for binding. Moreover, TSC1 migrated slower on the gel when bound to DYRK1A, possibly due to phosphorylation.

Interestingly, parallel experiments performed using the same set-up as described above but immunoprecipitating with a GFP-trap antibody instead showed a gel shift and decrease in TSC1

when it was co-expressed with GFP-DYRK1A lacking the N-terminal region (Fig.11C, co-expression with construct 2). This suggested a role for the N-terminal region in DYRK1A in TSC1 modification. To test this theory, various N-terminal constructs were overexpressed in T98G cells along with Myc-tagged TSC1 and V5-tagged TSC2 proteins. This was followed by immunoprecipitation using a GFP-trap antibody. Indeed, while constructs that lacked the kinase domain again failed to bind TSC1, it was also observed that TSC1 co-expressed with the N-terminal deletion construct of DYRK1A migrated differently on the gel (Fig. 11E). No binding or difference in modification was seen when TSC1 was overexpressed with only the N-terminal domains of DYRK1A (Fig. 11E). This difference in the amount of protein seen when the immunoprecipitations are performed by different antibodies (TSC1 *vs*. DYRK1A) could be reflecting the total amount of each protein present in the cell extract at a given time, leading to differences in binding patterns for the same protein. Also, the interaction between TSC1 and TSC2 proteins was observed irrespective of the presence or absence of DYRK1A binding. It is also essential to note that two of the N-terminal fragments of DYRK1A when overexpressed failed to bind to TSC1 but bound to TSC2, which contradicts our previous observation that DYRK1A binds primarily to TSC1. It is yet to be seen whether this is a result of the V5 tag sticking to the beads or is a true interaction. Studies are underway to confirm this effect.



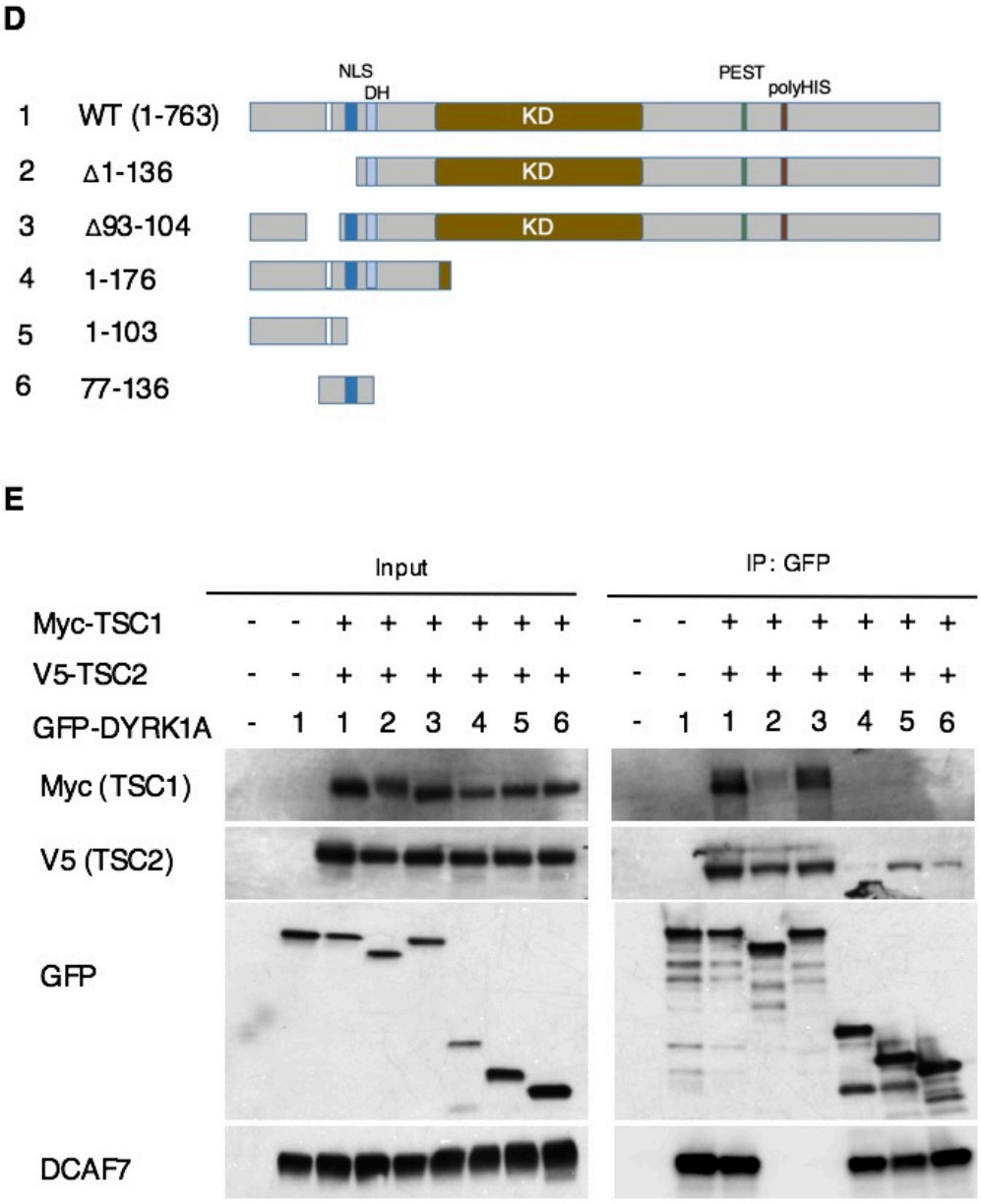
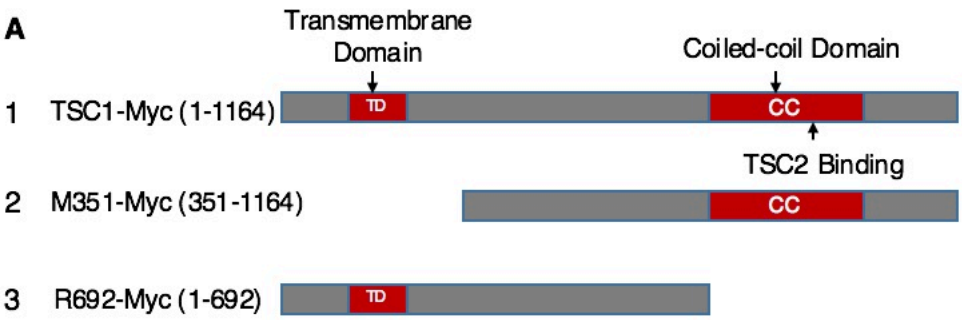


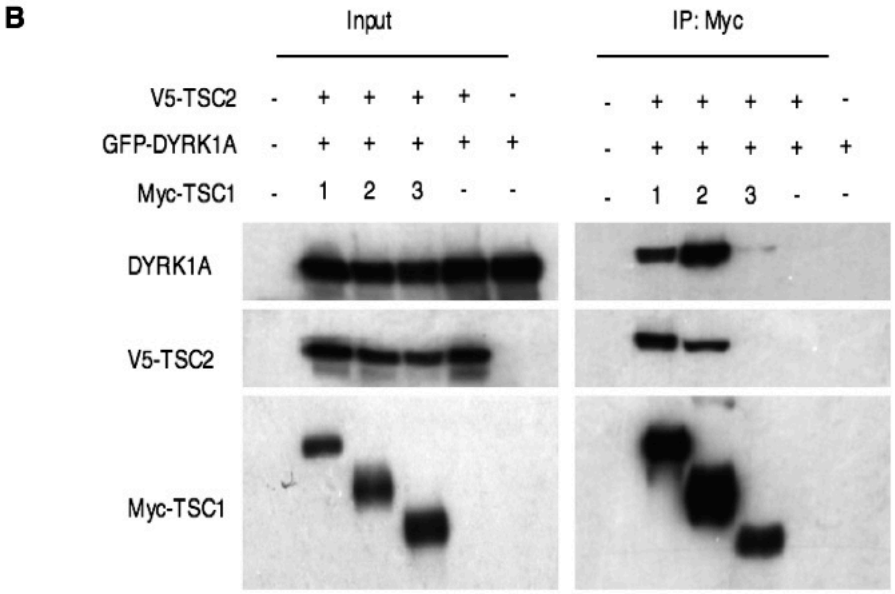
Fig.11. The kinase domain is required for DYRK1A binding to the TSC1. A: A schematic for different GFP-tagged mouse DYRK1A constructs used in the study. The numbers correspond to mouse isoforms. **B:** Myc-TSC1, HA-TSC2 and GFP-DYRK1A (mouse isoforms) were co-expressed in T98G Cells. The cells were then immunoprecipitated with GFP and probed for the indicated antibodies. **C:** Myc-TSC1, HA-TSC2 and GFP-DYRK1A (mouse isoforms) constructs were co-expressed in T98G Cells. The cells were then immunoprecipitated with Myc-TSC1 and probed for the indicated antibodies. **D:** A schematic for different GFP-tagged rat DYRK1A constructs used in the study. The numbers correspond to rat isoforms. **E:** Myc-TSC1, V5-TSC2 and GFP-DYRK1A were co-expressed in T98G Cells. The cells were then immunoprecipitated with GFP and probed for the indicated antibodies. DCAF7 is a DYRK1A binding protein that is used as a control to confirm proper DYRK1A pull-down.

1.3. TSC1 interacts with DYRK1A at its C-terminal region

Since we have shown in the previous sections that TSC1 seems to mediate the binding between DYRK1A and TSC2, we next wanted to characterize the domains that are involved in this interaction. For this purpose, full length, N-terminal deleted and C-terminal deleted mutants of TSC1 were co-expressed with V5-tagged TSC2 and GFP-tagged DYRK1A. TSC1 was then immunoprecipitated using a myc tag antibody and the resulting gels were probed for DYRK1A and TSC2 binding. As observed previously, no interaction was observed between overexpressed TSC2 and DYRK1A in the absence of TSC1. TSC1 binding to DYRK1A was lost when the C-terminal region was deleted, indicating that DYRK1A binds TSC1 at this region (Fig.12). Loss of the C-terminal region also leads to a decrease in TSC2 binding, which is again consistent with previous reports on mapping the TSC1/TSC2 binding domains [58].

Combined with the results seen with the Tsc1 and Tsc2 MEFs, our data so far supports the conclusion that DYRK1A interaction with TSC is mediated mainly through the TSC1 protein and requires the kinase domain. Consequently, our further studies focused on deciphering the role of the interaction between TSC1 and DYRK1A.





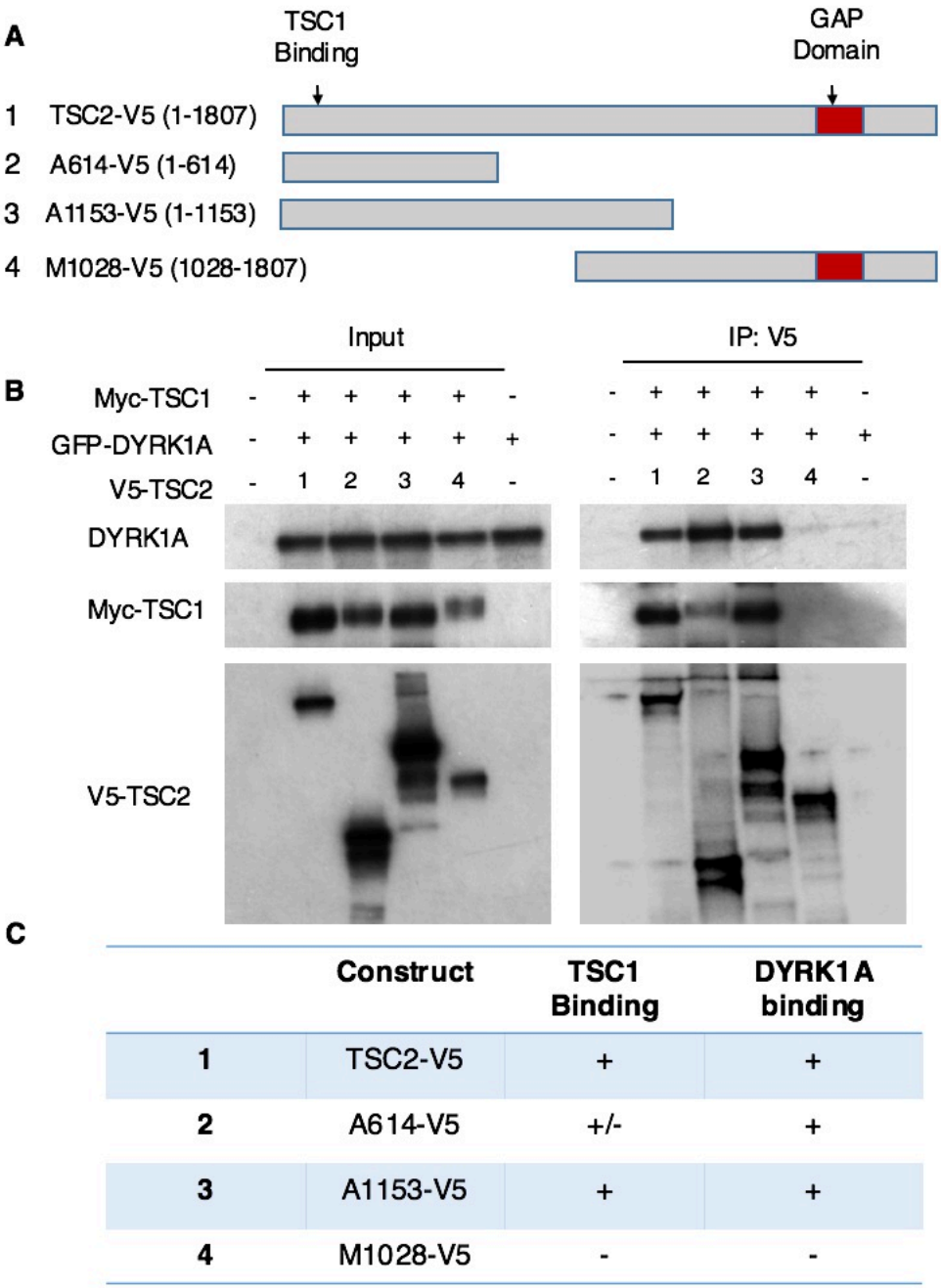
C

	Construct	TSC2 Binding	DYRK1A binding
1	TSC1-Myc	+	+
2	M351-Myc	+	+
3	R692-Myc	-	+/-

Fig.12. TSC1 interacts with DYRK1A through its C-terminal region. A: Schematic of different Myc-tagged TSC1 constructs used for domain mapping studies in Fig.1B. **B:** GFP-DYRK1A and V5-TSC2 were co-expressed with Myc-TSC1 (Full length), TSC1-M351 (*Δ*N-TSC1), and TSC1-R692 (*Δ*C-TSC1) constructs. The proteins were immunoprecipitated using Myc antibody and the gels were probed for the indicated proteins. **C:** Summary of the results seen in the binding experiment from 12B.

