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CONTRACTING ORGANIZATION: King's College, London, UK

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14. ABSTRACT Tuberous sclerosis complex (TSC) is a dominant genetic disorder caused by mutations in the genes <i>TSC1</i> and <i>TSC2</i> and characterised by benign tumours in multiple organs. The neurological manifestations of TSC, including epilepsy and autism, have a particularly early onset and have the greatest morbidity. Mutations in <i>Tsc1/2</i> result in activation of the highly conserved mechanistic target of rapamycin (mTOR) pathway. We recently identified the protein Unkempt as the first downstream component of the mTOR pathway to regulate neuronal differentiation in <i>Drosophila</i> . In this project we are testing the hypothesis that Unkempt is a key downstream regulator of mTOR complex 1 (mTORC1) in the developing mammalian nervous system and that mis-regulation of Unkempt contributes to the neurological manifestations of TSC. During this research period we have validated an Unkempt phospho-specific antibody that we have generated and made progress towards testing the role of Unkempt phosphorylation in vivo and analysing Unkempt phosphorylation in a mouse model of TSC.					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	5
2. Keywords	5
3. Accomplishments	5
4. Impact	6
5. Changes/Problems	6
6. Products	7
7. Participants & Other Collaborating Organizations	7
8. Special Reporting Requirements	9
9. Appendices	9

1. INTRODUCTION

Tuberous sclerosis complex (TSC) is a dominant genetic disorder caused by mutations in the genes *TSC1* and *TSC2* and characterised by benign tumours in multiple organs. The neurological manifestations of TSC, including epilepsy and autism, have a particularly early onset and have the greatest morbidity. Mutations in *Tsc1/2* result in activation of the highly conserved mechanistic target of rapamycin (mTOR) pathway. We recently identified the protein Unkempt as the first downstream component of the mTOR pathway to regulate neuronal differentiation in *Drosophila*. We now have strong evidence that Unkempt is a downstream regulator of mTOR complex 1 (mTORC1) signaling in mammals and that mis-regulation of Unkempt contributes to the neurological manifestations of TSC. We will test the hypothesis that Unkempt is a key downstream regulator of mTORC1 in the developing nervous system and that mis-regulation of Unkempt contributes to the neurological manifestations of TSC.

2. KEYWORDS

TSC; mTOR; Unkempt; signaling; neurogenesis

3. ACCOMPLISHMENTS

- What were the major goals of the project?

Major goals as stated in the approved SOW:

Specific Aim 1: To determine the mechanism by which mTORC1 regulates Unkempt in neurons	Timeline	% completion
Major Task 1: Investigating the mTOR-dependent phosphorylation of Unkempt.	Months	
Subtask 1: LC/MS/MS identification of mTORC1-dependent Unkempt phosphorylated residues	1-12	100%
Subtask 2: mTORC1 <i>in vitro</i> kinase assays with Unkempt as the substrate (in collaboration with Dr Tee)	1-12	100%
Subtask 3: Generation of Unkempt phospho-specific antibodies	12-18	100%
Major Task 2: Testing the function of mTOR dependent Unkempt phosphorylation <i>in vivo</i>		
Subtask 1: <i>In utero</i> electroporation of CD-1 mouse embryos with Unkempt phospho-mutant constructs (initial training from Dr Hindges and collaboration to use his equipment for all utero electroporation experiments). Unkempt shRNA and RNAi resistant plasmids provided by Professor Shi. 40 embryos will be used.	6-24	100%
Specific Aim 2: To analyze the neurodevelopmental and behavioral phenotypes of Unkempt knock-out mice as a new animal model of TSC.		
Major Task 3: Determining the neurogenic phenotype of Unkempt knock-out mice		
Subtask 1: Immunohistochemical analysis of <i>Unk^{tm1c/tm1c};nestin-Cre⁺</i> mice neurodevelopmental phenotypes. 40 mouse embryos and 20 P21 adults.	12-36	100%
Subtask 2: Single neural progenitor cell labelling and analysis in <i>Unk^{tm1c/tm1c};nestin-Cre⁺</i> mice. 20 mouse embryos.	12-36	100%
Subtask 3: <i>In utero</i> electroporation of Cre into neural progenitors in <i>Unk^{tm1c/tm1c}</i> mice to produce localized loss of Unkempt expression. 20 mouse embryos.	12-36	100%
Major Task 4: Analysis of behavioral phenotypes in Unkempt knock-out mice		
Subtask 1: Analysis of <i>Unk^{tm1c/tm1c};nestin-Cre⁺</i> mice behavior at 6 weeks (open-field and accelerating rotarod tests, social approach and social novelty tests, reversal learning assay in the Morris Water Maze), in collaboration with Dr Cathy Fernandes. 48 adult mice.	12-36	100%
Specific Aim 3: To test whether overexpression of Unkempt can prevent the neurodevelopmental defects in mouse models of TSC.		
Major Task 5: Overexpression of Unkempt in TSC neurodevelopmental models		
Subtask 1: Overexpression of Unkempt by <i>in utero</i> electroporation in neural progenitors in <i>Tsc1^{fl/fl}</i> mice. 14 adult mice. <i>Tsc1^{fl/fl}</i> mice provided by Professor Bordey.	9-30	100%

Subtask 2: Overexpression of Unkempt in neural progenitors by co-in utero electroporation with Rheb ^{CA} vector. 14 adult mice. Rheb ^{CA} vector provided by Professor Bordey.	9-30	100%
Subtask 3: <i>In utero</i> electroporation of Unkempt mTORC1-dependent phosphorylation site mutants (from Specific Aim 1) into <i>Tsc1^{fl/fl}</i> mice or together with Rheb ^{CA} vector to test if phosphorylation is required to prevent neurodevelopmental phenotypes in TSC models. 28 adult mice.	18-36	100%
Major Task 6: Analysis of Unkempt expression and phosphorylation in the brain in a TSC mouse model		
Subtask 1: Immunostaining, immunoblot and qRT-PCR analysis of Unkempt expression and phosphorylation status in embryonic brains from <i>Tsc1^{fl/fl};nestin-Cre⁺</i> mice.	12-30	100%

- **What was accomplished under these goals?**

Major activities:

All of the major activities of the project have been achieved. Highlights of the results are described in previous annual reports and the details described and findings discussed in the 2 publications included in Appendix 1 and 2.

Specific objectives:

All specific objectives have been achieved, see above.

Significant results or key outcomes:

Overall, we have shown that Unkempt is a novel substrate of mTORC1 in neurons and phosphorylation of Unkempt regulates cellular morphogenesis. We have also analysed the neurodevelopmental and behavioral phenotypes of Unkempt conditional knockout mice. See previous annual reports and Appendices 1 and 2.

- **What opportunities for training and professional development has the project provided?**

See previous annual reports. Training and professional development opportunities in the last 12 months of the project were very limited due to Covid-19.

- **How were the results disseminated to communities of interest?**

Ongoing results have been presented at internal seminars and discussed with collaborators and other colleagues. Dr Baskaran and Dr Bateman attended the 2021 International Tuberous Sclerosis Complex Research Conference (online) in June 2021. Dr Baskaran was selected to give an oral presentation at the conference. The conference will be an excellent opportunity to disseminate the results of the project. Results of the projected have also been disseminated through primary research papers-see Appendix 1 and 2.

- **What do you plan to do during the next reporting period to accomplish the goals?**

N/A

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

The project has been extended in an ongoing collaboration with Prof Jernej Murn at UC Riverside. His lab have used the Unkempt phospho-mutants we generated during the project and shown that phosphorylation of Unkempt is required to regulate cellular morphogenesis. These data are included in the manuscript currently under review (see Appendix 2). The project has also led to a new collaboration between Prof Bateman and Dr Sila Ultanir at the Francis Crick Institute. We were awarded a joint KCL/Crick PhD studentship to characterise the mTOR phosphoproteome during neurodevelopment. We were also recently awarded a TSC Alliance

Biosample Seed Grant to analyse the mTOR dependent phospho—proteome in brain tissue from TSC patients. We expect that these projects will provide important new insight into the neurological manifestations of TSC.

- **What was the impact on other disciplines?**

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. **CHANGES/PROBLEMS**

Our research institute was shut-down in mid-March 2020 to due COVID-19 and partially re-opened in July 2020. However, the postdoc working the project, Dr Baskaran, took maternity leave from June 2020 to February 2021. We received a no cost extension to enable completion of the project.

6. **PRODUCTS**

- **Publications, conference papers, and presentations**

Dr Baskaran gave an oral presentation on the project at the 2021 International Tuberous Sclerosis Complex Research conference (online). Dr Bateman gave a seminar on the work at the Cardiff University Divisional seminar series in February 2021.

- **Journal publications**

In a paper in *Scientific Reports* we described the characterisation of the Unkempt cKO mice generated during the project-see Appendix 1. In a paper currently under review in *Journal of Biological Chemistry* we have described our results showing that Unkempt is a novel substrate of mTORC1 and that phosphorylation of Unkempt regulates cellular morphogenesis-see Appendix 2 (BioRxiv manuscript).

- **Books or other non-periodical, one-time publications**

Nothing to report.

- **Other publications, conference papers, and presentations**

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Dr Joseph Bateman
Project role:	PI
ORCID ID:	0000-0003-0754-1785
Nearest person month worked:	2 months
Contribution to project:	Manage project; coordinate with collaborators; supervise postdoc
Funding support:	KCL

Name:	Dr Pranetha Baskaran
Project role:	Postdoc
ORCID ID:	0000-0001-9927-1684
Nearest person month worked:	12 months
Contribution to project:	Planned and performed experiments, analysed data.
Funding support:	TSCRIP Idea Development Award

Name:	Mr Carl Hobbs
Project role:	Departmental histology and imaging manager
ORCID ID:	
Nearest person month worked:	1 month
Contribution to project:	Histology training and support
Funding support:	KCL

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

- **Organization Name:** Cardiff University
- **Location of Organization:** UK
- **Partner's contribution to the project:**
 - **Collaboration:** Assisted with in vitro kinase assay and Raptor overlay assays.
- **Organization Name:** UC Riverside
- **Location of Organization:** USA
- **Partner's contribution to the project:**
 - **Collaboration:** Performed cellular morphogenesis assays of Unkempt phosphor mutants.

- **Organization Name:** Francis Crick Institute
- **Location of Organization:** UK
- **Partner's contribution to the project:**
 - **Collaboration:** Performed western blot analysis of Unkempt phosphorylation in Tsc1 cKO mice.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

Appendix 1: Vinsland et al 2021.

Appendix 2: Baskaran et al 2022.



OPEN

The zinc finger/RING domain protein Unkempt regulates cognitive flexibility

Elin Vinsland^{1,8}, Pranetha Baskaran¹, Simeon R. Mihaylov¹, Carl Hobbs², Hannah Wood³, Ihssane Bouybayoune⁴, Kriti Shah⁶, Corinne Houart⁴, Andrew R. Tee⁵, Jernej Murn⁶, Cathy Fernandes^{3,7} & Joseph M. Bateman¹✉

Correct orchestration of nervous system development is a profound challenge that involves coordination of complex molecular and cellular processes. Mechanistic target of rapamycin (mTOR) signaling is a key regulator of nervous system development and synaptic function. The mTOR kinase is a hub for sensing inputs including growth factor signaling, nutrients and energy levels. Activation of mTOR signaling causes diseases with severe neurological manifestations, such as tuberous sclerosis complex and focal cortical dysplasia. However, the molecular mechanisms by which mTOR signaling regulates nervous system development and function are poorly understood. Unkempt is a conserved zinc finger/RING domain protein that regulates neurogenesis downstream of mTOR signaling in *Drosophila*. Unkempt also directly interacts with the mTOR complex I component Raptor. Here we describe the generation and characterisation of mice with a conditional knockout of Unkempt (*Unk^{CKO}*) in the nervous system. Loss of Unkempt reduces Raptor protein levels in the embryonic nervous system but does not affect downstream mTORC1 targets. We also show that nervous system development occurs normally in *Unk^{CKO}* mice. However, we find that Unkempt is expressed in the adult cerebellum and hippocampus and behavioural analyses show that *Unk^{CKO}* mice have improved memory formation and cognitive flexibility to re-learn. Further understanding of the role of Unkempt in the nervous system will provide novel mechanistic insight into the role of mTOR signaling in learning and memory.

The formation of the nervous system represents one of the fundamental challenges during development. Human brain development involves the generation of around 86 billion neurons and an equivalent number of non-neuronal cells that constitute the mature adult brain¹. Neurogenesis begins in the embryonic neuroectoderm, where neuroepithelial cells proliferate symmetrically in the early neural tube. Neuroepithelial cells transform into radial glial cells, which divide asymmetrically to generate intermediate progenitor cells or neurons². Disturbances during brain development can cause cerebral malformations and functional impairments that result in developmental neuropathology³. Neurodevelopmental disorders affect around 1–2% of the population and may last the lifetime of the individuals affected.

Mechanistic target of rapamycin (mTOR) is a large serine/threonine protein kinase that forms the catalytic subunit of two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)⁴. mTORC1 integrates signaling inputs from cellular nutrients, growth factors, energy, and stress to regulate a wide range of anabolic processes. mTOR signaling plays a fundamental role in mammalian neurogenesis^{5,6}. Hyperactivation of mTORC1,

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through loss-of-function mutations in the genes *TSC1* or *TSC2*, causes the multisystem disorder tuberous sclerosis complex (TSC). Patients with TSC have benign tumours in multiple organs, including the brain, which can result in epilepsy, autism and intellectual disability⁷. Activating mutations in other components of the mTORC1 pathway cause focal cortical dysplasia, hemimegalencephaly and other epilepsy syndromes⁶.

mTOR also plays a crucial role in regulating translation at the synapse and as a result in synaptic plasticity. Long term modification of synaptic strength, or long term potentiation (LTP), requires increased local translation. Early studies showed that the mTORC1 substrate 4E-BP1 and its binding partner eIF4E colocalise with post-synaptic markers⁸. Moreover, mTORC1 regulates the local translation of the elongation factor eEF1A in dendrites to promote LTP⁹. More recently, evidence from patients and animal models has shown that increased synaptic translation due to upregulation of mTORC1 activity contributes to epilepsy associated and autism spectrum disorders, such as fragile X syndrome, Angelman syndrome and tuberous sclerosis complex^{10,11}.

Unkempt is a highly conserved zinc finger/RING domain protein that was originally identified in *Drosophila*, where it was shown to be expressed in the developing embryonic nervous system and to be important for tissue patterning. *Drosophila* null mutants in Unkempt are developmentally lethal, while hypomorphic mutants are viable but have an 'unkempt' phenotype, with roughened eyes, splayed wings and crossed scutellar bristles¹². We previously showed that Unkempt acts genetically downstream of mTOR to regulate differentiation of photoreceptors in the developing retina in *Drosophila*^{13–15}. In nutrient rich conditions, loss of Unkempt does not affect cell proliferation, but alters the timing of *Drosophila* photoreceptor differentiation^{13,16}. Loss of Unkempt causes precocious differentiation of photoreceptor neurons and patterning defects in the adult eye¹³. Recently we also showed that Unkempt is strongly expressed in the larval nervous system in *Drosophila*, where it negatively regulates the cell cycle in neural progenitor cells¹⁷.

Mammalian Unkempt is most strongly expressed in cell lines with a neuronal origin and in vivo its expression is strongest in the developing central nervous system¹⁸. Unkempt expression in the developing brain peaks between embryonic days 12 and 18 and is particularly abundant in Tuj-1 expressing neurons¹⁸. In vitro experiments have shown that mammalian Unkempt binds mRNAs through its zinc finger domain to regulate their translation^{18,19}. Moreover, both *Drosophila* and mammalian Unkempt physically interact with the mTORC1 component Raptor^{16,20}. However, the role of mammalian Unkempt in vivo is largely uncharacterised. To assess the role of Unkempt in vivo we generated a nervous system-specific Unkempt knockout mouse. Loss of Unkempt in the developing nervous system causes a reduction in the expression of the mTORC1 component Raptor but surprisingly does not affect neural progenitor proliferation. The overall development of the nervous system is also unaffected by loss of Unkempt in this model. However, expression studies show that Unkempt is strongly expressed in the adult cerebellum and hippocampus, and behavioural analyses show that Unkempt knockout mice have improved reversal learning. Thus, loss of Unkempt improves cognitive flexibility.

Results

Loss of Unkempt causes reduced expression of Raptor in the developing brain. Unkempt was originally identified in *Drosophila* as a zinc finger/RING domain protein essential for developmental viability and patterning (Fig. 1A,B)¹². Unkempt is expressed ubiquitously in *Drosophila* but enriched in developing nervous system, where it acts as a component of the mTOR pathway to regulate neurogenesis^{12,13,17}. The mTOR pathway has important roles in nervous system development and hyperactivation of the mTOR pathway causes neurological diseases associated with aberrant intellectual development, epilepsy and autism^{5,6}. Unkempt is conserved in mammals (Fig. 1A,C) and most strongly expressed in developing neurons but its role in the mammalian nervous system is largely unknown¹⁸. To address this, we generated a conditional allele of *Unkempt* in which exons 3 and 4 are flanked by loxP sites (Fig. 1D). Removal of exons 3 and 4 is predicted to generate a premature stop codon after 113 amino acids (Supplemental Figure S1). We crossed these mice to Nestin-Cre expressing mice²¹, to generate animals in which *Unkempt* is knocked-out in neural progenitors (*Unk^{CKO}*) from around embryonic day (E) 10.5 (Fig. 1D–F and Supplemental Figure S2A). qRT-PCR analysis of E16.5 brain tissue using two primer sets, both within the deleted genomic region, showed a dramatic reduction in Unkempt transcript in *Unk^{CKO}* embryos (Fig. 1G). At E16.5 homozygous *Unk^{CKO}* embryos also had no detectable Unkempt protein expression in the brain, while heterozygotes had around 50% Unkempt expression levels compared to littermate controls (Fig. 1H; Supplemental Figure S2A).

In mammals, Unkempt has a paralog called Unkempt like (Unkl), which also contains N- and C-terminal zinc finger and RING domains respectively²². To test whether there is cross regulation between Unkempt and Unkempt like we analysed Unkempt like expression in *Unk^{CKO}* embryos. Unkempt like expression was unaltered in E16.5 *Unk^{CKO}* embryos (Fig. 1I, J, K; Supplemental Figure S2B), indicating that loss of Unkempt does not affect Unkempt like expression in the developing nervous system.

Proteomic analysis of the mTOR pathway identified physical interactions between Unkempt and several mTORC1 components in *Drosophila* cultured cells²⁰. Subsequent co-immunoprecipitation studies have shown that both *Drosophila* and mammalian Unkempt interact specifically with the mTORC1 component Raptor¹⁶. To confirm these findings, we used a Raptor overlay assay as a complementary approach. This assay uses direct association of the purified candidate protein to Raptor or Raptor mutant 4, which retains the ability to interact with mTOR but not does not interact with mTOR substrates^{23,24}. Using the Raptor overlay assay we found that, like the mTORC1 substrate 4E-BP1, Unkempt bound avidly to wild-type HA-Raptor but binding to HA-Raptor mutant 4 was greatly reduced (Fig. 2A; Supplemental Figure S3A). These data confirm that Unkempt directly interacts with Raptor in vitro.

