AWARD NUMBER: W81XWH-17-1-0082

TITLE: Establishing the Molecular Basis of the Neurodevelopmental Features of TSC.

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REPORT DATE: April 2020

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
APRIL 2020	Annual	**15th March 2019-14th March 2020**

4. TITLE AND SUBTITLE
Establishing the Molecular Basis of the Neurodevelopmental Features of TSC

5a. CONTRACT NUMBER
W81XWH-17-1-0082

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
King's College London, London SE5 9NU, UK

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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*. 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 performed in depth characterisation of Unkempt phosphorylation by mTORC1, generated phospho-specific antibodies against Unkempt and analysed/quantitied the expression of neurodevelopmental markers in Unkempt conditional knockout mice.

15. SUBJECT TERMS
TSC; mTOR; Unkempt; signaling; neurogenesis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	**12**	**19b. TELEPHONE NUMBER** *(include area code)*

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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 *in vitro* 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	90%
Major Task 2: Testing the function of mTOR dependent Unkempt phosphorylation *in vivo*		
Subtask 1: *In utero* 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	50%
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 $Unk^{tm1c/tm1c}$;*nestin-*Cre^{+} mice neurodevelopmental phenotypes. 40 mouse embryos and 20 P21 adults.	12-36	90%
Subtask 2: Single neural progenitor cell labelling and analysis in $Unk^{tm1c/tm1c}$;*nestin-*Cre^{+}mice. 20 mouse embryos.	12-36	40%
Subtask 3: *In utero* electroporation of Cre into neural progenitors in $Unk^{tm1c/tm1c}$ mice to produce localized loss of Unkempt expression. 20 mouse embryos.	12-36	40%
Major Task 4: Analysis of behavioral phenotypes in Unkempt knock-out mice		
Subtask 1: Analysis of $Unk^{tm1c/tm1c}$;*nestin-*Cre^{+} 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 in utero electroporation in neural progenitors in $Tsc1^{fl/fl}$ mice. 14 adult mice. $Tsc1^{fl/fl}$ mice provided by Professor Bordey.	9-30	40%

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: *In utero* electroporation of Unkempt mTORC1-dependent phosphorylation site mutants (from Specific Aim 1) into $Tsc1^{fl/fl}$ 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	20%
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 $Tsc1^{fl/fl}$;*nestin-*Cre^{+} mice.	12-30	30%

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

Major activities:

1. In depth characterization of Unkempt phosphorylation.
2. Generation of Unkempt phospho-specific antibodies.
3. Immunohistochemical analysis of $Unk^{tm1c/tm1c}$;*nestin-*Cre^{+} mice neurodevelopmental phenotypes.

Specific objectives:

1. Deletion analysis of Unkempt to identify the mTORC1-dependent phosphorylated region.
2. Point mutation analysis of Unkempt to validate the LC-MS/MS data.
3. Generation of Unkempt phospho-specific antibodies against Ser606 and Ser611.
4. Quantification of $Unk^{tm1c/tm1c}$;*nestin-*Cre^{+} mice neurodevelopmental phenotypes at E16.5.

Significant results or key outcomes:

We have successfully identified mTORC1-dependent phosphorylated residues in Unkempt using LC-MS/MS (see 2nd year report and Figure 1A). These residues are clustered in a 173 amino acid region that is extremely rich in serine residues (41 (24%) serines; Figure 1A). We have generated deletions in this 'serine rich region', tested these constructs and constructs containing deletions of conserved domains for their potential to be phosphorylated by mTORC1. Interestingly, deletion of part, or all, of the serine rich region partially or completely prevents mTORC1-dependent phosphorylation of Unkempt (Figure 1A-C). Whereas deletion of the zinc finger domain or RING domain does not affect phosphorylation (Figure 1A-C). To further characterize the mTORC1-dependent residues we have generated a series of point mutants in which serine/threonine residues identified by LC-MS/MS were mutated to alanine. Mutation of phosphorylated residues from S467 to S546 to alanine did not affect mTORC1-dependent phosphorylation of Unkempt (Figure 1A, D, E; Appendix 1). However, additional mutation of phosphorylated residues between S565-S578, S596-S611 and S631-S640 to alanine either strongly reduced or completely prevented mTORC1-dependent phosphorylation of Unkempt (Figure 1A, D, E; Appendix 1). Specific serine/threonine residues within the serine rich region therefore mediate the phosphorylation of Unkempt by mTORC1. We are now generating alanine and aspartate mutants of these mTORC1-activity dependent residues in Unkempt to test in the *in-utero* electroporation experiments (Major Task 2, Subtask 1 and Major Task 5, Subtask 3).