1.4. TSC2 interacts with DYRK1A through its N-terminal region

Our data in the previous section showed that DYRK1A interacts with TSC1 and TSC2 through its kinase domain. Next, we wanted to characterize which domain of TSC2 is required for binding to DYRK1A. For this purpose, various N-terminal (M1028-V5) and C-terminal (A614, A1153) deletion mutants of V5-tagged TSC2 were co-expressed with Myc-tagged TSC1 and GFP-tagged DYRK1A in T98G glioblastoma cells. TSC2 was then immunoprecipitated using a V5-tag antibody and the resulting gels were probed for DYRK1A and TSC1 binding. It was observed that the N-terminal deletion mutant did not bind to both TSC1 and DYRK1A (Fig.13). This finding is consistent with previous reports that show that TSC1 binds to TSC2 through its N-terminal region [59]; and further supports our model that TSC1 mediates the binding between DYRK1A and TSC2.



C

	Construct	TSC1 Binding	DYRK1A binding
1	TSC2-V5	+	+
2	A614-V5	+/-	+
3	A1153-V5	+	+
4	M1028-V5	-	-

Fig.13. TSC2 Interacts with DYRK1A through its N-terminal region. A: Schematic of the different V5-tagged TSC2 constructs used for domain mapping studies in Fig.13B. **B:** GFP-DYRK1A and Myc-TSC1 were co-expressed with V5-TSC2(Full length), TSC2-A614, TSC2-A1153, and TSC2-M1028 constructs. The proteins were immunoprecipitated using V5 tantibody and were probed for the indicated proteins. **C:** Summary of the results seen in the binding experiment from 13B.

1.5. DYRK1A phosphorylates TSC1 in-vitro

In our domain mapping studies, we noticed that TSC1 undergoes a change in gel migration when it was co-expressed with DYRK1A. Since DYRK1A is a kinase, we wanted to investigate whether TSC1 is a substrate of DYRK1A.

Preliminary studies in the lab performed by Dr. Vijay Menon showed that TSC1 co-transfected with GFP-DYRK1A migrated slower as compared to TSC1 co-trasnsfected with a GFP control vector. This effect was abrogated by treatment wth λ-phosphatase, indicating that the effect on migration was due to phosphorylation (Fig. 14A). A similar effect on migration was seen when WT, N-terminal deletion mutant (M351) and C-terminal deletion mutant (R692) of TSC1 were co-transfected with either GFP control or GFP-DYRK1A (Fig. 14C, courtesy of Dr. Vijay Menon). This data was suplemented by an *in-vitro* radioactive kinase assay using TSC1-M351 and TSC1-R692 deletion mutants that were overexpressed and immunoprecipitated under denaturing conditions using RIPA to eliminate any associated kinases. The *in-vitro* kinase assay showed that both the N-terminal deletion mutant as well as the C-terminal deletion mutant were phosphorylated by purified GST-DYRK1A (Fig. 14D). Moreover, the M351 fragment was found to be more strongly phosphorylated as compared to the R962 fragment. This could be due to the fact that R692 binds very weakly to DYRK1A as compared to M351.

Since DYRK1A phosphorylates most of its substrates on a proline directed **RPX(S/T) P** motif, the TSC1 protein sequence was analyzed for this specific consensus. However, no specific consensus sequence was present in TSC1 and therefore we hypothesized that TSC1 is phosphorylated by DYRK1A on non-consensus sites. The next step was to identify these sites.

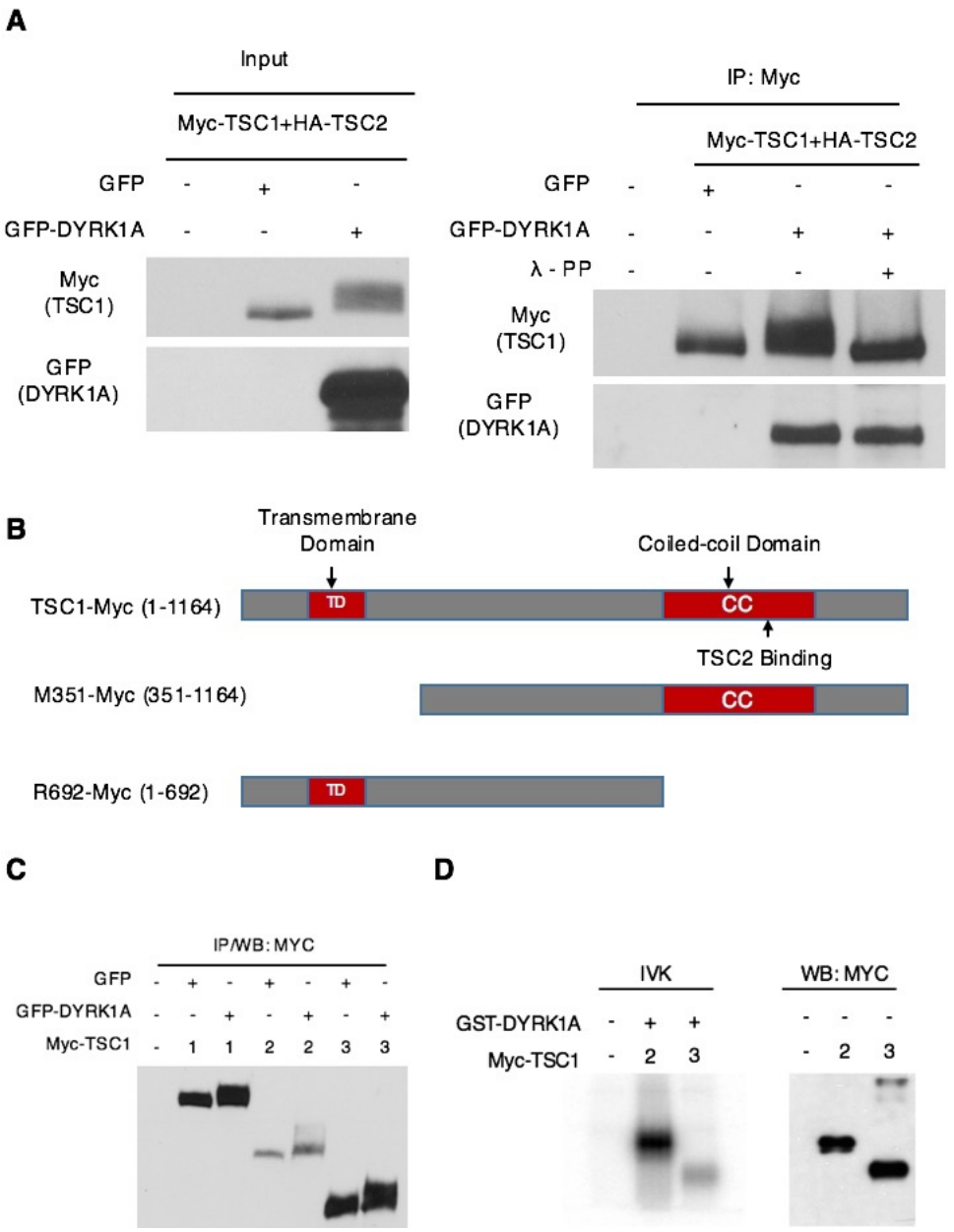


Fig. 14. DYRK1A phosphorylates TSC1 in vitro. A: Co-transfection of TSC1 with DYRK1A leads to a slower migration of TSC1; slower migration is abrogated with the use of λ-phosphatase indicating that the shift was due to phosphorylation. **B:** Schematic for the Myc-tagged TSC1 constructs used in the experiments shown in **C** and **D**. **C:** TSC1 constructs shown in **B** were co-transfected with either GFP control or GFP-tagged DYRK1A wt constructs. Co-transfection of TSC1 with DYRK1A leads to a slower migration of TSC1 as compared to TSC1 cotransfected with GFP controls. **D:** TSC1-ΔC (R692) and TSC1-ΔN (M351) constructs were overexpressed in HEK293T cells, Immunoprecipitated and used as a substrate for DYRK1A in an in-vitro kinase reaction. Autoradiogram shows stronger phosphorylation of the TSC1-ΔN fragment compared to TSC1-ΔC. Western blot shows equal expression of both TSC1 fragments.

1.6. Identification of DYRK1A specific phosphorylation sites in TSC1

TSC1 is a large protein of approximately 150 KDa. Thus, in order to more easily identify the DYRK1A specific phosphorylation sites on TSC1, specific staggered PCR primers were designed in order to create 3 overlapping fragments of TSC1. These fragments were then cloned into an expression vector pRSET-A (2.9kb). The expression constructs obtained by this method were then expressed in bacterial cells and purified to use as a substrate. Purified TSC1 fragments were then incubated with and without DYRK1A and the resulting reaction samples were sent for Mass Spectrometry (MS) analysis in order to identify DYRK1A specific sites (the purification and MS analysis were performed by Dr. Anton Chestukhin and Charles Lyons from Massey Cancer Center). Initial MS analysis revealed three potential DYRK1A specific phosphorylation sites for TSC1- a Serine 505 (S505), a Threonin 547 (T547) and a Threonine 773 (T773). All of these three sites were found in Fragment 2. The next step was to confirm whether these sites contributed to the gel shift observed upon co-expression of TSC1 and DYRK1A.

For this purpose, the above three sites were mutated using site directed mutagenesis to form an alanine i.e S505A, T547A, T773A in order to abrogate any potential phosphorylation that can occur on these sites. These mutant TSC1 fragments were then used in gel migration assays in order to detect any changes in phosphorylation. (Fig.15).

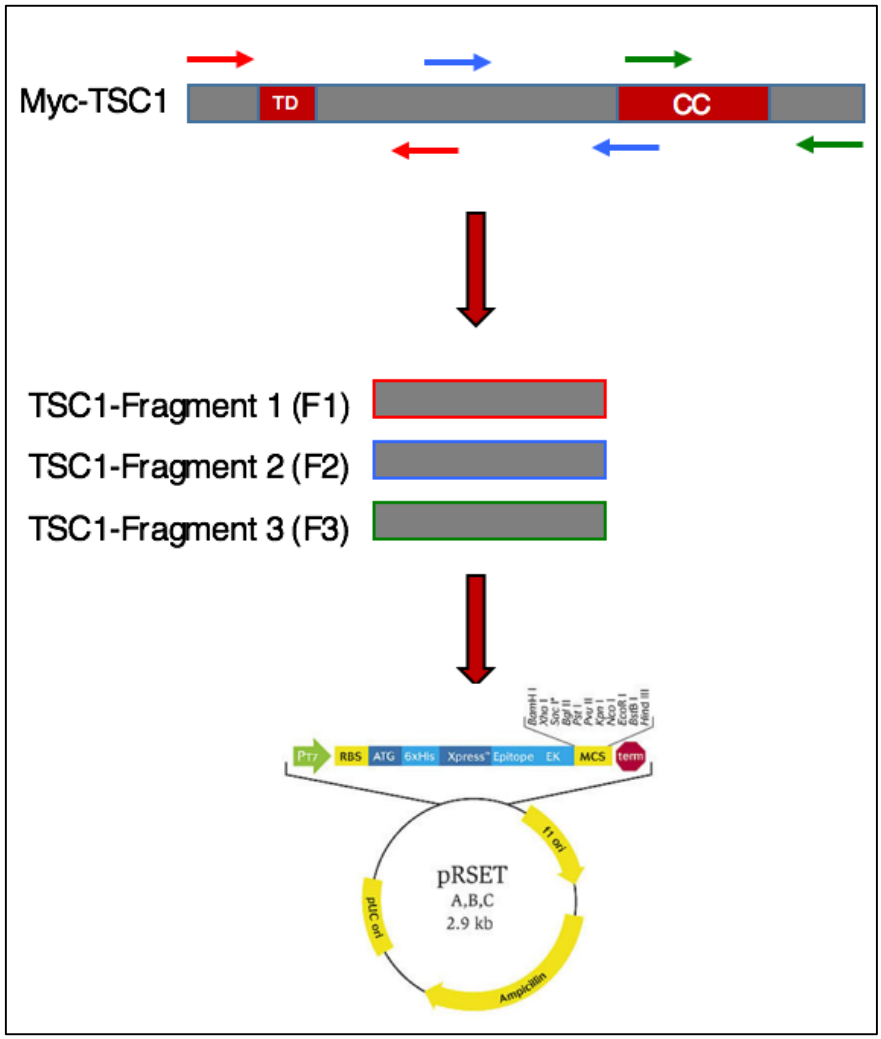


Fig.15. Identification of DYRK1A specific phosphorylation sites in TSC1. A: Design of the overlapping PCR primers encompassing the entire TSC1 protein. These PCR fragments were then cloned into pRSET-A expression vector, purified and sent for Mass Spectromtery analysis after incubation with DYRK1A and ATP.

For the gel migration assay, Myc-tagged TSC1-M351 fragments were co-transfected with either GFP-control vector or with GFP DYRK1A in T98G cells and run on a 7.5% polyacrylamide gel in order to observe TSC1 migration with and without DYRK1A co-expression. WT TSC-M351 transfected with and without DYRK1A was used as a control. Mutation in each of the three predicted phosphorylation sites i.e. S505, T547 and T773, resulted in decreased gel shift as compared to wt controls. (Fig.16)

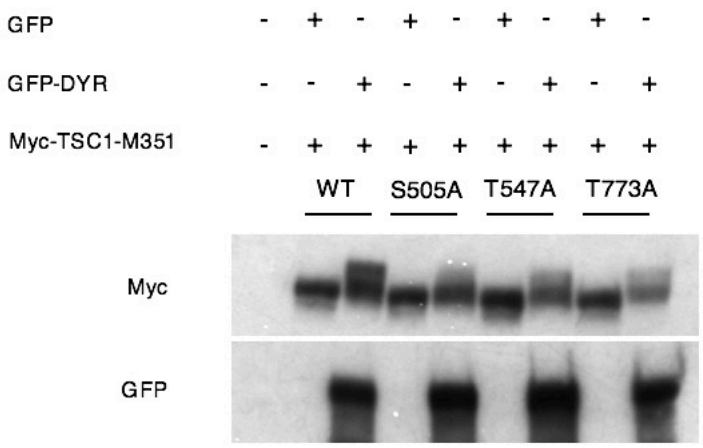


Fig.16. Validation of DYRK1A phosphorylation sites in TSC1. Myc-tagged TSC1 WT and mutant M351 constructs were co-transfected with and without DYRK1A in T98G cells followed by Western blot analysis for detection of gel shift.