We next tested whether loss of Unkempt affects the expression of Raptor in the developing nervous system. Interestingly, *Unk^{CKO}* embryos (Fig. 2B,C; Supplemental Figure S3B) had a significantly reduced level of Raptor in the brain (Fig. 2B,D). To determine whether the reduction in Raptor perturbs mTORC1 signaling, we analysed

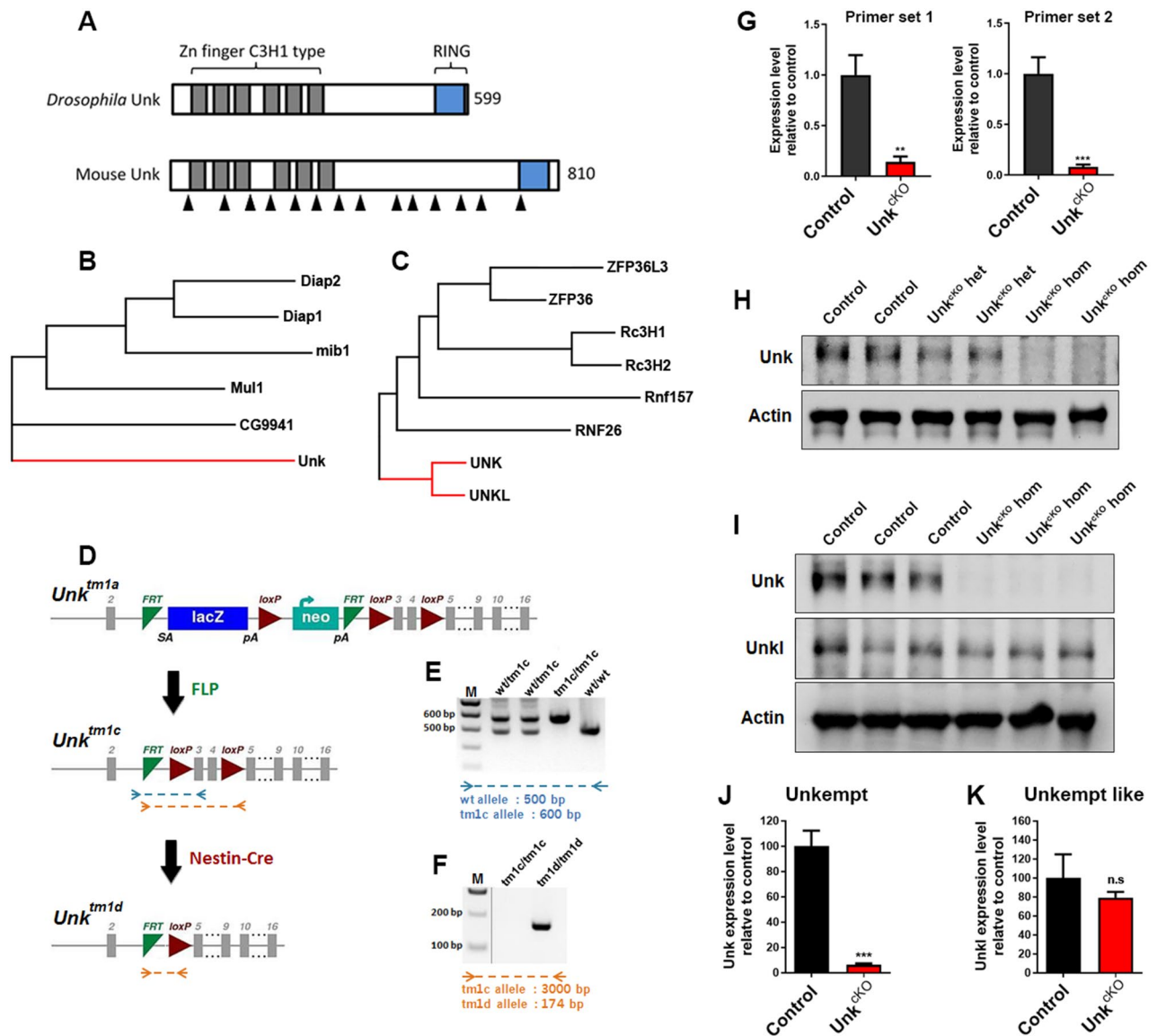


Figure 1. Generation of an Unkempt nervous system conditional knockout mouse. (A) Schematic of the primary structure of *Drosophila* and mouse Unkempt proteins. (B,C) Dendrograms showing evolutionary relationship between *Drosophila* Unkempt and closely related proteins (B) and mouse Unkempt and Unkempt like and closely related proteins (C). (D) Generation of the *Unk^{CKO}* allele. The *Unk^{tm1a}* knockout first conditional ready cassette, which contains a lacZ reporter and neomycin (neo) resistance gene flanked by FRT sites, was converted to the *Unk^{tm1c}* allele by crossing to mice expressing FLP recombinase, and further converted to the conditional allele *Unk^{tm1d}* (*Unk^{CKO}*) by crossing to mice expressing Nestin-Cre recombinase. Grey boxes represent exons. SA = splice acceptor site, pA = poly-adenylation tail. Dotted lines represent primer binding sites for PCR reactions shown in (E) and (F). (E) Example genotyping of the *Unk^{tm1c}* allele. The remaining FRT and loxP sites increase the size of the *Unk^{tm1c}* allele PCR product by 100 bp compared to wild-type. (F) Example genotyping of the *Unk^{tm1d}* allele. Removal of the critical exons creates a PCR product of 174 bp in size. M = molecular weight marker. (G) qRT-PCR analysis of E16.5 CNS tissue from control (*Unk^{tm1c/tm1c}*) and *Unk^{CKO}* homozygous (*Unk^{CKO} hom*; *Nestin-Cre*, *Unk^{tm1d/tm1d}*) embryos using 2 primers sets, both within the deleted genomic region. Control n = 5, *Unk^{CKO}* n = 5. (H) Western blot of Unkempt (Unk) expression in E16.5 CNS tissue from control (*Unk^{tm1c/tm1c}*), *Unk^{CKO}* heterozygous (*Unk^{CKO} het*; *Nestin-Cre*, *Unk^{tm1d/+}*) and *Unk^{CKO}* homozygous (*Unk^{CKO} hom*; *Nestin-Cre*, *Unk^{tm1d/tm1d}*) embryos. (I) Western blot analysis of Unkempt (Unk) and Unkempt like (Unkl) expression in control and *Unk^{CKO}* E16.5 CNS tissue. (J,K) Quantification of Unkempt (Unk) and Unkempt like (Unkl) expression. Data are presented as mean ± SEM. Student's t test, n.s. not significant, ***p* < 0.05, ****p* < 0.001. Control n = 3, *Unk^{CKO}* n = 3.

the expression and phosphorylation of ribosomal protein S6 (rpS6) and eukaryotic translation initiation factor 4E-binding protein 2 (4E-BP2), the predominant form of 4E-BP in the brain²⁵. Total rpS6 and phospho-rpS6

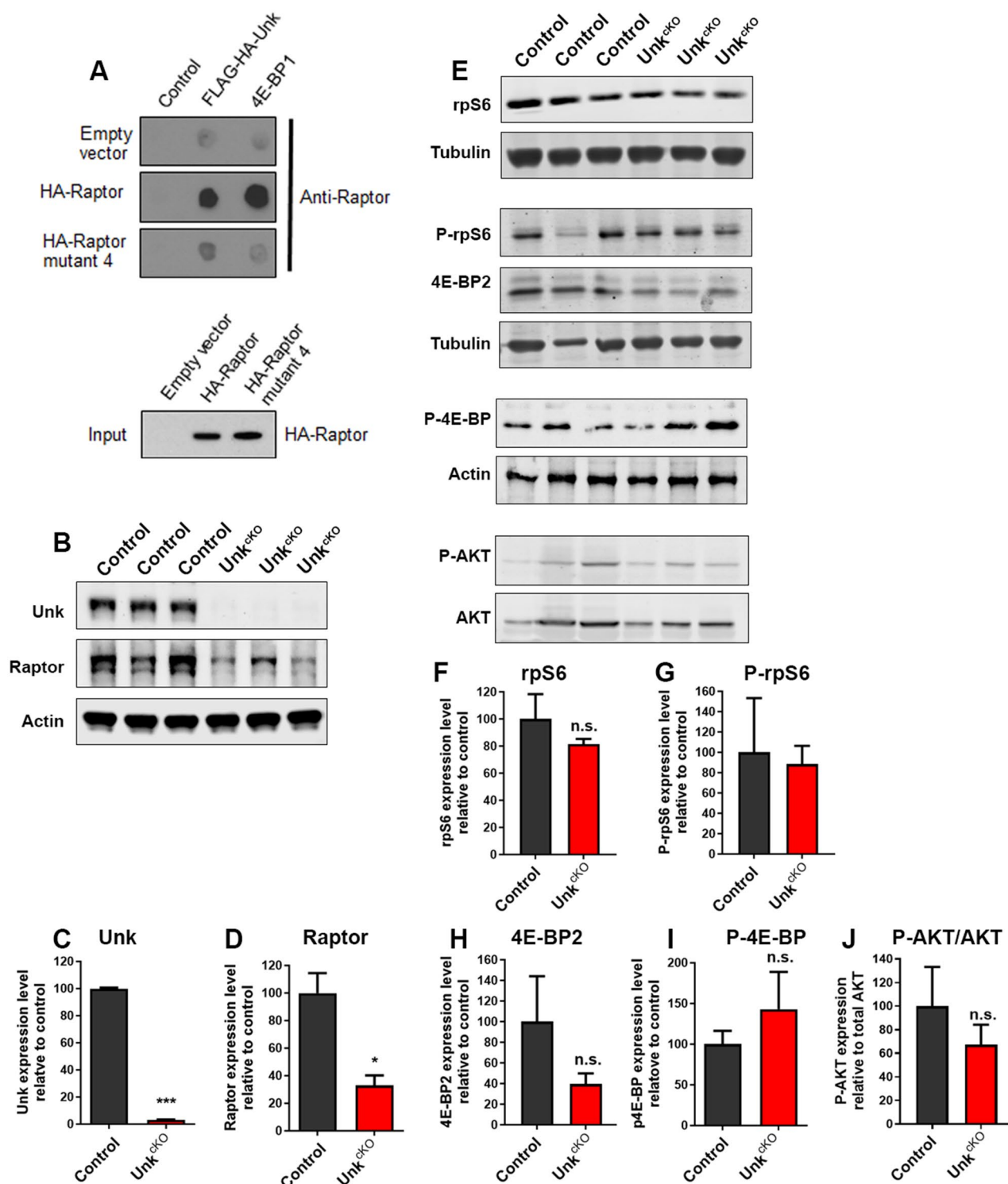


Figure 2. *Unk^{KO}* mice have reduced Raptor levels in the developing nervous system. (A) Unkempt binds to Raptor but not Raptor mutant 4 in a Raptor overlay assay. HA-Raptor and HA-Raptor mutant 4 expression levels were determined by western blot (lower panel). (B) Western blot of Raptor expression in *Unk^{KO}* E16.5 embryonic brain tissue. (C,D) Quantification of Unkempt and Raptor expression in *Unk^{KO}* E16.5 embryonic brain tissue. (E) Western blot of rpS6, phospho-rpS6 (P-rpS6), 4E-BP2 and phospho-4E-BP (P-4E-BP) expression in *Unk^{KO}* E16.5 embryonic brain tissue. (F–J) Quantification rpS6, P-rpS6, 4E-BP2, P-4E-BP and P-AKT expression in *Unk^{KO}* E16.5 embryonic brain tissue. Data are represented as mean ± SEM. Student's t test, **p* < 0.05, ****p* < 0.001, n.s. not significant. Control *n* = 3, *Unk^{KO}* *n* = 3.

(P-rpS6) levels in the brain were similar in controls and *Unk^{KO}* embryos (Fig. 2E–G; Supplemental Figure S3C). Total 4E-BP2 and phospho 4E-BP (P-4E-BP) levels in the brain were also not significantly different between

controls and *Unk^{ckO}* embryos (Fig. 2E,H,I; Supplemental Figure S3C). We also used phosphorylation of AKT at S473 as a readout of mTORC2 activity. Phosphorylation of AKT at S473 was not altered in *Unk^{ckO}* embryos (Fig. 2E,J, Supplemental Figure S3D), indicating that loss of Unkempt does not affect mTORC2 activity. These data indicate that Unkempt is necessary to maintain normal levels of Raptor expression during nervous system development. However, the reduced Raptor expression resulting from loss of Unkempt does not negatively impact mTORC1 signaling.

Loss of Unkempt does not affect nervous system development. To investigate whether loss of Unkempt affects neurodevelopment we analysed neuroanatomy and neurogenesis in *Unk^{ckO}* embryos. Overall neuroanatomy of the brain of *Unk^{ckO}* mice at E16.5 appeared normal compared to littermate controls (Fig. 3A,B). To assess the requirement for Unkempt in early cortical development we stained brains from E16.5 *Unk^{ckO}* embryos with markers of different neural sub-types. Expression of the neuronal markers NeuN and DCX, as well as the cortical layer specific markers Tbr1, Tbr2 and Ctip2 were largely normal in *Unk^{ckO}* embryos (Fig. 3C,D). We also stained for and quantified the expression of markers of cell proliferation and mitosis (Ki67, phosphohistone 3 (PH3) and BrdU incorporation), as well as Mash1-expressing transit amplifying cells in the subventricular zone of *Unk^{ckO}* embryos at E16.5. Expression of all these neurogenic markers were not significantly different from controls in *Unk^{ckO}* mice (Fig. 3E–P). Overall expression of Ki67 in the developing olfactory bulb and medial region of the brain in *Unk^{ckO}* mice at E16.5 was also similar to control (Supplemental Figure S4). Thus, although Raptor expression is reduced in the brain of *Unk^{ckO}* embryos, neurogenesis and overall brain development appear unimpeded.

Unkempt is strongly expressed in the adult cerebellum and hippocampus. Unkempt is strongly expressed in the developing nervous system in both *Drosophila* and mice but whether its expression continues in the adult nervous system is unknown^{17,18}. Weak Unkempt protein expression was detectable in whole brain lysate at post-natal day (P) 60 and, as expected, was not expressed in *Unk^{ckO}* mice brain tissue (Fig. 4A; Supplemental Figure S3E). In situ hybridisation analysis of Unkempt mRNA expression from the Allen Brain Atlas shows that at P56 Unkempt is very weakly expressed in most brain regions (Fig. 4A)²⁶. However, Unkempt mRNA expression is increased in the olfactory bulb, and is strongly expressed in the molecular layer of the cerebellum, as well as the pyramidal layer and dentate gyrus granule cell layer of the hippocampus (Fig. 4B,C).

***Unk^{ckO}* mice have improved cognitive flexibility.** Hyperactivation of the mTOR pathway in the brain can cause seizures, increased anxiety and memory impairment in animal models, and in patients can cause epilepsy, autism and intellectual disability^{5,27}. Conversely, chronic inhibition of mTOR signaling is associated with decreased anxiety, improved learning and memory in animal models^{28,29}. *Unk^{ckO}* mice were born at the expected Mendelian ratios (Table 1) but adult mice weighed slightly less than controls (Fig. 5A). Overall neuroanatomy of the mature *Unk^{ckO}* brain appeared normal (Fig. 5B,C, Supplemental Figure S5). We then used a battery of tests to determine whether loss of Unkempt affects behaviour in adult mice. To assess anxiety and locomotor activity open field and elevated plus maze tests were used^{30,31}. *Unk^{ckO}* mice had similar locomotor activity to littermate controls (Fig. 5D,E). However, *Unk^{ckO}* mice showed a trend towards decreased anxiety, as they spent more time in the central zone of the open field arena (Fig. 5F) and in the elevated plus maze test spent more time on the open arms (Fig. 5G,H), although these differences were not statistically significant.

We next assessed the requirement for Unkempt in learning and memory. *Unk^{ckO}* mice performed similar to controls in the Y maze (Fig. 6A), a test of spontaneous spatial novelty preference that measures rapidly acquired, short-term spatial memory³². Analysis of spatial learning and reference memory using the Morris water maze³³, showed that both controls and *Unk^{ckO}* mice had a significant reduction in the latency to find the platform over the hidden days 1–6 (H1–H6) and spent a significantly increased time in the platform-containing quadrant at the end of day 6 (Fig. 6B,C), indicating normal acquisition learning and reference memory.

Activation of mTOR signaling impairs the ability to re-learn in ‘reversal learning’ behavioural paradigms, which test cognitive flexibility^{34,35}. We used the Morris water maze to test reversal learning: after the six (hidden) days (Fig. 6B,C), the platform was moved and testing continued for a further five days (the reversal task, R1–R5). In the reversal learning test *Unk^{ckO}* mice showed a significantly reduced latency to find the platform and spent more time in the probe quadrant compared to controls (Fig. 6B,D). *Unk^{ckO}* mice also turned significantly more acutely towards the probe quadrant (Fig. 6E,F). *Unk^{ckO}* mice swim speed was not significantly different from controls (Fig. 6G,H) and so did not confound their ability to find the platform. Thus, spatial learning acquisition and retention of spatial memory over time is unchanged in *Unk^{ckO}* mice, but they show enhanced memory formation and cognitive flexibility to re-learn.

Discussion

Unkempt is a highly conserved zinc finger/RING domain protein. *Drosophila* and mouse Unkempt have the same overall primary structure and 60% identity in the zinc finger domain. Unkempt has been characterised genetically in *Drosophila* as a regulator of neurogenesis and growth control, acting downstream of mTOR^{13,16,17}. In mammals, mechanistic studies have revealed the role of the zinc finger domain of Unkempt. Unkempt regulates the translation of several hundred target mRNAs, in both cultured cells and the developing brain, by binding a specific U/A-rich motif through its zinc fingers^{18,19}. The requirement for Unkempt in the mammalian nervous system has not been previously determined. Given its essential role in *Drosophila* development our study shows, surprisingly, that conditional knockout of Unkempt in the developing nervous system is not overtly detrimental. However, loss of Unkempt leads to improved cognitive flexibility in adult mice.

