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DYRK1A Interacts with the Tuberous Sclerosis Complex and Promotes mTORC1 Activity

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Abstract

DYRK1A, a ubiquitously expressed kinase, is linked to the dominant intellectual developmental disorder, microcephaly and Down syndrome in humans. It regulates numerous cellular processes such as cell cycle, vesicle trafficking and microtubule assembly. DYRK1A is a critical regulator of organ growth; however, how it regulates organ growth is not fully understood. Here, we show that the knockdown of *DYRK1A* results in reduced cell size, which depends on mTORC1. Using proteomic approaches, we found that DYRK1A interacts with the Tuberous sclerosis complex (TSC) proteins, namely TSC1 and TSC2, which negatively regulate mTORC1 activation. Further, we show that DYRK1A phosphorylates TSC2 at T1462, a modification known to inhibit TSC activity and promote mTORC1 activity. We also found that the reduced cell growth upon knockdown of DYRK1A can be rescued by overexpression of RHEB, an activator of mTORC1. Our findings suggest that DYRK1A inhibits TSC complex activity through inhibitory phosphorylation on TSC2, thereby promoting mTORC1 activity. Further, using the *Drosophila* neuromuscular junction as a model, we show that the *mnb,* the fly homologues of *DYRK1A*, is rescued by RHEB overexpression, suggesting a conserved role of *DYRK1A* in TORC1 regulation.

eLife assessment

This **fundamental** study identifies the kinase DYRK1A as a novel component of the tuberous sclerosis complex (TSC) protein complex, which is central to cellular growth and cell size. The findings presented here have broad implications for how cell size and growth is regulated. The methodology and analysis are **convincing** and support the findings.

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1. Introduction

The *Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A (DYRK1A)* is a ubiquitously expressed kinase that belongs to the CMGC group (Cyclin-dependent kinases, Mitogen-activated protein kinase, Glycogen synthase kinases and CDK-like kinases group). *DYRK1A*, is located within the Down syndrome Critical Region (DSCR). The phenotypes associated with Down syndrome (1), including abnormal neurodevelopment (2, 3), and increased susceptibility to acute megakaryoblastic leukaemia (4), have been reasoned to be due to an increased expression of *DYRK1A*. On the other hand, haploinsufficiency of *DYRK1A* causes DYRK1A syndrome, a rare autosomal dominant disease characterized by intellectual disability, intrauterine growth retardation, microcephaly and stunted growth (5–7). Mouse models heterozygous for *Dyrk1a* also show a reduction in brain and body size (8, 9). In *Drosophila*, loss of *mnb*, the *Drosophila* homolog of DYRK1A, results in reduced organ size, including brain, legs and wings (10, 11). Therefore, *DYRK1A* is a critical regulator of organ growth (12); however, the cellular processes by which *DYRK1A* promote organ growth are unclear.

DYRK1A and its homologs have also been implicated in many cellular processes, such as cell cycle, signal transduction, gene expression, vesicle trafficking and microtubule assembly (13). DYRK1A has been shown to extend the G1 phase of the cell cycle in neuroblastoma cells by phosphorylation and subsequent degradation of cyclin D1 (14). In glioblastoma cells, DYRK1A phosphorylates ubiquitin ligase CDC23, which mediates mitotic protein degradation, thereby promoting tumor growth (15). Further, DYRK1A expression positively correlates with EGFR levels, and DYRK1A inhibitors reduce EGFR-dependent glioblastoma growth (16). DYRK1A promotes the expression of several genes required for ribosomal biogenesis and protein translation (17, 18); for example, RPS6 ribosomal protein S6, a component of the 40S subunit, and translation initiation factor, EIF4A3 (19). However, if the regulation of these genes by DYRK1A is physiologically significant, it is currently unknown.

The Mechanistic Target of Rapamycin Complex 1 (mTORC1) is a key energy sensor and a master regulator of anabolic processes (20). The mTORC1 promotes anabolic processes such as protein, nucleotide and lipid synthesis and represses catabolic processes such as autophagy, thereby promoting cellular growth (21, 22). The mechanism of mTORC1-mediated regulation of multiple anabolic pathways has been studied extensively (20–22). The Tuberous Sclerosis Complex (TSC), consisting of TSC1, TSC2 and TBC1D7 (TBC1 domain family member 7), integrates various extracellular signals and negatively regulates mTORC1 complex activity (20, 23). TSC2 is a GTPase-activating protein (GAP) and promotes the conversion of GTP-bound RHEB (Ras Homolog Enriched in Brain) to the GDP-bound state, thereby inhibiting it. When TSC2 is inhibited, the GTP-bound RHEB activates mTORC1 (24, 25). The mTORC1 activation is controlled by extracellular stimuli, nutrients, energy status, and stress. For example, in response to insulin stimulation, AKT phosphorylates TSC2 at several sites, including S939 and T1462, suppressing its

inhibitory effect on RHEB-GTP recycling, thus activating mTORC1 (26). Similarly, in response to growth factors, Extracellular signal-Regulated Kinase (ERK) (27) and p90 Ribosomal S6 Kinase 1 (RSK1) phosphorylate TSC2 and activate mTORC1 (28). MK2 (MAP kinase-activated protein kinase 2/ MAPKAPK2) phosphorylates TSC2 at yet another site, S1210, in the presence of serum and leads to the inhibition of TSC2 activity, which allows the activation of mTORC1 (24). Catabolic signals, such as low energy levels during glucose scarcity, drive AMPK-mediated phosphorylation of TSC2 that promotes its GAP function and suppresses mTORC1 activity (29). Several kinases regulating the TSC activity underscores the importance of investigating novel kinases involved in its regulation, which may regulate mTORC1 activity in distinct cellular contexts.

In this study, we discover that DYRK1A interacts with TSC1 and TSC2. We show that DYRK1A phosphorylates TSC2 at T1462 and is a possible positive regulator of mTORC1 activity and cell growth. Further, we show that in *Drosophila*, both Mnb, the DYRK1A-homolog, and mTORC1 are required to promote Neuromuscular Junction (NMJ) growth. Moreover, the activation of mTORC1 can rescue the NMJ growth phenotype of *mnb* loss of function, suggesting Mnb may regulate mTORC1 to promote neuromuscular junction morphology. We propose that DYRK1A-mediated regulation of mTORC1 activity is a conserved mechanism to regulate cell size and development.

2. Results

2.1 DYRK1A promotes cell growth

To study the function of *DYRK1A*, we knocked down DYRK1A in multiple cell lines, including in HEK293 cells (18). We noticed that DYRK1A-depleted cells were smaller than the control cells. To explore if DYRK1A has a role in maintaining cell size, we performed a systematic analysis of the cell size phenotypes after transient knockdown of *DYRK1A* in HEK293 and observed a reduction in cell size (**Figure 1A-B**). Since mutations in *DYRK1A* are associated with stunted growth and microcephaly, we wondered if neuronal cells require *DYRK1A* to maintain proper cell size. Therefore, we knocked down *DYRK1A* in the human neuroblastoma cell line SH-SY5Y and observed a significant reduction in cell size (**Figure 1 C-D**). Further, we tested if the observed cell size phenotype is conserved in mice. We targeted *Dyrk1a* in NIH-3T3 using CRISPR/Cas9 and observed a significant reduction in cell size (**Figure 1E-F**). Our data suggests that *DYRK1A* promotes cell size across cell lines derived from different models and tissues. Thus, we tested if the overexpression of *DYRK1A* can promote cell size. Since strong overexpression of *DYRK1A* leads to cell cycle exit (2, 30, 31), we used the Doxycycline-inducible system to express low levels of *Flag-DYRK1A* in HEK293 cells (32). We noted a change in cell size with an increasing dosage of Doxycycline, which reached a saturation state at 100ng/ml Doxycycline in our system (**Supplementary figure 1**). We observed a significant increase in cell size at 40 ng/ml Doxycycline treatment for 48 hours when the *DYRK1A* mRNA levels were approximately twice that of control (**Figure 1G-H**). At this level of *DYRK1A* mRNA expression, overexpressed Flag-DYRK1A was undetectable by western blot. Overall, our knockdown and overexpression experiments in different cell lines, including both human and mouse, suggest that *DYRK1A* promotes cell size.