It is interesting to note that out of the three potential sites, Serine 505 and Threonine 547 have both been reported to be phosphorylated by other groups. The site S505 especially has been reported in over 84 high throughput proteomics studies and was found to be conserved in humans, mice and rats. T773 however, was entirely specific to our study. More importantly, the gel shift data shows the highest effect with the mutation of this site as compared to other sites. Additionally, more recent mass spectrometric analysis performed by Charles Lyons at VCU, has detected several additional DYRK1A specific phosphorylation sites, many of which have been reported previously by large-scale proteomic studies, but whose function is unknown. These additional sites are yet to be validated and studied further. A comprehensive list of all the sites identified in this study is presented in Supplementary Table.1.

Conclusion: MudPIT results showing the binding of DYRK1A to the TSC proteins were confirmed under in-vitro conditions. TSC1 was found to bind DYRK1A both under endogenous

and overexpressed conditions while TSC2 binds to DYRK1A only under overexpressed conditions. Additionally, the kinase domain but not the kinase activity of DYRK1A is required for binding to the TSC1 complex. The N-terminal region of TSC2 and the C-terminal region of TSC1 are required for binding to DYRK1A. The interaction between TSC2 and DYRK1A requires TSC1 whereas the binding between TSC1 and DYRK1A is TSC2-independent. Furthermore, mass spec analysis of the TSC1 protein revealed three potential sites that are specific to DYRK1A phosphorylation- Serine 505, Threonine 547 and Threonine 773 as well as more potential sites from recent studies. Further studies rare under way to determine their functional significance.

PART II: FUNCTIONAL CHARACTERIZATION OF THE TSC/DYRK1A INTERACTION

Chapter 1: Role of TSC/DYRK1A interaction in regulation of mTORC1 and protein synthesis

Introduction: The mTORC1 pathway, the primary downstream effector of the TSC, controls multiple steps in the protein biosynthesis machinery including initiation and elongation factors as well as ribosomal biosynthesis. Precise regulation of these processes is required for safeguarding cells from expending the energy and resources under unfavorable conditions.

One way in which mTORC1 controls translation is by phosphorylation of the eIF4E binding proteins (4EBP1). eIF4E is a translation initiation factor that binds the 5'-cap structure of the mRNA and a multicomponent translation initiation complex eIF4G, and thus activates translational initiation. eIF4E binds to 4EBP1 at the same region as eIF4G. Thus, the binding of eIF4E to 4EBP1 prevents translation initiation [87]. mTORC1 phosphorylates 4EBP1 and prevents it from binding to eIF4E thus allowing activation of translation initiation.

Another way in which mTORC1 regulates protein synthesis is through activating phosphorylation of the p70S6K protein, a ribosomal protein kinase. Activated p70S6K catalyzes the phosphorylation of the S6K protein (rpS6), a part of 40S eukaryotic ribosomal subunit. This protein is crucial for both protein synthesis and regulation of cell size. Specifically, p70S6K activates the translation of a class of mRNAs with a 5'-oligo pyrimidine tract. rpS6 null mice were found to be smaller in size than wild type mice and also showed impaired glucose tolerance, indicating a role for this protein in both cell size and glucose homeostasis [88, 89]. The p70S6K protein has multiple phosphorylation sites, the most important ones being Thr229, Thr389 and

Ser404. Historically, the Thr389 site, that is phosphorylated by mTOR, is used as an indicator for activation of the mTORC1 pathway [90, 91].

The mTORC1 mediated phosphorylation events described above are tightly regulated by the presence of nutrients and growth factors. When cells are abundantly supplied with growth factors and nutrients such as amino acids and glucose, mTORC1 actively phosphorylates its downstream effectors 4EBP1 and p70S6K and continues to activate the protein synthesis machinery. Under unfavorable conditions, this phosphorylation is abrogated, which further stalls protein synthesis.

Yet another mechanism of mTORC1 control is the localization of its major inhibitory protein TSC2. When nutrients are abundantly available, TSC2 is mainly localized in the cytoplasm. Under cellular stress, TSC2 is phosphorylated and translocated to the lysosomal membrane where its GAP domain catalyzes the conversion of its target protein Rheb from an active GTP bound state to an inactive GDP-bound state, and thus inactivates the mTORC1 kinase (Fig.17). Hence, localization of TSC2 is also an important indication of the status of the mTORC1 pathway.

Since the TSC is an important regulator of the mTORC1 pathway which regulates protein synthesis, and due to its interaction with the DYRK1A kinase summarized in Part I of this dissertation, it was initially hypothesized that DYRK1A may be a novel regulator of the mTORC1 complex through its interaction with TSC. Specifically, it was hypothesized that with depletion of DYRK1A, cells will have a sustained activation of the mTORC1 pathway under conditions of stress.

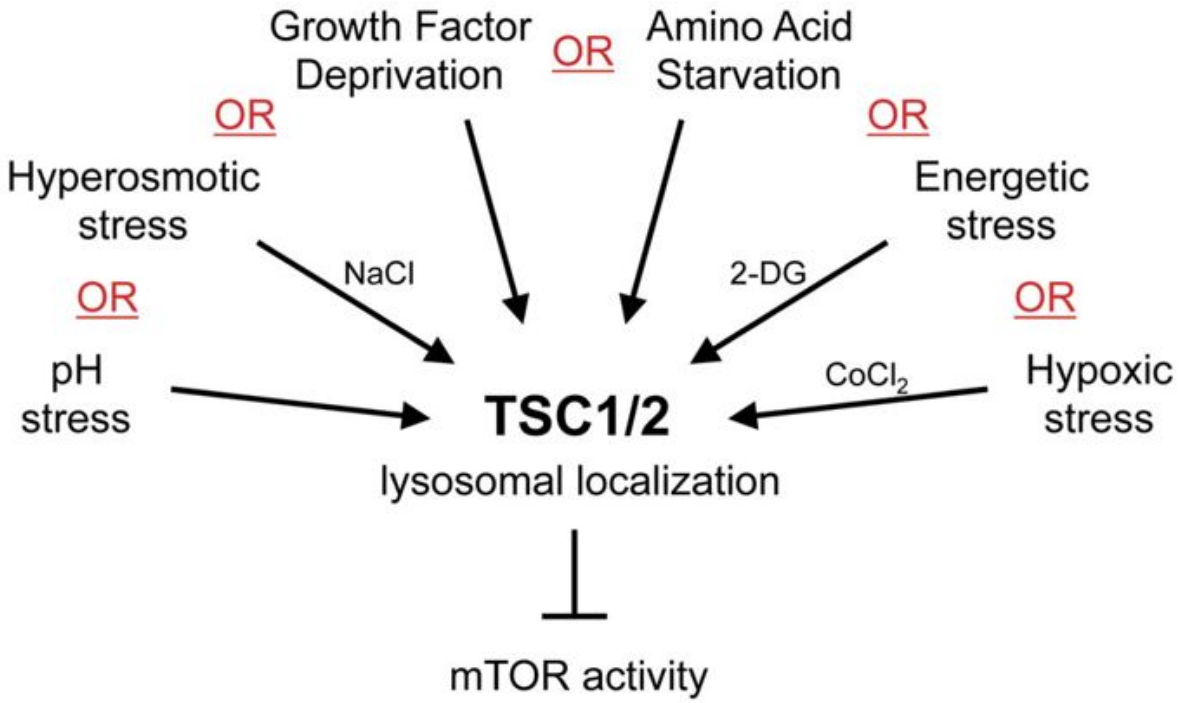


Fig.17. Lysosomal localization is a response to cellular stress. Adopted from- Demetriades C, Plescher M, Teleman AA. Lysosomal recruitment of TSC2 is a universal response to cellular stress. *Nat Commun*. 2016;7:10662. Published 2016 Feb 12. doi:10.1038/ncomms10662

This next section outlines the effect of DYRK1A on mTORC1 dependent and independent functions of the TSC, focusing on TSC1 specific effects.

1.1. Loss of DYRK1A has no effect on the mTORC1 pathway

A preliminary experiment performed in the lab by Dr. Vijay Menon indicated that under stress conditions, TSC2 localizes to the lysosomes to a greater extent in T98G shControl cells as compared to shDYRK1A cells (Fig.18A). In accordance with this, the mTORC1 pathway was found to be slightly activated in these stable DYRK1A knockdown cells, under stress conditions (Fig.18B). This led to further exploration of the effect of DYRK1A on the mTORC1 pathway.

In order to assess the effect of DYRK1A on the mTORC1 pathway, DYRK1A was first knocked down in HCT116 cells (colorectal carcinoma cells) using a DYRK1A specific silencer RNA (siRNA). A non-specific silencer RNA was used as a control. The cells were kept post-transfection for 48 hours and were then serum starved for 0 h, 6 h, 12 h and 24 h respectively. The control and starved cells were then collected and lysed using RIPA buffer and were probed for a panel of mTORC1 effector proteins. Both siNT and siDYRK1A HCT116 cells showed the expected decrease in the expression of p-p70S6K and p-4EBP1 with no significant difference between them (Supplemental figure 1). This was inconsistent with our initial hypothesis that loss of DYRK1A would lead to constitutively active mTORC1 under stress conditions. To further confirm this effect, a similar experiment was carried out with T98G cells, in which the DYRK1A gene was deleted by the CRISPR-Cas9 technique [92, 93]. In accordance with the results obtained with transient DYRK1A knockdown cells, no significant difference was observed in the phosphorylation patterns of the mTORC1 downstream effector proteins in DYRK1A WT and KO cell lines (Fig. 18C).

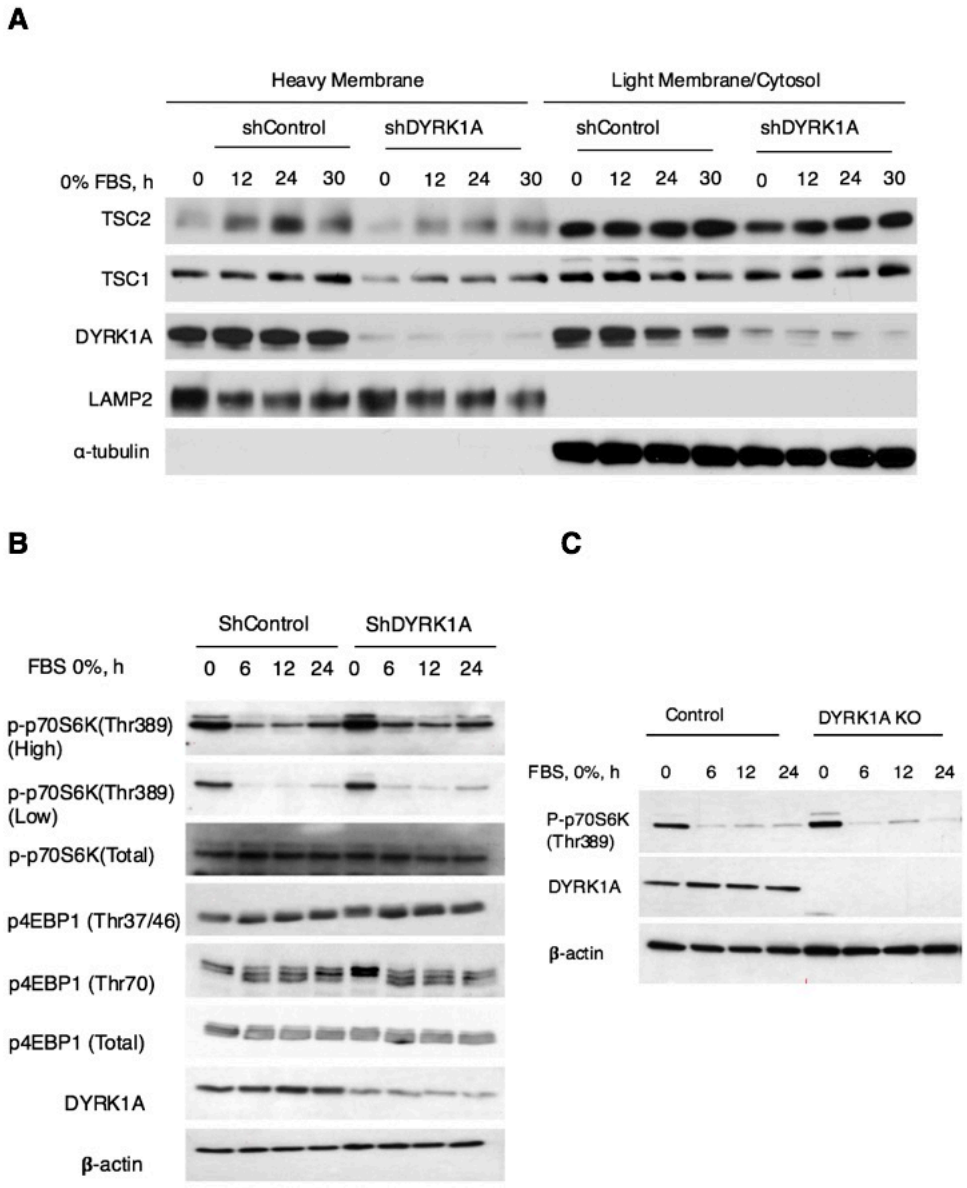


Fig.18. DYRK1A co-localizes with the TSC components but does not affect mTORC1. A: T98G control and shDYRK1A stable cell lines were serum starved for indicated time points, and fractionated into the heavy and light membrane/cytosolic components. Note that DYRK1A is detected in the heavy membrane fractions, as well as in the cytosol. LAMP2 and tubulin serve as fractionation controls. **B:** T98G control and shDYRK1A stable cell lines were serum starved for indicated time points and the mTORC1 pathway activity was measured by detecting phosphorylation of 4EBP1 and p70S6K using Western blotting. **C:** Control and CRISPR Cas9 DYRK1A-knocknout (KO) T98G glioblastoma cell lines were starved for the indicated time points, and used to detect phosphorylation of p70S6K using Western blotting.