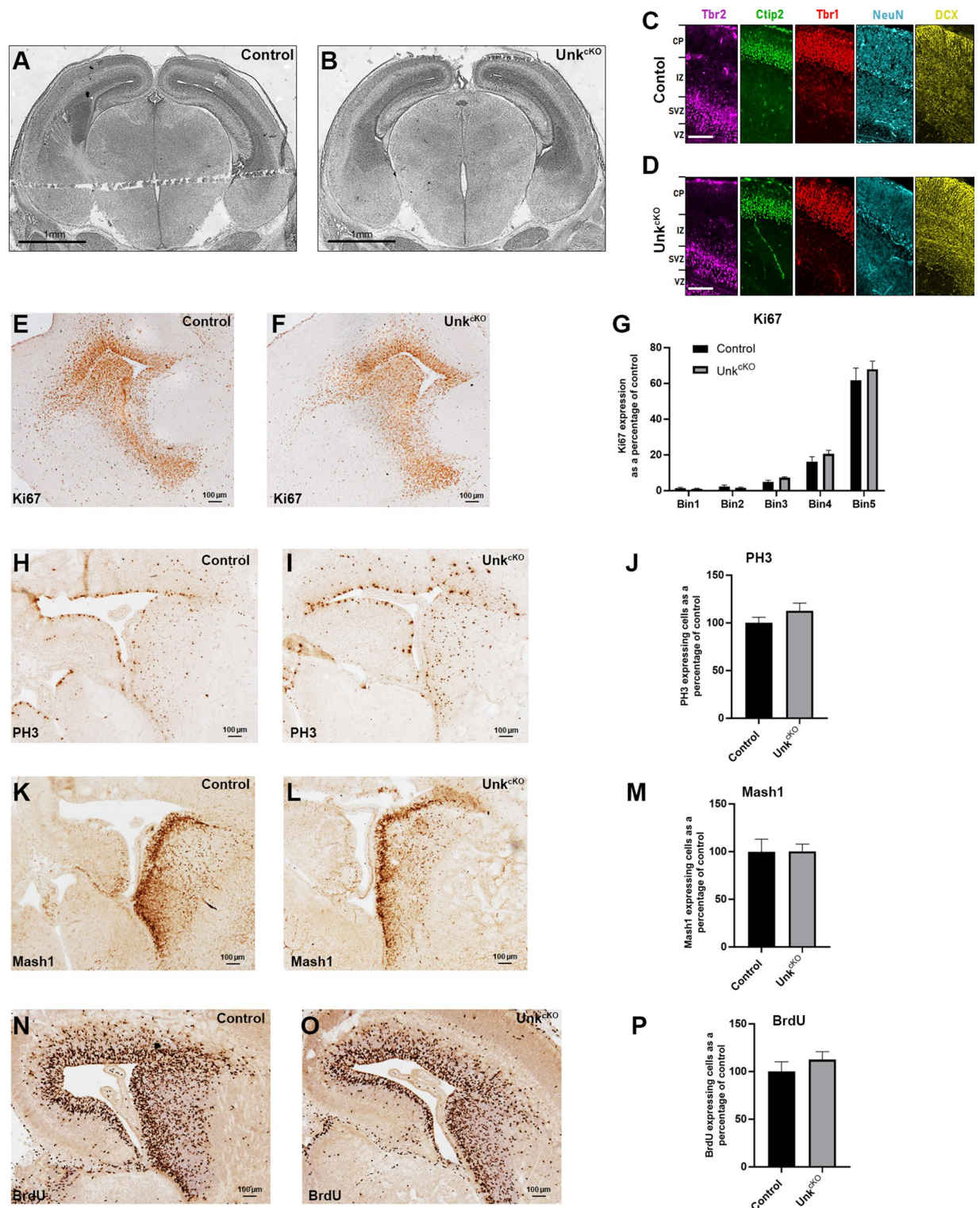


Figure 3. Normal neurodevelopment in *Unk^{CKO}* mice. (A,B) Coronal sections of the brains from control (A) and *Unk^{CKO}* (B) mice stained with haematoxylin and eosin at E16.5. (C,D) Expression of cortical layer markers Tbr2 (magenta), Ctip2 (green), Tbr1 (red), NeuN (cyan), and DCX (yellow) in the developing cortex from control (C) and *Unk^{CKO}* (D) mice at E16.5. CP = cortical plate, IZ = intermediate zone, SVZ = subventricular zone, VZ = ventricular zone. Scale bars: 100 μ m. (E–P) Coronal sections of the subventricular zone from control and *Unk^{CKO}* embryos at E16.5 stained for Ki67 (E,F), PH3 (H,I), Mash1 (K,L) and BrdU incorporation (N,O). Quantifications shown in (G) control $n=5$, *Unk^{CKO}* $n=5$; (J) control $n=7$, *Unk^{CKO}* $n=9$; (M) control $n=7$, *Unk^{CKO}* $n=9$; and (P) control $n=5$, *Unk^{CKO}* $n=5$. Data are presented as mean $\pm$ SEM.

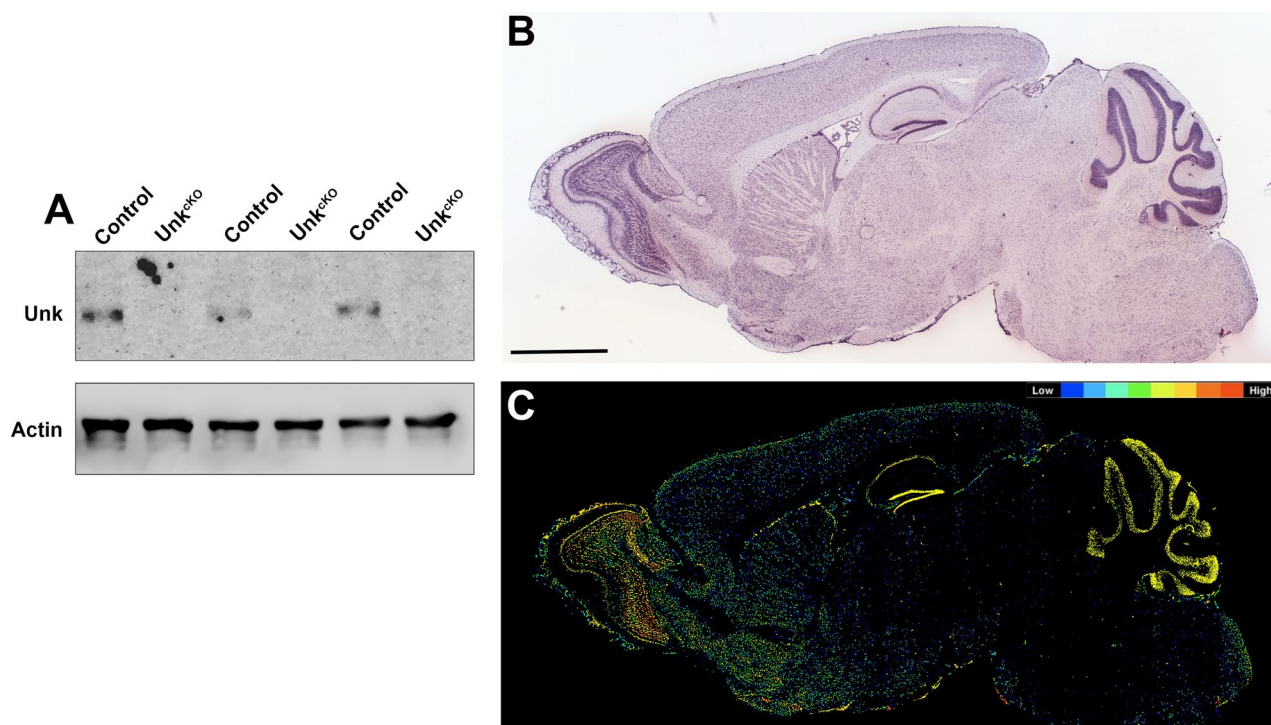


Figure 4. Unkempt is strongly expressed in the adult cerebellum and hippocampus. (A) Western blot of Unkempt (Unk) expression in P60 CNS tissue from control and *Unk^{KO}* mice. (B) Unkempt mRNA in situ hybridisation of a P56 C57BL/6 J mouse brain sagittal section. Image credit: Allen Institute. Scale bar: 2 mm. (C) Expression mask image of Unkempt mRNA expression. Image credit: Allen Institute.

Genotype	Observed	Expected	Frequency observed (%)	Frequency expected (%)
Control (<i>Unk^{tm1c/tm1c}</i>)	69	64.5	26.74	25
Control (<i>Unk^{tm1c/+}</i>)	58	64.5	22.48	25
<i>Unk^{KO}</i> heterozygous (<i>Nestin Cre, Unk^{tm1d/+}</i>)	77	64.5	29.84	25
<i>Unk^{KO}</i> homozygous (<i>Nestin Cre, Unk^{tm1d/tm1d}</i>)	54	64.5	20.93	25
Total	258	258	100	100

Table 1. *Unk^{KO}* mice are born at expected Mendelian ratios. The table shows the number of pups born for each genotype, from 32 litters. Statistical significance was calculated with Pearson's Chi-square test, Chi-square value = 5.1, *p* = 0.165.

A previous study used in utero electroporation to knockdown Unkempt in the developing murine cortex¹⁸. Knockdown of Unkempt perturbed neuronal migration and caused aberrant neuronal morphology, which was rescued by expression of an RNAi-resistant Unkempt construct. Given these findings, and that Unkempt regulates neurogenesis and is essential for viability in *Drosophila*, it is remarkable that knockout of Unkempt did not affect neurodevelopment. There are several potential explanations for the absence of a neurodevelopmental phenotype in *Unk^{KO}* mice. Firstly, the paralog Unkempt like may compensate for the loss of Unkempt during nervous system development. Unkempt like is expressed in the developing neurogenic niche¹³ and we find that Unkempt like is still robustly expressed in the developing nervous system in *Unk^{KO}* mice. The function of Unkempt like has not been studied in vivo but it may act redundantly with Unkempt. Future double knockout studies may test this hypothesis. Secondly, expression of Unkempt before E10.5, when Nestin-Cre expression begins, in our knockout model may mask the full requirement for the protein during nervous system development. Unkempt is expressed in the nervous system at E10¹⁸, but protein levels earlier in neurodevelopment have not been analysed. Interestingly, recent single cell RNA-sequencing shows that Unkempt mRNA is expressed in the developing brain as early as E6-E7, and from E8-E10 in ectoderm, neural crest, and radial glial cells (<http://mousebrain.org/development>)³⁶. Thus, perdurance of Unkempt mRNA or protein expressed prior to E10 may partially rescue the neurodevelopmental phenotype in *Unk^{KO}* mice. However, redundancy with Unkempt like or perdurance do not necessarily explain the difference between *Unk^{KO}* mice and Unkempt knockdown; redundancy with Unkempt like should also apply with the knockdown approach, which was performed at E14.5¹⁸. Although the phenotypes were rescued by expression of an RNAi-resistant Unkempt¹⁸, the migration and morphology phenotypes

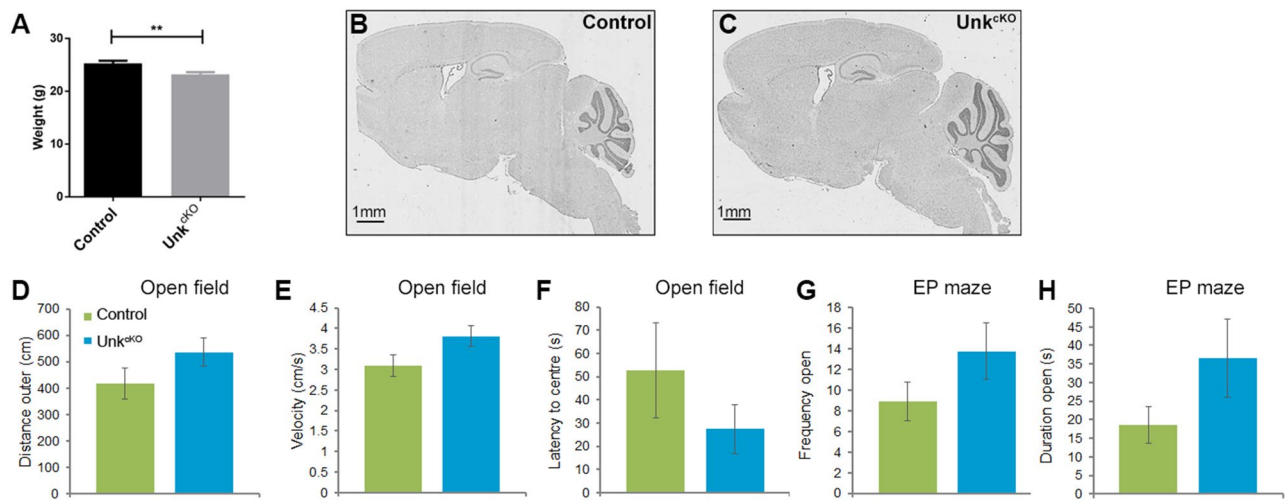


Figure 5. *Unk^{KO}* mice have normal locomotor activity and show a trend towards decreased anxiety. (A) Body weight of control and *Unk^{KO}* mice at 12 weeks. (B,C) Sagittal sections of control (B) and *Unk^{KO}* (C) mice at P20 stained with haematoxylin and eosin. (D,E) Open field test measure of distance (D) and velocity (E) of movement in the outer ring of the arena. (F) Open field test, latency to centre. (G,H) Elevated plus (EP) maze test, number of entries to open arms (G), time spent in open arms (H). Control $n = 11$, *Unk^{KO}* $n = 8$. Data are presented as mean $\pm$ SEM. Students t -test, $**p < 0.01$ compared to control.

resulting from Unkempt knockdown may still be artefactual due to off-target effects or toxicity. Alternatively, acute knockdown of Unkempt during development may reveal phenotypes before mechanisms that compensate for loss of Unkempt expression have time to become activated. Future studies using the Unkempt conditional allele we have generated together with acute expression of Cre during neurodevelopment will test this possibility.

Our Raptor binding assay data supports previous evidence that Unkempt physically interacts with Raptor^{16,20}. The precise function of the interaction between Unkempt and Raptor is currently unknown. Inhibition of mTOR signaling abrogates the physical interaction between Unkempt and Raptor in *Drosophila* cultured cells¹⁶. Moreover, the mTOR pathway negatively regulates Unkempt protein levels in the *Drosophila* developing retina and so Raptor may regulate the stability of Unkempt¹³. Interestingly, we found a strong reduction in Raptor levels in the embryonic brain of *Unk^{KO}* mice, suggesting a reciprocal requirement for protein stability between Unkempt and Raptor. Although Raptor levels were reduced, expression of the canonical readouts of mTORC1 activity, phospho-rpS6 and phospho-4E-BP, were not altered in *Unk^{KO}* mice. Therefore, Unkempt function likely diverges downstream of mTORC1, potentially acting as a branchpoint in the mTOR pathway (Fig. 6I).

Knockout of Raptor or mTOR in the developing brain causes microcephaly, reduced neural progenitor proliferation and postnatal lethality, while knockout of *Tsc1* or *Tsc2* causes macrocephaly and premature neural progenitor differentiation^{6,37,38}. This contrasts to knockout of Unkempt in the developing nervous system, which leads to reduced Raptor levels, but does not have an obvious effect on cortical neurogenesis or viability. Similar to *Unk^{KO}* mice, mice with a brain-specific knockout of FKBP12, which causes partial activation of mTORC1, and mice overexpressing the mTORC1 effector eIF4E, are viable, healthy and have normal spatial learning but have impaired reversal learning^{34,35}. Moreover, inhibition of mTOR signaling by chronic rapamycin treatment has been reported to enhance spatial learning, and chronic dietary restriction inhibits mTORC1 to enhance memory performance in young mice^{28,29}. The improved reversal learning phenotype in *Unk^{KO}* mice is therefore consistent with models of decreased mTOR pathway activity in the brain. Taken together, these studies show that chronic inhibition of mTOR signaling in the brain improves learning and memory. Moreover, manipulation of non-essential mTORC1 components and downstream factors, such as FKBP12, eIF4E and Unkempt, specifically affect cognitive flexibility.

Regulation of local translation by mTORC1 is crucial for synaptic plasticity³⁹. Unkempt has been proposed to be a “regulator of regulators”, as it controls the translation of proteins that themselves regulate translation, including mTOR, eIF4 and p70S6K pathway associated proteins, suggesting cross-talk between Unkempt and mTORC1 regulated mRNAs¹⁸. Loss of Unkempt and the resulting altered translation of its target mRNAs may affect local synaptic translation in hippocampal circuits involved in learning and memory (Fig. 6I). The resulting increased synaptic plasticity in the dentate gyrus may lead to improved cognitive flexibility in *Unk^{KO}* mice. Conversely, misregulation of Unkempt by hyperactive mTORC1 may contribute to the neurological manifestations of tuberous sclerosis complex and focal cortical dysplasia. Targeting Unkempt may therefore be a novel therapeutic strategy for neurological diseases associated with activated mTOR signaling. Future studies will elucidate the key role of Unkempt in cognition and its intersection with mTOR signaling in learning and memory.

Materials and methods

Western blot analysis. For western blot analysis cell extracts were denatured and reduced in 1× sample buffer (500 mM Tris pH 6.8, 40% (v/v) glycerol, 0.2% (w/v) SDS, 2% (v/v) β -mercaptoethanol, and 0.02% (w/v) bromophenol blue) and boiled at 98 °C for 10 min. Proteins were separated by SDS–polyacrylamide gel electro-

phoresis (PAGE). Proteins were then transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were blocked in 10% fat-free milk powder in tris buffered saline (TBS) (50 mM Tris pH 7.4, 150 mM NaCl) and probed overnight at 4 °C with primary antibodies in 5% (w/v) bovine serum albumen (BSA; Fisher) TBS-T (TBS + 0.1% (v/v) Tween 20). Following four 10 min washes in TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:4000) in 5% milk TBS-T for one hour at room temperature. Finally, blots were treated with enhanced chemiluminescence reagents (ECL, GE Healthcare) and imaged using a Kodak or Bio-Rad ChemiDoc system imaging system. For detecting ubiquitinated proteins the nitrocellulose membrane was blocked with 1% (w/v) BSA in phosphate buffered saline (Oxoid), 0.1 (v/v) Tween 20 (Sigma; PBS-T), the primary antibody diluted in 1% (w/v) BSA TBS-T, secondary antibody in 0.05% (w/v) BSA in PBS-T, and washed with PBS-T. To quantifying protein expression, densitometry of western blot bands was performed using ImageJ and values were normalised to β -Actin.

Primary antibodies used were rabbit anti-Unkempt (1:1000, HPA023636, Cambridge Bioscience), rabbit anti-Unkempt like (1:1000, HPA055801, Sigma), rabbit anti-Raptor (1:1000, #2280, Cell Signaling), rabbit anti-S6 (1:1000, #2217, Cell Signaling), rabbit anti-phospho-S6 (Ser235/236, 1:1000, #2211, Cell Signaling), rabbit anti-4E-BP2 (1:1000, #2845, Cell Signaling), rabbit anti-phospho-4E-BP1 (Thr37/46, 1:1000, #2855, Cell Signaling), rabbit anti- β -actin (1:5000, #4967, Cell Signaling), rabbit anti-phospho AKT (Ser473, 1:1000, #4060T, Cell Signaling), rabbit anti-AKT (1:1000, #4691T, Cell Signaling).

Quantitative reverse transcription PCR (qRT-PCR) analysis. cDNA was generated using PrimeScript RT-PCR Kit (Takara, RR014B) and qRT-PCR was performed using PowerUp™ SYBR Green Master Mix (Applied biosystems, A25741). The Ct values were normalized to RPS18.

The primers used were:

Ms_qPCR Unk F1: 5'-ACAGCCCGAGAAACCGCAGCACTA-3'
 Ms_qPCR Unk R1: 5'-GCAGGAATGGGCACTCGTCGC-3'
 Ms_qPCR Unk F2: 5'-CGGTGGCAAGAGACTGCTTAT-3'
 Ms_qPCR Unk R2: 5'-ACCTGTATTTGTGCTTCCGGG-3'
 RPS18-F: 5'-CGGAAAATAGCCTTCGCCATCAC-3'
 RPS18-R: 5'-ATCACTCGCTCCACCTCATCCT-3'.

Raptor overlay assay. HeLa S3 cells stably expressing doxycycline inducible FLAG-HA-Unkempt¹⁸ were used to purify FLAG-HA-Unkempt as follows. Cells from three 15 cm dishes per condition were scraped and pooled together. They were lysed for 20 min on ice in 10 ml lysis buffer (25 mM Tris pH 8.0, 150 mM NaCl, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail and 0.2 mM PMSF). Lysates were centrifuged at 20,000×g for 20 min at 4 °C and the cleared supernatant was incubated with 70 μ l anti-FLAG M2 affinity agarose gel rotating end over end overnight at 4 °C. Samples were then washed twice in lysis buffer followed by three washes in high salt buffer (50 mM Tris pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 1% (v/v) Triton X-100) and three times in wash buffer (50 mM Tris pH 8.0, 150 mM NaCl). Recombinant FLAG-HA-Unkempt was eluted in 80 μ l wash buffer supplemented with 0.2 mg/ml 3×FLAG peptide (Generon) rotating end over end for 30 min at 4 °C. Purified Unkempt was collected using 30G needle, flash-frozen in liquid nitrogen and stored at −80 °C until required.

The Raptor overlay assay was performed as described previously²³. Briefly, purified FLAG-HA-Unkempt, mock purified extract from un-induced HeLa S3 cells (control), or recombinant 4E-BP1 were dotted onto PVDF membrane. The membrane was then incubated with lysate from HEK293 cells overexpressing HA-Raptor, HA-Raptor mutant 4²⁴, or carrying empty vector. The membrane was then probed with an anti-Raptor antibody. HA-Raptor and HA-Raptor mutant 4 expression levels were determined by western blot.