2.2 DYRK1A physically interacts with the Tuberous Sclerosis Complex

To understand how *DYRK1A* promotes cell growth, we investigated DYRK1A protein-interactors. Previously, we have identified protein-interactors of wild-type and kinase-dead DYRK1A (K188R) from HEK293 cells using affinity purification and mass spectrometry (18, 32). In these purifications, we have observed significant enrichment of DCAF7, ARIP4, RB1, EP300, CREBBP, TRAF2, TRAF3, FAM53C, RNF169, many of which have also been identified by many other groups as bona-fide DYRK1A interacting proteins (33–37). We also observed significant enrichment

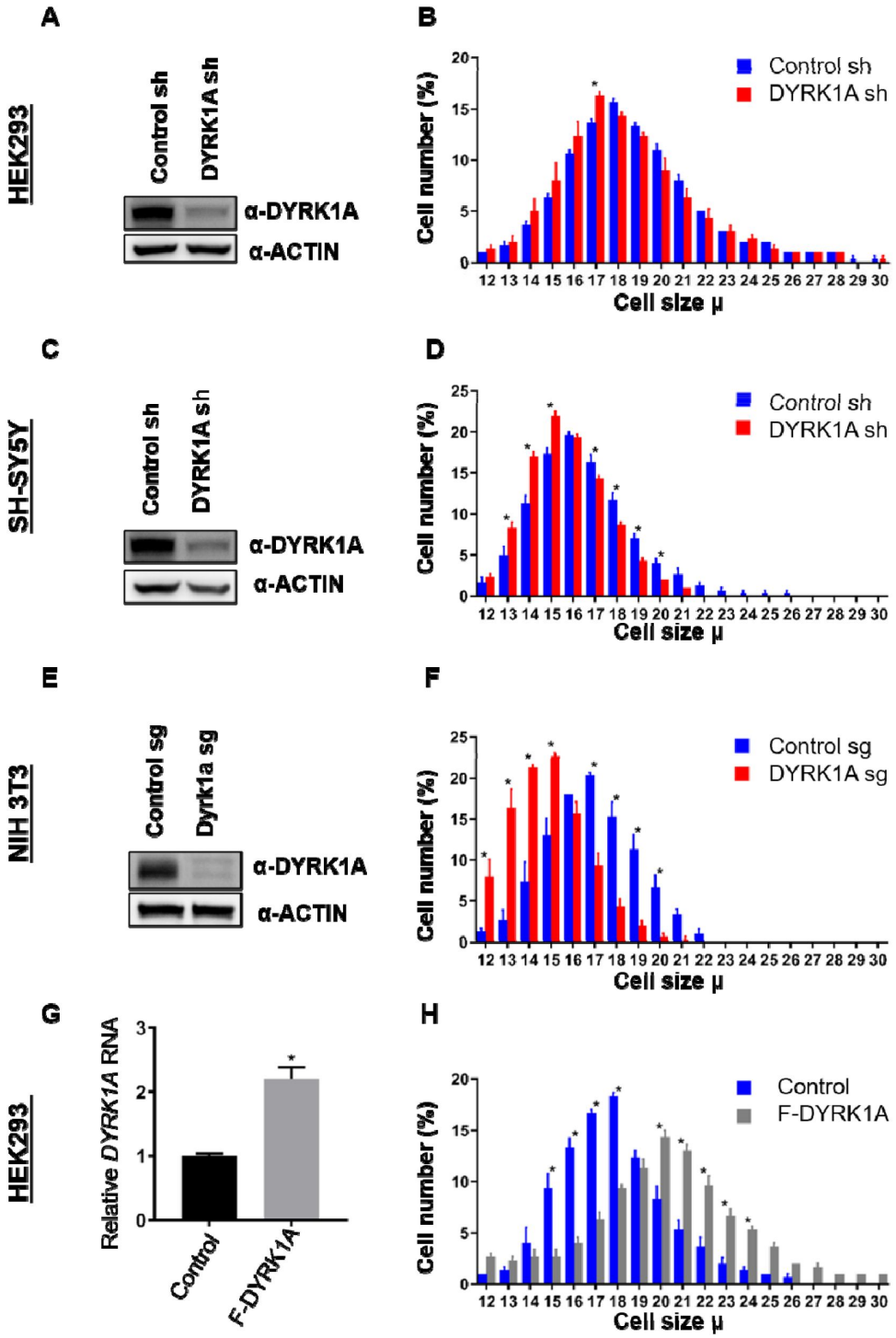

Figure 1.

DYRK1A regulates cell size

shRNA-mediated knockdown of *DYRK1A* was performed in **(A-B)** HEK293 and **(C-D)** SH-SY5Y cells using lentivirus. Transduced cells were selected for four days before analysis. Western blot shows the efficiency of *DYRK1A* knockdown. **(E-F)** NIH3T3 cells were treated with *Dyrk1a*-targeting sgRNA expressing lentivirus and selected for four days before analysis. Western blot shows the efficiency of *DYRK1A* knockdown. **(G-H)** HEK293 cells expressing *Flag-DYRK1A* and the parental cells were treated with 40ng/ml Doxycycline for 48 hours and analyzed for cell size. (G) Overexpression was analyzed by qRT-PCR. GAPDH mRNA was used to normalize RNA in q-RT-PCR samples. Data represent the mean ± SD (n = 3 biological replicates).

of TSC components TSC1 and TSC2 among proteins co-purified with wild-type and kinase-dead DYRK1A (K188R) (**Figure 2A**). Interestingly, a few peptides of TBC1D7, the third component of TSC, were also detected in the wild-type pull-down (see dNSAF values in **Figure 2 A**), suggesting that DYRK1A interacts with the TSC complex. This interaction is intriguing as TSC is a negative regulator of the mTORC1, which is known to promote cell growth (38–40).

To ascertain the interaction between DYRK1A and TSC, we expressed *Flag-DYRK1A* in HEK293 cells through the Doxycycline-inducible system and performed Flag affinity purification. Surprisingly, when we used HEPES buffer containing either 0.5% NP40 or 1% 5 Tween 20, we did not detect co-purification of TSC2 with Flag-DYRK1A (data not shown). However, when we prepared extracts by lysing the cells in hypotonic buffer without detergent followed by Dounce homogenization, we found that both TSC1 and TSC2 co-purify with Flag-DYRK1A (**Figure 2B**). Further, we tested the interaction between endogenous DYRK1A and TSC by immunoprecipitating DYKR1A; we observed co-purification of both TSC1 and TSC2 (**Figure 2C**). Immuno-affinity purification of overexpressed HA3-TSC1 and Flag-TSC2 was also able to pull down endogenous DYRK1A (Supplementary Figure 2). These results confirmed that DYRK1A physically interacts with the TSC complex. We then sought to identify the domains of DYRK1A that interact with TSC1, as TSC1 is the scaffolding protein in the TSC (20). We co-overexpressed HA3-TSC1 and various truncated forms of Flag-DYRK1A in HEK293 cells (**Supplementary Figure 3**). We found that interaction between DYRK1A and TSC1 was abolished in all the truncated forms that lack DYRK1A kinase domain, which suggests that DYRK1A kinase domain is required for the interaction (**Supplementary Figure 3**). We further confirmed that DYRK1A interacts with TSC1 through its kinase domain by co-expressing the FLAG-tagged DYRK1A kinase domain and HA3-TSC1, followed by immunoprecipitation using anti-FLAG antibodies (**Figure 2D**). Taken together, our results show that DYRK1A physically interacts with the TSC.

2.3 DYRK1A promotes mTORC1 activity

Next, we sought to test the implications of TSC and DYRK1A interaction on mTORC1 activity. mTORC1 is known to be induced in HEK293 cells by treatment with fetal bovine serum (FBS), insulin, and many growth factors following serum starvation (21, 22, 41, 42). We performed similar experiments, where we knocked down *DYRK1A* followed by serum starvation and tested for levels of pS6K, a reporter for mTOR activity. We did not observe a significant reduction in basal phosphorylation levels on S6K and S6. However, 10 and 20 min after FBS treatment, we observed significantly reduced levels of Phospho-S6K and Phospho-S6 in *DYRK1A* KD compared to control HEK293 cells (**Figure 3A-C**). Further, we tested the levels of pS6K and pS6 in NIH3T3 after CRISPR-mediated targeting of *Dyrk1a*. Interestingly, we found significant reductions in pS6K and pS6 compared to controls (**Figure 3 D-F**). Overall, our results indicate that DYRK1A is required to promote mTORC1 activity.

2.4 DYRK1A phosphorylates TSC2 at T1462

Next, we investigated if DYRK1A phosphorylates TSC1/TSC2, which is known to regulate mTORC1 activity. For this, we first knocked down *DYRK1A* in HEK293 cells, co-expressed *HA3-TSC1* and *Flag-TSC2* and performed immunoprecipitation followed by LC/tandem MS (MS/MS) to identify the phosphorylation status of TSC1 and TSC2 in these cells. We observed that *DYRK1A* knockdown samples have reduced TSC2 phosphorylation at T1462 (T1462) compared to the control samples. T1462 has been previously identified as a site phosphorylated by various kinases that activate the mTORC1 pathway, including AKT (26), ERK (27), p90 ribosomal S6 kinase 1 (RSK1) (28), IκB kinase β (IKKβ) (43), and MAPKAPK2 (MK2) (24) in response to insulin and other growth factors. To ascertain that indeed, TSC2 T1462 is the site phosphorylated by DYRK1A, we performed *in-vitro* kinase assays using purified wild-type and kinase-dead DYRK1A. As TSC2 is about 200KDa protein, we purified transiently overexpressed HA3-TSC1 and Flag-TSC2 from HEK293 cells and performed *in vitro* kinase assay on beads. Wild-type DYRK1A autophosphorylates and runs as

A DYRK1A Protein Interactions

	FLAG-DYRK1A		FLAG-DYRK1A-KD		FLAG-Control (x2)
Proteins	Peptides	dNSAF	Peptides	dNSAF	Peptides
DYRK1A	39	0.0174	35	0.0472	0
DCAF7	27	0.1925	23	0.1850	0
ARIP4	43	0.0033	20	0.0020	0
RB1	14	0.0020	18	0.0075	0
EP300	26	0.0009	31	0.0038	0
CREBBP	23	0.0004	34	0.0012	0
TRAF2	15	0.0056	13	0.0055	0
TRAF3	6	0.0008	12	0.0049	0
FAM53C	14	0.0137	11	0.0107	0
RNF169	7	0.0011	1	0.0002	0
TSC1	11	0.0007	23	0.0039	0
TSC2	6	0.0002	33	0.0048	0
TBC1D7	2	0.0005	0	0	0