1.2. Reverse phase protein array analysis (RPPA) showed no effect on mTORC1 pathway after loss of DYRK1A.

Although we did not observe changes in the phosphorylation of p70S6K and 4EBP1, other components of mTORC1 pathway could be deregulated upon DYRK1A loss. For this purpose, T98G CRISPR control and DYRK1A KO cell lines were analyzed using Reverse Phase Protein Array analysis (RPPA). RPPA is a robust and sensitive quantitative antibody based protein assay, which can be used to analyze a large number of samples for assessment of key proteins in important functional pathways such as protein synthesis, cell cycle progression, apoptosis, etc. Proteins that were assessed in the RPPA analysis include the eukaryotic translation initiation factor 4E (eIF4E) and calnexin, which are involved in protein synthesis and protein folding pathways respectively; insulin-like growth factor binding protein (IGFBP2/3) involved in glucose/energy metabolism; as well as the components of the mTORC1 pathway including p-4EBP1, p-p70S6K, TSC1 and TSC2. Cell cycle regulating proteins such as CDK1, p27 and Cyclin D1 were also analyzed. A total of almost 500 antibody targets were analyzed in the samples provided. The end result of the assay is the creation of a proteomic and phospho-proteomic signature for specific samples that have been analyzed. The RPPA analysis for this project was performed by the RPPA core facility at MD Anderson Institute, Houston, Texas, following data analysis by Dr. Mikhail Dozmorov (VCU). The analysis showed no significant difference in both upstream and downstream of the mTORC1 pathway factors between the control and KO samples (Fig. 19). This was consistent with our western blot analysis. Proteins that were found to be differentially expressed and are relevant to this project are discussed below.

Calnexin: Calnexin (CANX) was found to be significantly downregulated in the DYRK1A KO cells. This protein is a calcium-binding, endoplasmic reticulum (ER)-associated molecular

chaperone that binds to newly synthesized N-linked glycoprotein molecules and facilitates their folding and assembly [94]. Proper protein folding and assembly are essential for protein function.

IGFBP2/3: Insulin like growth factor binding proteins 2 and 3 were found to be significantly upregulated in DYRK1A KO cells. IGFBP2 binds insulin, IGF1 and IGF2, and modulates cell proliferation, survival and differentiation. Moreover, it has been identified as a biomarker in a variety of cancers such as gastric cancer, ovarian cancer, colorectal cancer, and glioblastoma [95].

Autophagy proteins: Two proteins important for autophagy were identified to be downregulated in DYRK1A KO cells, including ULK1 and ATG5. ATG5 is essential for autophagic vesicle formation while ULK1 is a serine/threonine kinase important for the formation of autophagophores (precursors to autophagosome) [96]. It has been previously reported that autophagy is deregulated in both Alzheimer's disease and Down syndrome, suggesting a role for DYRK1A in this pathway [97]. It will thus be interesting to explore the effect of DYRK1A loss and TSC/DYRK1A interaction on this pathway in the future.

Cytoskeletal proteins: A large number of cytoskeletal proteins such as myosin, vinculin, paxillin etc., were found to be modulated in the DYRK1A KO cells as compared to the control cells. Regulation of these proteins not only modulate the cell size and shape, but are also important for determining cancer cell malignancy through regulation of cell motility. These observations might explain some of the phenotypic effects such as decrease in cell size that have been seen with the CRISPR cell lines (will be further discussed in the next section).

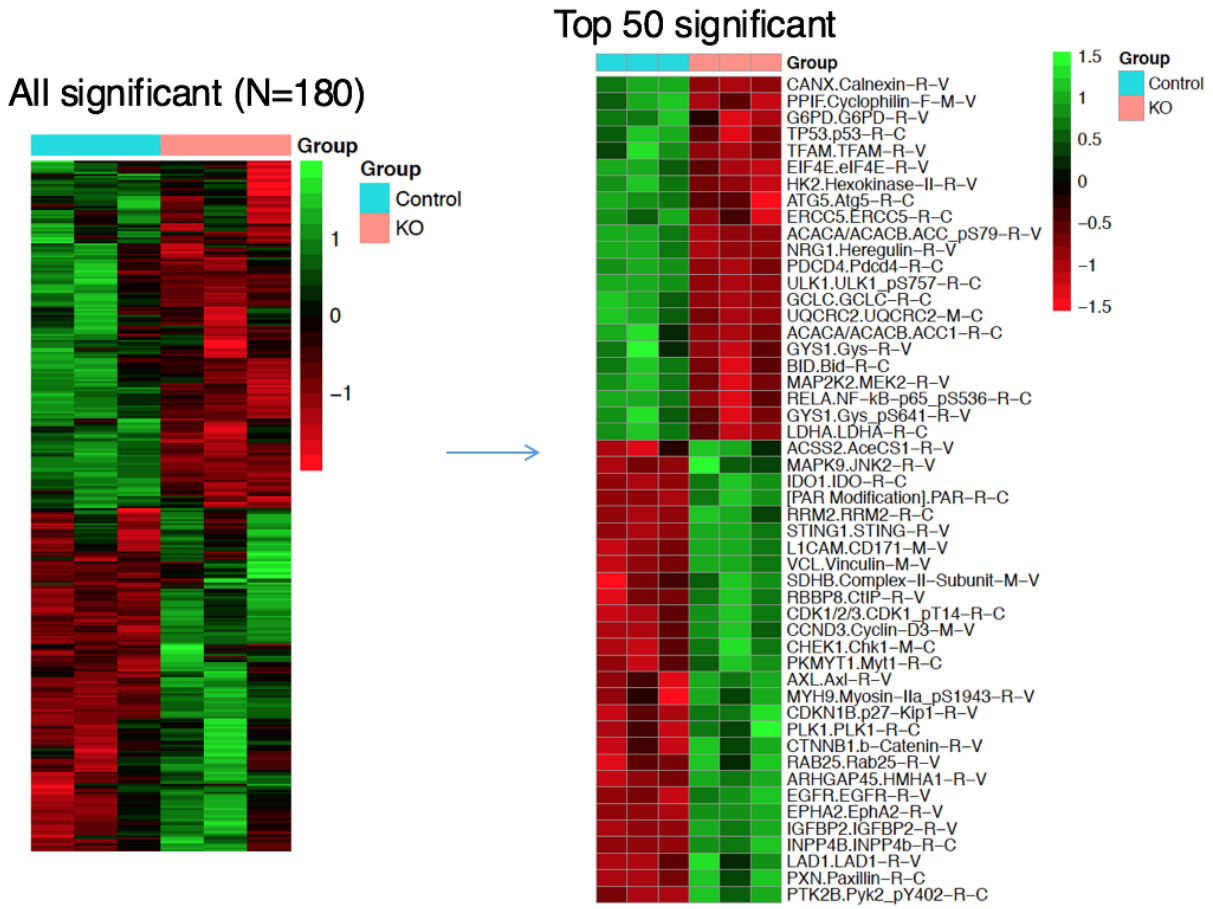


Fig.19. Reverse phase protein array analysis of DYRK1AKO cells. The left panel compiles the total number of differentially regulated proteins that were significantly modulated between control and DYRK1A KO cells. The right panel displays the top 50 proteins that were significantly differentially expressed between the control and the DYRK1A KO cells. Figure prepared by Dr. M. Dozmorov.

1.3. Loss of DYRK1A results in reduced cell size.

As previously reported, the mTORC1 pathway plays a role in the control of cell size and cell shape. Specifically, inhibition of the mTORC1 pathway has been shown to reduce cell size through a reduction in the phosphorylation of its downstream components 4EBP1 and p70S6K [98]. An interesting observation was made with T98G glioblastoma and NIH3T3 mouse fibroblast cell lines that had a knockout of DYRK1A using the CRISPR-Cas9 technique. In both cases, the DYRK1A KO cells appeared smaller in size as compared to the control cells. In order to confirm and quantify

this observation, T98G cells and NIH3T3 cells were analyzed by flow cytometry analysis. A Forward Scatter versus Side Scatter analysis of both these cell lines showed that DYRK1A KO cells were significantly smaller as compared to the CRISPR Control cells (Fig.20).

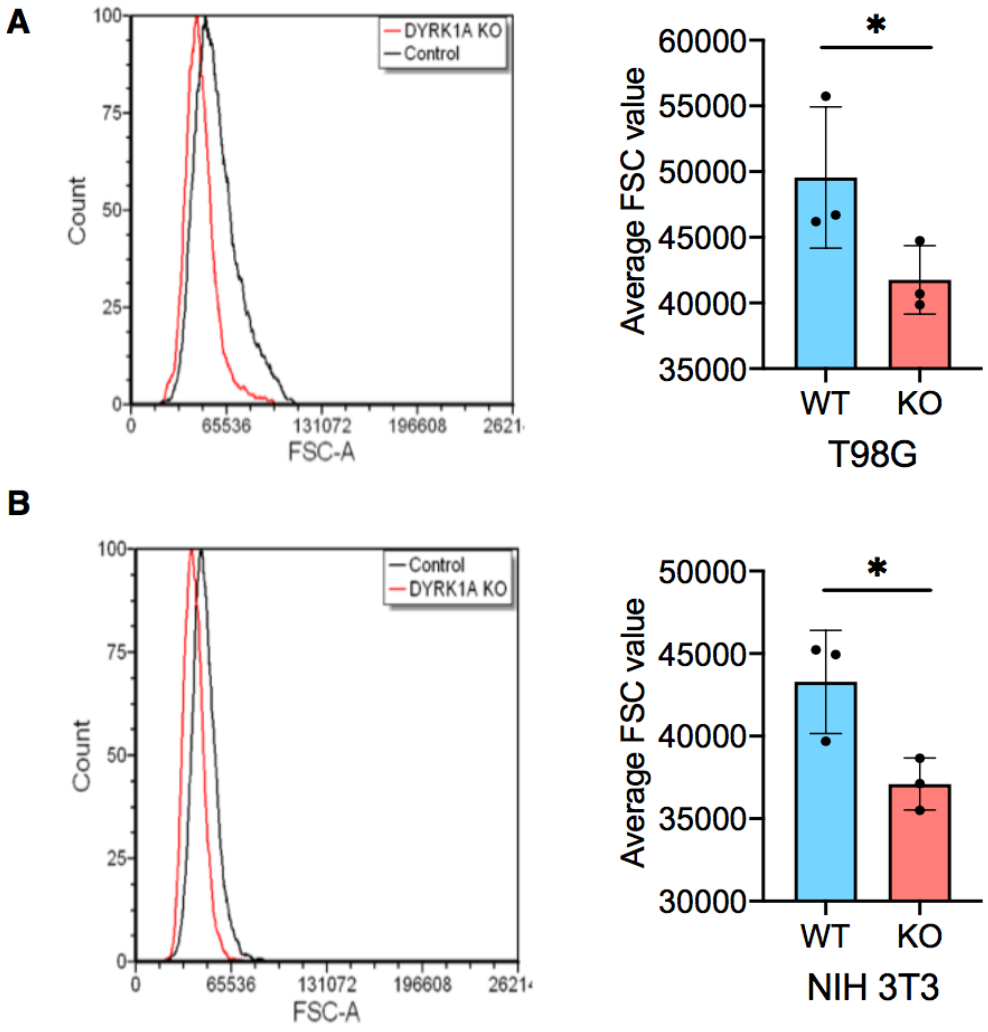
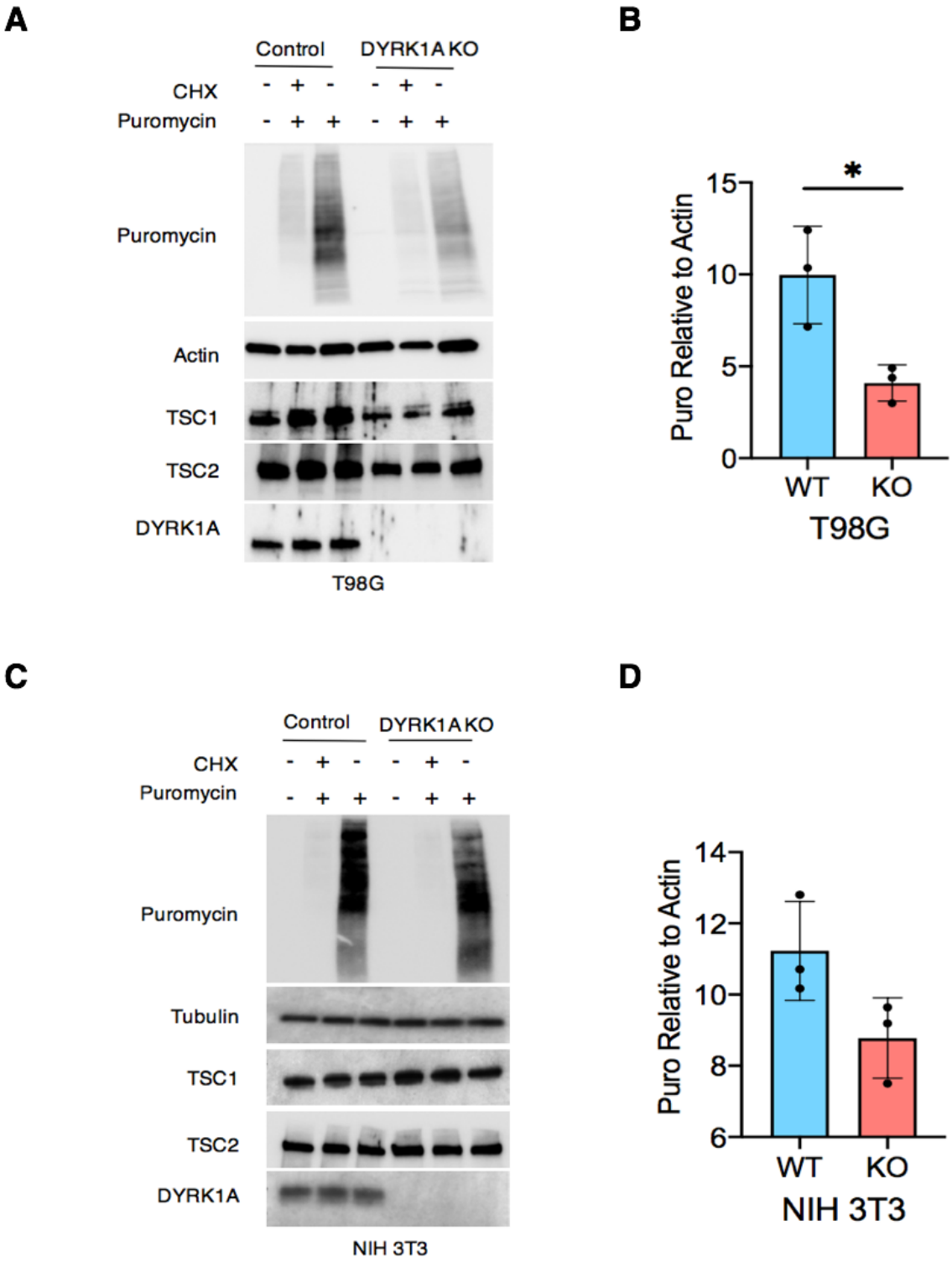


Fig. 20. Loss of DYRK1A results in reduced cell size. Representative histogram plots and quantification of the FCS values for the control (WT) or DYRK1A CRISPR-Cas9 knockout (KO) cell lines analyzed by flow cytometry. **A:** T98G cells. **B:** NIH 3T3 cells. Graphs show average ± stdev (N=3, * - p-value < 0.05 using Student's two-tailed t-*test)*.