Animal models. C57BL/6 J mice were used for all experiments and were housed under a 12 h light/dark cycle with ad libitum access to food and water. The mouse studies were carried out in accordance with UK Home Office regulations and the UK Animals (Scientific Procedures) Act of 1986 (ASPA) under a UK Home Office licence (PPL 70/8719) and approved by the King's College London Ethical Review Committee. Animal studies were performed in accordance with ARRIVE guidelines (<https://arriveguidelines.org>). All mice were housed in individually-ventilated cages except for the mice used in the behavioural study, which were housed in standard, open-top cages (32 × 16 × 14 cm). All cages contained sawdust, a cardboard shelter, and extra bedding material (Datesand Ltd, Manchester). Mice were maintained at a standard temperature (21 °C) and humidity (45%), and in pathogen-free conditions kept under a regular 12 h light/12 h dark schedule, with ad libitum access to water and food (5053 – PicoLab Rodent Diet 20, Lab Diet, St. Louis, USA). *Unk^{tm1a(KOMP)Wtsi} (Unk^{tm1a})* mice were generated by the Wellcome Trust Sanger Institute as part of the.

European Conditional Mouse Mutagenesis Program and the Deciphering Mechanisms of Developmental Disease programme⁴⁰ and distributed by the European Mouse Mutant Archive. *Unk^{tm1a}* were generated by blastocyst injection of targeted ES cells using standard techniques^{41,42} and germline transmission of the *Unk^{tm1a}* allele was confirmed by PCR genotyping⁴¹. The *Unk^{tm1a}* colony was maintained on a mixed genetic C57BL/6N; C57BL/6N-A^{tm1Brd/a} background. Presence of the *Unk^{tm1a}* allele was confirmed by PCR genotyping using Unk mutant primers (5'-CATGTGCTGTACCGTCCTGT-3' and 5'-TCGTGGTATCGTTATGCGCC-3', which produces a 327 bp product).

Unk^{tm1a} mice were crossed to FLP recombinase mice (Gt(ROSA)26Sortm1(FLP1)Dym, #003956 the Jackson Laboratory, kindly provided by Karen Steel) to delete the FRT-site-flanked cassette. UnkWT primers (5'-CATGTGCTGTACCGTCCTGT-3' and 5'-TAGGCTTCTGAGAGGGGTCA-3') were used to identify mice with the

Unk^{tm1c} allele (600 bp product compared to 491 bp product in wild-type). *Unk^{tm1c}* mice were then crossed with Nestin-Cre mice (B6.CgTg(Nes-cre)1Kln/J, #003771, The Jackson Laboratory, kindly provided by Oscar Marín) and conversion to the *Unk^{tm1d}* allele confirmed with PCR genotyping with *UnkTm1d* primers (5'-AAGGCGCAT AACGATACCAC-3' and 5'-ACTGATGGCGAGCTCAGACC-3' which produces a 174 bp product). Cre primers (5'- GTTATTCGGATCATCAGCTACACC-3' and 5'-GTCCAATTTACTGACCGTACACC-3' which produces a 650 bp product) were used to confirm presence of Nestin-Cre.

Ear-clipped tissue was digested at 55 °C overnight in 50 µl of lysis buffer (100 µM Tris pH 8, 5 mM EDTA, 200 µM NaCl, 0.2% (w/v) SDS + 2.5 mg/ml proteinase K). The digested tissue was diluted by the addition of 100 µl distilled water, then a 1:20 dilution made and 2 µl was used for the PCR reaction. PCR reactions were performed using GoTaq Green Master Mix (Promega) or Reddymix (Thermo Fisher Scientific) with 10 µM forward and reverse primers in a total reaction volume of 20 µl.

For dendrograms, sequences were aligned with MUSCLE (MEGA-X also has MUSCLE alignment tool implemented) and dendrograms generated with MEGA-X software using the Maximum Likelihood model.

Immunofluorescence and Immunohistochemistry. For immunofluorescence, embryonic brains were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 24 h at 4 °C while rotating, then cryoprotected by immersion in PBS 15% sucrose for 24 h at 4 °C, and PBS 30% sucrose for 24 h, then embedded in O.C.T Compound (VWR) and stored at -80 °C. O.C.T embedded brains were cut using a cryostat into 10–20 µm sections on Superfrost plus slides (Thermo Fisher) and dried overnight at room temperature. For better adhesion of sections, the slides were further dried at 50 °C for one hour. Citric acid (pH 6) was pre-heated to 90 °C in a convection oven, and then slides put inside for 45 min for heat-induced epitope retrieval. Slides were left to cool in the same solution in a fume hood, then rinsed three times in 1X TBS (pH 7.6). Slides were drained and a ring drawn around sections with a liquid blocker pen (Agar Scientific). Sections were covered in blocking solution (2% BSA TBS; Sigma-Aldrich) for 5–10 min. Blocking solution was removed and primary antibody added (diluted in blocking buffer) for 16 h at room temperature. Slides were rinsed three times in TBS and incubated with the appropriate Alexa-conjugated secondary antibody (ThermoFisher, 1:300 in blocking buffer) with DAPI for one hour at room temperature. Slides were rinsed three times in TBS and mounted using an aqueous based fluorescence mounting medium (Sigma-Aldrich).

For 3,3'-diaminobenzidine (DAB) staining, embryonic brains were fixed with 4% PFA for 24 h at room temperature and dehydrated using increasing concentrations (70%, 90% and 100%) of ethanol. Dehydrated brains were cleared in xylene and immersed in molten paraffin wax at 64 °C. Brains were then orientated coronally and embedded in moulds (VWR) of fresh molten wax and left to set at 4 °C. Post-setting, the blocks were removed, trimmed to remove excess wax and serially sectioned (6 µm) on to Superfrost slides (ThermoFisher) using a microtome. Slides were dried overnight, then heated at 60 °C for one hour in a convection oven. Slides were dewaxed with xylene (2 × 5 min) and 100% ethanol (2 × 5 min). Slides were washed under running tap water and endogenous peroxidase activity was blocked with 3% hydrogen peroxide (10 min, room temperature, Sigma). Heat-induced epitope retrieval was performed using preheated citric acid (pH 6.4) in a pressure cooker for five minutes prior to incubation with primary antibodies. Slides were incubated in blocking solution (2% (w/v) BSA in 1 × TBS, sodium azide, pH 7.6) for five minutes and incubated in primary antibody for 16 h at room temperature. Slides were washed in TBS and incubated for one hour at room temperature in biotinylated secondary antibody (Vector Labs, 1:500) diluted in blocking solution. Slides were then incubated with StreptABC-HRP (Vector Labs) for 30 min at room temperature and developed in DAB (Sigma) solution for 10 min with gentle agitation. Slides were washed under running water until clear and dehydrated with methylated spirits, cleared with xylene and mounted using DPX (Sigma).

For staining adult brains, transcardial perfusion of adult mice was performed by injection of 50 ml PBS, 12.5 mM EDTA and then 4% (w/v) PFA in PBS through the left ventricle. The brains were then excised and further fixed for 24 h in 4% PFA and then dehydrated and embedded in paraffin.

For BrdU incorporation in embryos, pregnant dams were injected intraperitoneally with BrdU in 0.9% NaCl (100 mg/kg, Sigma) at E16.5. Mice were culled two hours post-injection and embryonic heads were fixed in 4% (w/v) PFA for 24 h, dehydrated and embedded in paraffin. For adult BrdU incorporation, P20 mice were injected intraperitoneally with BrdU in 0.9% NaCl (100 mg/kg, Sigma) three times at two-hour intervals. 24 h later, the animals were anesthetized and fixed by transcardial perfusion using 4% PFA.

Primary antibodies for immunofluorescence were mouse anti-NeuN (1:2500, ab104224, Abcam), rabbit anti-DCX (1:5000, ab18723, Abcam), rabbit anti-TBR1 (1:1000, ab31940, Abcam), rabbit anti-Tbr2 (1:1000, ab23345, Abcam), rat anti-Ctip2 (1:500, ab18465, Abcam), mouse anti-Ki67 (#556003, BD Pharmingen). Primary antibodies for DAB staining were sheep anti-BrdU (1:1000, ab1893, Abcam), rabbit anti-phospho-Histone H3 (1:2000, 06-570, Cell Signaling), rabbit anti-Ki67 (1:700, RM9106, LabVision), mouse anti-Mash1 (1:5, a gift from François Guillemot), rabbit anti-DCX (1:5000, ab18723, Abcam), mouse anti-NeuN (1:2500, ab104224, Abcam).

Brightfield images were taken on a Zeiss Axioskop microscope and quantified using Image J. For each animal, staining in four consecutive 6 µm sections was quantified and averaged. Colour deconvolution was applied to the images, and the same brightness and contrast settings were used for each experiment. Intermodos thresholding was applied and expression per mm² calculated using the analyze particles tool for embryonic Ki67, Mash1 and BrdU. For Ki67 staining, the neocortex was divided into five bins in Image J using the Bin division plugin. For embryonic PH3 numbers of cells expressing these markers per mm² were counted manually. Raw numbers were then converted to percentage as a proportion of control.

Immunofluorescence imaging was performed using a Zeiss LSM710 confocal microscope with Zen 2012 LSM software. Imaging of controls and experimental samples was performed using identical confocal microscope settings.

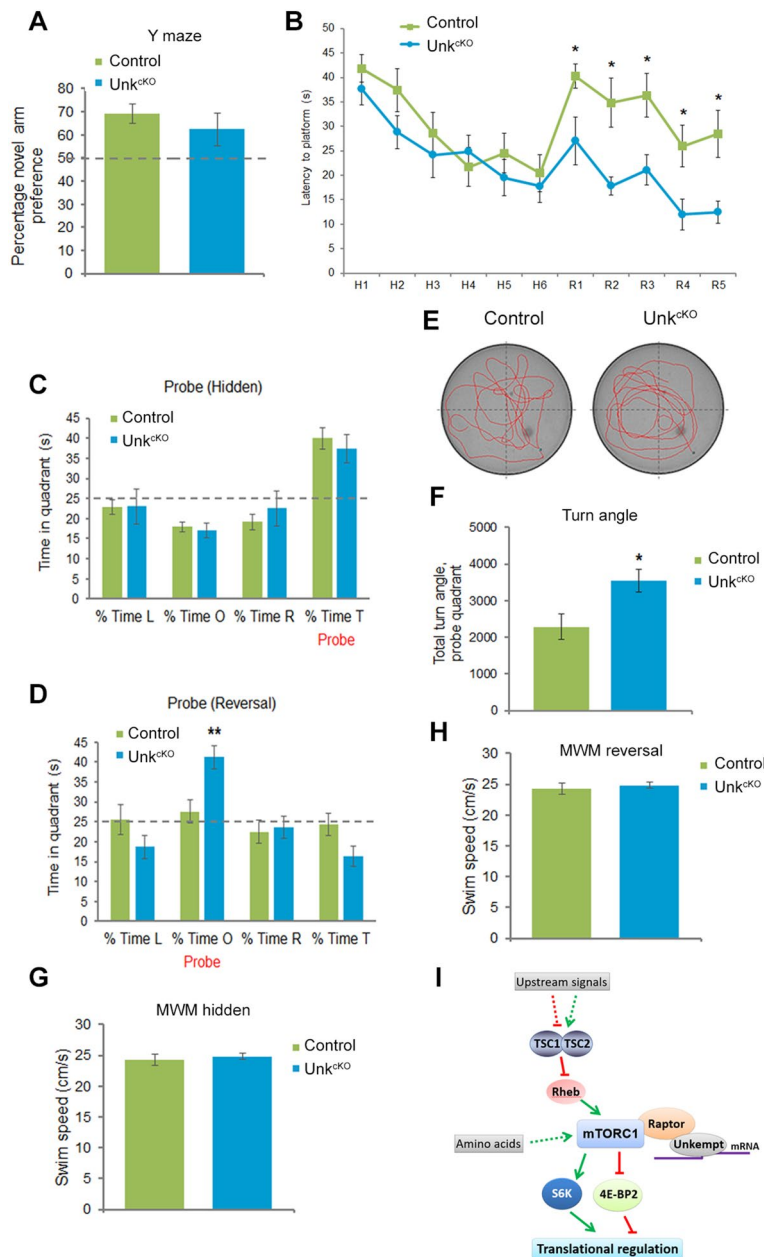


Figure 6. Loss of Unkempt improves reversal learning. **(A)** Y maze, preference for novel arm. **(B)** *Unk^{cKO}* mice show enhanced cognitive flexibility. The Morris water maze test was carried out by measuring the latency to find the hidden platform for six hidden sessions (H1-6) and then the location of the platform was changed to measure re-learning for another five reversal sessions (R1-5). All mice showed significant spatial learning across the hidden task (session factor $F(5, 85) = 10.0$, $p < 0.0001$), but there was no genotype effect (genotype factor $F(1, 17) = 1.2$, $p = 0.3$). In the reversal task, all mice showed significant reversal learning (session factor $F(4, 68) = 6.7$, $p < 0.01$) but there was a significant genotype effect (genotype factor $F(1, 17) = 14.3$, $p = 0.001$) with *Unk^{cKO}* mice displaying much greater cognitive flexibility than control mice. **(C,D)** Probe trial data showing the percentage of time spent in each quadrant after the hidden **(C)** and reversal **(D)** trials. L = left, O = opposite, R = right, T = target quadrant. *Unk^{cKO}* mice showed a significant preference for the opposite quadrant, indicating reference memory in the reversal task ($t(17) = -3.3$, $p < 0.01$). **(E)** Representative tracing of control and *Unk^{cKO}* mice in the probe trial of the Morris water maze reversal task. The platform is located in the bottom right quadrant. **(F)** Turn angle (degrees) made by mice during the probe trial for the reversal task was significantly increased in *Unk^{cKO}* compared to controls ($t(17) = 2.6$, $p < 0.05$). **(G,H)** Swim speed during hidden **(G)** and reversal **(H)** Morris water maze (MWM) probe trials. Control $n = 11$, *Unk^{cKO}* $n = 8$. Statistical significance for genotype effects for each session (genotype factor) and between the daily sessions (session factor) of the Morris water maze were calculated using two-way ANOVA. Comparisons between *Unk^{cKO}* and control mice were made using a Student's t -test, $*p < 0.05$, $**p < 0.01$ compared to control. **(I)** A model for mTORC1 signaling in the brain.

Unkempt in situ hybridisation data is available at <https://mouse.brain-map.org/experiment/show/69013684>. The Expression Energy was calculated as follows: Within a given area A (voxel or structure), expression energy = (sum of intensity of expressing pixels in A) / (sum of all pixels in A).

Behavioural testing. For behavioural analysis, only male mice were used, and the experimenter was always blinded to the genotypes. The mice were singly housed one week prior to behavioural testing, and throughout the test period, to avoid any potential confounds from social hierarchies, which could influence the controlled assessment of social behaviours⁴³. Sawdust was changed every other week but never on the day before, or the day of testing and the enrichment (nesting material and house) was changed less regularly to minimize the disruption to the animals. Testing was performed when the mice were 12–20 weeks old. Tests were recorded with a camera above the test arenas and the mice were tracked using Ethovision software (Noldus Information Technologies bv, Wageningen, The Netherlands). Urine and boli was removed, and the arena cleaned with 1% Anistel® solution (high level surface disinfectant, Trisel Solution Ltd, Cambridgeshire, UK) between each trial to remove odours. Mice were returned to their home cage after testing.

For the open field test³¹ the arena consisted of a circular open field (40 cm diameter) enclosed by walls. The light intensity in the room was set to 25 lx by a light-adjustable floor lamp. The mouse was placed inside the arena next to the wall (always in the same starting location) and left to explore the arena for 10 min. Two virtual zones within the arena were defined on Ethovision; a 'central zone' (20 cm diameter) and the 'outer zone' (remainder of arena). The latency(s) to enter, and the time(s) spent in, the central zone of the arena and the mean velocity (cm/s) of the mice in the outer zone were extracted by Ethovision.

For the elevated plus maze test³⁰ the arena consisted of four arms; two opposing open arms with a 0.5 cm ledge around, and two opposing closed arms enclosed by a 15 cm high Perspex wall. All arms were 30 × 5 cm, and the whole maze elevated 40 cm above the ground. The light intensity in the open arms was 40 lx, and 20 lx in the closed arms. Each mouse was placed in the centre of the platform, and its movement tracked for five minutes undisturbed before being taken out. The duration(s) and frequency of entry in the open arms, was extracted using Ethovision.

The spontaneous spatial novelty preference test was conducted using a perspex Y-maze³². Each arm was 22 cm long, 7 cm wide, with 20 cm-high walls. One entry into an arm was defined as placement of two paws into that arm. For the first trial, one of the arms of the Y-maze was closed, therefore mice could either go left or right according to a pseudorandom sequence (equal numbers of left and right arms were blocked in total sessions), mice could also move in the central arm for five minutes. In the second trial one hour later to assess short term memory, the test was repeated with access to both arms. The time(s) spent in each arm was extracted from Ethovision. For trial 2, a percentage preference for the novel arm [$100 \times (\text{time spent in the novel arm} / (\text{time spent in the novel arm} + \text{time spent in the familiar arm}))$] was calculated.

For the Morris water maze test³³ the pool arena was made of white acrylic, with a diameter of 1 m, and 30 cm deep. The pool was filled with a non-toxic, white aqueous emulsion (Acusol OP301 Opacifier, Rohm & Haas, Landskrona, Sweden). In the middle of one of the quadrants a platform with 10 cm diameter was located 1 cm below the surface. During testing, the room was lit with white light (100 lx) using four lamps pointing upwards at each corner. The pool was surrounded by cream-coloured curtains from which distinct spatial cues were suspended to help the mice navigate around the pool. Four equidistant positions around the pool walls were designated as Target (T), Opposite (O), Left (L) and Right (R), dividing the arena into four virtual quadrants. The mice were placed at these alternate locations for the successive trials, which were run in a pseudorandom manner. Each mouse therefore underwent four trials per day, and the latency to find the platform was calculated as an average of these four trials.

Mice were run in squads of six mice, with four trials each. A new trial started when all mice in the squad had finished. Between trials, mice were returned to their home cages and at the end of each session of four trials, mice were returned to the housing room. The trials were started by placing the mouse into the pool close to the wall in one of the four start locations (each trial was started in a different quadrant). Each mouse was given 60 s to swim to the platform and remained on the platform for 10 s before being removed. The latency to reach the platform was manually recorded from when the mouse was put into the pool to when all four paws of the mouse were on the platform. If the mouse did not reach the platform after 60 s it was guided to the platform and left there for 10 s. The same protocol was used over the subsequent six days of hidden sessions. On the final day of hidden platform training, a probe task was run. This was done by removing the platform and allowing the mice to swim in the pool for 60 s. Then, the platform was moved to the quadrant opposite the target quadrant for five days of reversal training. On the final day of reversal training, another 60 s probe task was run. To assess the retention of spatial memory, the average latencies to find the platform were recorded over sessions (days), and by comparing the time spent in the quadrant that contains the platform (target quadrant) with the time spent in other quadrants. The swim speeds (cm/s) and angular velocity were extracted by Ethovision during the probe trials.

Statistical analyses. Data were analysed using GraphPad Prism version 7 (GraphPad Software) or Statistica software (Version 5.5, StatSoft, Inc., Tulsa, OK). Data distributions were assessed for normality, then the effects of genotypes were analysed using a Student's t-test or two-way ANOVA, as appropriate. For two-way ANOVA, the between-factors were always genotype, and the within-factors were sessions.

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Author contributions

Conceptualization, J.M.B.; Methodology, E.V., J.M., C.F., C.H., J.M.B.; Formal analysis, E.V., P.B., C.F.; Investigation, E.V., P.B., S.R.M., A.R.T., H.W., I.B., C.H., K.S.; Writing – Original Draft, J.M.B.; Writing – Review & Editing, J.M.B., E.V., C.F., A.R.T.; Visualization, E.V., P.B., J.M.B.; Funding Acquisition, J.M.B., A.R.T. and C.H.; Supervision, J.M.B., C.H., C.F.