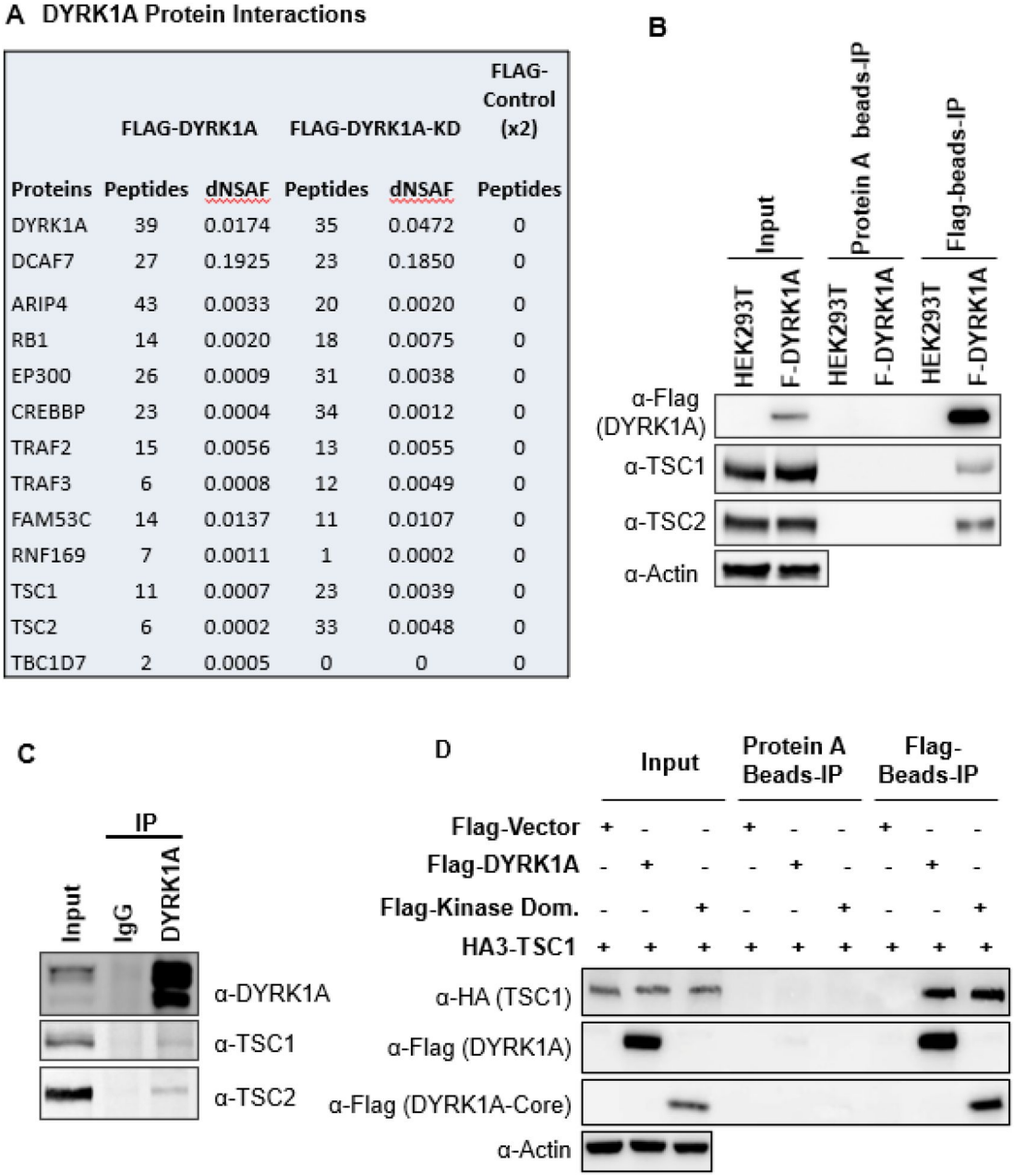

Figure 2.

DYRK1A interacts with the Tuberous Sclerosis Complex

(A) The MS/MS datasets previously acquired by MudPIT analyses of FLAG-DYRK1A affinity purifications and negative FLAG controls (18) were searched against the most recent releases of the human protein sequence databases (built by collating and removing redundant entries from NCBI *Homo sapiens* RefSeq *GCF_000001405.40_GRCh38.p14* and *GCF_009914755.1_T2T-CHM13v2.0*). Highly enriched proteins include known and novel DYRK1A-interacting partners and are reported with their peptide counts and distributed Normalized Spectral Abundance Factor (dNSAF) values, which reflect their relative abundance in the samples (57). **(B)** Flag beads were used to pull-down Flag-DYRK1A from whole cell extracts of HEK293 transfected with Flag-DYRK1A, Protein A beads were used as a control. The blots were probed with TSC1 and TSC2 antibodies. Actin was used to normalize the lysates inputs. **(C)** endogenous DYRK1A was immunoprecipitated with DYRK1A antibody from HEK293 cytoplasmic fraction generated using the Dignam protocol (18) and probed with antibodies against endogenous DYRK1A, TSC1 and TSC2. Rabbit IgG was used as the IP control **(D)** Flag-DYRK1A and Flag-DYRK1A kinase domain constructs were affinity purified using Flag-beads from HEK293 cells co-transfected with HA3-TSC1 and probed with α-HA and α-Flag antibodies. Actin was used as the loading control.

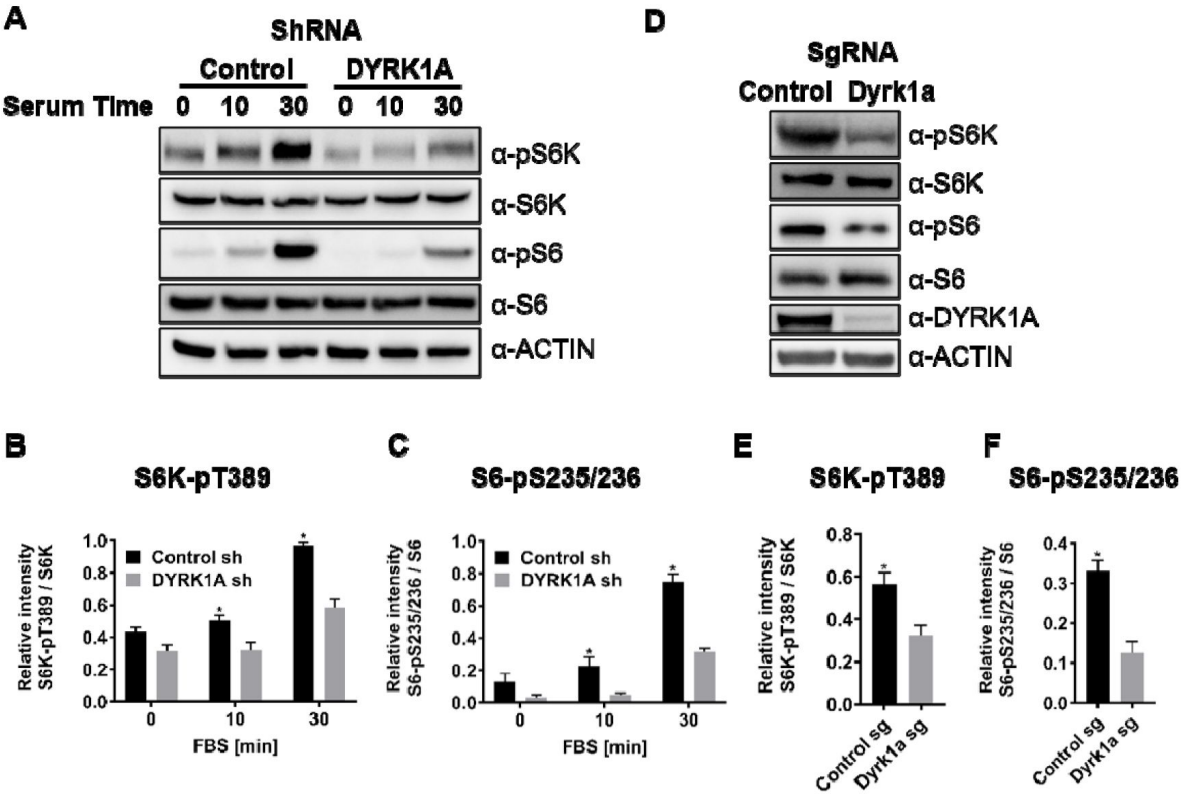

Figure 3.

DYRK1A promotes the activation of mTORC1 pathway in human and mouse cells

(A) HEK293 cells treated with *DYRK1A* shRNA or control shRNA were serum starved for 12 hours before being activated with serum for the indicated times. Cells were then harvested, lysates and probed with the indicated antibodies. Actin was used as the loading control. **(B - C)** Quantification of proteins in A, levels of pS6K (T389), S6K, pS6 (pS235/236) and S6 were quantified using Image J software and the ratio of pS6K/S6K and pS6/S6 were plotted (n = 3 biological replicates). **(D)** NIH3T3 cells were treated with sgRNA-targeting *Dyrk1a* or non-targeting control and selected for four days with Puromycin before harvesting. Lysates were probed with indicated antibodies. **(E - F)** Quantification of proteins in D, levels of pS6K (T389), S6K, pS6 (pS235/236) and S6 were quantified (as described for B and C) and ratios were plotted (n = 3 biological replicates). Student's *t* tests were done to compare samples. *p* value = * ($p < 0.05$).

fuzzy bands around 90-100Kda in polyacrylamide gels, whereas kinase-dead DYRK1A runs only as a single sharp band (**Figure 4A**). In our assay, we did not observe an increase in phosphorylation at S1387 or Serine 939 in the presence of ATP and DYRK1A (**Figure 4A**). However, immunopurified TSC2 is heavily phosphorylated at Serine 1387 and Serine 939, which are two important sites that are phosphorylated by AKT(26, 41, 43). Therefore, we cannot rule out if the immunopurified TSC2 was fully phosphorylated at S1387 and S939. However, it is important to note that we did not observe any differential loss of phosphorylation in TSC2 at S1387 in our mass spectrometry phosphorylation analysis. Interestingly, we found T1462 was strongly phosphorylated by wild-type DYRK1A, not by kinase-dead DYRK1A (**Figure 4A**). Taken together, these experiments demonstrate that DYRK1A phosphorylates TSC2 on T1462. This phosphorylation is known to be an inhibitory modification and it is a key site that gets phosphorylated by various other key regulators of the mTORC1 pathway (43). We could not perform in-vitro tests to check if DYRK1A phosphorylates TSC1 or other sites on TSC2 due to the lack of appropriate antibodies. The overall data suggest that DYRK1A mediates TSC2 T1462 phosphorylation to inhibit TSC activity, promoting TORC1 activity.