Based on the LC-MS/MS analysis of Unkempt we have identified Ser611 as a high confidence mTORC1-dependent substrate residue (see 2nd year report and Appendix 1). Together with CovalAb UK Ltd, we have generated phospho-peptides containing P-Ser606 (which is mTORC1 independent; see Appendix 1), P-Ser611 (which is mTORC1 dependent; see Appendix 1) and both P-Ser606/PSer-611. We have used these peptides as antigens to generate phospho-specific antibodies. The purified antibodies have very good immunoreactivity by ELISA (data not shown). We have been unable to test the antibodies yet by western due to the COVID-19 shutdown but will do as soon as we restart in the lab.

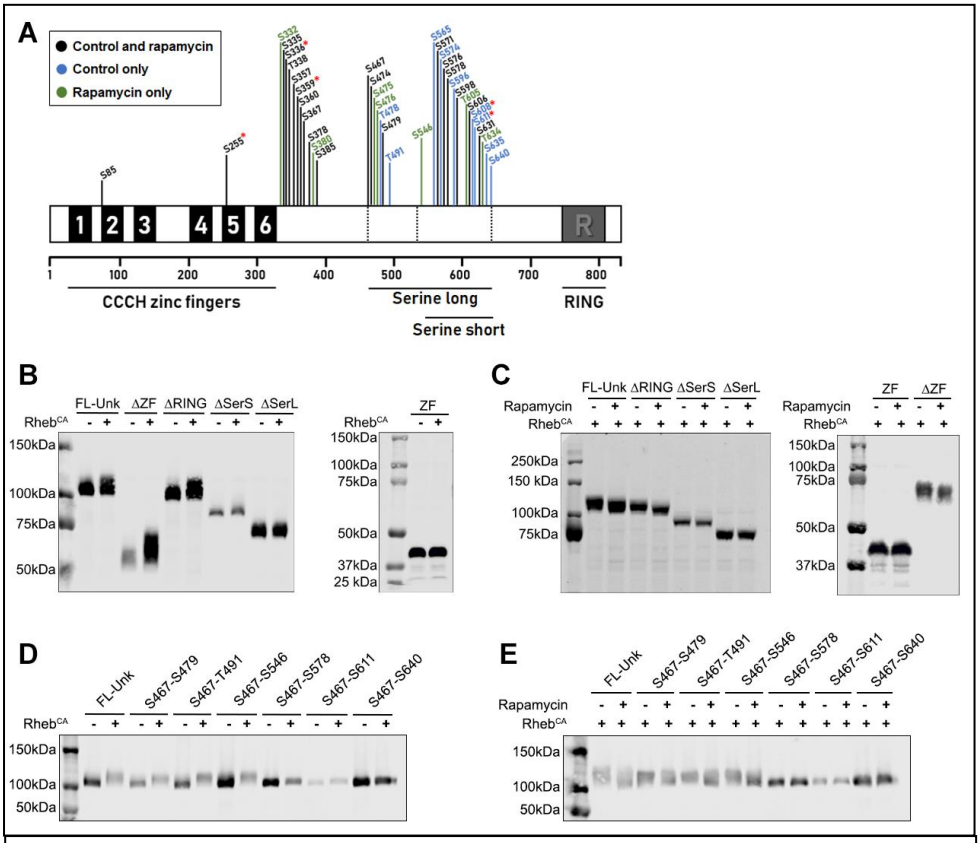

Figure 1. Unkempt is phosphorylated by mTORC1 in the C-terminal serine rich region. (A) A schematic of the phosphorylated residues identified in Unkempt by LC-MS/MS. Phosphorylated residues identified in both control and rapamycin treated cells shown in black; phosphorylated residues identified only 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 Appendix 1 for details. (B, C) Full length V5-Unkempt (FL-Unk), or deletion mutants lacking the zinc finger domain (ΔZF) or the RING domain (ΔRING) are phosphorylated in HEK293 cells expressing constitutively active Rheb ($Rheb^{CA}$) (B) and dephosphorylated in these cells treated with rapamycin (C). Phosphorylation is absent with zing finger domain alone (ZF) and completely or partially absent with deletion mutants lacking whole serine rich region (ΔSerL) or part of the serine rich region (ΔSerS). (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 between S467-S578, S467-S611 and S467-S640 partially or completely prevent phosphorylation by constitutively active Rheb (D) and dephosphorylation by rapamycin (E). See Appendix 1 for details of mutations.