1.4. Loss of DYRK1A results in reduced protein synthesis and is independent of TSC1.

Another important factor to note is that cell size partially depends on the rate of protein synthesis. For example, induction of mTORC1 signaling leads to an increase in the rate of protein synthesis and larger cell size. Inhibition of mTORC1 on the other hand may cause a decrease in protein synthesis and a decrease in cell size [61, 99]. Hence, we next investigated whether DYRK1A KO leads to a decrease in the rate of protein synthesis. For this purpose, the rate of protein synthesis was assessed in T98G cells and NIH3T3 cells using the puromycin-tagging assay. In this assay, cells are given a short pulse of puromycin, which attaches to all actively translating protein molecules. Cyclohexamide (CHX) blocks protein translation, thereby making it an effective negative control. The proteins tagged with puromycin can be detected using an anti-puromycin antibody by western blot analysis [100, 101]. In agreement with the effect observed on cell size, we saw a significant decrease in the rate of protein synthesis in the DYRK1A KO T98G cells and a similar trend of decrease in protein synthesis in the DYRK1A KO NIH3T3 cells. To determine whether this effect can be rescued by loss of TSC1 (if TSC1 is downstream of DYRK1A), we transiently knocked down TSC1 in T98G cell lines using TSC1 specific siRNA. When TSC1 was knocked down in these cells, followed by the puromycin tagging assay, it was observed that knockdown of TSC1 neither increased nor rescued the phenotype observed in DYRK1A KO cell lines. (Fig. 21) These results suggested that loss of global protein translation in DYRK1A KO cell lines were independent of TSC1. An alternate explanation for this observation could be that TSC1 played a role upstream of DYRK1A rather than downstream, thus showing no effect in cells that already had a knockout of DYRK1A in them. This result will be further validated in the future using more rigorous quantitative techniques such as ribosome profiling [102].



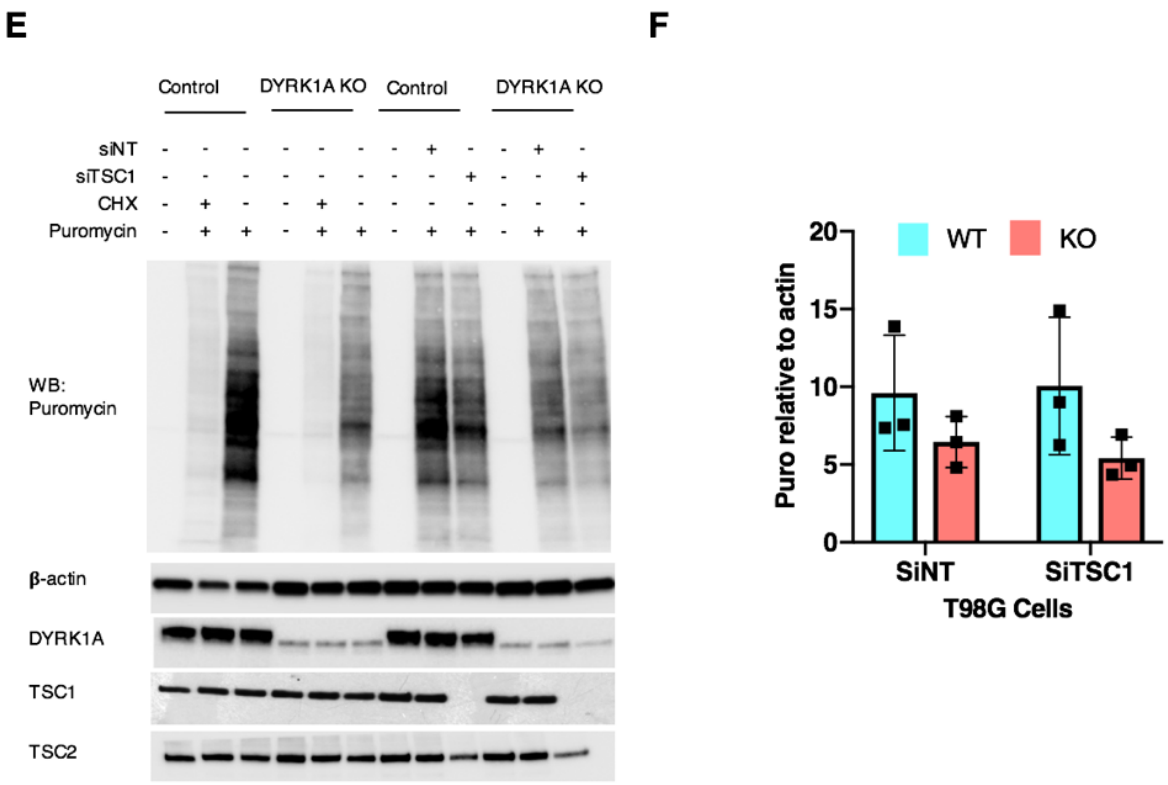


Fig.21. Decreased protein synthesis in DYRK1A-KO cells is not mediated by TSC1. A, C: Control or the CRISPR-Cas9 knockout cell lines were incubated for 15 min with 10 µg/ml puromycin, harvested and analyzed by immunoblotting with anti-puromycin antibody. Cyclohexamide (CHX) treatment for 1 hr before puro pulse was used to block protein synthesis and to confirm the assay specificity. **B, D:** Graphs show quantification of the data in A and B (average ± stdev of N=3, * - p-value: 0<0.05, Student's two-tailed t-test). **E:** Control and CRISPR-Cas9 knockout cell lines were transfected with a non-specific siNT (control) or with siTSC1 and treated with puromycin the same way as **A, C. F:** Graphs show quantification of the data in E (average ± stdev of N=3).

1.5. Loss of DYRK1A shows some effect on regulation of the actin cytoskeleton

Apart from its role in the regulation of the mTORC1 pathway, TSC components, especially TSC1, also play an important role in the regulation of the actin cytoskeleton. Previous reports have suggested that TSC1 can regulate the distribution of actin fibres through spatial regulation of the Rho family of GTPases [103]. Moreover, in our studies, loss of DYRK1A not only showed a change in cell size, but our RPPA analysis also revealed significant differences in various actin cytoskeletal proteins. Thus, we investigated whether loss of DYRK1A could have any effect on the actin cytoskeleton and its related functions such as wound healing and adhesion. As shown in

Supplementary Fig. 2, we indeed observed an effect of DYRK1A loss on cytoskeletal functions. For instance, we observed a decrease in the wound healing ability of DYRK1A KO cells as compared to CRISPR controls. However, it was also seen that these T98G cell lines overall showed inefficient and slow wound healing, therefore more studies will be needed with a better model for wound healing experiments. Moreover, an increase in stress fiber formation was found in DYRK1A KO cells but this effect was again highly variable and inconsistent. More studies need to be performed in order to verify whether the interaction between TSC/DYRK1A can have any effect on the regulation of the actin cytoskeleton.

Conclusion: Section I of this dissertation described the biochemical relationship between the TSC proteins and the DYRK1A protein kinase. The first part of Section II is focused on deciphering the epistatic relationship between the TSC and DYRK1A proteins. One of the major roles of the TSC proteins is inhibition of the mTORC1 pathway. Hence, the effect of loss of DYRK1A on the mTORC1 pathway downstream effector proteins was first analyzed. The results indicated that while TSC1 and TSC2 proteins are present in the same molecular compartment as the DYRK1A protein, only a modest activation of the mTORC1 activity was seen in shDYRK1A cells under stress conditions. This functional effect could be due to a decrease in the recruitment of TSC2 protein to the lysosomal membrane fraction of DYRK1A-depleted T98G cells under conditions of serum starvation induced stress. However, this effect could not be replicated in cells that had a CRISPR-Cas9 knockout of DYRK1A or in cells with DYRK1A transiently knocked down. One reason for the variability in these effects could be dosage sensitivity of the DYRK1A protein. T98G cells contain three copies of the DYRK1A gene, similar to what is seen in a Down syndrome model. A gene knockout in these cells leads to a complete loss of DYRK1A expression. On the

other hand, shDYRK1A cells show only a partial loss in total DYRK1A levels, which may result in the variability seen in the mTORC1 effector proteins in these cell lines.

Since the effects on mTORC1 effector proteins were inconclusive, it was important to carry out further functional assays in order to assess the status of the mTORC1 pathway in cells lacking DYRK1A. Interestingly, cells that had a CRISPR knockout of DYRK1A were significantly smaller in size as compared to their controls. Moreover, DYRK1A KO cells also showed a significant decrease in the global protein translation rate as compared to control cells. These results further imply that mTORC1 was not constitutively activated in DYRK1A KO cells as we originally hypothesized. It was also seen that the decrease in protein synthesis was unaffected by a knockdown of the TSC1 protein, the major binding partner of the DYRK1A kinase indicating that TSC1 may not be responsible for this particular phenotype seen in the DYRK1A KO cell lines. Further studies need to be carried out in order to decipher the mechanisms responsible for decreased protein synthesis in the DYRK1A KO cell lines.

Finally, investigation of the effect of loss of DYRK1A on the regulation of the actin cytoskeleton showed some potentially interesting effects. Further work needs to be done in order to confirm these preliminary observations.

Chapter 2: Role of the TSC/DYRK1A interaction on DYRK1A regulated cell cycle control

Introduction: DYRK1A is an important protein for embryonic development; specifically, in neurological development by regulating the balance between proliferation and differentiation of neural progenitors [23, 104]. DYRK1A plays an important role in the regulation of tumorigenesis, cell cycle and survival, in a dosage dependent manner. However, the ultimate readout of DYRK1A overexpression and inhibition depends strongly on cellular context, as well as the substrates that are targeted by this kinase in different cell lines and under different conditions. These complexities in DYRK1A regulation mean that it can act both as an oncogene as well as a tumor suppressor depending on the cellular context. Considering the above factors, it is not surprising that one of the most well studied function of DYRK1A has been its role in the regulation of the cell cycle.

One of the ways in which DYRK1A controls the cell cycle is through the regulation of the DREAM complex assembly. Briefly, the cell cycle can be repressed by the assembly of the DREAM complex and subsequent repression of cell cycle dependent genes. The DREAM complex consists of Retinoblastoma-like protein (RB-like) p130, E2F4, DP1 and a MuvB core complex that includes LIN52 protein. The MuvB core complex can also interact with B-MYB which can then activate G2/M regulating genes and promote proliferation. The assembly of DREAM complex requires DYRK1A-mediated phosphorylation of LIN52 at the S28 position. In the absence or inhibition of DYRK1A using various DYRK1A kinase inhibitors such as Harmine or CX4945, LIN52 is not phosphorylated, DREAM complex is not assembled, and the MuvB core can now interact with B-MYB allowing it to activate early phase cell cycle genes and promote proliferation (Fig. 22A). Thus, phosphorylation of LIN52 can be used as a measure of DYRK1A kinase activity. First, an in-vitro kinase assay was performed using T98G DYRK1A WT and DYRK1A KO CRISPR cell

lines, in order to confirm that DYRK1A is a major LIN52 protein kinase in the cell (Fig.22B) and this effect has also been previously published by the lab [92].

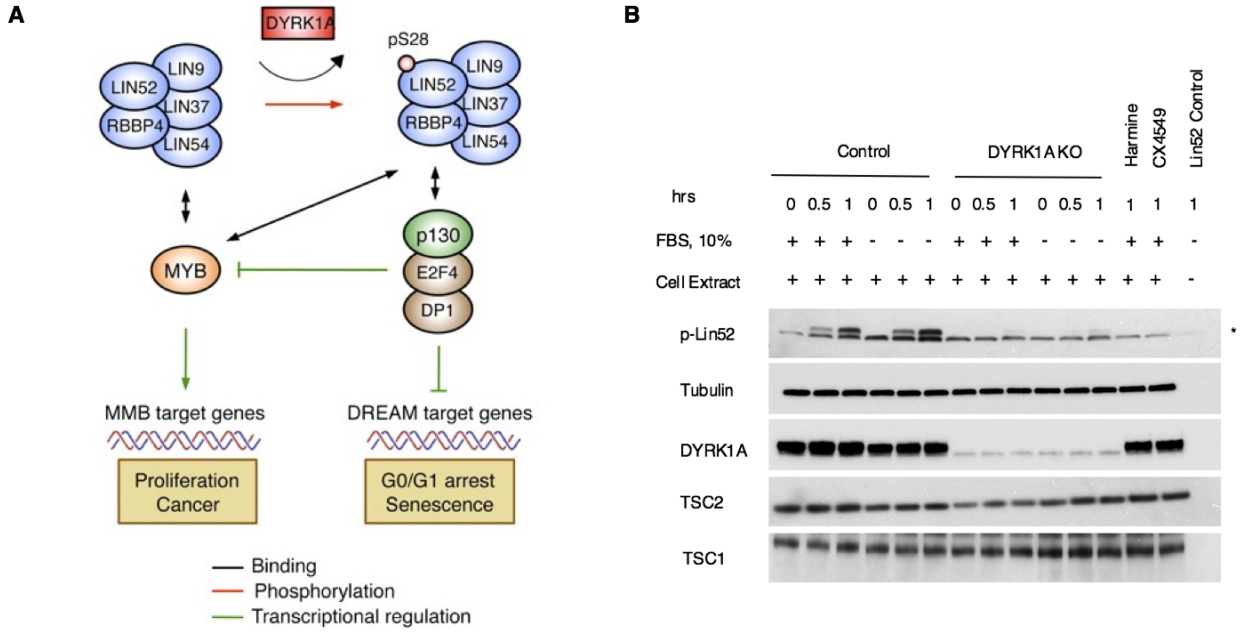


Fig.22. Loss or inhibition of DYRK1A leads to loss of LIN52 phosphorylation. A: Cartoon showing the role of DYRK1A kinase in DREAM regulation, *adopted from*: Litovchick L, Florens LA, Swanson SK, Washburn MP, DeCaprio JA. DYRK1A protein kinase promotes quiescence and senescence through DREAM complex assembly. Genes Dev. 2011;25(8):801–813. **B:** In-vitro kinase experiment with T98G WT and DYRK1A KO CRISPR cell lines. T98G cell lysates treated with or without DYRK1A kinase inhibitors Harmine and CX4945 were incubated with GST-LIN52 for the indicated time points. The lysates were then probed for the indicated proteins. LIN52 phosphorylation was abrogated in DYRK1A KO and DYRK1A inhibitor treated cells.