2.5 RHEB overexpression can rescue cell growth phenotype of *DYRK1A* knockdown

TSC phosphorylation has been shown to inhibit TSC, and activate mTORC1 via RHEB, where the activation of TSC leads to the hydrolysis of GTP-bound RHEB, which in turn leads to mTORC1 inhibition (20). We hypothesized that DYRK1A-mediated phosphorylation of TSC2 may promote mTORC1 activation in a RHEB-mediated manner. Therefore, we reasoned that overexpression of *RHEB* will rescue the cell size phenotype caused by *DYRK1A* knockdown. We overexpressed *RHEB* in *DYRK1A* knockdown cells and found a partial rescue of cell size, suggesting that DYRK1A indeed plays a role in cell size regulation at least partly upstream to RHEB (**Figure 4B** and **C**). Further, inhibition of mTOR with either Rapamycin or Torin1 rescued the increased cell size phenotype mediated by overexpression of *DYRK1A* in the inducible HEK293A-Flag-DYRK1A model (Supplementary Figure 4). These data suggest that DYRK1A phosphorylates TSC2, which inhibits its action, leading to the activation of mTORC1 via RHEB.

2.6 *Drosophila mnb* mutants show reduced neuromuscular junctions, which can be rescued by RHEB overexpression

Since the TSC-TOR pathway is conserved in *Drosophila* (44), we sought to test if the DYRK1A-mediated regulation of the TSC-TOR pathway is also conserved. The TOR pathway is shown to promote neuromuscular junction (NMJ) development in flies (45, 46) (**Figure 5B, G**). Similarly, *mnb*, the fly homolog of DYRK1A, is also required for NMJ growth (47); however, the mechanism of *mnb*-mediated NMJ growth is unknown. Based on our finding that *DYRK1A* promotes TOR activity, we hypothesized that *mnb* promotes larval NMJ growth through TOR. To test this hypothesis, we systematically compared the NMJ growth phenotype of *mnb* mutant with that of loss or gain of TOR activity at larval NMJ. Since TOR mutants are early larval lethal, we expressed dominant-negative TOR (*TOR.Ted*) in motor neurons using the UAS-Gal4 system (*D42Gal4>UAS-TOR.Ted*) (48). To activate the TOR pathway, we overexpressed *RHEB* in motor neurons (*D42Gal4>UAS-RHEB*) (49). We stained NMJ using anti-HRP (to mark neurons) and anti-DLG (to mark boutons where synaptic connections are formed). As shown in **Figure 5 (A, B, G)**, the loss of *mnb* causes a reduction in the NMJ bouton numbers compared to the wild-type control (WT, Canton S)— similarly, the expression of TOR.TED in motor neurons (*D42Gal4>UAS-Tor.Ted*) also causes a reduction in NMJ bouton numbers (Supplementary Figure S5 D-F). In contrast, overexpression of *mnb* in motor neurons increases the bouton numbers (**Figure 4 C, D, H**). Similarly, the activation of the TOR pathway by overexpression of *RHEB* in motor neurons or mutations in *gigas* (*gig*, the fly homolog of *TSC2*) increases the number of boutons (**Fig 5 E, F, I**, Supplementary Figure S5 C). The similarities in the TOR and *mnb* phenotypes suggest that Mnb

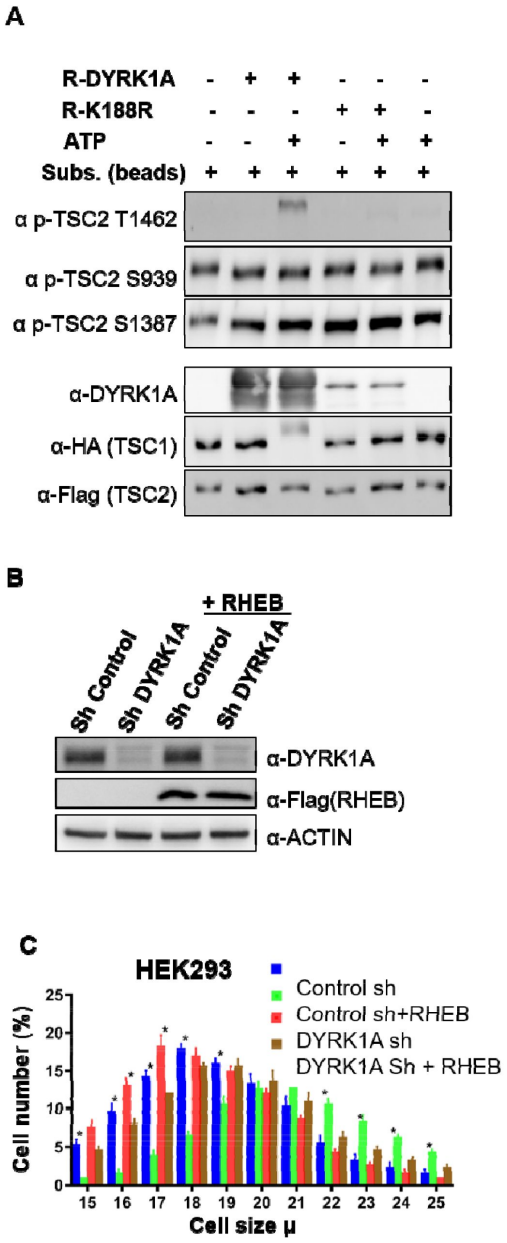

Figure 4.

DYRK1A phosphorylates TSC2 at T1462 *in vitro*, and RHEB overexpression rescues mTORC1 activity in cells

(A) An in-vitro kinase assay was performed using DYRK1A and kinase-dead DYRK1A (K188R) that were purified from bacteria. Flag-TSC2 and HA3-TSC1 were co-expressed in HEK293 cells and purified using a combination of (1:1) of HA and Flag beads. Beads were equilibrated with kinase assay buffer before the reactions were initiated on beads. After incubation for 30 min at 30°C, reactions were stopped by the addition of SDS loading buffer. Since bacterially purified DYRK1A is autophosphorylated, it exhibits a fuzzier signal, whereas kinase-dead DYRK1A is incapable of phosphorylation and appears as a sharp signal. (B-C) *RHEB* overexpression partially rescues the size of HEK293 cells. HEK293 cells were first transduced with shRNA lentivirus targeting DYRK1A or control and selected with 1 ug/ml Puromycin for three days, after which they were re-transduced with lentivirus expressing Flag-RHEB. The concentration of Puromycin was raised to 2ug/ml for the next 48 hours in order to select for the second round of transduction. **(B)** shows knockdown efficiency of DYRK1A and overexpression of RHEB. (C) Lower panel shows cell size analysis. Data represent the mean ± SD (n=3 biological replicates). Student's t-test was done to compare samples. Significant difference in p value = * ($p < 0.05$)

may positively regulate TOR activity and promote NMJ growth. To test this hypothesis, we asked whether or not the NMJ phenotype of the *mnb* mutant is rescued by the activation of TOR. As shown in **Figure 5L**, overexpression of *RHEB* rescues the NMJ bouton numbers in *mnb* mutants. These results indicate that *RHEB* functions downstream to *mnb* to regulate the NMJ development. Taken together, the results in mammalian cells and flies suggest that the role of DYRK1A/Mnb in regulating the TOR pathway is conserved.

3 Discussion

This work shows that DYRK1A promotes cell size as a reduction in *DYRK1A* levels in both human and mouse cells results in small cell size (**Figure 1A-D**). mTORC1 is a key energy sensor and a master regulator of cell growth (21, 22). We found that reduced levels of *DYRK1A* in cells result in decreased S6K and S6 phosphorylation, suggesting that DYRK1A positively regulates mTORC1 (**Figure 3**). One of the key regulators of mTORC1 is the TSC complex, consisting of TSC1, TSC2 and TBC1D7 (TBC1 domain family member 7), which integrates various extracellular signals and negatively regulates mTORC1 complex activity (20, 23). DYRK1A physically interacts with TSC complex through its kinase domain (**Figure 2**). TSC2 is a GTPase-activating protein (GAP) that promotes the conversion of GTP-bound RHEB to the GDP-bound state, thereby inhibiting it. When TSC2 is inhibited, the GTP-bound RHEB activates mTORC1 (25, 41). Phosphorylation at TSC2 at T1462 has been shown to inhibit TSC and promote TORC1 activity through RHEB (43, 50). Our study, for the first time, shows that DYRK1A phosphorylates TSC2 at the T1462 site, thereby promoting the mTORC1 pathway. We also show that the overexpression of *RHEB* could partially rescue the cell growth phenotypes exhibited by *DYRK1A* knockdown cells, indicating that the DYRK1A works upstream to RHEB.