We have now completed the immunohistochemical analysis of neurodevelopment in *Unk*^{cKO} mice. We stained for and quantified the expression 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 (Figure 2). Therefore, Unkempt may act redundantly with another protein (potentially its paralog Unkempt like) to control embryonic neurogenesis downstream of mTORC1. The improved cognitive flexibility in *Unk*^{cKO} mice (see 2nd year report) may be due to altered neurogenesis in a specific temporal or spatial niche. We have isolated and stained for several neuronal markers in adult brain tissue from *Unk*^{cKO} mice and littermate controls at P21. We are currently imaging and quantifying these data.

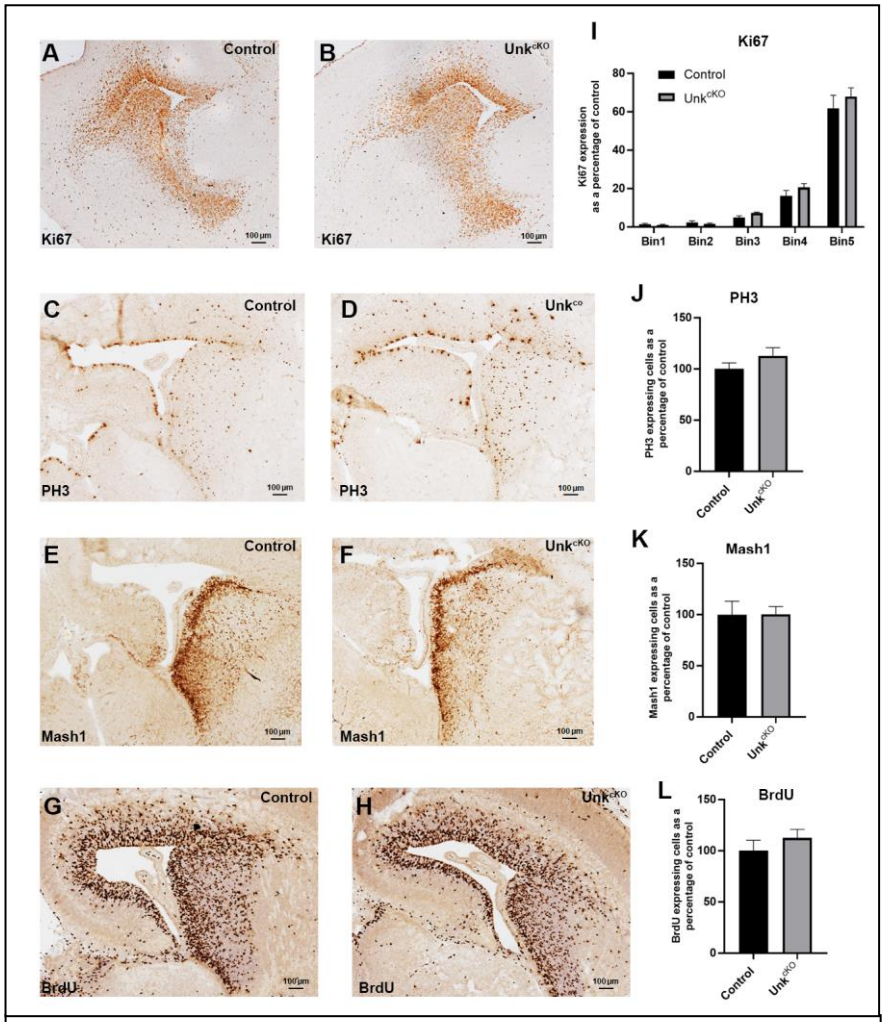

Figure 2. Characterisation neurodevelopment in Unk^{cKO} *mice.* (A-H) Coronal sections of the subventricular zone from control (*Unk*$^{tm1c/tm1c}$) and *Unk*^{cKO} (*Nestin-Cre, Unk*$^{tm1d/tm1d}$) embryos at E16.5 stained for Ki67 (A, B), PH3 (C, D), Mash1 (E, F) and BrdU incorporation (G, H). Quantifications shown in (I) control n=5, *Unk*^{cKO} n=5; (J) control n=7, *Unk*^{cKO} n=9; (K) control n=7, *Unk*^{cKO} n=9; and (L) control n=5, *Unk*^{cKO} n=5. Data are presented as mean ± SEM.

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

The postdoc on the project, Dr Baskaran, has been trained by Mr Carl Hobbs, our departmental histology and imaging manager, to perform fixation, embedding, sectioning and immunostaining of mouse brain tissue. This is a highly skilled technique which Dr Baskaran has mastered and is now proficient in this method.