The previous section of this dissertation showed that the interaction between DYRK1A and TSC does not significantly affect the mTORC1 pathway, the major downstream effector of the TSC. Since it was possible that the binding of these two proteins may have an effect on the DYRK1A kinase protein rather than on TSC functions, we hypothesized that DYRK1A-TSC binding may have an effect on the DYRK1A regulated functions such as cell cycle control. This hypothesis was further influenced by the fact that Tsc1 null mouse embryonic fibroblasts progressed through the cell cycle faster as compared to Tsc1 wt cells (Fig.23). This is an effect we would expect to see if loss of TSC1 had an effect on the functions of the DYRK1A kinase.

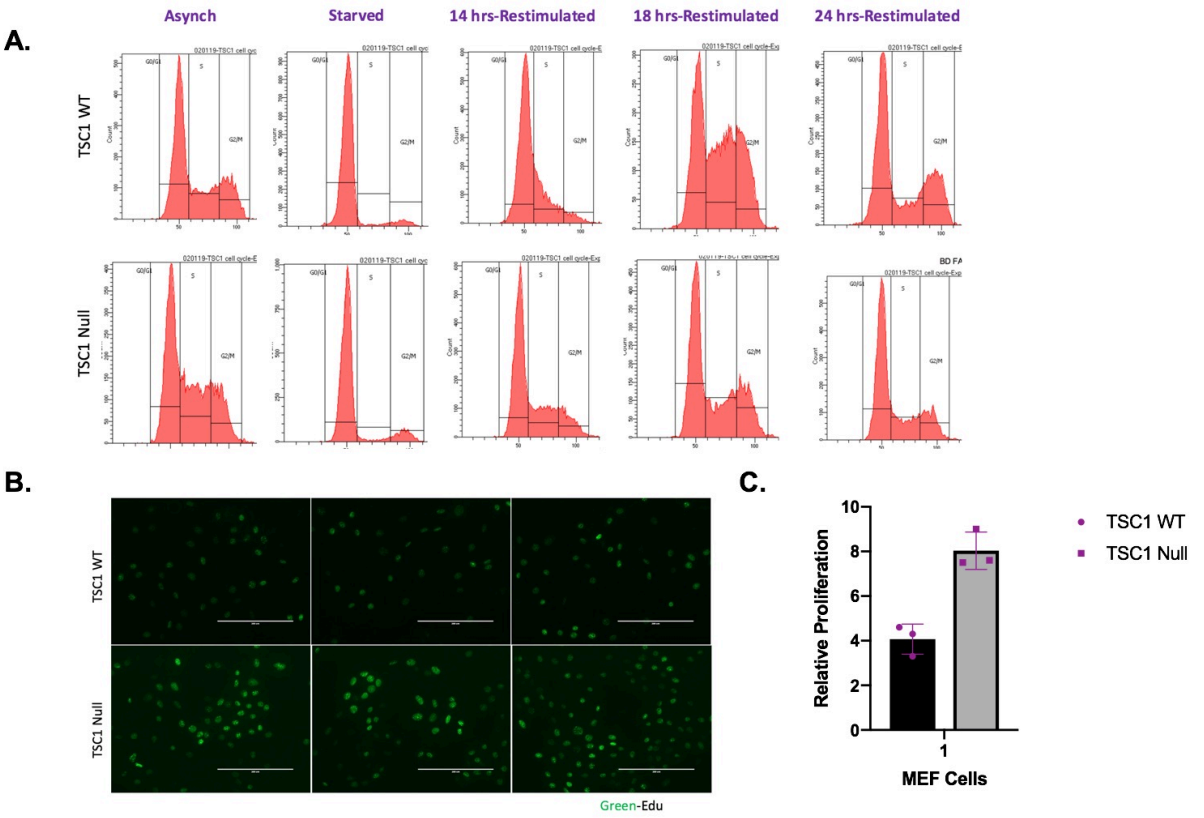


Fig.23. Tsc1 null cells progress faster through the cell cycle then Tsc1 wt cells. A: Tsc1 wt and Tsc1 null MEFs were starved for 24 hrs with 0% FBS, and restimulated for the indicated time-points. Asynchronous (cycling), Starved and re-stimulated Tsc1 wt and null cells were collected and stained with propidium iodide to assess cell cycle distribution. **B:** Cycling Tsc1 wt and null cells were stained for EdU (5'-ethynyl-2'-deoxyuridine) to detect newly synthesized DNA (green). Three representative fields are shown here. **C:** Proliferation assay using direct counting of trypan blue stained cells.

The effects shown in the figures above provide the rationale for exploring the effect of TSC1 on the DYRK1A kinase function.

2.1. Tsc1 null mouse embryonic fibroblasts have decreased DYRK1A activity.

In order to analyze the effect of TSC1 on DYRK1A kinase function, wt and Tsc1 null mouse embryonic fibroblasts (MEFs) were tested for DYRK1A kinase activity using the in-vitro kinase experiment that was described above in Fig. 23B. Specifically, asynchronous or starved Tsc1 wt and null cells were first collected and lysed using EBC lysis buffer. The resulting lysates were then incubated with GST-LIN52 for 0 min, 30 min and 1 h time-points. The wt cells were also pre-incubated with a DYRK1A kinase inhibitor CX-4945, as a negative control. DYRK1A specific

LIN52 phosphorylation was found to be decreased in Tsc1 null cells. Moreover, starvation did not have a major effect on LIN52 phosphorylation in both wt and null Tsc1 cells. Interestingly, DYRK1A protein expression was also significantly reduced in the Tsc1 null cells as compared to controls. Another intriguing effect was the increase in both DYRK1A and TSC2 protein expression in Tsc1 wt starved cells as compared to asynchronous controls, although this effect was not significantly different in three biological repeats (Fig. 24). Neither protein was upregulated by starvation in the Tsc1 null cells. Such an effect has not been seen before with other transformed and non-transformed cell lines.

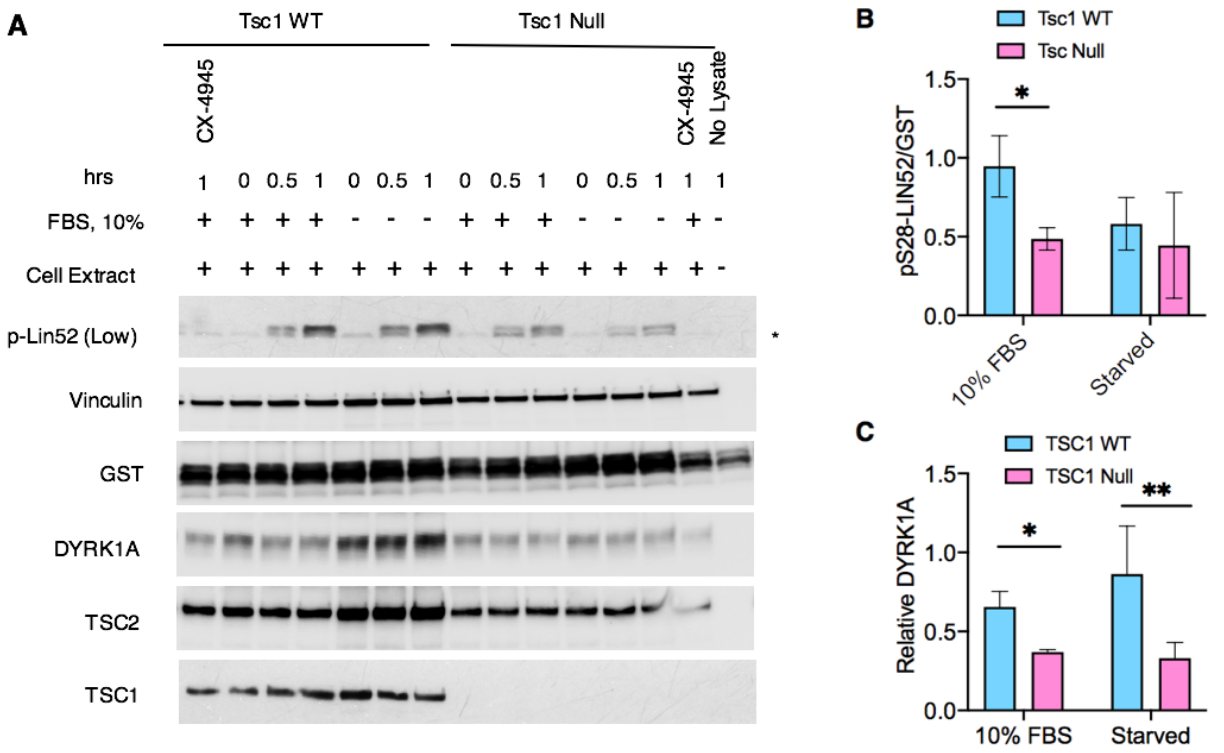


Fig.24. TSC1 affects DYRK1A expression and kinase activity in mouse cells. A: Tsc1 WT and Tsc1-null mouse embryonic fibroblasts were grown in the presence or absence of serum for 12 hours, and harvested. The resulting lysates were incubated with ATP, kinase buffer and GST-tagged purified LIN52 for 0, 30 and 60 minutes followed by detection of phospho-S28-LIN52 by western blotting. **B:** Quantitation of the data shown in A. Graphs show normalized band density for pLin52 (N=3; average ± stdev; * - $p<0.05$). **C:** Quantitation of the data shown in A. Graphs show band density for DYRK1A relative to vinculin (N=3; average ± stdev; * - $p<0.05$).

Consequently, next step was to investigate whether this effect can be replicated and observed in additional cells lines. For this purpose, TSC1 was first stably knocked down in U-2 OS osteosarcoma cells and BJ-hTERT fibroblasts. Additionally, TSC1 was also stably and transiently downregulated in T98G cell lines (data not shown). The in-vitro kinase assays to observe the effect on DYRK1A kinase function were then repeated with these additional cell lines. However, the effect seen with the Tsc1 null MEFs could not be replicated in these additional cell lines indicating that the effect may have been limited to the mouse embryonic fibroblasts. (Fig. 25)

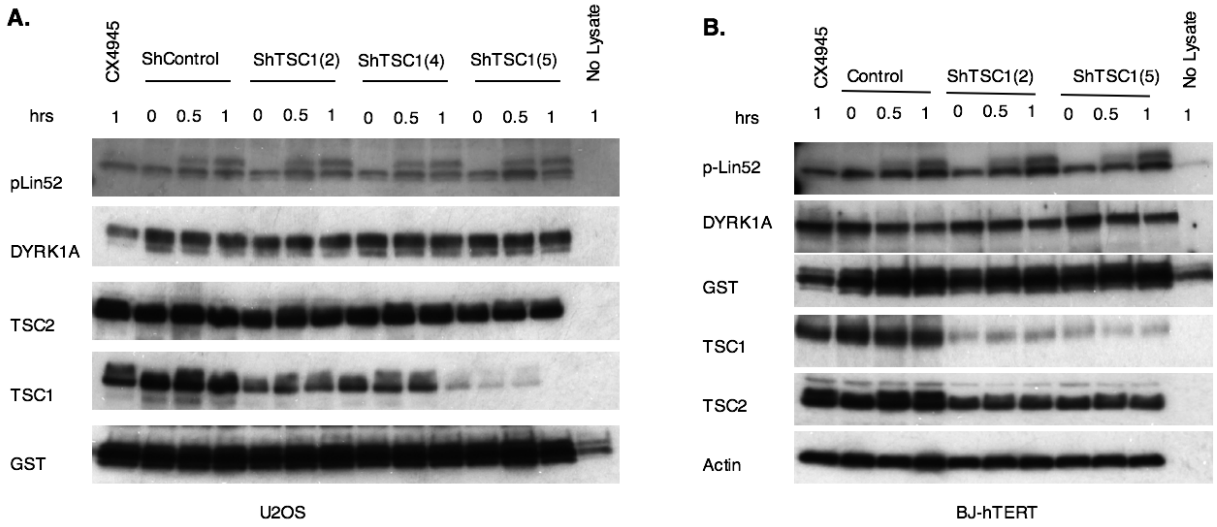


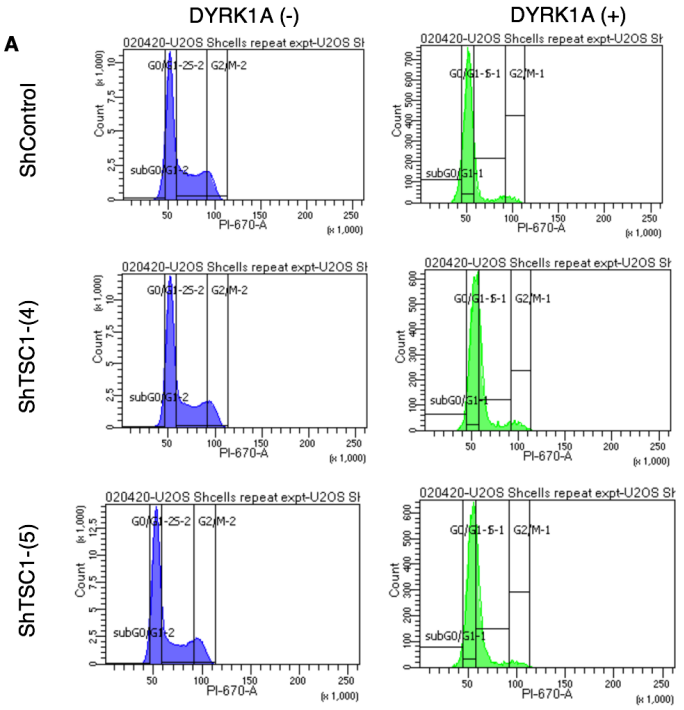
Fig.25. Knockdown of TSC1 in U2OS and BJ-hTERT cell lines does not decrease DYRK1A dependent LIN52 phosphorylation. A, B: U-2 OS and BJ-hTERT cells with knockdown of TSC1 were serum starved for 12 hours. The resulting lysates were treated with GST-tagged LIN52 for 0,30 and 60 minutes followed by detection of LIN52 phosphorylation at the DYRK1A dependent S28 position by western Blotting.