In DYRK1A syndrome, loss of one copy of *DYRK1A* leads to stunted growth and microcephaly in humans (7). A recent report showing a reduction in mTOR signaling in mice cortex lacking *Dyrk1a* indicates that the DYRK1A-mTORC1 axis may regulate brain development (51). In *Drosophila*, *mnb* is widely expressed in the developing central nervous system and has been linked to post-differentiation roles in neurons. For example, in flies, *mnb* has been linked to long-term memory formation (52). In humans and mice, *DYRK1A* has been linked to the neurite spine morphogenesis, and it has been suggested that the neurite spine phenotype may contribute to intellectual disability in Down syndrome (53–55). The phenomenon of NMJ morphogenesis in fruit flies is analogous to neurite spine morphogenesis. The TOR pathway is shown to promote neuromuscular junction (NMJ) development in flies (45, 46) (**Figure 5B, G**). Similarly, *mnb* is also required for NMJ growth (47); however, the mechanism of *mnb*-mediated NMJ growth was unknown. Our results show that the reduction in NMJ size in *mnb* mutants can be rescued by *RHEB* overexpression, suggesting that Mnb positively regulates mTORC1. Our results are also supported by a study in *Arabidopsis* that showed YAK1, a DYRK kinase, regulates meristem activity through the mTOR pathway (56). Overall, our results suggest that the role of Mnb/DYRK1A in the regulation of mTORC1 pathway is evolutionarily conserved, and the DYRK1A-mTORC1 axis plays a crucial role in neuronal morphogenesis and cellular growth.

Methods

Expression plasmids and cell culture

pcDNA3 -HA3-TSC1 (Yue Xiong; Addgene #19911), pcDNA3 Flag TSC2 (Brendan Manning: Addgene #14129), LentiCRISPR v2 (Feng Zhang; Addgene #52961) plasmids were procured from Addgene. For expressing cDNA using lentivirus, LentiCRISPR v2 was modified by removing the U6 promoter and tracer RNA, and cDNA were cloned in place of SpCas9, and named LentiExp vector. *RHEB*

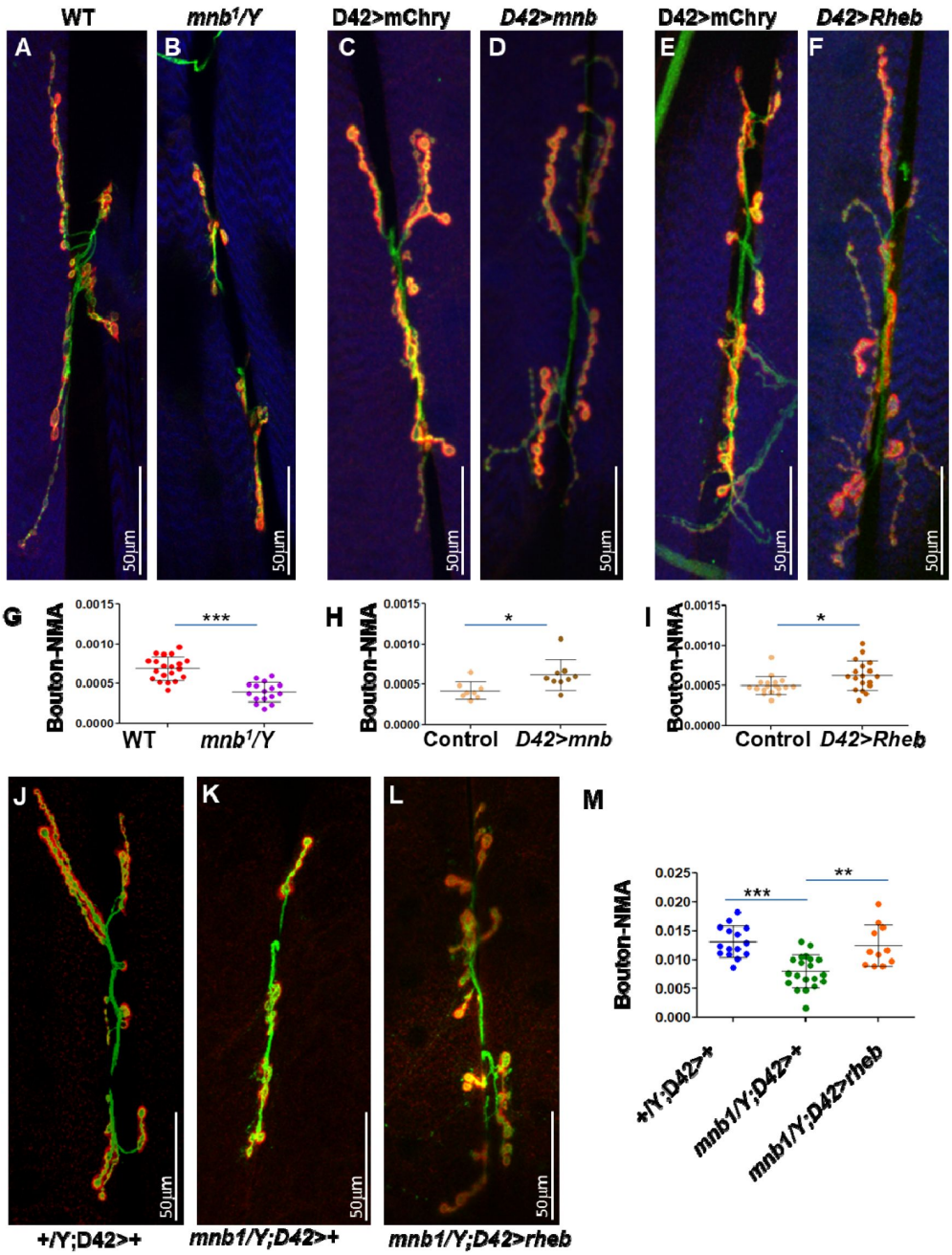

Figure 5.

mnb mutant phenotype can be rescued by TOR activation in flies

(A-F and J-L) 3rd instar larval NMJ (muscles 6/7) were stained using anti-HRP (Green) and anti-Dlg (Red). Muscles are stained with phalloidin (Blue, **A-F**). HRP (green) stains the entire neuron and Dlg (red) stains only boutons (Red+Green). **(G-I, M)** Quantification of bouton numbers, normalized to muscle area (Bouton-NMA). Error bars represent standard deviation. Statistical significance (*p*-values: *** < 0.001; ** < 0.01; * < 0.05) is calculated by unpaired student's t- test. **(A, B, G)** mnb^1 alleles show fewer boutons numbers as compared to wild-type (WT, Canton S) control **(B)**. Data are quantified in **G**. **(C, D, H)** *mnb* overexpression (*D42-Gal4*>UAS-*mnb,* **D**) increases bouton numbers as compared with mCherry overexpression (*D42-Gal4*>UAS-mCherry, Control, C). D42-Gal4 is a motor-neuron specific driver. Data are quantified in H. **(E, F, I**) Rheb overexpression (*D42-Gal4*>UAS-Rheb, F) increases bouton numbers as compared with mCherry overexpression (*D42-Gal4*>UAS-mCherry, Control, **E**). Data are quantified in **I**. **(J-M)** *Rheb* overexpression in *mnb* mutant (mnb^1/Y *D42-Gal4*>UAS-Rheb, **L**) suppressed bouton phenotype as compared to *mnb* mutant (mnb^1/Y *D42-Gal4*/+, **K**). Wild type is heterozygous *D42-Gal4* (+/Y; D42-Gal4/+, J). Data is quantified in **M**.

sequence from pRK7-RHEB (John Blenis; Addgene #15888) was cloned into the LentiExp vector by replacing SpCas9. Expression was confirmed by western blot analysis. *DYRK1A* expression vector, *DYRK1A* shRNA, and control shRNA have been previously described (18). All constructs were confirmed by DNA sequencing. Expression of Flag-tagged proteins was induced with 250ng/ml Doxycycline for 36 to 48 h. HEK293, HEK293T and NIH3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). All cell lines were obtained from the American Type Culture Collection (ATCC). Initially frozen stocks were tested for mycoplasma after thawing. Cell lines were maintained at 37°C under 5% CO_2.

Antibodies

Antibodies against Actin (AC026) and HA (AE008) were from Abclonal, anti-DDDDK from MBL (M185-3L), TSC1 (4906), TSC2 (4308), S6K (2708), pS6K (9205), S6 (2317), pS6 (4856) were from CST. DYRK1A antibody was generated in house and has been described before (18).

Cell extracts and Immunoblotting

For immunoblotting, total cell extracts were prepared. Briefly, collected cells were washed once with cold PBS and lysed in cold NP-40 lysis buffer (40mM HEPES, pH 7.4, 120mM NaCl, 1mM EDTA, 1% NP-40 [Igepal CA-630], 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 0.5 mM sodium orthovanadate and 1:100 protease inhibitors [Solarbio, P6730]) for 15 min on ice. Lysates were cleared by centrifugation, and protein concentration was measured using BCA kit. Western blot signals were quantified using ImageJ.

shRNA mediated knock down and overexpression of RHEB

Short hairpin RNA (shRNA) targeting *DYRK1A* have been previously described (18). Lentiviral particle preparation and infection were performed as previously described (18). Lentivirus-infected HEK293 and SH-SY5Y cells were selected with 1 µg/mL(HEK293) or 2 µg/mL (SH-SY5Y) Puromycin respectively for four days.