- **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 2019 International Tuberous Sclerosis Complex conference in Toronto. Dr Baskaran was awarded a travel award to attend the conference and was selected to give an oral presentation. The conference was excellent, and we received extremely useful feedback on the project.

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

We are on schedule with the goals described in the SOW and so we will continue the project as described in the Project Narrative. We will test the Unkempt phospho-specific antibodies we have generated together with CovalAb UK Ltd. We have now identified several mTORC1 dependent phosphorylation sites in Unkempt and so will test these functionally. We will complete the immunohistochemical analyses of Unkempt cKO mice at P21.

4. IMPACT

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

Nothing to report.

- **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**

We have had problems with the in utero electroporation experiments using the *Unk*$^{tm1c/tm1c}$ mice (Major task 3, subtasks 2&3) and the *Tsc1*$^{fl/fl}$ mice (Major task 5, subtasks 1&3). This is because the background strain for these mice (C57BL/6) have far fewer embryos (3-8 embryos per litter) than the CD1 strain (10-16 embryos per litter) we have used previously. Also, the C57BL/6 dams are more sensitive to the surgical procedure. We therefore frequently lose litters or have few embryos per litter. We have spoken to other labs who have had similar problems, so this appears to be a general limitation with this strain rather than something specific to our laboratory.

Our research institute was shut-down in mid-March 2020 to due COVID-19. Currently, we do not know when we will be allowed back and able to re-start experiments.

6. **PRODUCTS**

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

Dr Baskaran gave an oral presentation on the project at the 2019 International Tuberous Sclerosis Complex conference in Toronto.

- **Journal publications**

We submitted a manuscript describing the biochemical analysis of Unkempt and the knockout mouse to PNAS and the manuscript is currently under review.

- **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:	TSCRP Idea Development Award

Name:	Mr Carl Hobbs
Project role:	Departmental histology and imaging manager
ORCID ID:	
Nearest person month worked:	2 months
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 projec**t:
        - **Collaboration**: Assisted with in vitro kinase assay and Raptor overlay assays.

8. **SPECIAL REPORTING REQUIREMENTS**

Not applicable.

9. **APPENDICES**

Appendix 1.

	IP HeLa cells									
	No. times identified		*In vitro* kinase assay		Serine/threonine mutated to alanine					
Residue	Control	Rapamycin	Control	Rapamycin/ FKBP12	S467-S479	S467-T491	S467-S546	S467-S578	S467-S611	S467-S640
S85	2	1								
S255	2	2	YES	NO						
S332	0	2								
S335	3	3	YES	YES						
S336	2	2	YES	NO						
T338	3	3	YES	YES						
S357	3	3	YES	YES						
S359	2	3	YES	NO						
S360	3	3								
S367	2	1								
S378	3	3	YES	YES						
S380	0	1								
S385	2	2								
S467	1	1			X	X	X	X	X	X
S474	2	1			X	X	X	X	X	X
S475	0	1			X	X	X	X	X	X
S476	0	1			X	X	X	X	X	X
T478	1	0			X	X	X	X	X	X
S479	2	2			X	X	X	X	X	X
T491	1	0				X	X	X	X	X
S546	0	1					X	X	X	X
S565	1	0						X	X	X
S571	1	1						X	X	X
S574	1	0						X	X	X
S576	1	3						X	X	X
S578	3	3	YES	YES				X	X	X
S596	1	0							X	X
S598	3	3							X	X
T605	0	1	YES	YES					X	X
S606	2	2							X	X
S608	1	0	YES	NO					X	X
S611	3	0	YES	NO					X	X
S631	3	1								X
T634	0	1								X
S635	1	0								X
S640	1	0								X
mTORC1-dependent phosphorylation					YES	YES	YES	STRONGLY REDUCED	STRONGLY REDUCED	NO

Table 1. mTORC1 dependent and independent phosphorylated residues in Unkempt. (A) Columns 1-3 show a summary of the number of times each phosphorylated residue was identified by LC-MS/MS in FLAG-HA-Unkempt immunoprecipitated from vehicle (DMSO) control and rapamycin treated HeLa S3 cells in three biological replicates. Columns 4 and 5 show phosphorylated residues identified by LC-MS/MS of purified FLAG-HA-Unkempt following an *in vitro* kinase assay with reconstituted mTORC1/Rheb^{CA} in control or rapamycin/FKBP12 conditions. Columns 6-11 show the serine and threonine residues mutated to alanine in the constructs used in Figure 1D, E and their effect on phosphorylation.