In addition to TSC1 knockdown cell lines, the effect of loss of Tsc2 on DYRK1A kinase activity was also assessed using in-vitro kinase assays on mouse embryonic fibroblasts that were wt and null for the Tsc2 protein. Again, no significant effect of loss of Tsc2 was seen on both DYRK1A kinase activity as well as DYRK1A protein expression, indicating that the effect observed in Tsc1 null cells was limited to only those cell lines (data not shown). The difference between the wt and

Tsc1 null cells could be because they are both different cell lines obtained from two separate mouse embryos, or because of the incomplete knockdown of TSC1 in the shRNA-depleted cells.

2.2. Downregulation of TSC1 has a modest impact on DYRK1A mediated cell cycle arrest.

DYRK1A plays an important role in cell cycle arrest. Moreover, it was observed that TSC1 knockout in mouse embryonic fibroblasts leads to increase in proliferation and faster cycling through the cell cycle. Hence, the next step was to investigate whether knockdown of TSC1 showed any effect on DYRK1A mediated cell cycle arrest. For this purpose, stable cell lines with a knockdown of TSC1 were created in U2OS osteosarcoma cell lines using a lentiviral vector system. U-2 OS cells were chosen for their ease in transfection as well as because it was previously shown that DYRK1A causes G0/G1 cell cycle arrest in these cells [19]. These cells were then transfected with GFP-tagged DYRK1A and analyzed for cell cycle profiles 3 days' post-transfection using the PI staining of DNA followed by FACS analysis. As expected, there was an increase in the proportion of G0/G1 phase cells in both the shControl and the shTSC1 cells upon over-expression of DYRK1A. Also expected, was the decrease in the proportion of G2/M phase cells in both the shControl and the shTSC1 cell lines. Specifically, it was observed that there was a substantial difference (~23%) in the proportion of S-phase cells between DYRK1A GFP-positive (12%) and negative (35%) U2OS shControl cells. However, this difference was reduced to ~10% or less in cells depleted of TSC1. Additionally, shTSC1 cells consistently showed a higher proportion of S-phase cells in DYRK1A overexpressed cells as compared to shControl cells. This experiment was repeated once more with a similar result (data not shown). These observations indicate that shTSC1 cells may be more resistant to DYRK1A mediated cell cycle arrest as compared to shControl U-2 OS cells. (Fig. 26)



B

	ShControl	
	DYR (-)	DYR (+)
G0/G1	46	69.1
S	35	12
G2/M	8.5	4.4

	ShTSC1-(4)	
	DYR (-)	DYR (+)
G0/G1	50.3	65.4
S	31.4	21.2
G2/M	10	5.3

	ShTSC1-(5)	
	DYR (-)	DYR (+)
G0/G1	52.6	64.3
S	29.8	24.3
G2/M	11.6	4.1

Fig.26. Role of TSC1 in DYRK1A-mediated G0/G1 arrest. A: Cell cycle profiles for stable control and stable TSC1 knockdown U2OS cell lines. B: Quantitative values of the cell cycle profiles shown in part A, showing that TSC1 knockdown U-2 OS cells are more resistant to DYRK1A mediated cell cycle arrest as seen by the increase in the proportion of S-phase cells in DYRK1A overexpressing cells.

Conclusion: In this section of the study we observed that loss of TSC1 in mouse embryonic fibroblasts leads to a subsequent loss of DYRK1A expression as well as DYRK1A mediated LIN52 phosphorylation. This effect could not be confirmed or replicated in additional cell lines. However, we also observed that cells with a downregulation of the TSC1 protein may attenuate DYRK1A mediated cell cycle arrest. This result however needs to be confirmed and replicated in additional cell lines.

IV. DISCUSSION

The dual specificity tyrosine regulated protein kinase DYRK1A is encoded by an evolutionary conserved gene that is highly interesting because of its specific location in the Down syndrome critical region on Chromosome 21. DYRK1A has been implicated in a variety of pathological manifestations including neurological diseases and diabetes. This study introduces the Tuberous Sclerosis Complex (TSC) as a novel binding partner of DYRK1A. TSC is the major upstream inhibitor of mTORC1 kinase, which in turn, regulates multiple cellular processes such as cell proliferation, ribosomal biosynthesis, protein translation, autophagy and others. Briefly, in this study, we observed a strong TSC1 mediated interaction of TSC with DYRK1A. Attempts to decipher the mechanisms that are regulated by this interaction showed that the interaction may not affect the mTORC1 pathway directly, but may affect protein translation through an as yet unidentified mechanism. Moreover, TSC1 could contribute to DYRK1A-mediated cell cycle arrest in certain cell lines, although more data is needed to support this conclusion. Additionally, we also report that TSC1 is a substrate of the DYRK1A kinase, at non-canonical sites that have been identified, but need to be further confirmed by additional experiments.

1. The biochemical interaction between TSC and DYRK1A.

The interaction between the main components of TSC was discovered over two decades ago [58]. Moreover, the exact domains of TSC1 and TSC2 that interact with each other have also been thoroughly studied. The TSC also contains a third subunit, the Tre2-bub2-cdc16 domain family member 7 (TBC1D7) protein [105]. Our initial proteomic analysis suggested that TBC1D7 is not a major DYRK1A interacting protein and hence it was not the focus of this study. Additionally, mutations in TBC1D7 do not lead to the formation of the hamartomas that characterize tuberous sclerosis. Both TSC1 and TSC2 proteins are required for complete activity of TSC- mediated

mTORC1 inhibition. As described in the introductory paragraphs, while the presence of the catalytic GTPase domain in the TSC2 protein makes it clear why TSC2 is essential, less is known about the exact roles of the TSC1 protein. TSC1 is mainly involved in stabilization of the TSC2 protein and has also been predicted to help stabilize various other proteins by acting as a chaperone for proper folding. Additionally, TSC1 has been found to interact with multiple proteins, and the physiological significance of these interactions is yet unknown. In this study, DYRK1A is introduced as a novel interactor of TSC. Specifically, it is shown that DYRK1A interacts with the TSC primarily through the TSC1 protein. The specific domains that are required for the interaction between TSC1, TSC2 and DYRK1A have also been mapped using various deletion constructs of the three proteins.

One major observation from this part of the study was that the kinase domain, but not kinase activity is required for the interaction of DYRK1A with TSC1 and TSC2. We also found that DYRK1A binds to the same domain in TSC1 that binds TSC2. One implication of this observation is that the interaction between these proteins might render both TSC1 and DYRK1A unavailable to perform their regular functions of regulation of protein synthesis and cell cycle arrest. If this scenario is true, the fraction of TSC1 that is bound to DYRK1A would be unavailable for inhibition of mTORC1 even under stress conditions, and this could have an effect on both the physiological pathways controlled by mTORC1, as well as the pathology of tuberous sclerosis.

Loss of function mutations of TSC1 and TSC2 are mainly responsible for the neuropathology seen in patients with tuberous sclerosis. However, the observations made in our study could reveal a unique mechanism by which TSC components may be hindered from performing their function. This explanation could also account for the reports of patients who do not show a mutation in either *TSC1* or *TSC2* [106]. One way to investigate this could be by analyzing the binding between

TSC1 and DYRK1A in cell culture models of tuberous sclerosis. Recent advances using human-induced pluripotent stem cells derived from patient samples combined with CRISPR-Cas9 techniques, provide a way to potentially establish cell culture models of TS that can then be used for binding studies [107]. It is important to note however that this line of thought clashes with the observation that DS patients show a lower incidence of solid tumors. However, since DS patients also show a higher incidence of Acute Lymphoblastic Leukemia (ALL) as compared to the normal population, it is possible that these effects are highly selective and cell type specific.

Both TSC1 and TSC2 (data not shown) are DYRK1A substrates in *in-vitro* kinase assays. In this study, there was more emphasis on determining the phosphosites on TSC1, since TSC1 was the major DYRK1A binding partner, and because TSC1 had no known DYRK1A consensus sequences. Using mass spec analysis, several potential sites for DYRK1A mediated TSC1 phosphorylation was identified. Historically, regulation of the activation and the inhibition of the TSC components is largely controlled by their phosphorylation at specific sites by various upstream kinases. Thus, it is important to explore the function of these identified sites, and determine their role in regulation of TSC/mTORC1 pathway. Studies that are currently ongoing involve creation of a triple mutant TSC1 construct (using the sites that were identified in our first round of mass spec analysis i.e. S505, T547 and T773), with an alanine mutation in all three sites. The expectation is that we would see a more dramatic loss of gel-shift with a mutation in all three sites as compared to the partial loss that we see with single mutants. Moreover, yet another method would be to perform an in-vitro kinase assay with radioactive ATP and a recombinant GST-DYRK1A kinase so as to confirm specific phosphorylation by DYRK1A. A third method would be to separate distinct phospho forms using Phos-tag gels, which may give a better idea about the effect of these mutants on phosphorylation. Similar experiments will need to be repeated in order

to confirm the other sites that have been identified by mass spec analysis. The next step, as discussed, would be to analyze the specific function of these sites. One way to investigate this would be to determine whether localization of unphosphorylated mutant TSC1 proteins changes as compared to phosphorylated WT proteins.

Finally, the localization of TSC components is an important factor controlling mTORC1 regulation. Thus, apart from the binding analysis performed in our studies, further studies are needed to verify the localization of TSC components, especially TSC1, in the presence and absence of DYRK1A.

2. The role of DYRK1A in canonical and non-canonical functions of the TSC.

The observations of this study indicate that DYRK1A interaction with TSC may not necessarily have an effect on the function of TSC as a major inhibitor of the mTORC1 complex. This leads us to question as to what exactly may be the role of this interaction. One of the most interesting observations from this study was the decrease in total protein synthesis that was observed in DYRK1A null CRISPR cell lines, that seems to be independent of TSC1 expression. An alternate mechanism that could be involved in the effect we see on protein synthesis in the DYRK1A KO cell lines, is through the effect of amino acids on the mTORC1 pathway. In this study, when we checked the effect of DYRK1A loss on the mTORC1 pathway, stress induction was done by means of serum starvation. When we serum starve the cells, we deprive it of all essential nutrients and other growth factors, but not necessarily all amino acids. Amino acids being the essential building blocks of all proteins, can have a great impact on multiple steps of protein translation. Moreover, amino acid sensing is one of the major conserved regulatory pathways feeding into the mTORC1 pathway [108]. Previous studies have shown that the amino acid sensing arm of the mTORC1 upstream pathway, does not pass through the TSC, as seen with other nutrients and growth factor

proteins [109]. Instead, the primary function of amino acids is to regulate the intracellular localization of mTORC1 so that it colocalizes with the Rheb protein at the lysosomal membrane, a step that is required for a functional mTORC1 pathway [110, 111]. This was the major reason why initial experiments in the lab did not involve studying the effects of amino acids specifically. Previous reports combined with our data showing decreased protein synthesis in DYRK1A KO cells, however, calls into question whether the effect on protein translation that we see in DYRK1A KO cells is entirely independent of the TSC and is based on the amino acid sensing pathway instead.

In an attempt to decipher what mechanisms could be responsible for this decrease in cell size and decrease in protein synthesis, T98G control and DYRK1A KO CRISPR cell lines were sent for reverse phase protein array (RPPA) analysis. The results from this assay are still being investigated. However, it is important to note that the RPPA analysis showed no difference in the mTORC1 pathway effectors. Interestingly, a variety of cytoskeleton proteins were found to be affected with DYRK1A knockout. This could be an alternate explanation to the smaller size of the DYRK1A KO CRISPR cell lines. Thus, results from the RPPA analysis, especially with regards to the cytoskeletal proteins such as vinculin, myosin and paxillin need to be further validated in multiple cell lines, in order to investigate whether the effects on cell size seen in DYRK1A KO cells may be entirely independent of mTORC1 or could be due to changes in the cytoskeleton instead. Future studies in this section should include DYRK1A rescue cell lines, in order to check for changes in protein synthesis and cell size, as well to check whether differences seen in the cytoskeletal proteins are abrogated with RPPA analysis in rescue cell lines as well. These rescue cell lines can include both DYRK1A WT rescue cells as well as DYRK1A-Kinase dead (KD)

rescue cells, in order to differentiate whether the effects observed are only based on DYRK1A binding or are based on DYRK1A phosphorylation.

Additionally, the TSC/DYRK1A interaction could have an effect on the non-canonical functions of the TSC which includes cytoskeletal regulation. Preliminary studies performed in the lab were inconclusive as to the effects of TSC-DYRK1A interaction on cytoskeletal regulation, but it may be a good idea to revisit this hypothesis in the future. One of the major issues that was observed while investigating the cytoskeletal effects of DYRK1A loss, was the use of the T98G CRISPR cell lines as a model system. These cells are poor models for assays such as wound healing, mainly due to their inability to complete close the wound, even 72 hrs post wound induction. Moreover, these cell lines were created from a single cell clone and it is possible that these cells developed alternate mechanisms that compensate for the loss of DYRK1A, leading to the masking of physiological effects that would otherwise have been seen with loss of DYRK1A. Future studies to investigate the role of TSC/DYRK1A interaction in regulation of the actin cytoskeleton should also focus on studying effects on cell motility, and formation of cell protrusions, both of which are mechanisms regulated by TSC1. These mechanisms in turn have an important impact on malignancy.