For *RHEB* overexpression rescue of *DYKR1A* shRNA-mediated knockdown HEK293 cells, cells were first infected with lentivirus expressing shRNAs targeting control and *DYRK1A*, and then selected with 1µg/mL Puromycin for 48 hours. After selection cells were transduced with lentivirus expressing *Flag-RHEB*. These cells were further selected with 2µg/mL Puromycin for 48 hours before harvesting.

Serum starvation

shRNA treated HEK293 cells selected for 4 days with 1ug/ml puromycin were washed once with PBS and then DMEM without serum added. Serum starvation was performed for 12 hours. For stimulation with FBS (10%), pre-warmed FBS was directly added to the serum-free media for the time periods indicated.

CRISPR/Cas9 mediated *Dyrk1a* knockdown

NIH3T3 cells were transduced with lentiCRISPR v2 (Addgene plasmid #52961) containing either no sgRNA (control) or sgRNA targeting *Dyrk1a* (58). Transduced cells were selected with 3µg/mL Puromycin for 4 days before harvesting. sgRNA targeting mouse *Dyrk1a* is provided in supplemental table S1.

Cell size measurements

Cell size was measured and analyzed with a JIMBIO FIL electronic cell counter following the manufacturer's protocol. In short, 2.5×10^5 cells were seeded in 6-well plates. After 24 hours of culture, cells were trypsinized and taken up in full DMEM. Each cell line was measured three times; each measurement comprised three cycles of cell counts with intervals of 1 µ ranging from

4 to 30 µ. The sum of counts of viable cells in the range of 12 to 30 µm was plotted and quantified. Three biological replicates were performed per cell type (i.e., HEK293, SH-SY5Y and NIH3T3 cells [KD/KO and corresponding Control]). Control and corresponding KD/KO were compared with multiple unpaired t-tests. *p* values are presented as stars above the corresponding bar graphs; *, $p < 0.05$. HEK293 *Flag-DYRK1A* cell size was measured as above, except cells were induced with 40ng/mL Doxycycline for 48 hours.

Quantitative reverse transcription polymerase chain reaction (q-RT-PCR) assays

2.5×10^5 cells were seeded in 6-well plates. After 48 hours of 40ng/mL Doxycycline induction, total RNA from fresh cells were extracted using RNA-easy Isolation Reagent (Vazyme) as per the manufacturer's instructions. After determining the total RNA concentration and quality using a Nanodrop (Thermofisher), 1 µg RNA was used in 20 µL reaction mixture to reverse transcribe RNA using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme R212) and stored at –20˚C. 2µL was used to perform qPCR using ChamQ Universal SYBR qPCR Master Mix (Vazyme Q711) with 10 µM forward and reverse gene specific primers. The cycling consisted of 2 min at 95˚C, followed by 40 cycles of 5 s at 95˚C and 10 s at 60. Following completion of the final cycle, a melting curve analysis was performed to monitor the purity of the PCR products. Each sample was analyzed in triplicate. Quantification was performed by means of the comparative Ct method ($2^{-\ CT}$). The mRNA expression of target genes was normalized to that of GAPDH, and the data are represented as the mean ± SEM of biological replicates.

Mass Spectrometry Analysis for identification of phosphorylated peptides of TSC1/TSC2

Protein precipitation and digestion: Proteins were precipitated with TCA (trichloroacetic acid). The protein pellet was dried in Speedvac. The pellet was subsequently dissolved with 8 M urea in 100 mM Tris-Cl (pH 8.5). TCEP (final concentration is 5 mM; Thermo Scientific) and Iodoacetamide (final concentration is 10mM; Sigma) were added to the solution and incubated at room temperature for 20 and 15 minutes for reduction and alkylation respectively. The solution was diluted four times and digested with Trypsin at 1:50 (w/w; Promega).

LC/tandem MS (MS/MS) analysis of peptide: The peptide mixture was loaded on a home-made 15 cm-long pulled-tip analytical column (75 µm i.d.) packed with 3µm reverse-phase beads (Aqua C18, Phenomenex, Torrance, CA) connected to an Easy-nLC 1000 nano HPLC (Thermo Scientific, San Jose, CA) for mass spectrometry analysis. Data-dependent tandem mass spectrometry (MS/MS) analysis was performed with a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA). Peptides eluted from the LC system were directly electrosprayed into the mass spectrometer with a distal 1.8-kV spray voltage. One acquisition cycle includes one full-scan MS spectrum (m/z 300-1800) followed by top 20 MS/MS events, sequentially generated on the first to the twentieth most intense ions selected from the full MS spectrum at a 27% normalized collision energy. MS scan functions and LC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific).

Data Analysis

The acquired MS/MS data were analyzed against a UniProtKB *Homo sapiens* database using Integrated Proteomics Pipeline (IP2, *http://integratedproteomics.com/*). In order to accurately estimate peptide probabilities and false discovery rates, we used a decoy database containing the reversed sequences of all the proteins appended to the target database. Carbamidomethylation (+57.02146) of cysteine was considered as a static modification and lysine phosphorylation (+79.9663) of STY as a variable modification.

Flag Immunoprecipitation

With detergent: 1×10^6 cells were seeded in 10 cm plates. After 24 hours of culture, cells were co-transfected with 5 g pcDNA-*Flag-DYRK1A*/deletion and 5µg *HA-TSC1* using PEI. After 48h, collected cells were washed once with cold PBS, and lysed in cold NP-40 lysis buffer (40mM HEPES, pH 7.4, 120mM NaCl, 1mM EDTA, 1% NP-40 [Igepal CA-630], 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 0.5 mM sodium orthovanadate and 1:100 protease inhibitors [Solarbio, P6730]) for 15 min on ice. Lysates were cleared by centrifugation, and protein concentration was measured using BCA kit. The supernatant was then incubated with Flag beads (Smart-Lifesciences, SA042005) for ~4 h at 4 Finally, beads were washed with a lysis buffer without NP-40 three times and resuspended in a 1× SDS loading buffer. Samples were heated for 10 min at 95 and separated by SDS-PAGE.

Overexpressed *DYRK1A* immunoprecipitation without detergent

Approximately 10^8 HEK293-*Flag-DYRK1A* (with 250ng/ml Doxycycline treatment), and parental cells were induced for 48h were collected and washed with PBS. Cells were swollen for 15 min in hypotonic buffer (Buffer A: 10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT supplemented with freshly prepared protease inhibitors, Solarbio, P6730). Swollen cells were dounced 20 times in a Wheaton Dounce homogenizer till about 90% of cells were lysed (as observed in a microscope). Lysates were then centrifuged at 5000-6000 g for 10 min at 4. The supernatant was transferred to a new tube, and 0.11 volume of Buffer B (0.3M HEPES pH 7.9, 1.4M KCl, 0.03M $MgCl_2$) was added; the supernatant was then centrifuged at 12000g for 30 min. The supernatant was transferred to a new tube and incubated with Flag-beads (Smart-Lifesciences; SA042005) for ~4 h at 4. Finally, beads were washed three times with wash buffer containing 150 mM NaCl and 0.2% Triton X-100 and resuspended in a 1× SDS loading buffer and analyzed by SDS-PAGE.

Endogenous *DYRK1A* immunoprecipitation without detergent

Approximately 10^8 HEK293 cells were swollen for 15 min in hypotonic buffer (Buffer A: 10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT supplemented with freshly prepared protease inhibitors, Solarbio, P6730). Swollen cells were dounced 20 times in a Wheaton Dounce homogenizer and centrifuged at 16000g for 30 min. Supernatant was transferred to a new tube, and 0.11 volume of Buffer B (0.3M HEPES pH 7.9, 1.4M KCl, 0.03M $MgCl_2$) was added and incubated with Protein A/G magnetic beads (Smart-Lifesciences, SM015001) for ~4 h at 4. Finally, beads were washed three times with a wash buffer containing 150 mM NaCl and 0.2% Triton X-100 and resuspended in a 1× SDS loading buffer and analyzed by SDS-PAGE.

Kinase Assay

GST-DYRK1A and GST-DYRK1A K188R were cloned in pGEX-6p1vector, proteins were expressed in BL21 strain and purified using GST beads. FLAG-TSC2 and HA3-TSC1 were co-expressed in HEK293 cells, and Flag-Affinity purified. After wash, beads were rinsed with 1× kinase assay buffer (25 mM HEPES, pH 7.0, 5 mM MgCl2, 0.5 mM DTT), and kinase assay was performed on beads bound with FLAG-TSC2 and HA-TSC1. Equal amounts of GST-DYRK1A or GST-K188R were added to the Flag beads in presence/absence of ATP (100µM), and incubated at 30°C for 30 min. Reactions were stopped by addition of 5× SDS loading dye and analyzed by SDS PAGE.

Drosophila culture

Flies were grown in standard fly food at 25°C. Following genotypes were used in this study: Canton S (Bloomington Stock Center), *mnb[1]* (FBal0012364, (11), gift from Francisco J. Tejedor), UAS-*mnb* (FBtp0114512, (59), gift from Francisco J. Tejedor), UAS-*RHEB* (FBst0009688, Bloomington

Stock Center), D42-Gal4 (FBti0002759, Bloomington Stock Center (49), P[(45)w[+mC]=UAS-Tor.TED]II (FBti0026636, (60), Bloomington Stock Center), UAS-mCherry (FBti0147460, Bloomington Stock Center).