Competing interests

The authors declare no competing interests.

Additional information

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Phosphorylation of the novel mTOR substrate Unkempt regulates cellular morphogenesis

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Key words

Unkempt, mTOR, cellular morphogenesis, Raptor, intrinsically disordered region, phosphorylation

Abstract

Mechanistic target of rapamycin (mTOR) is a protein kinase that integrates multiple inputs to regulate anabolic cellular processes. mTOR complex I (mTORC1) has key functions in growth control, autophagy and metabolism. Much less is known about the signalling components that act downstream of mTORC1 that regulate cellular morphology, a vital determinant of cellular function. Here we show that the RNA-binding protein Unkempt, a key regulator of cellular morphogenesis, is a novel substrate mTORC1. We find that Unkempt phosphorylation is regulated by nutrient levels and growth factors via mTORC1. Furthermore, Unkempt physically interacts with and is directly phosphorylated by mTORC1 through binding to the regulatory-associated protein of mTOR, Raptor. Phosphorylation of Unkempt, which we find is mTORC1-dependent in cultured mammalian cell lines as well as in primary tissues, occurs largely within the highly serine-rich intrinsically disordered region of Unkempt. Importantly, mutation analysis of this region indicates that phosphorylation inhibits the ability of Unkempt to induce a bipolar morphology. Our findings reveal a novel molecular link between mTORC1 signalling and cellular morphogenesis.

Introduction

Mechanistic target of rapamycin (mTOR) is a large serine/threonine kinase that forms the catalytic subunit of two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Saxton & Sabatini, 2017). mTORC1 integrates signalling inputs from cellular nutrients, growth factors, energy, and stress to regulate a range of anabolic processes. Hyperactivation of mTORC1 signalling is implicated in myriad disease contexts including cancer, metabolic, immune and neurological diseases (Liu & Sabatini, 2020; Tee *et al*, 2016). mTORC2 was first shown to regulate the actin cytoskeleton via the AGC kinase PKC α and was subsequently shown to have additional roles in cell proliferation and survival (Jacinto *et al*, 2004; Sarbassov *et al*, 2004; Saxton & Sabatini, 2017).

mTOR regulates the phosphorylation of several hundred proteins, dependent on the cell type (Battaglioni *et al*, 2022; Demirkan *et al*, 2011; Hsu *et al*, 2011; Robitaille *et al*, 2013; Yu *et al*, 2011). Relatively few direct mTOR substrates have however been characterised in detail. Well characterised mTORC1 substrates include S6K, 4E-BP1 and the adaptor protein Grb10, all regulators of cell growth and the protein kinase ULK1, which regulates autophagosome biogenesis (Ben-Sahra *et al*, 2013; Egan *et al*, 2011; Hsu *et al*, 2011; Saxton & Sabatini, 2017; Yu *et al*, 2011). mTORC2 phosphorylates AKT and SGK1 to control cell proliferation and survival (García-Martínez & Alessi, 2008; Sarbassov *et al*, 2004; Sarbassov *et al*, 2006). mTORC2 also phosphorylates multiple PKC isoforms, including PKC α , PKB β II and PKC ζ , to regulate actin cytoskeletal dynamics (Baffi *et al*, 2021; Ikenoue *et al*, 2008; Li & Gao, 2014).

In contrast to mTORC2, the role of mTORC1 in cellular morphogenesis is much less well understood. We previously showed that the zinc finger/RING domain protein Unkempt acts genetically downstream of mTOR in the developing retina and central nervous system in *Drosophila* (Avet-Rochex *et al*, 2014; Bateman, 2015; Bateman & McNeill, 2004; Maierbrugger *et al*, 2020). Mammalian Unkempt is a key regulator of cell morphogenesis and, through its zinc finger domain, binds mRNAs involved in control of protein synthesis and cell shape to affect their translation and to induce the establishment of the early neuronal morphology (Murn *et al*, 2016; Murn *et al*, 2015).

Here we show that mammalian Unkempt is abundantly phosphorylated and that phosphorylation is acutely sensitive to mTORC1 activity. Unkempt physically interacts with

mTORC1 by binding to regulatory-associated protein of mTOR (Raptor), which mediates direct phosphorylation by mTOR at multiple serine/threonine residues in the highly serine-rich intrinsically disordered region (IDR). Using mice with conditional knockout of *Tsc1* to activate mTORC1 in the nervous system, we find that Unkempt phosphorylation is mTORC1 dependent *in vivo*. Finally, we show that phosphorylation in the serine rich region suppresses the capacity of Unkempt to induce a bipolar morphology, a critical cellular activity of Unkempt.

Results and Discussion

mTORC1 activity regulates Unkempt phosphorylation

Unkempt is a highly conserved RNA-binding protein that plays critical roles in controlling cell proliferation and differentiation in *Drosophila* and cellular morphogenesis in mammals (Avet-Rochex *et al.*, 2014; Li *et al.*, 2019; Maierbrugger *et al.*, 2020; Murn *et al.*, 2015). How the activity of Unkempt might be regulated remains unknown. Recent studies have suggested that Unkempt acts genetically downstream of mTORC1 (Avet-Rochex *et al.*, 2014; Li *et al.*, 2019; Maierbrugger *et al.*, 2020), but it is unclear whether mTORC1 signalling might be linked to the function of Unkempt. Mammalian Unkempt is either not expressed or expressed at very low levels in non-neural cell lines but is abundant in cells of neuronal lineages (Murn *et al.*, 2015). We therefore used human neuroblastoma SH-SY5Y cells to determine whether Unkempt is regulated in an mTORC1-dependent manner.

In serum-starved neuroblastoma SH-SY5Y cells we found that stimulation of the mTORC1 pathway with insulin or serum re-addition caused a mobility shift of Unkempt to a higher resolved band, whereas direct inhibition of mTORC1 kinase activity with rapamycin prevented this shift (Figure 1A). A strict correlation between inhibition of mTORC1, as judged by phosphorylation of ribosomal protein S6 (rpS6), and decreased mobility of Unkempt was observed over a wide range of rapamycin concentrations (Supplemental Figure 1A). Moreover, a phospho-specific antibody against Unkempt (see details below and Figure 3D) detected reduced levels of phosphorylated Unkempt in rapamycin treated cells (Supplemental Figure 1A). Rapamycin treatment also caused increased phosphorylation of AKT at Ser473 as a result of the established feedback loop between S6K and the insulin receptor substrate (IRS) (Harrington *et al.*, 2004; Shah *et al.*, 2004) (Supplemental Figure 1A).

Unkempt showed a similar mTORC1-dependent mobility shift in response to rapamycin in mouse N2a neuroblastoma cells (Figure 1B) and in mouse E14.5 primary cortical cells (Figure 1C). After insulin stimulation, lambda protein phosphatase treatment of the SH-SY5Y cell lysate converted a higher resolving Unkempt band to a lower resolving species, below that in unstimulated cells (Figure 1D). Together, these data suggest that Unkempt is phosphorylated in an mTORC1-dependent manner and exhibits a basal level of phosphorylation in unstimulated cells.

mTORC1 can be regulated by multiple signalling inputs, including insulin, amino acids and ATP levels (Saxton & Sabatini, 2017). We found that addition of amino acids alone in the media did not suffice to activate mTORC1, as judged by phosphorylation of S6K, or increase Unkempt phosphorylation (Figure 1E, compare lanes 1 and 4). However, the presence of amino acids amplified the effect of insulin stimulation, as seen both by increased mTORC1 activity and enhanced phosphorylation of Unkempt (Figure 1E, compare lanes 2 and 5). Similarly, increased glucose concentration, while having little effect on its own (Figure 1E, compare lanes 8 and 12), potentiated mTORC1 activity and the phosphorylation of Unkempt primed by insulin stimulation in the presence of amino acids (Figure 1E, compare lanes 10 and 13). The effects of amino acids and glucose were mTORC1-dependent, as rapamycin prevented the phosphorylation of Unkempt in the presence of amino acids, glucose, and insulin together (Figure 1E, compare lanes 10 and 11). Unkempt phosphorylation is therefore acutely sensitive to nutrients and growth factors that regulate mTORC1.

mTOR is a component of both mTORC1 and mTORC2. Prolonged rapamycin treatment can inhibit mTORC2 (Sarbasov *et al.*, 2006) but short-term (one hour) rapamycin treatment of SH-SY5Y cells did not inhibit mTORC2, as judged by AKT phosphorylation at Ser473 (Supplemental Figure 1A). Moreover, treatment of SH-SY5Y cells with a dual mTORC1/mTORC2 inhibitor, KU0063794 (Figure 1F, lane 3), or rapamycin and KU0063794 together (Figure 1F, lane 4), produced a similar reduction in phosphorylation of Unkempt as treatment with rapamycin alone (Figure 1F, lane 5), further evidence that it is the activity of mTORC1 but not mTORC2 that regulates Unkempt phosphorylation. Furthermore, treatment of SH-SY5Y cells with a range of concentrations of the highly selective mTORC1 inhibitor DL001 (Schreiber *et al.*, 2019), which does not inhibit mTORC2-dependent AKT phosphorylation, decreased the mobility of Unkempt and reduced the recognition by an Unkempt phospho-specific antibody (Supplemental Figure 1B). We also asked whether

Unkempt phosphorylation might be regulated at the level of S6K, however, treatment of SH-SY5Y cells with the S6K inhibitor PF4708671 had no noticeable effect (Figure 1G). Together, these data confirm that Unkempt phosphorylation is mTORC1-dependent.

Unkempt physically interacts with mTORC1

Unkempt has been previously shown to physically interact with Raptor (Li *et al.*, 2019; Vinsland *et al.*, 2021). To further test whether Unkempt physically interacts with mTORC1 we used HeLa S3 cells with a doxycycline (DOX)-inducible FLAG-HA-Unkempt to immunoprecipitate Unkempt (Murn *et al.*, 2015). Rapamycin treatment showed that phosphorylation of FLAG-HA-Unkempt is mTORC1-dependent (Figure 1H). Endogenous Raptor and mTOR co-immunoprecipitated with FLAG-HA-Unkempt (Figure 1H). Unkempt therefore physically interacts with mTORC1.

We also noticed that the co-immunoprecipitation of mTOR in this experiment was sensitive to the type of detergent, whereas pulldown of Raptor was not (Figure 1H). This is in line with a known property of the zwitterionic detergent CHAPS to preserve and the non-ionic detergent Triton X-100 to disrupt the interaction between Raptor and mTOR (Kim *et al.*, 2002), consistent with Unkempt interacting with mTOR indirectly via Raptor. These co-immunoprecipitation data further validate the previously observed physical interaction of Unkempt with Raptor (Li *et al.*, 2019; Vinsland *et al.*, 2021) and show that Unkempt physically interacts with a functional mTORC1.

mTORC1 phosphorylates Unkempt at multiple sites in its intrinsically disordered region

Using mass spectrometry (MS) of FLAG-HA-Unkempt immunoprecipitated from cells treated or not with rapamycin we identified 31 phosphorylated serines and five phosphorylated threonines grouped in three clusters within a predicted intrinsically disordered region (IDR) of Unkempt (Figure 2A, B, Supplemental Figures S2, S3). About 24% of the C-terminal portion of the IDR between amino acids 467 and 640 consists of serine residues (41 residues) and harbours the majority of phosphorylation sites (phosphosites), including all of the phosphosites that were never detected in rapamycin-treated cells (Figure 2A, blue residues, Supplemental Figures S2, S3). To determine if any of the phosphosites might be direct substrates of mTORC1 we used an *in vitro* kinase assay with purified reconstituted mTORC1 and GTP-bound Rheb. We found that purified Unkempt was indeed

directly phosphorylated by mTORC1/Rheb-GTP (Figure 2C). MS identified five serine residues in Unkempt that were *in vitro* phosphorylated in the control condition but not in the rapamycin/FKBP12 treated condition (addition of the rapamycin binding partner FKBP12 is necessary for mTORC1 inhibition *in vitro* (Dunlop *et al.*, 2009); Figure 3A, red asterisks; Supplemental Figures S2, S3). Four of these serines have a proline or leucine at the +1 position and so adhere to the predicted mTORC1 phosphorylation motif consensus sequence (Hsu *et al.*, 2011; Robitaille *et al.*, 2013), while two (S608 and S611) are also mTORC1-dependent in rapamycin-treated cells (Figure 2A, Supplemental Figures S2, S3).

Unkempt phosphorylation at S611 was reproducibly rapamycin dependent in cells (in three biological replicates) and in the *in vitro* kinase assay (Supplemental Figure S3). S611 also has a leucine residue at the +1 position and hydrophobic residues at -1 (glycine) and -4 (alanine) and so adheres to the mTORC1 phosphorylation motif consensus sequence (Hsu *et al.*, 2011; Robitaille *et al.*, 2013) (Supplemental Figure S2). We generated a phospho-specific antibody against a peptide containing rapamycin sensitive phospho-S611 and nearby rapamycin insensitive phospho-S606, which was also reproducibly identified in cells by MS (Supplemental Figure S3). This antibody recognised Unkempt in serum stimulated SH-SY5Y cells but not in cells that were treated with rapamycin (Figure 2D, see also Supplemental Figure S1A). Taken together, these data provide evidence that Unkempt is an mTORC1 substrate that is phosphorylated at multiple residues, located mostly in the serine-rich region of the IDR.

To independently define the region of Unkempt that is targeted by mTORC1-dependent phosphorylation we performed deletion analysis of Unkempt transiently expressed in HEK293 cells. Co-expression of constitutively active Rheb (Rheb^{CA}), which directly hyperactivates mTORC1 (Hartman *et al.*, 2013), caused phosphorylation of full-length Unkempt (FL-Unk) in the absence but not in the presence of rapamycin (Figure 3A, B). Unkempt deletion mutants lacking either the zinc finger domain (Δ ZF) or the RING domain (Δ RING) retained mTORC1-dependent phosphorylation potential, while a mutant containing just the zinc finger domain alone (ZF) was not phosphorylated by mTORC1 hyperactivation (Figure 3A, B). Notably, removal of the whole serine-rich region (Δ SerL), or of a shorter region between amino acids 539 and 640 (Δ SerS) almost completely prevented phosphorylation (Figure 3A, B). This deletion analysis confirms that the serine-rich region in Unkempt contains the majority of the mTORC1-dependent phosphosites.

MS analysis showed that specific serine and threonine residues in the IDR of Unkempt are targets of mTORC1-dependent phosphorylation (Figure 2A). To test these data further we mutated clusters of MS-identified phosphorylated serine and threonine residues in the IDR to alanine (Figure 3C). Mutation of phosphosites from S467 to S546 to alanine had no obvious effect on mTORC1-dependent phosphorylation of Unkempt (Figure 3D, E). However, additional mutation of phosphosites between S565-S578, S596-S611 and S631-S640 to alanine either strongly reduced or completely prevented mTORC1-dependent phosphorylation of Unkempt (Figure 3D, E). Based on this, we then tested mutants that only affect phosphosites located exclusively within the serine-rich region of with the IDR (Figure 3C). Interestingly, mutations of groups of these phosphosites (S565-S578, S596-S611 or S631-S640), or their combinations (S596-S640 or S565-S640), reduced but did not completely prevent the overall mTORC1-dependent phosphorylation of Unkempt (Figure 3C, F, G). These mutagenesis data validate the MS analyses and point to the serine-rich region of the IDR as the hotspot of mTORC1-dependent phosphorylation of Unkempt.

Unkempt is phosphorylated by mTORC1 in vivo

In mice, Unkempt protein is expressed in the developing nervous system from at least as early as E10 (Murn *et al.*, 2015). To analyse the phosphorylation status of Unkempt by mTORC1 *in vivo* we utilised *Nestin-Cre;Tsc1^{fl/fl}* mice, in which *Nestin* promoter-driven Cre recombinase is expressed in neural progenitors beginning at E10.5 causing loss of TSC1 expression throughout the developing brain (Anderl *et al.*, 2011). Consequently, *Nestin-Cre;Tsc1^{fl/fl}* mice exhibit activated mTORC1 signalling in the brain, macrocephaly and die at P0, likely due to malnutrition, hypoglycemia and hypothermia (Anderl *et al.*, 2011). The activated mTORC1 signalling and perinatal death of these mice can be suppressed by a single dose of rapamycin between E15-E17 (Anderl *et al.*, 2011). We confirmed hyperactivation of mTORC1 via increased phosphorylation of rpS6 in the brain of E16.5 *Nestin-Cre;Tsc1^{fl/fl}* mice embryos (Figure 4A, B). Although we did not detect a change in the electrophoretic mobility of Unkempt protein, using the phospho-specific antibody we observed a dramatic increase in Unkempt phosphorylation in brain tissue from *Nestin-Cre;Tsc1^{fl/fl}* embryos (Figure 4A, C).

To test whether inhibition of mTORC1 affects Unkempt phosphorylation during nervous system development, mice pregnant with *Nestin-Cre;Tsc1^{fl/fl}* embryos were injected with

rapamycin or vehicle at E15.5. Twenty four hours after rapamycin injection, rpS6 phosphorylation was dramatically reduced in the embryonic brain (Figure 4E, F). Rapamycin treatment also significantly decreased phosphorylation of Unkempt (Figure 4E, G). To determine whether prolonged rapamycin treatment affected mTORC2 signalling (Sarbasov *et al.*, 2006), we analysed PKC α phosphorylation (Huang *et al.*, 2009). PKC α phosphorylation was unaffected in the brain of *Nestin-Cre;Tsc1^{fl/fl}* embryos compared to controls (Figure 4A, D) and did not change with twenty-four-hour rapamycin treatment (Figure 4E, H), indicating that mTORC2 signalling was unaffected. These data show that Unkempt phosphorylation is mTORC1 dependent in the developing brain.

Phosphorylation of Unkempt affects its capacity to remodel cellular morphology

In addition to being required for the early morphology of neurons, Unkempt also possesses a remarkable capacity to establish a similar, early neuronal-like bipolar morphology in cells of non-neuronal origin, indicating that Unkempt regulates a specific program of cell morphogenesis (Murn *et al.*, 2016; Murn *et al.*, 2015). To examine whether phosphorylation affects the morphogenetic activity of Unkempt, we inducibly expressed in HeLa cells GFP either alone or together with wild-type or phosphosite-mutant Unkempt and monitored the effects on cell shape. With clusters of phosphosites turned to either alanines or aspartates to prevent or mimic phosphorylation, respectively, we focused on the serine-rich region of IDR, in particular its C-terminal region (S596-S640), as the hotspot for mTORC1-dependent phosphorylation of Unkempt (Figure 4C-G, Figure 5A). Strikingly, whereas alanine mutations largely preserved the morphogenetic activity of Unkempt, aspartate mutations severely compromised or abolished cell polarization by Unkempt (Figure 5B-D). All mutants showed similar gross RNA-binding to wild-type Unkempt (Supplemental Figure S4), suggesting that the morphogenetic effects of these mutations are unlikely to be due to differences in RNA binding, a key requirement for Unkempt-induced cell morphogenesis (Murn *et al.*, 2016; Murn *et al.*, 2015). Together, these data point to a critical role of phosphorylation in regulating the activity of Unkempt in cellular morphogenesis (Figure 5E).

We have shown that Unkempt phosphorylation is exquisitely sensitive to mTORC1 activity and is modulated by a range of inputs including glucose, amino acids and insulin signalling. mTORC1 regulates the phosphorylation of hundreds of different proteins (Battagioni *et al.*, 2022; Hsu *et al.*, 2011; Robitaille *et al.*, 2013; Yu *et al.*, 2011) with roles in growth control, autophagy, nucleotide and lipid metabolism but the control of cellular morphogenesis by

mTORC1 is less well characterised. The microtubule associated proteins CLIP-170 and Tau are known to be direct substrates of mTORC1 but do not have the Unkempt's profound ability to reorganise cell morphology (Choi *et al*, 2002; Tang *et al*, 2013).