With the above observations, it is also important to take note of one of the potential drawbacks of the study. Specifically, a majority of the experiments were performed using T98G glioblastoma cell lines, that have a mutant p53. It has been observed that deletion or mutation of the p53 molecule leads to loss of TSC2 from the lysosomal membrane and an increase in lysosomal Rheb, leading to hyperactivation of the mTORC1 pathway [112]. This means that these cell lines have a much higher baseline level of mTORC1 activity as compared to cell lines with p53 wild type protein; and this could have masked the finer changes that might have otherwise been seen on the

pathway with a loss of the DYRK1A protein. A further complicating factor with these cell lines is the fact that these cells contain 3 copies of DYRK1A, leading to a 1.5x increase in protein expression as compared to cell lines with normal content of DYRK1A. Again, knowing that DYRK1A is highly dosage sensitive, this factor could also have had an impact on the overall results obtained in the study. Moreover, T98G cells are hyperpentaploid and contain several deletions and translocation, which possibly contributed to some of the variability seen in our results. Thus, future studies could include U-2 OS, HCT116 and MCF7 cells as model system to see whether the results can be replicated in these cells. Again, a caution while using U-2 OS cells would be to note that while U-2 OS cells have a WT p53, there is just one copy of DYRK1A.

Finally, the decrease in the rate of protein synthesis, if confirmed by a much more sensitive assay such as ribosome profiling, also has profound implications for the pathology seen in Down syndrome patients. It has been reported that a transgenic model of DS (Ts65Dn) shows a decrease in protein translation through the activation of the Integrated Stress Response pathway (ISR) [113]. ISR regulates proteostasis in response to stress conditions by regulating the rate of protein synthesis [114]. A similar activation of the ISR was seen in post-mortem brain samples from human individuals with DS. DYRK1A KO mice models are embryonically lethal while DYRK1A haploinsufficiency in humans has been reported to be pathogenic showing effects such as microcephaly, ID and speech impairment. If our observations on decreased protein synthesis in DYRK1A KO cells can be further confirmed in additional cell models, it might shed further light not only on the dosage sensitivity of DYRK1A but also on the fact that haploinsufficiency as well as overexpression of DYRK1A give rise to neurological defects possibly because the same pathway is being dysregulated under both conditions. Whether or not TSC1 plays a role in these pathways is yet to be seen.

Additionally, recent reports have suggested that mTORC1 is hyperactivated in DS patients [115]. The use of Rapamycin (mTORC1 inhibitor) has long been suggested as a therapeutic strategy for treatment of DS. The effects seen in the mTORC1 pathway response in our studies further support the use of mTORC1 inhibition as a therapeutic target for treatment of DS.

3. Effect of TSC/DYRK1A interaction on DYRK1A mediated cell cycle control

While the effects of the TSC-DYRK1A interaction on the functions of the TSC proteins was the first pathway that was looked at in this study, it is possible that TSC1 acts upstream of the DYRK1A signaling pathway and has an effect on the functions of DYRK1A kinase. Additionally, it was observed that TSC1 null MEFs showed an increased rate of proliferation and passed more quickly through the cell cycle as compared to the WT controls. In line with this hypothesis, in-vitro kinase assays with Tsc1 wt and null MEFs showed a decrease in LIN52 phosphorylation, a marker for DYRK1A kinase function. A decrease in the expression of DYRK1A was also observed in the Tsc1 null cells. However, since this effect could not be repeated in other cell lines including Tsc2 null MEFs, it was concluded that this effect was cell type specific. Moreover, the Tsc1 WT and Null MEFs were obtained and established from two separate mouse embryos. Thus, the differences observed in LIN52 phosphorylation and DYRK1A expression in these lines could just be cell line specific. In order to confirm whether this effect is indeed due to a loss of Tsc1, Tsc1 rescue cell lines can be created using the Tsc1 Null cells. These cells can then be tested for DYRK1A expression and activity. An increase in LIN52 phosphorylation in these cells would confirm that the effects seen are due to Tsc1, specifically, and not just cell line specific.

Interestingly, when U-2 OS cells with a knockdown of TSC1 were used for analyzing the effect of DYRK1A mediated cell cycle arrest, it was observed that TSC1 null cell lines were more resistant to DYRK1A mediated cell cycle arrest compared to control cells. This suggests that TSC1 may be

a new effector of DYRK1A induced cell arrest in addition to DREAM. However, this effect needs to be further confirmed with repeats of the cell cycle assay in U-2 OS cells and also by replicating it in other additional cell lines. As noted in case of the T98G cell lines above, it is important to note that U-2 OS cells contain a single copy of DYRK1A. Again, based on the dosage dependent nature of this protein, it is possible that these cell lines are much more sensitive to the downregulation of TSC1 as compared to other cell lines. Thus further studies should include both T98G (three copies of DYRK1A) and a non-transformed cell line such as BJ-hTERT in order to establish both the effect of TSC1 as well the effect of DYRK1A status on the effect of TSC1 down-regulation. Additionally, established DS cell line models can be utilized in order to obtain a more clinically relevant picture of the effect of TSC1 on DYRK1A mediated cell cycle arrest.

V. APPENDIX

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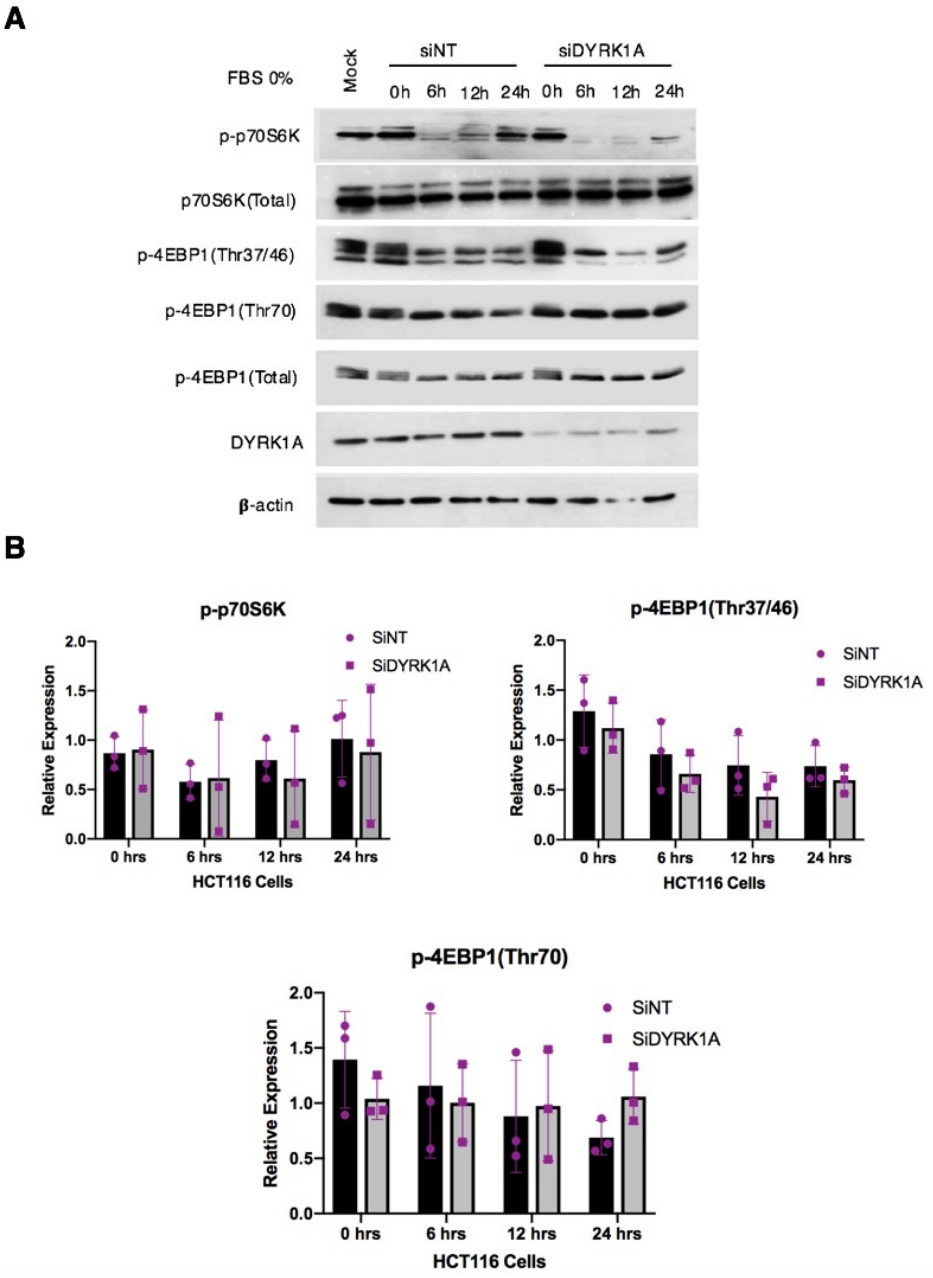
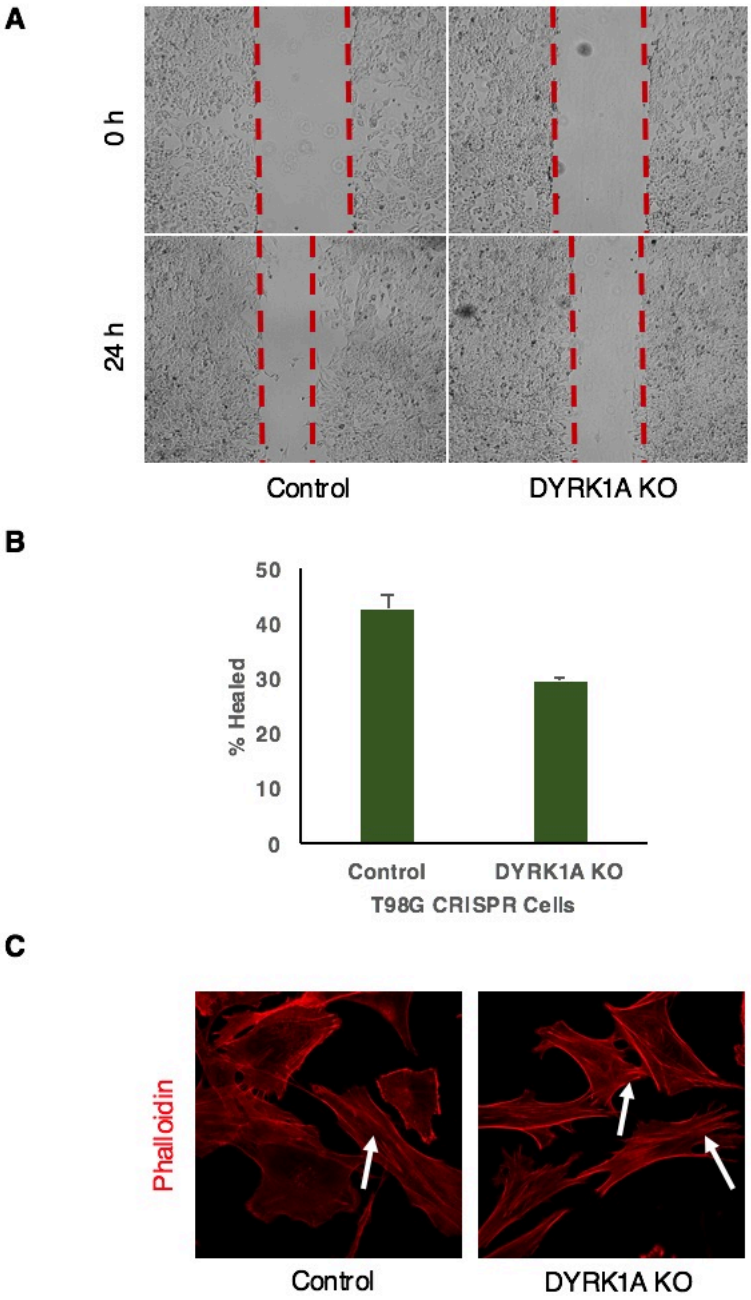


Supplementary Fig.1. Inactivation of DYRK1A does not have a major effect on mTORC1 signaling. **A:** HCT116 colorectal carcinoma cells were transiently transfected with siRNA oligo's and serum starved for the indicated time points. The mTORC1 pathway activity was measured by detecting phosphorylation of 4EBP1 and p70S6K using Western blotting**. B:** The graphs show the cumulative quantifications of three biological repeats for the expression of p-p70S6K and p-4EBP1 normalized to total protein levels.



Supplementary Fig.2. Effect of loss of DYRK1A on regulation of the actin cytoskeleton. A: Knockdown of DYRK1A in T98G cells causes a decrease in cell migration. Representative Images from Wound Scratch experiment to assess cell migration (N=3). Red dotted lines show the boundaries of the wound. **B:** Quantitation of the rate of wound-healing from 3 experiments. **C:** Representative images for staining of Control and DYRK1A KO T98G cells for actin fibers using Phalloidin (red).

Fragment	Peptide sequence	Sites	PhosphositePlus
1	SADVTtsPYADTQNSYGcATSTPYSTSR	T294; S295	No Yes
	VFGTTAGGKGtPLGTPATSPPPAPLIR	T386	Yes
	VFGTTAGGKGTPLGtPATsPPPAPLIR	S394	Yes
2	RGGFDsPYRD	S505	Yes
	LGPDtPK	T547	Yes
	DTmVtKLHSQIR	T773	No
	KtHsAAsssQGAsVNPEPLHSSLDK (1 site)	T519; S521; S524; S525; S526; S530;	Yes Yes No No No No
3	GssGsRGGGGsSSSSSELSTPEKPPHQR (1 site)	S1029; S1030; S1032; S1038	Yes Yes No Yes
	IPLNLDGPHPsPPtPDSVGQLHIMDYNE-THHEHS	S1141; T1144	Yes Yes
	WEttMGEAsAsIPTTVGSLPSSK (1 site)	T1065; T1066; S1071; S1073	No No No No

Supplementary Table 1. Compilation of Phosphosites on TSC1. Phospho-sites identified by Mass Spectrometry Analysis by Charles Lyons at Massey Caner Center, VCU.

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