Drosophila NMJ immunofluorescence staining and quantification

For Immunofluorescence staining of NMJ the third instar larvae were fixed in 4% paraformaldehyde (Himedia Cat#TCL119) for 20 min at room temperature and washed in 1X PBST (0.2% Triton X-100). Antibodies were used at the following dilutions: Mouse anti-DLG 1:500 (DSHB 4F3, (59), Rabbit anti-HRP conjugated with alexa488 (dilution 1:500, Jackson 123-545-021), Goat anti-mouse conjugated with Alexa 555 (Dilution 1:500, Invitrogen A28180). Samples were imaged with a confocal microscope (Leica Stellaris 5 (PL APO 40X/1.30 oil objective) or Olympus FV3000 (UPLFLN 40X/1.30 oil objective). Images were documented and quantified using ImageJ. We marked the HRP-labeled boutons to quantify NMJ phenotypes and manually counted the number. The number of boutons was then normalized to the muscle area (muscle 6/7), and the normalized data were compared between genotypes.

Statistical analysis

GraphPad Prism version 7.00 was used for statistical and statistical presentation of quantitation. *p* values are presented in the figures above or below the compared conditions. Two-way ANOVA followed by a Sidak's multiple comparisons test was applied to cell size data, p-S6 and p-S6k. Unpaired two-tailed Student's *t*-test was applied to DYRK1A protein expression. The data are represented as the mean ± SEM of three biological replicates.

Mass spectrometry dataset accessibility

Mass spectrometry data files for FLAG-DYRK1A affinity purifications and negative controls are available from Massive at */ftp://massive.ucsd.edu/MSV000085815/and* ProteomeXchange (PXD020533). Original mass spectrometry data underlying this manuscript can be accessed after publication from the Stowers Original Data Repository at *http://www.stowers.org/research/publications/libpb-1722*.

Materials availability statement

Reagents created for this study are available from the authors upon request.

Supporting Information

Supporting Information is available online on elife website.

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Editors

Reviewer #2 (Public Review):

This study reports a physical interaction between the kinase DYRK1A and the Tuberous Sclerosis Complex (TSC) protein complex (TSC1, TSC2, TBC1D7). Furthermore, this study demonstrates that DYRK1A, upon interaction with the TSC proteins, regulates mTORC1 activity and cell size. Additionally, this study identifies T1462 on TSC2 as a phosphorylation target of DYRK1A. Finally, the authors demonstrate that DYRK1A impacts cell size using human, mouse and Drosophila cells.

The interaction described here is highly impactful to the field of mTORC1-regulated cell growth and uncovers a previously unrecognized TSC-associated interacting protein. DYRK1A and its regulation of mTORC1 activation may have an impact for multiple diseases in which mTORC1 is hyperactivated.

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Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
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> ***Reviewer #1 (Public Review):***
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> *In this manuscript, Wang et al. demonstrate that knockdown of DYRK1A results in reduced cell size, which is mediated by mTORC1 activity. They found that DYRK1A interacts with TSC1/TSC2 proteins which leads to the phosphorylation of TSC2 at T1462. Phosphorylation of TSC2 at T1462 inhibits TSC2 activity leading to the activation of mTORC1. The authors complement their findings by demonstrating that overexpression of RHEB (positive regulator of mTORC1) rescues the phenotype of DYRK1A (mnb in flies) mutation in the NMJ.*
>
> *The authors' findings on the regulation of cell size and mTORC1 activity by DYRK1A reflect the previous findings of Levy et al. (PMID: 33840455) that cortical deletion of Dyrk1a in mice causes decreased neuronal size associated with a decreased activity of mTORC1 that can be rescued by the inhibition of Pten or supplementation of IGF1.*

> *The authors demonstrate that T1462 phospho-site at TSC2 is phosphorylated in response to the overexpression of WT but not kinase-dead DYRK1A. However, the authors do not provide any evidence that the regulation of mTORC1 is mediated via phosphorylation of this site. In addition, T1462 site is known to be phosphorylated by Akt. There is a possibility that Akt was co-purified with TSC1/TSC2 complex and DYRK1A promotes phosphorylation of TSC2 indirectly via the activation of AKT that can be tested by using AKT depleted cells.*

We thank the reviewer for reviewing this manuscript and the critical comments. Various groups have reported the significance of the Phosphorylation of TSC2 T1462, along with four other phosphorylation sites, in regulating mTORC1, and therefore, we did not deal with this in the current manuscript (Manning et al. PMID: 12150915, Inoki et al. PMID: 12172553, Zhang et al. PMID: 19593385). Regarding co-purification of AKT with TSC1/TSC2 - AKT phosphorylates T1462, S939 and S1387 (Manning et al. PMID: 12150915, Inoki et al. PMID: 12172553, Zhang et al. PMID: 19593385). However, in in vitro kinase assay, signal intensities of anti-TSC2 S939 and S1387, with or without ATP, showed no significant difference, suggesting that AKT is not pulled down with TSC1 or TSC2. DYRK1A and Kinase dead DYRK1A were expressed and purified from bacteria. Moreover, multiple studies have purified TSC1 and TSC2 and reported no AKT co-purified (Menon et al. PMID: 24529379, Chong-kopera et al. PMID: 16464865).

> *RHEB is the most proximal regulator of mTORC1 and can activate mTORC1 even under amino acid starvation. The fact that RHEB overexpression rescues the cell size under* DYRK1A *depletion or* mnb *(*DYRK1A *in* Drosophila*) mutant phenotype does not prove that DYRK1A regulates the cell size via TSC1 as it would rescue any inhibitory effects upstream to mTORC1.*

We agree with the reviewer that overexpression of RHEB may rescue any inhibitory effects upstream to mTORC1. In the results and discussion sections (Page number 7, last 3 lines), we mentioned that Rheb overexpression only supports our suggestion that DYRK1A likely works upstream to RHEB. We, however, have performed another experiment to strengthen our hypothesis. We show that increased cell size phenotype due to DYRK1A overexpression can be suppressed by inhibiting the TORC1 pathway, suggesting that mTORC1 is necessary for DYRK1A-mediated cell growth. These results are presented in Supplementary Figure 4. The results of two reciprocals of experiments (Suppression of DRYK1A/Mnb loss of function phenotypes by RHEB overexpression and suppression of rescue of DYRK1A Gain of function phenotypes) along with and regulation of TSC phosphorylation by DYRK1A strongly suggests that DYRK1A positively regulates TSC pathway.

> ***Reviewer #2 (Public Review):***
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> *This study aims to describe a physical interaction between the kinase DYRK1A and the Tuberous Sclerosis Complex proteins (TSC1, TSC2, TBC1D7). Furthermore, this study aims to demonstrate that DYRK1A, upon interaction with the TSC proteins regulates mTORC1 activity and cell size. Additionally, this study identifies T1462 on TSC2 as a phosphorylation target of DYRK1A. Finally, the authors demonstrate the role of DYRK1A on cell size using human, mouse, and Drosophila cells.*
>
> *This study, as it stands, requires further experimentation to support the conclusions on the role of DYRK1A on TSC interaction and subsequently on mTORC1 regulation. Weaknesses include, 1) The lack of an additional assessment of cell growth/size (eg. protein content, proliferation), 2) the limited data on the requirement of DYRK1A for TSC complex stability and function, and 3) the limited perturbations on the mTORC1 pathway upon DYRK1A deletion/overexpression.*

We thank the reviewer for reviewing this manuscript and the comments. We have previously analyzed the effect of DYRK1A knockdown in the proliferation of THP cells (human leukemia monocytic cell line) (Li Shanshan et al. PMID: 30137413) and have shown that DYRK1A knockdown negatively affects cell proliferation. Other studies have also shown a role for DYRK1A in cell proliferation, including in foreskin fibroblasts (Chen et al. PMID: 24119401) and HepG2 cells (Frendo-Cumbo et al. PMID: 36248734). mTORC1 regulates several pathways, including protein synthesis, lipid synthesis, nucleotide synthesis, autophagy, and stress responses. We have not done the protein content as this parameter is directly affected by TORC1 activation and may not be a suitable measure for cell growth. A large number of studies involving mTORC1 regulation analyze the levels of S6K and S6 phosphorylation, as these are direct readouts of mTORC1 function (Prentzell et al. PMID: 33497611, Zhang et al. PMID: 17052453, Ben-Sahra et al, PMID: 23429703, Düvel et al. PMID: 20670887, Zhang et al. PMID: 2504303). Therefore, we used these markers to assess the status of the mTORC1 pathway.

> *(2) ..the limited data on the requirement of DYRK1A for TSC complex stability and function,*

We agree with this limitation in our study. We have not seen a significant difference in TSC1 or TSC2 protein levels in DYRK1A knockdown or overexpressing cells, so we did not follow up on this aspect.

> *..and 3) the limited perturbations on the mTORC1 pathway upon DYRK1A deletion /overexpression.*

We have performed an additional experiment where we overexpressed DYRK1A and showed that increased cell size phenotype due to DYRK1A overexpression can be suppressed by inhibiting the TORC1 pathway, suggesting that mTORC1 is necessary for DYRK1A-mediated cell growth. These results are presented in Supplementary Figure 4. The results of two reciprocals of experiments (Suppression of DRYK1A/Mnb loss of function phenotypes by RHEB overexpression and suppression of Rescue of DYRK1A Gain of function phenotypes) along with and regulation of TSC phosphorylation by DYRK1A suggests that DYRK1A positively regulates TSC pathway.