Raptor physically interacts with Unkempt, enabling mTOR to phosphorylate multiple residues within the serine-rich region of the Unkempt IDR. Unkempt phosphorylation is complex and includes mTORC1-dependent and independent serine and threonine residues, suggesting the involvement of additional kinases. The number and proximity of phosphosites in the IDR also implies potential flexibility in the regulation of Unkempt phosphorylation. *In vivo* phosphorylation of Unkempt may depend on the context, both spatially and temporally. Thus, through phosphorylation, the IDR of Unkempt might serve as a rheostat that integrates inputs from multiple signalling pathways to fine-tune the activity of Unkempt (Figure 5E). This might not be limited to the control of the cellular morphogenesis but could extend to other known roles of Unkempt in neuronal differentiation and cell proliferation (Avet-Rochex *et al.*, 2014; Li *et al.*, 2019; Lores *et al*, 2010; Maierbrugger *et al.*, 2020; Mohler *et al*, 1992; Vinsland *et al.*, 2021).

It remains unclear how phosphorylation might regulate the activity of Unkempt at the molecular level. Thus far, the most extensively documented molecular property of Unkempt is its capacity to bind mRNA, which occurs in an RNA sequence-specific manner and is mediated by a tandem array of six highly conserved CCCH-type zinc fingers located at the N-terminus (Murn *et al.*, 2016; Murn *et al.*, 2015). Through RNA binding, Unkempt is thought to primarily regulate the translation of several hundred of its target mRNAs involved in processes including control of protein synthesis and cell morphogenesis, although the mechanistic basis for translational control and potential other modes of post-transcriptional processing by Unkempt remains unknown. Given the physical separation of the zinc finger domain from the heavily phosphorylated serine-rich region in the IDR, but also because the zinc finger domain alone suffices for high-affinity RNA binding, it seems unlikely that phosphorylation in the serine-rich region would affect RNA binding by Unkempt (Murn *et al.*, 2016; Murn *et al.*, 2015). Indeed, our mutagenesis analysis coupled with CLIP data suggest that phosphorylation exerts no overt effect on Unkempt-RNA interactions. Instead, we propose that phosphorylation by mTORC1 acts to modulate protein-protein interactions (PPIs) between the IDR and partner proteins, in keeping with the known propensity of IDRs to act as protein rheostats by forming transient PPIs regulated by phosphorylation (Wright &

Dyson, 2015). Phosphorylation-sensitive PPIs might entail interactions of Unkempt with a large number of cytosolic RNA granule components and other effectors of post-transcriptional processing identified via unbiased screens (Go *et al*, 2021; Havugimana *et al*, 2012; Youn *et al*, 2018). Such a mechanism could provide a novel route for the mTOR pathway to control morphogenesis and other cellular processes, beyond those regulated by 4E-BP/eIF4E and S6K (Thoreen *et al*, 2012). Overall, this study paves the way for investigation of the mechanistic principles by which Unkempt and its phosphorylation by mTORC1, and potentially other kinases, regulate cellular morphogenesis.

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Author contributions

Conceptualization, J.M.B.; Methodology, E.V., J.M., J.M.B.; Formal analysis, E.V., P.B. S.R.M., K.S.; Investigation, E.V., P.B., S.R.M, K.S., A.R.T.; Writing – Original Draft, J.M.B.; Writing – Review & Editing, J.M.B, P.B, E.V., S.R.M., S.U., J.M.; Visualization, E.V., P.B., S.R.M., J.M.B; Funding Acquisition, S.U., J.M.B, A.R.T., J.M; Supervision, S.U., J.M., J.M.B.

Competing interests

The authors declare no competing interests.

Materials and methods

Animal models

Mouse studies were carried out in accordance with UK Home Office regulations and the UK Animals (Scientific Procedures) Act of 1986 (ASPA) under a UK Home Office licence (PPL 70/8719) and approved by the King's College London Ethical Review Committee. *Nestin-Cre* mice (obtained from Jackson, stock number 003771) and *Tsc1^{fl/fl}* mice (obtained from Jackson, stock number 005680) were maintained in a C57BL/6 background. Mice were housed under a 12 hour light/dark cycle with *ad libitum* access to food and water. *Tsc1^{fl/fl}* mice were genotyped using primers 5'-GTC ACG ACC GTA GGA GAA GC-3' and 5'-GAA TCA ACC CCA CAG AGC AT-3'. *Nestin-Cre* mice were genotyped using primers 5'-TTGCTAAAGCGCTACATAGGA-3' and 5'-GCCTTATTGTGGAAGGACTG-3'.

Pregnant dams were injected intraperitoneally with 1mg/kg rapamycin (Fluorochem Ltd) at E15.5, embryos removed at E16.5, embryonic brains dissected and immediately frozen on dry ice.

Cell culture, immunoprecipitation and western blot analysis

SH-SY5Y, Neuro-2a (N2a) and HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Sigma-Aldrich) supplemented with 10% (v/v) foetal bovine serum (FBS; Sigma-Aldrich) and 1% (w/v) penicillin/streptomycin (Sigma-Aldrich). Cells were trypsinized and sub-cultured twice a week and incubated in 5% CO₂ at 37°C throughout the experiments unless stated otherwise.

For insulin and rapamycin treatment cells were seeded at 7 x 10⁵ cells per well in a six-well plate (Thermo Scientific) in DMEM + 10% FBS, then serum-starved for 16 hours in DMEM alone. Unless stated otherwise, cells were then incubated in DMEM ± 1µM insulin (Sigma-Aldrich), or DMEM + 10% FBS ± 1µM rapamycin (LC Laboratories), or another inhibitor, for one hour. 10µM S6K inhibitor (PF4708671, Sigma-Aldrich), 1µM mTOR kinase active site inhibitor (KU0063794, Sigma-Aldrich) and DL001 (Aeovian Pharmaceuticals Inc. at the concentrations shown in Supplemental Figure 1B) were used. Cells were lysed with 0.2% (w/v) SDS (Sigma-Aldrich), 10mM EDTA, 1x protease inhibitor cocktail (Roche), and 1x phosphatase inhibitor cocktail (Sigma-Aldrich) unless stated otherwise.

C57BL/6J mouse primary cortical cells were extracted at E14.5 and one million cells were plated per well in a six well plate in Neurobasal medium (2% (v/v) B27, 2mM glutamine,

0.3% (w/v) glucose, 37.5mM NaCl, 5% (v/v) FBS; ThermoFisher). 48 hours later the media was replaced with 5% (v/v) FBS $\pm$ 1 μ M rapamycin for one hour and lysed as above.

For lambda protein phosphatase treatment cells were serum starved overnight, stimulated with DMEM $\pm$ 1 μ M insulin, then lysed in 50mM Tris pH 7.5, 150mM NaCl, 0.1% (v/v) Triton, 1X protease inhibitor cocktail $\pm$ lambda protein phosphatase (New England Biolabs) as per the manufacturer's instructions and incubated at 30°C for one hour.

For glucose and amino acid studies, SH-SY5Y cells were seeded and serum starved as above. This was followed by a 2.5 hour incubation in Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich) to starve cells of amino acids and to reduce the concentration of glucose (to 1g/l) and in some conditions a 30 minute pre-incubation of 1 μ M rapamycin. Trophic factors were then added to the starved cells for 25 minutes with a combination of glucose, amino acids, FBS (all from Sigma-Aldrich), DMEM no glucose (ThermoFisher), insulin (1 μ M), or rapamycin (1 μ M), in continued HBSS or DMEM high glucose medium.

For western blot analysis cell extracts were denatured and reduced in 1x sample buffer (500mM Tris pH 6.8, 40% (v/v) glycerol, 0.2% (w/v) SDS, 2% (v/v) β -mercaptoethanol, and 0.02% (w/v) bromophenol blue) and boiled at 98°C for 10 minutes. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were blocked in 10% fat-free milk powder in tris buffered saline (TBS) (50mM Tris pH 7.4, 150mM NaCl) and probed overnight at 4°C with primary antibodies in 5% (w/v) bovine serum albumen (BSA; Fisher) TBS-T (TBS + 0.1% (v/v) Tween 20). Following four 10 minute washes in TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:4000) in 5% milk TBS-T for one hour at room temperature. Finally, blots were treated with enhanced chemiluminescence reagents (ECL, GE Healthcare) and imaged using a Kodak or Bio-Rad ChemiDoc system imaging system.

Embryonic brains were lysed in urea lysis buffer (8M urea, 50mM HEPES pH8.2, 10mM glycerol-2-phosphate, 50mM NaF, 5mM sodium pyrophosphate, 1mM EDTA, 1mM sodium orthovanadate, 1mM DTT, cOmplete Protease inhibitor cocktail, Sigma phosphatase inhibitor cocktail III, 500nM okadaic acid) and sonicated briefly. Protein concentration was measured using Pierce BCA Assay kit. Twenty micrograms of brain lysate per embryo were denatured and reduced for 10min at 70°C in 1X Pierce LDS sample buffer supplemented with 100mM DTT. Denatured samples were loaded onto pre-cast NuPAGE 4-12%, Bis-Tris protein gel

(Invitrogen). Proteins were then transferred onto PVDF membrane using transfer buffer (25mM Tris pH8.3, 192mM glycine, 20% v/v methanol) for 1.5hrs at 310mA. Membranes were blocked in 10% w/v skimmed-milk (Marvel) dissolved in TBS-T for 1 hour. Membranes used for immunoblotting with phospho-specific antibodies were washed with TBS-T prior to incubation at 4°C overnight with primary antibodies in 5% w/v BSA dissolved in TBS-T. Overnight membranes were then washed three times with TBS-T and incubated with an appropriate HRP-conjugated secondary antibody diluted in 5% w/v milk. After washing the PVDF membranes with TBS-T, they were incubated with enhanced ECL solution (Cytiva) and imaged using Amersham Imager 600 (GE Healthcare). Western blot images were quantified using the Amersham Imager 600 Software.

Primary antibodies used were rabbit anti-Unkempt (1:1000, HPA023636, Cambridge Bioscience), rabbit anti-mTOR (1:1000, #2972, Cell Signalling), rabbit anti-Raptor (1:1000, #2280, Cell Signalling), rabbit anti- β -actin (1:5000, #4967, Cell Signalling), rabbit anti-S6K (1:1000, #9202, Cell Signalling), rabbit anti-phosphoS6K (Thr389, 1:1000, #9234, Cell Signalling), rabbit anti-S6 (1:1000, #2217, Cell Signalling), rabbit anti-phospho-S6 (Ser235/236, 1:1000, #2211, Cell Signalling), rabbit anti-4E-BP1 (1:1000, #9644, Cell Signalling), rabbit anti-phospho-4E-BP1 (Thr37/46, 1:1000, #2855, Cell Signalling), rabbit anti-FLAG (1:2000, F7425, Sigma-Aldrich), rat anti-FLAG (1:2000, #200473-21, Agilent), mouse anti-V5 (1:1000, R960-25, ThermoFisher), mouse anti-HA (1:1000, sc-7392, Santa Cruz), rabbit anti-phosphoUnkempt (1:200, S606, S611, this study), rabbit anti-phospho AKT (Ser473, 1:1000, 4060T, Cell Signalling), rabbit anti-AKT (1:1000, 5691T, Cell Signalling), mouse anti- α -tubulin (1:10000, T9026, clone DM1A, Sigma-Aldrich), rabbit anti-hamartin (TSC1) (1:1000, ab270967, clone EPR24364-109, Abcam), rabbit anti-PKC α (1:1000, ab32376, clone Y124, Abcam), rabbit anti-phospho-PKC α (Ser657, 1:1000, ab180848, clone EPR1901(2), Abcam).

Expression vectors, cloning, derivation of inducible cell lines, and transfections

Full length mouse Unkempt was amplified from cDNA using primers 5'-CACCAGATATCCAATGTCGAAGGGCCCCGGGCCCCG-3' and 5'-GACGACTCTAGATCAGACTGGAGGGCATGGGCCC-3' and cloned into pENTR/D-

TOPO (ThermoFisher) according to the manufacturer's instructions to create pENTR-Unk. Primers used to generate the Unkempt deletions and zinc finger constructs in pENTR-Unk were the same as used previously (Murn *et al.*, 2015). Primers used to generate the SerL deletion in pENTR-Unk were 5'-GGGCCAGGGGCTGCTGAGCT-3' and 5'-GCCACAGGGAGGATGCCCA-3' and to generate the SerS deletion in pENTR-Unk were 5'-GGGCCAGGGGCTGCTGAGCT-3' and 5'-GTTATCAAAAGTCTTCTCTAGG-3'. These constructs were then recombined into pcDNA3.1/nV5-DEST (ThermoFisher). pCAG-Rheb^{CA} (also known as pCAG-Rheb^{S16H}), was a gift from Angelique Bordey (Hartman *et al.*, 2013).

The alanine and aspartate substitution mutations in Unkempt were created by PCR, using the SuperFi mastermix (Thermo Fisher) as per the manufacturer's instructions using pENTR-Unk as a template. The PCR products were digested using DpnI (NEB) at 37°C for 90 minutes and then transformed into DH5α chemically competent cells. The following primers were used:

S467-S479>A (S467A, S474A, S475A, S476A, T478A, S479A)

Forward primer – 5'

GACTTCCAGCATCGCTGCCGCTATTGCCGCCAGCTTGGCAGCCACTCCC 3'

Reverse primer – 5'

GCTGGAGGTAATAGCGGCAGCGATGCTGGAAGTCAGGGGGGCGCCCACAGG 3'

S467-S479>D (S467D, S474D, S475D, S476D, T478D, S479D)

Forward primer – 5'

GACTTCCAGCATCGATGACGATATTGACGACAGCTTGGCAGCCACTCCC 3'

Reverse primer – 5'

GCTGTTCGTCATATCGTCATCGATGCGGAAGTCAGGGGGTGCAGCCACAGG 3'

T491A

Forward primer – 5' CTGCAGGCGCCAACAGCACCCCTGGCATGAATGC 3'

Reverse primer – 5' GCTGTTGGCGCCTGCAGGGCTGGGGGGAG 3'

T491D

Forward primer – 5' CTGCAGGCGACAACAGCACCCCTGGCATGAATGC 3'

Reverse primer – 5' GCTGTTGTGCGCTGCAGGGCTGGGGGGAG 3'

S546A

Forward primer – 5' CACCCCAGCGCCGTCACAATCGGTGGC 3'

Reverse primer – 5' CGATTGTGACGGCGCTGGGGTGGGGCACTGC 3'

S546D

Forward primer – 5' CACCCCAGCGACGTCACAATCGGTGGC 3'

Reverse primer – 5' CGATTGTGACGTCGCTGGGGTGGGGCACTGC 3'

S565-S578>A (S565A, S571A, S574A, S576A, S578A)

Forward primer – 5'

CAGCTCAGCTGCCTTCCACGCTGCTGCTCCAGCCCCTCCCGTCAGCCTCTCC 3'

Reverse primer -

5'

GGGAGGGGCTGGAGCAGCAGCGTGGAAGGCAGCTGAGCTGCCCAGGGCGCCAG
GGATG 3'

S565-S578>D (S565D, S571D, S574D, S576D, S578D)

Forward primer – 5'

CAGCTCAGCTGACTTCCACGATGCTGATCCAGACCCTCCCGTCAGCCTCTCC 3'

Reverse primer – 5'

GGGAGGGTCTGGATCAGCATCGTGGAAGTCAGCTGAGCTGCCCAGGTCGCCAGG
GAT 3'

S596-S611>A (S596A, S598A, T605A, S606A, S608A, S611A)

Forward primer – 5'

CGTTTTTGGGGGCCGACGACGACACATGGAGCTTTGGGTCTGAACGGG 3'

Reverse primer -

5'

CCAAAGCTCCATGTGCTGCTGCGGCCCCCAAAAACGTGTTTTCTGCCTGAGCCAA
GTGGCC 3'

S596-S611>D (S596D, S598D, T605D, S606D, S608D, S611D)

Forward primer – 5'

CGTTTTTGGGGGACGACGACGACCATGGAGATTTGGGTCTGAACGGG 3'

Reverse primer – 5'

CCAAATCTCCATGGTCTGCGTCGTCCCCCAAAAACGTGTTTTCTGCCTGATCCAA
GT 3'

S631-S640>A (S631A, T634A, S635A, S640A)

Forward primer – 5' CCAGGCGCTGCCCCTGCCTTCCTAGCAGGGCCAGGGGCTGC 3'

Reverse primer -

5' CCCTGCTAGGAAGGCAGGGGCAGCGCCTGGGGCGAAGCTTCCAGAGGC 3'

S631-S640>D (S631D, T634D, S635D, S640D)

Forward primer – 5' CCAGGCGATGACCCTGCCTTCCTAGCAGGGCCAGGGGCTGC 3'

Reverse primer – 5'

CCCGTCTAGGAAGGCAGGGTCATCGCCTGGGTCTGAAGCTTCCAGAGGC 3'

Doxycycline-inducible HeLa cells used for the analyses of cellular morphogenesis and for CLIP experiments were generated as described previously (Murn *et al.*, 2015). Briefly, stably rtTA3-expressing cells were infected ecotopically with retroviruses for TREtight-driven expression of GFP alone (pTt-IGPP) or GFP and either wild-type Unkempt protein or any of the Unkempt alanine or aspartate substitution mutants indicated in Figure 6 and created as described above (pTtight-X-IGPP, where X is either wild-type or mutant Unkempt). To

induce transgene expression, double-selected cells were treated with doxycycline (Sigma-Aldrich) at 1 $\mu\text{g/ml}$.

For transient transfections, HEK293 cells were seeded at 2×10^5 cells per well in six well plates (Thermo Fisher) in DMEM/10% FBS. The following day, cells were transfected with V5-tagged Unkempt plasmids at 1 $\mu\text{g/well}$, using polyethylenimine (Sigma) at 7 $\mu\text{g/well}$ and 200 $\mu\text{l/well}$ of OptiMem (Sigma). For co-transfections, Rheb^{CA} was used at 0.5 $\mu\text{g/well}$ and the V5-tagged Unkempt constructs at 1 $\mu\text{g/well}$. Cells were lysed 48 hours post transfection using 0.2% (w/v) SDS (Sigma-Aldrich), 10mM EDTA, 1x protease inhibitor cocktail (Roche), and 1x phosphatase inhibitor cocktail (Sigma-Aldrich).

Co-immunoprecipitation experiments and liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis

To induce overexpression of and immunoprecipitate FLAG-HA-Unkempt, previously described HeLa S3 cells stably expressing doxycycline-inducible FLAG-HA-Unkempt (Murn *et al.*, 2015) were used. They were cultured in DMEM (high glucose; Sigma) supplemented with 10% (v/v) FBS (Sigma) and 1% penicillin/streptomycin (Lonza) in 15 cm dishes (Nunc, ThermoFisher) to 90% confluency and induced with doxycycline hyclate (Sigma-Aldrich) at 1 $\mu\text{g/ml}$ for 24 hours. Non-induced cells were also cultured in parallel to use as a negative control. 16 hours prior to co-immunoprecipitation, cells were serum starved in DMEM, in the presence of doxycycline. 10% FBS was then re-introduced to culture media and cells were treated with 1 μM rapamycin (LC Laboratories) or DMSO for one hour. Dishes were then washed with ice-cold PBS and cells were scraped and lysed on ice for 15 minutes in 3 ml lysis buffer.