> *Finally, this study would benefit from identifying under which nutrient conditions DYRK1A interacts with the TS complex to regulate mTORC1. The interaction described here is highly impactful to the field of mTORC1-regulated cell growth and uncovers a previously unrecognized TSC-associated interacting protein. Further characterization of the role that DYRK1A plays in regulating mTORC1 activation and the upstream signals that stimulate this interaction will be extremely important for multiple diseases that exhibit mTORC1 hyper-activation.*

We agree that identifying nutrients (or physiological conditions) that affect DYRK1A-mediated TSC regulation will be important to understanding the additional complexity in context-dependent mTORC1 activation/deactivation. This study has not addressed those issues, particularly due to DYRK1A's pleiotropic nature. DYRK1A has many substrates, and both overexpression and loss of DYRK1A lead to multiple phenotypes. Identifying nutrient conditions or growth factors that can regulate the activation of DYRK1A is not yet known and would require an independent investigation.

> ***Reviewer #3 (Public Review):***
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> *The manuscript describes a combination of in vitro and in vivo results implicating Dyrk1a in the regulation of mTORC. Particular strengths of the data are this combination of cell*

> *and whole animal (drosophila) based studies. However, most of the experiments seem to lack a key additional experimental condition that could increase confidence in the authors' conclusions. Overall some tantalizing data is presented. However, there are several issues that should be clarified or otherwise addressed with additional data.*

We thank the reviewer for reviewing and commenting on this manuscript.

> *(1) In Figure 1G, why not test overexpression levels of Dyrk1a via western rather than only looking at the RNA levels?*

Induced overexpression of DYRK1A was probed by analyzing mRNA levels, as the concentration of Doxycycline used (0-100 ng/ml) did not produce enough protein that could be detected by anti-flag antibody in a western blot. We have modified the sentence (page 5, paragraph 1).

> *(2) In Figure 2, while there is clearly TSC1 protein in the Dyrk1a and FLAG-Dyrk1a IPs that supports an interaction between the proteins, it would be good to see the reciprocal IP experiment wherein TSC1 or TSC2 are pulled down and then the blot probed for Dyrk1a.*

In the revised manuscript, we have provided evidence that TSC1 and TSC2 can interact with endogenous DYRK1A. We have performed immunoprecipitation of affinity-tagged TSC1 or TSC2 and have probed for the enrichment of DYRK1A (Supplementary Figure S2).

> *(3) Figures 3 A and D tested the effects of Dyrk1a knockdown using different methods in different cell lines. This is a reasonable approach to ascertain the generalizability of findings. However, each experiment is performed differently. For example, in 3A, the authors found no difference in baseline pS6, so they did a time course of treatment to induce phosphorylation and found differences depending on Dyrk1a expression. In 3D, they only show baseline effects from the CRISPR knockdown. Why not do the time course as well for consistency? Also, why the an inconsistency in approaches wherein one shows baseline effects and the other does not? The authors could also consider the pharmacologic inhibition of Dyrk1a activity as well.*

We agree that different methods were used in different cell lines to assess the effect of DYRK1A. Since *DYRK1A* is a pleiotropic gene, its manipulation has diverse effects on different cell lines. Also, not all cell types have similar levels of mTORC activity. Hence, we had to adapt to different strategies in different cell types, which accounted for the inconsistency in the methodology. However, various groups have used these methods to determine the activity of mTORC1 by S6 and S6K phosphorylation by both starvations, followed by the stimulation and direct estimation methods in cycling cells (Prentzell et al. PMID: 33497611, Zhang et al. PMID: 17052453, Ben-Sahra et al, PMID: 23429703, Düvel et al. PMID: 20670887, Zhang et al. PMID: 25043031). ShRNA-mediated knockdown in HEK293 cells does not change S6 or S6K phosphorylation levels in actively growing cells, whereas cycling NIH3T3 cells shows a significant reduction in S6 and S6K phosphorylation. As suggested, we used pharmacological inhibition of DYRK1A and 1uM Harmine to treat the HEK293 cells and perform starvation. However, cells treated and starved start to float and die in large numbers. Thus, we did not follow this experiment further.

> *(4) In Figure 4, RHEB overexpression increases cell size in both Dyrk1a wt and Dyrk1a shRNA treated cells, although the magnitude of the effect appears reduced in Dyrk1a shRNA cells. However, there is the possibility here that RHEB acts independently of Dyrk1a. Why not also do the experiment of Figure 1 wherein Dyrk1a is overexpressed and then knockdown RHEB in that context? If the hypothesis is supported, then RHEB knockdown should eliminate the cell size effect of Dyrk1a overexpression.*

We thank the reviewer for suggesting this experiment. We have overexpressed DYRK1A using the inducible HEK293A-Flag-DYRK1A overexpression system and treated cells with mTOR inhibitors (Rapamycin or Torin1). The results are added to the supplementary figure S4. Our results show that the increased cell size phenotype due to DYRK1A overexpression can be suppressed by inhibiting the TORC1 pathway. This suggests that mTORC1 is necessary for DYRK1A-mediated cell growth. This data further supports the hypothesis that DYRK1A is a positive regulator of the mTORC1 pathway.

> *(5) The discussion should incorporate relevant findings from other models, such as Arabidopsis. Barrada et al., Development (2019), 146 (3).*

We have incorporated the findings from Arabidopsis (Barrada et al., Development (2019), 146 (3) PMID: 30705074) in the last paragraph of the discussion section.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations For The Authors):***
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> *(1) To demonstrate that DYRK1A can phosphorylate T1462 phospho-site at TSC2 in the absence of Akt using genetic and pharmacological approaches (by using pan-Akt small molecule inhibitors).*

We have performed *in vitro* kinase assay using recombinant DYRK1A, and affinity purified TSC1/TSC2 from HEK293 cells. However, we have not been able to perform this experiment by overexpression of DYRK1A in human cells, as 1) strong overexpression of DYRK1A leads to cell cycle exit, as demonstrated by various laboratories (Soppa et al. PMID: 24806449, Hämmerle et al PMID: 21610031, Najas et al. PMID: 26137553, Park et al. PMID: 20696760) and our observations, and 2) T1462 Antibody signal is weak and cannot be seen in cellular extracts. We have attempted this experiment with at least three different batches of T1462 antibody from CST without success.

> *(2) To demonstrate that endogenous phosho-mutant/mimetic substitution of T1462 phospho-site at TSC2 is sufficient to prevent the regulation of cell size/NMJ phenotype in Drosophila by DYRK1A (mnb).*

This is an interesting experiment, and we thank the reviewer for this suggestion. However, we are skeptical about interpreting the possible results. Since T1462 substitution will also block the regulation by other kinases, e.g., Akt, and it may constitutively suppress the mTORC1, any interpretation will be confusing.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *(1) In section 2.1 the authors claim that DYRK1A down-regulation enhances cell growth. An additional assessment of cell growth or size would strengthen this statement. Is total protein content also increased upon DYRK1A overexpression? Does DYRK1A KD also increase cell proliferation? In Figure 1, providing the median or mean size of cells in each condition will help the reader understand the impact of DYRK1A on cell size. In Supplementary Figure 1, the important statistical differences should be highlighted.*

We have not claimed that down-regulation of DYRK1A enhances cell growth. We have not tested the protein content in a cell directly. Knockdown of DYRK1A leads to a reduction in cell proliferation, as shown by various groups, including ours (Shanshan Li PMID: 30137413, Luna et al. PMID: 30343272). Cell size is a very dynamic process and is variable within the population. All the studies measuring cell size show the size using assays on a population of

cells. We have not been able to figure out a way to display the median or mean cell size that accurately reflects the cell size of the whole population.

> *(2) In section 2.2 the authors describe the interaction between DYRK1A and the TSC proteins. Do the DYRK1A mutants impact interaction with TSC2 and TBC1D7 or is this specific to TSC1?*

We have not tested this possibility.

> *(3) In section 2.3, more detailed perturbations of the mTORC1 pathway are needed. Is the mTORC1 activation observed sensitive to rapamycin treatment? Since mTORC1 regulates cell size via S6 ribosomal protein and transcription via 4EBP1, phosphorylation of 4EBP1 should also be considered. In Figure 3A, what is the level of DYRK1A down-regulation? It is unclear how many shRNA constructs were used or whether these were pooled constructs or single clones. If one shRNA/sgRNA is used, it would be very helpful to validate some of the key findings of this study with at least one more clone.*

Many research studies have measured the activity of various mTORC1 substrates, the most commonly used being the phosphorylation of S6 and S6K. We agree that analyzing 4EBP1 would make the study more comprehensive, but to complete the study with our limited resources and in a limited time, we have not attempted to establish the 4EBP1 phosphorylation status. We have used a previously described and validated DYRK1A shRNA (as mentioned in the methods section).

> *(4) In section 2.3 is T1462 an activating or inhibiting phosphorylation event? If DYRK1A phosphorylates and activates mTORC1 via RHEB, shouldn't that result in the inhibition of mTORC1?*

Multiple laboratories have demonstrated that T1462 phosphorylation leads to a reduced TSC complex activity and, hence, increased mTORC1 activity (Manning et al. PMID: 12150915, Inoki, PMID: 12172553, Zhang PMID: 19593385).

> *(5) In section 2.4, what is the status of AKT phosphorylation? Would an AKT inhibitor be useful in this scenario?*

AKT phosphorylates T1462, S939 and S1360, as demonstrated by others. However, in our in vitro assay kinase assay, the following facts suggest that AKT is not involved in T1462 phosphorylation we observed:

(1) Signal intensities of anti-TSC2 S939 and S1387 with or without ATP, do not show any significant differences, suggesting that AKT is not pulled down with TSC1 or TSC2.

(2) Multiple studies have performed phosphorylation studies of TSC1 and TSC2 and have not reported any co-purification of AKT.

> *(6) Very minor grammar errors were observed, mostly at the beginning of the manuscript.*

We tried our best to fix grammatical errors.

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