For co-immunoprecipitations, one 15 cm dish was used for each condition and cells were lysed in either CHAPS lysis buffer (120 mM NaCl, 40 mM HEPES pH 7.5, 1 mM EDTA, 0.3% (w/v) CHAPS, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail and 0.2 mM PMSF) or Triton X-100 lysis buffer (120 mM NaCl, 40 mM HEPES pH 7.5, 1 mM EDTA, 1% Triton X-100, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail and 0.2 mM PMSF). Samples were then centrifuged at $17,000 \times g$ for 10 minutes at 4°C . The supernatant was transferred to a new tube and protein concentration was determined using

Protein Assay Dye Reagent Concentrate (Bio-Rad) following the manufacturer's instructions. Samples were diluted and standardized to the condition with lowest protein concentration (≤ 2 mg/ml) using their respective lysis buffer. Equal volumes of diluted lysates were added to pre-washed 50 μ l anti-FLAG M2 affinity agarose gel slurry (Sigma-Aldrich) and incubated rotating end over end for two hours at 4°C. The agarose beads were then washed three times with lysis buffer followed by three additional washes with wash buffer (150 mM NaCl, 50 mM HEPES pH 7.5). Excess buffer was removed using an insulin needle (30G) and beads were resuspended in 60 μ l 2 x sample buffer and protein was eluted by heating at 95°C for 5 min. Co-immunoprecipitants were resolved on an 8% SDS-PAGE gel followed by protein transfer on nitrocellulose membrane (GE Healthcare Life Sciences). Western blot analysis was performed as described above.

For LC-MS/MS analysis of immunoprecipitated Unkempt, cells were cultured in two 15 cm dishes for each condition and samples were processed in a similar way to co-immunoprecipitation experiments but using a different lysis buffer (25 mM Tris pH 8.0, 150 mM NaCl, 5% (v/v) glycerol, 1% Triton (v/v) X-100, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail and 0.2 mM PMSF) and wash buffer (50 mM Tris pH 8.0, 150 mM NaCl). Sample dilution and standardization prior to incubation with anti-FLAG resin was omitted. Bound Unkempt was eluted with 40 μ l 2x sample buffer heated at 95°C for 5 minutes. 35 μ l of each eluate was resolved on a 4-12% Bis-Tris Novex NuPAGE gel (ThermoFisher) for coomassie staining and mass spec analysis. The remaining 5 μ l were resolved on the same gel and used for FLAG western blotting to confirm the immunoprecipitation and exact molecular weight of Unkempt. Half of the gel for LC-MS/MS analysis was fixed (7% (v/v) glacial acetic acid, 40% (v/v) methanol, 53% HPLC-grade water) for 30 minute shaking at room temperature. The fixed gel was stained overnight at room temperature (4 parts 1X Brilliant Blue-G Colloidal Concentrate (Sigma-Aldrich) and 1-part methanol, freshly mixed). The gel was destained three to four times (5% (v/v) glacial acetic acid, 25% (v/v) methanol, 70% HPLC-grade water) until background was significantly reduced and bands were clearly visible. Bands of the expected FLAG-HA-Unkempt size (~90 kDa) for both conditions, rapamycin and DMSO, were excised using sterile scalpel in a fume hood and stored in HPLC-grade water.

LC-MS/MS analysis was performed by the Cambridge Centre for Proteomics, University of Cambridge. Gel fragments were transferred into a 96-well PCR plate. The bands were cut into 1mm² pieces, destained with a solution 50% acetonitrile and 50mM ammonium

bicarbonate, reduced with 10mM DTT for one hour at 37°C and alkylated with 55mM iodoacetamide at room temperature in the dark for 45 minutes, then subjected to enzymatic digestion with 0.01µg/µl sequencing grade chymotrypsin (Promega V1061) overnight at 37°C. After digestion, the supernatant was pipetted into a sample vial and loaded onto an autosampler for automated LC-MS/MS analysis.

All LC-MS/MS experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific Inc, Waltham, MA, USA) system and a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). Separation of peptides was performed by reverse-phase chromatography at a flow rate of 300 nL/minute and a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2 µm particle size, 100Å pore size, 75 µm i.d. x 50cm length). Peptides were loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5 µm particle size, 100Å pore size, 300 µm i.d. x 5mm length) from the Ultimate 3000 autosampler with 0.1% (v/v) formic acid for three minutes at a flow rate of 10 µL/minute. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1% (v/v) formic acid and solvent B was 80% (v/v) acetonitrile, 20% water + 0.1% (v/v) formic acid. The linear gradient employed was 2-40% B in 30 minutes.

The LC eluant was sprayed into the mass spectrometer by means of an Easy-Spray source (Thermo Fisher Scientific Inc.). All m/z values of eluting ions were measured in an Orbitrap mass analyzer, set at a resolution of 70000 and was scanned between m/z 380-1500. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD, NCE:25%) in the HCD collision cell and measurement of the resulting fragment ions was performed in the Orbitrap analyser, set at a resolution of 17500. Singly charged ions and ions with unassigned charge states were excluded from being selected for MS/MS and a dynamic exclusion window of 20 seconds was employed.

Post-run, the data was processed using Protein Discoverer (version 2.1., ThermoFisher). Briefly, all MS/MS data were converted to mgf files and the files were then submitted to the Mascot search algorithm (Matrix Science, London UK) and searched against the UniProt_Mus_musculus_20180514 database (61295 sequences; 27622875 residues). The peptide and fragment mass tolerances were set to 20 ppm and 0.1 Da, respectively. A significance threshold value of $p < 0.05$ and a peptide cut-off score of 20 were also applied.

Following Mascot analysis, data were converted into .sf3 file and sent to user for further analysis.

Converted files were analysed using Scaffold4 Proteome Software (version 4.8.7). Protein threshold was set to 95% with minimum number of peptides at 2. Peptide threshold was relaxed and each phospho-peptide was examined manually accounting for accurate identification of amino acids, number of peptides with the same phosphorylation site and peptide identification probability percentage. All selected sites were less than 10 delta ppm.

Prediction disorder probability was performed using the online tool Protein DisOrder prediction System (PrDOS, <http://prdos.hgc.jp/cgi-bin/top.cgi>) (Ishida & Kinoshita, 2007) applying 5% prediction false positive rate. Mouse Unkempt protein sequence was used. Protein sequences were aligned using Clustal Omega.

In vitro mTORC1-Rheb kinase assays

HeLa S3 cells stably expressing doxycycline inducible FLAG-HA-Unkempt were used (Murn *et al.*, 2015). After serum starvation, stimulation and rapamycin treatment, as described above, cells from three 15 cm dishes per condition were scraped and pooled together. They were lysed for 20 min on ice in 10 ml lysis buffer (25 mM Tris pH 8.0, 150 mM NaCl, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail and 0.2 mM PMSF). Lysates were centrifuged at 20,000 x g for 20 minutes at 4°C and the cleared supernatant was incubated with 70 µl anti-FLAG M2 affinity agarose gel rotating end over end overnight at 4°C. Samples were then washed twice in lysis buffer followed by three washes in high salt buffer (50 mM Tris pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 1% (v/v) Triton X-100) and three times in wash buffer (50 mM Tris pH 8.0, 150 mM NaCl). Recombinant FLAG-HA-Unkempt was eluted in 80 µl wash buffer supplemented with 0.2 mg/ml 3 x FLAG peptide (Generon) rotating end over end for 30 min at 4°C. Purified Unkempt was collected using 30G needle, flash-frozen in liquid nitrogen and stored at -80°C until required.

Radiolabelled *in vitro* mTORC1 kinase assays with Rheb-GTP were performed as described previously (Dunlop *et al.*, 2009) using FLAG-HA-Unkempt purified as described above.

For the *in vitro* kinase assays for LC-MS/MS analysis, HEK293 cells grown in twelve 10 cm dishes were co-transfected with active myc-mTOR E2914K mutant (Addgene) and HA-

Raptor (Kim *et al.*, 2002), each at 9 µg per plate. In parallel another eight 10 cm dishes of HEK293 cells were transfected with 18 µg of the constitutively active mutant GST-FLAG-Rheb Q64L (Rheb^{CA}) (Long *et al.*, 2005). 48 hours later cells co-transfected with mTOR and Raptor were serum starved overnight and then stimulated with 100 nM insulin (Sigma-Aldrich) for 15 minutes. Cells were washed in ice-cold PBS and each plate was lysed in 1 ml mTOR lysis buffer (40 mM HEPES pH 7.4, 50 mM NaCl, 2 mM EDTA, 10 mM B-glycerophosphate, 0.3% (w/v) CHAPS) on ice for 10 minutes. Samples were centrifuged for 10 minutes at 17,000 x g at 4°C. Cleared supernatants were pooled together and incubated with 3 µl anti-c-Myc antibody (9E10, DSHB) per 1 ml lysate, rotating end over end for 1.5 hours at 4°C. The lysate was divided equally and incubated with 50 µl of prewashed 50% Protein G slurry (GE Healthcare) for each condition, rotating for one hour at 4°C. Beads were then washed once with low salt mTOR wash buffer (40mM HEPES pH 7.4, 150mM NaCl, 2mM EDTA, 10mM B-glycerophosphate, 0.3% (w/v) CHAPS), twice with high salt mTOR wash buffer (40mM HEPES pH 7.4, 400mM NaCl, 2mM EDTA, 10mM B-glycerophosphate, 0.3% (w/v) CHAPS), and twice with HEPES/KCl wash buffer (25mM HEPES pH 7.4, 20mM KCl). Cells transfected with GST-FLAG-Rheb Q64L were washed in ice-cold PBS and each plate was lysed in 1 ml Rheb lysis buffer (40 mM HEPES pH 7.4, 50 mM NaCl, 10 mM pyrophosphate, 10 mM glycerophosphate, 5 mM MgCl₂, 0.3% (w/v) CHAPS, 1X protease inhibitor cocktail) for 10 minutes on ice followed by centrifugation for 10 minutes at 17,000 xg at 4°C. Supernatants were incubated with 30 µl prewashed Glutathione sepharose 4B slurry (GE Healthcare) per 1 ml lysate, rotating end over end for two hours at 4°C. Beads were then washed twice with Rheb lysis buffer and once with Rheb storage buffer (20 mM HEPES pH 8.0, 200 mM NaCl, and 5 mM MgCl₂). The supernatant was removed with 30G needle and bound GST-Rheb was eluted in 40 µl Rheb elution buffer (20 mM HEPES pH 8.0, 200 mM NaCl, and 5 mM MgCl₂, 10 mM reduced Glutathione). To prepare the mTORC1 complex inhibitor, 5 µg FKBP12 was incubated in 50 µl of 30 mM rapamycin in 1x kinase assay buffer (25mM HEPES pH 7.4, 20mM KCl, 10mM MgCl₂) for five minutes in the dark at room temperature. For each condition, mTOR/Raptor immunoprecipitates (bound on beads) were incubated with 50 µl 3x kinase assay buffer, 25 µl GST-FLAG-Rheb Q64L, with or without 10 µl FKBP12:rapamycin complex, made up to 150 µl total reaction volume and incubated on ice for 20 minutes. The *in vitro* kinase assay was started by adding 50 µl assay start buffer (25mM HEPES pH 7.4, 10mM MgCl₂, 140mM KCl, 500uM ATP) and 1 µg purified FLAG-HA-Unkempt, or recombinant GST-4E-BP1 (Dunlop *et al.*, 2009) as a positive control. Samples were incubated for one hour shaking at 30°C. The reaction was

stopped with 4 x sample loading buffer and stored at -20°C. For mass spectrometry analysis, kinase assay reaction samples were diluted in 1 x kinase assay buffer/assay start buffer (3:1) and concentrated using Amicon Ultra-0.5 centrifugal filter unit with a 3 kDa cutoff. Concentrated samples were processed and analysed by LC-MS/MS as described above. Positive control samples were used for western blotting to confirm mTORC1 activity as assessed by phosphorylation of 4EBP-1. Primary antibodies used were mouse anti-c-Myc (1:1000, 9E10, DSHB), mouse anti-HA (1:1000, SC-7392, Santa Cruz), rat anti-FLAG (1:2000, #200473-21, Agilent), rabbit anti-4E-BP1 (1:1000, #9644, Cell Signalling), rabbit anti-phospho-4E-BP1 (Thr37/46, 1:1000, #2855, Cell Signalling).

UV-crosslinking and immunoprecipitation (CLIP) assays

CLIP experiments were carried out essentially as described in (Buchbender *et al*, 2020). GFP or GFP and Unkempt-inducible HeLa cells were grown in 10 cm plates and harvested at 80% confluency. Prior to harvest, the inducible HeLa cells were treated with doxycycline for 24 hours to induce transgene expression. Cells were washed, irradiated with UV-light (254 nm) at 150 mJ/cm² on ice, scraped into three 2 ml tubes, and spun down at 4°C. The supernatant was removed and the cell pellets were frozen at -80°C until use. Immunoprecipitation of the cross-linked Unkempt-RNA complexes was carried out using anti-Flag antibody (F1804, Sigma-Aldrich). Samples were processed exactly as described in (Buchbender *et al.*, 2020) until the autoradiography stage where the membrane was exposed to X-ray film (the 3' end dephosphorylation and first adapter ligation steps were omitted). The experiment was performed in three independent biological replicates. All replicates yielded comparable results.

Unkempt phospho-specific antibody generation

Three phospho-peptides were generated corresponding to residues 601-610 (containing phospho-serine 606), 607-615 (containing phospho-serine 611) and 601-615 (phospho-serines 606 and 611) in Unkempt. Rabbits were immunised with all three phospho-peptides and phospho-specific antibodies were affinity purified using the phospho-peptide corresponding to residues 601-615 (containing phospho-serines 606 and 611). Antibody generation was performed by Covalab.

Morphogenesis assays

Cellular morphologies were analysed as described previously (Schreiber *et al.*, 2019). Briefly, the morphology of each cell was evaluated by an axial ratio (y/x) where the length of the absolute longest cellular axis (y) was divided by the length of the longest axis perpendicular to the y -axis (x). Lengths of x - and y -axes of GFP-positive inducible HeLa cells were measured at 48 hours of treatment with doxycycline. At least 50 GFP-positive cells were quantified for each inducible cell line.

Statistical analyses

Data were analysed using GraphPad Prism version 7 (GraphPad Software). Data distributions were assessed for normality, then analysed using a Student's t -test. p values < 0.05 were considered significant.

Materials and Data Availability

All methods and data are described in the manuscript or supplemental materials.

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Figures

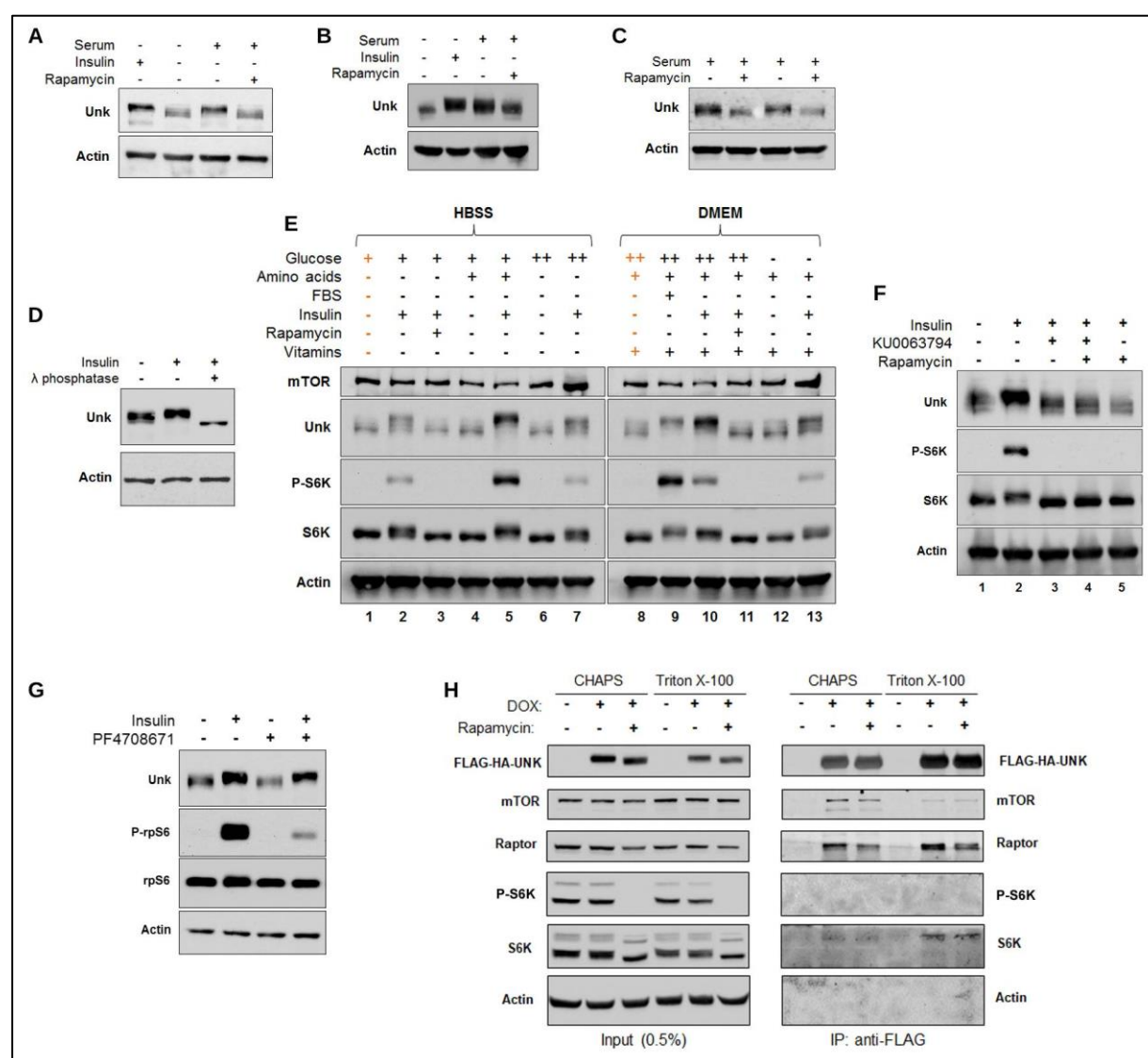


Figure 1. Phosphorylation of Unkempt is mTORC1 activity dependent and physically Unkempt interacts with Raptor. (A) Western blot showing serum starvation followed by insulin stimulation of SH-SY5Y cells increases, while rapamycin treatment decreases the electrophoretic mobility of Unkempt. (B, C) Rapamycin treatment decreases the electrophoretic mobility of Unkempt in mouse N2a cells and mouse E14.5 primary cortical cells. (D) Treatment of SH-SY5Y cell lysate with lambda protein phosphatase abrogates the

electrophoretic mobility shift in Unkempt. (E) Unkempt phosphorylation is highly sensitive to nutrient levels. SH-SY5Y cells were grown in minimal media lacking vitamins and amino acids (HBBS) or DMEM. Additional nutrients were added as indicated. Orange columns indicate control for that panel. (F) Treatment of SH-SY5Y cells with KU0063794 alone, rapamycin alone, or both KU0063794 and rapamycin all prevent the increase in Unkempt phosphorylation caused by insulin stimulation. (G) Inhibition of S6K with PF4708671 does not prevent the phosphorylation of Unkempt. (H) Unkempt physically interacts with Raptor and mTOR. Mock purified extract from un-induced HeLa S3 cells (DOX -) was used as a negative control.

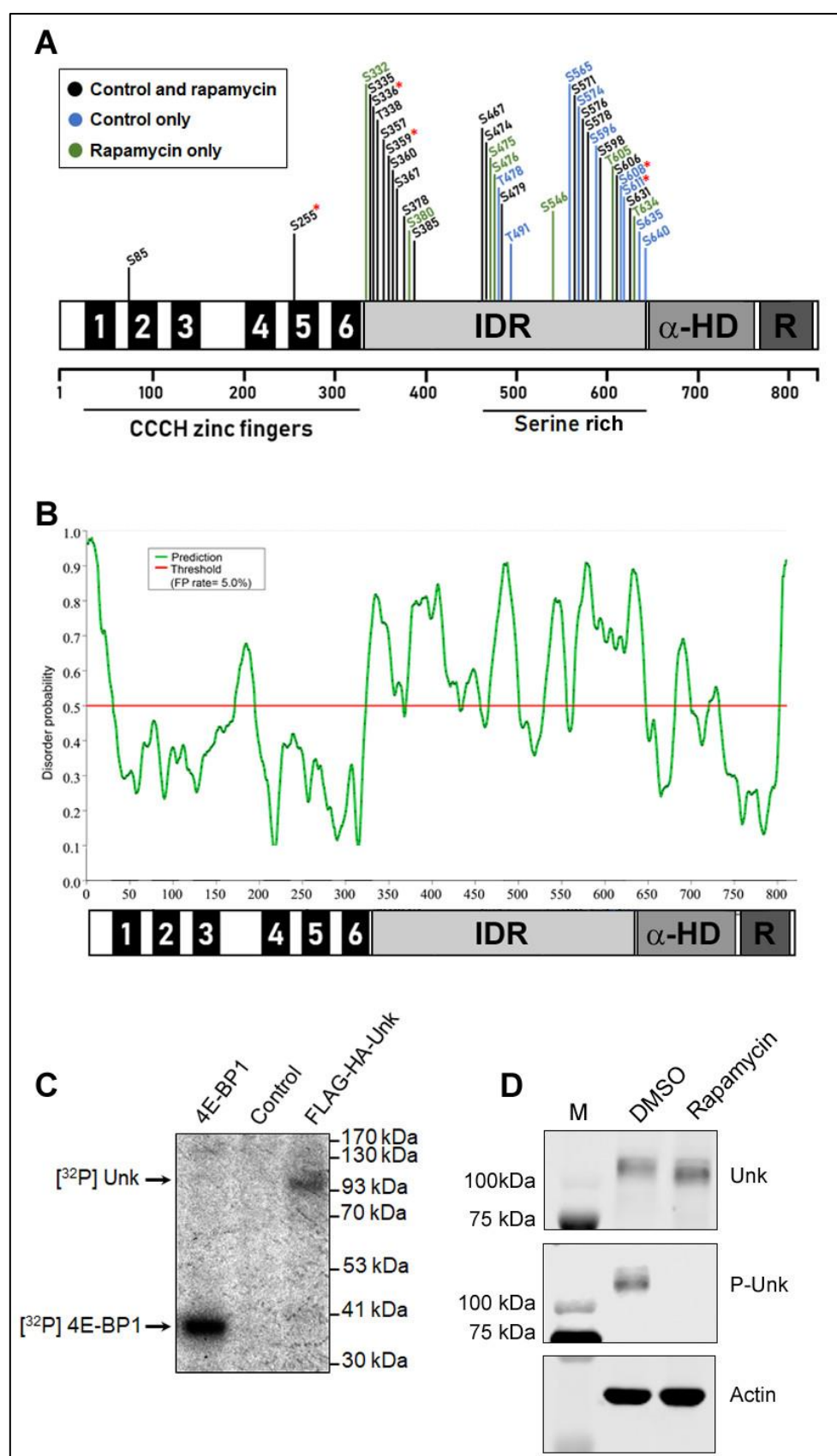


Figure 2. Unkempt is a direct substrate of mTORC1 and is phosphorylated in the IDR by mTORC1. (A) A schematic of the phosphorylated residues identified in Unkempt by LC-

MS/MS. Phosphorylated residues identified in both control and rapamycin treated cells shown in black; phosphorylated residues identified only in control cells shown in blue; phosphorylated residues identified only in rapamycin treated cells shown in green.

Phosphorylated residues identified only in the control *in vitro* kinase assay condition and not in the rapamycin/FKBP12 condition are highlighted with a red asterisk. See Supplemental Figures S2 and S3 for details. IDR: intrinsically disordered region, α -HD: alpha-helical domain, R: ring domain. (B) Prediction disorder probability plot of the primary sequence of mouse Unkempt. The serine-rich region (amino acids 467 to 640) is highly disordered. False positive rate of 5%. (C) FLAG-HA-Unkempt purified from HeLa S3 cells is phosphorylated *in vitro* by reconstituted mTORC1/Rheb-GTP. Recombinant 4E-BP1 was used as a positive control. Mock purified extract from un-induced HeLa S3 cells was used as a negative control. (D) A phospho-specific antibody raised against phospho-S606/ phospho-S611 in Unkempt does not recognise Unkempt from rapamycin treated SH-SY5Y cells. M: molecular weight marker.

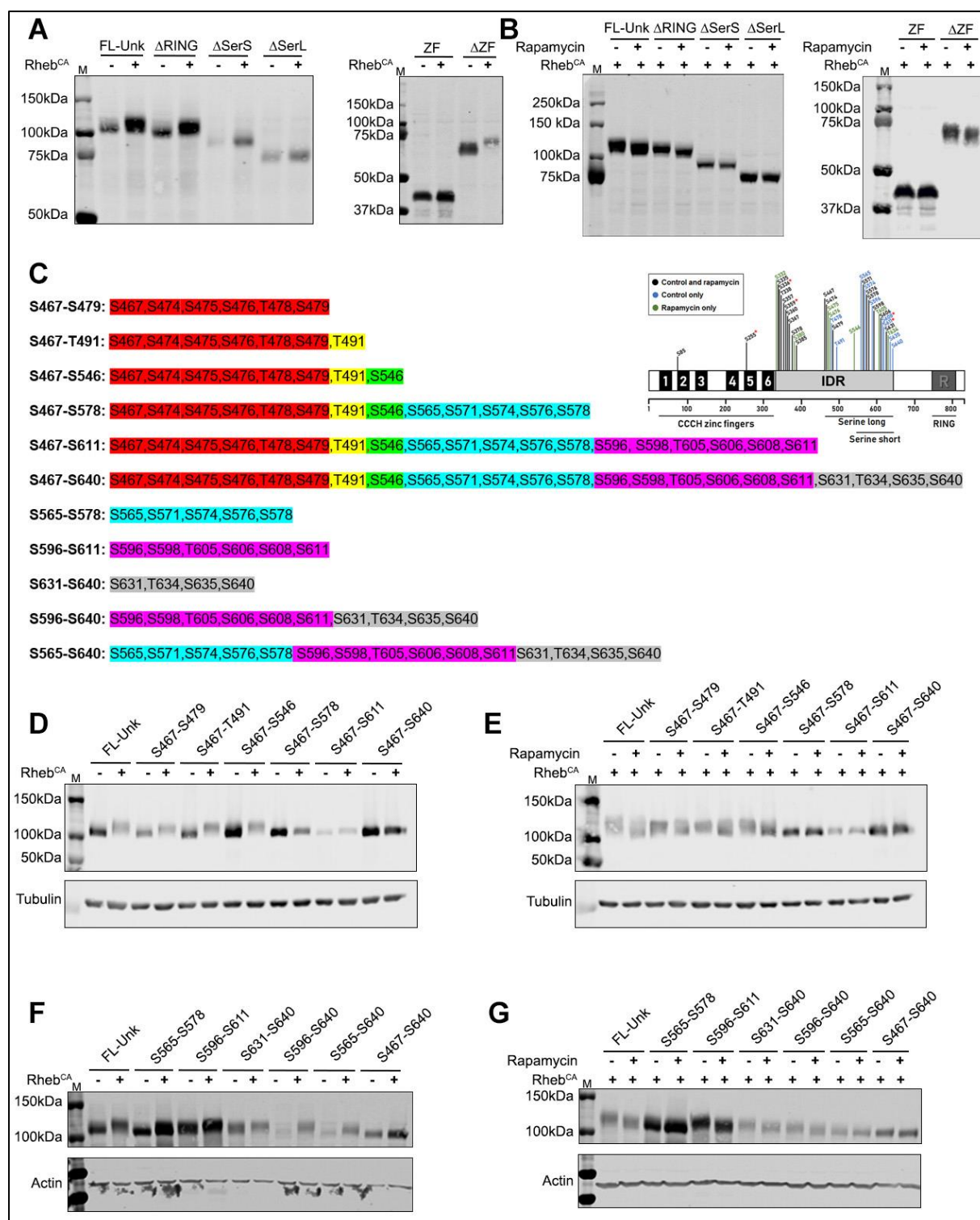


Figure 3. Phosphorylation within the serine-rich region of the IDR is necessary for mTORC1 dependent phosphorylation of Unkempt. (A, B) Full length V5-Unkempt (FL-Unk), or deletion mutants lacking the zinc finger domain (Δ ZF) or the RING domain (Δ RING) are phosphorylated in HEK293 cells expressing constitutively active Rheb (Rheb^{CA}) (A) and dephosphorylated in these cells treated with rapamycin (B). Phosphorylation is absent with zing finger domain alone (ZF) and completely or partially absent with deletion mutants

lacking whole serine-rich region (Δ SerL) or part of the serine-rich region (Δ SerS) (A, B). (C) A schematic of the phosphorylated residues identified in Unkempt by LC-MS/MS. Phosphorylated residues identified in both control and rapamycin treated cells shown in black; phosphorylated residues identified only in control cells shown in blue; phosphorylated residues identified only in rapamycin treated cells shown in green. Phosphorylated residues identified only in the control *in vitro* kinase assay condition and not in the rapamycin/FKBP12 condition are highlighted with a red asterisk. See Supplemental Figures S1 and S2 for details. (D, E) Full length V5-Unkempt (FL-Unk) or FL-Unk containing alanine mutations in phosphorylated serine and threonine residues between S467-S546 expressed in HEK293 cells are phosphorylated by constitutively active Rheb (Rheb^{CA}) (D) and dephosphorylated by rapamycin (E). Additional mutation of phosphorylated residues from S467-S578, S467-S611 and S467-S640 partially or completely prevent phosphorylation by constitutively active Rheb (D) and dephosphorylation by rapamycin (E). (F, G) Alanine mutants of Unkempt in phosphorylated serine and threonine residues between S565-S640 are phosphorylated by constitutively active Rheb (Rheb^{CA}) (F) and dephosphorylated by rapamycin (G). M: molecular weight marker.

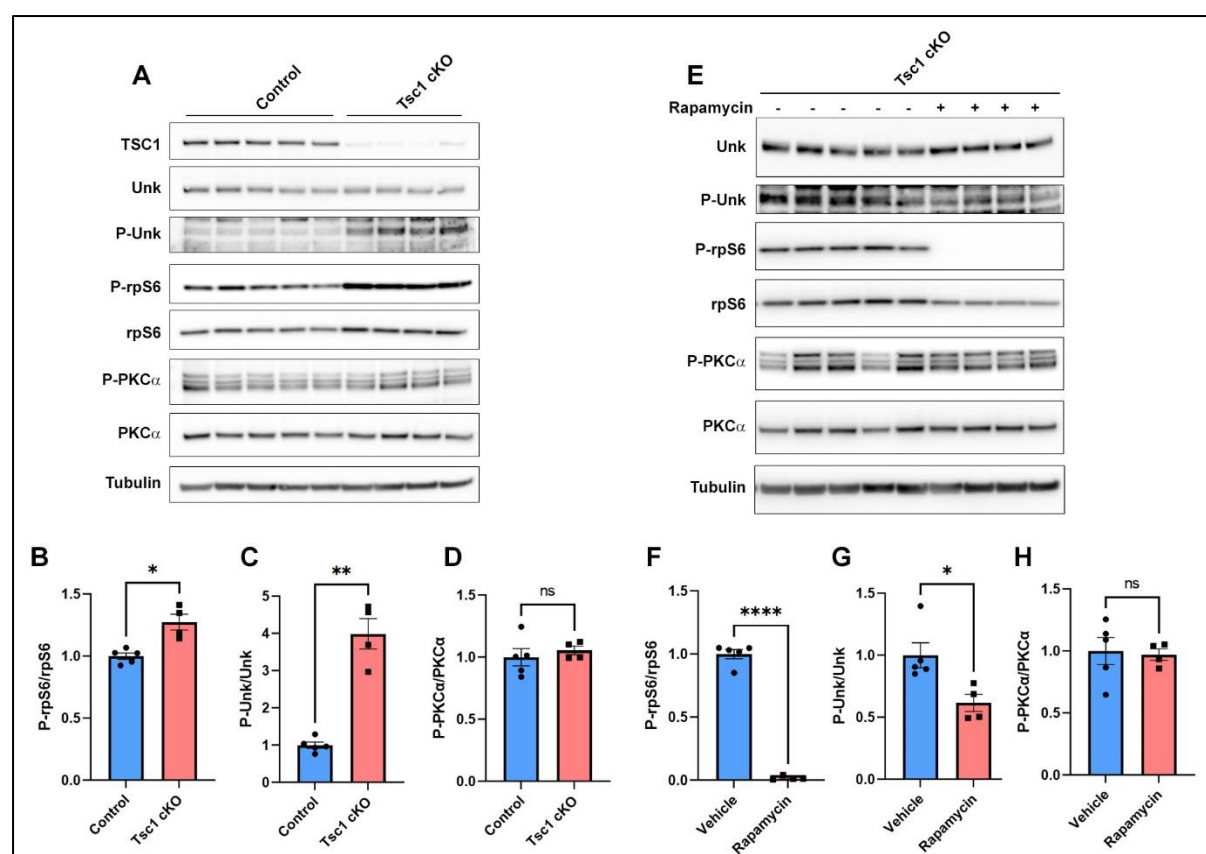


Figure 4. Unkempt is phosphorylated by mTORC1 during neurodevelopment. (A) Western blot analysis of E16.5 brain lysate from control (*Nestin-Cre;Tsc1^{fl/+}*) and Tsc1 cKO (*Nestin-Cre;Tsc1^{fl/fl}*) mice. (B-D) Quantification of phospho-rpS6 (B), phospho-Unkempt (C) and phospho-PKC α (D) expression. Control n=5, Tsc1 cKO n=4. (E) Western blot analysis of E16.5 brain lysate from Tsc1 cKO (*Nestin-Cre;Tsc1^{fl/fl}*) mice treated with vehicle or rapamycin for 24 hours. (F-H) Quantification of phospho-rpS6 (F), phospho-Unkempt (G) and phospho-PKC α (H) expression. Vehicle n=5, rapamycin treated n=4. Data are represented as mean $\pm$ SEM. Student's t test, ns not significant, *p<0.05, **p<0.01, ****p < 0.0001.

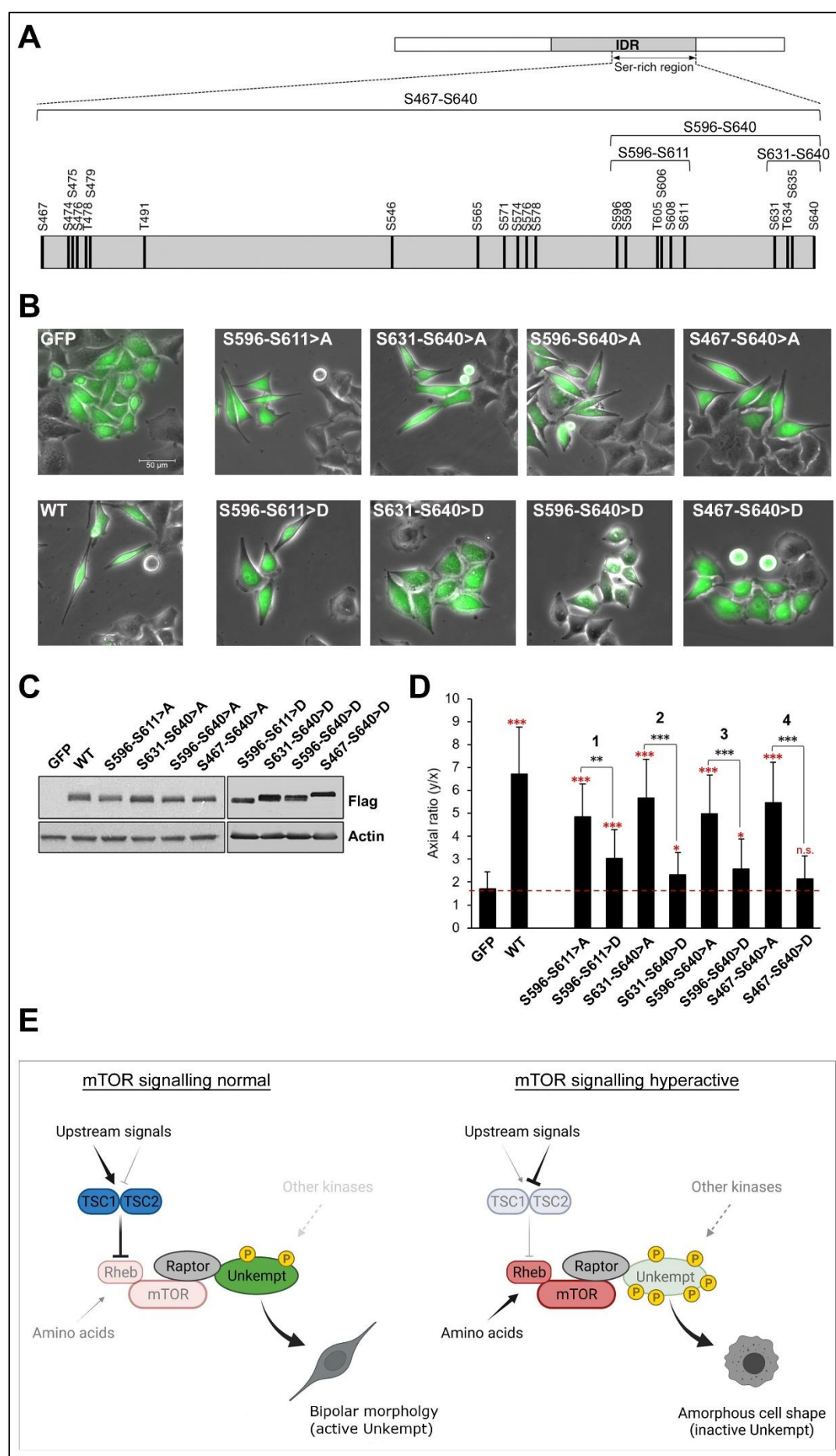


Figure 5. Phosphosite mutations in the serine-rich region compromise the morphogenetic activity of Unkempt. (A) Map of Unkempt protein (top) with the locations of all detected phosphosites within the serine-rich region of the IDR shown magnified on the bottom, drawn to scale. (B) GFP only-inducible or GFP and wild-type (WT) or phosphosite alanine (>A) or aspartate (>D) mutant Unkempt-inducible HeLa cells at 48 hours of treatment with doxycycline. Scale bar, 50 μ m. (C) Detection by immunoblotting of WT or the indicated mutant Unkempt proteins in total cell lysates of inducible HeLa cells at 48 hours of treatment with doxycycline. (D) The morphologies of the HeLa cell cultures indicated in (B) were quantified by calculating their axial ratios (y/x), as described previously (see Materials and methods (Murn *et al.*, 2015)). The results are compared with the GFP control (red asterisks) morphology, or mutant Unkempt-expressing cells are compared for each phosphosite region indicated in (A), (black asterisks). Red dashed line denotes the average axial ratio of the GFP control cells. Error bars indicate SD (n = 50). (*) $4.3\text{E-}5 < P < 5.2\text{E-}5$, (**) $P = 3.9\text{E-}6$, (***) $P < 1.3\text{E-}10$ (pertains to black and red asterisks), Student's *t*-test. (E) A model for the regulation of cellular morphogenesis by Unkempt acting downstream of mTORC1.