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PRINCIPAL INVESTIGATOR: Dr Joseph Bateman

CONTRACTING ORGANIZATION: King's College, London, UK

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Dr Joseph Bateman, Dr Pranetha Baskaran

E-Mail: joseph_matthew.bateman@kcl.ac.uk; Pranetha.baskaran@kcl.ac.uk

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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 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.

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

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TABLE OF CONTENTS

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	100%
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	80%
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	100%
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	60%
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	50%

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

Major activities:

1. Investigating the mTOR-dependent phosphorylation of Unkempt.
2. Overexpression of Unkempt in TSC neurodevelopmental models.
3. Analysis of Unkempt expression and phosphorylation in the brain in a TSC mouse model.

Specific objectives:

1. Validation of an Unkempt phospho-specific antibody.
2. In utero electroporation of Unkempt mTORC1-dependent phosphorylation site mutants together with RhebCA vector to test if phosphorylation is required to prevent neurodevelopmental phenotypes in TSC models.
3. Immunoblot analysis of Unkempt expression and phosphorylation status in embryonic brains from Tsc1fl/fl;nestin-Cre+ mice.

Significant results or key outcomes:

Based on the LC-MS/MS analysis of Unkempt we have identified Ser611 as a high confidence mTORC1-dependent substrate residue (see 3rd year report). Together with CovalAb UK Ltd, we have generated phospho-peptides containing P-Ser606 (which is mTORC1 independent), P-Ser611 (which is mTORC1 dependent) and both P-Ser606/P-Ser611. We have used these peptides as antigens to generate a phospho-specific antibody. We have tested this antibody in SH-SY5Y cells, which show that the antibody only recognizes Unk in serum stimulated cells, when Unk is phosphorylated (Figure 1). It fails to recognize Unk when SH-SY5Y cells are treated with the mTORC1 inhibitor rapamycin (Figure 1). Therefore, this antibody is specific for mTORC1-dependent phospho-Unk. This antibody will be a valuable tool for studying mTORC1 activity in the nervous system.

Based on our LC-MS phospho-proteomic analysis of Unkempt (see 3rd year report) we have recently generated pCAG plasmids containing serine to alanine and serine to aspartate substitutions in phosphorylated residues in Unkempt. We will now use these plasmids for in utero electroporation experiments to test whether these phospho-mutant versions of Unkempt rescue the neurodevelopmental phenotype caused by mTORC1 activation using RhebCA expression.

We are currently dissecting CNS tissue from E16.5 Tsc1fl/fl;nestin-Cre+ mice embryos. We will shortly analyse Unk expression and phosphorylation in this tissue.

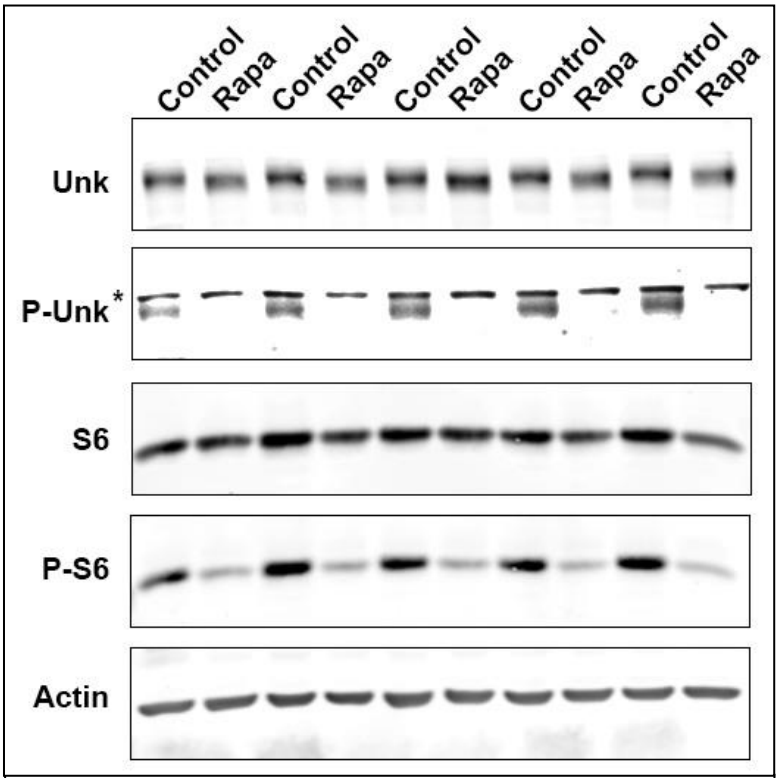

Figure 1. Validation of a phospho-specific Unkempt antibody. SH-SY5Y were cells were serum starved overnight and then incubated in DMEM+FCS+DMSO (Control) or DMEM+FCS+rapamycin (rapa) for 1h. Antibodies are against Unkempt (Unk), P-Ser606/P-Ser611 phospho specific Unkempt (P-Unk), rpS6 (S6), Ser235/236 phospho specific S6 (P-S6), and Actin. *non-specific band.

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

Due to maternity leave and Covid-19 shutdown this has not been possible.

- **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 will attend the 2021 International Tuberous Sclerosis Complex Research Conference (online) in June 2021. Dr Baskaran has been selected to give an oral presentation at the conference. The conference will be an excellent opportunity to disseminate the results of the project. We also have a manuscript currently in revision in Scientific Reports describing the characterisation of the Unkempt cKO mice generated during 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 complete the analysis of the Unkempt phospho-mutant constructs in vivo and test their ability to rescue neurodevelopmental phenotypes caused by mTORC1 activation using RhebCA

expression. We will complete the analysis of Unkempt expression and phosphorylation in E16.5 Tsc1fl/fl;nestin-Cre+ mice embryonic CNS tissue.

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

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 have received a no cost extension to enable us to complete the project.

6. PRODUCTS

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

Dr Baskaran will give 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**

We have a manuscript currently in revision in Scientific Reports describing the characterisation of the Unkempt cKO mice generated during the project.

- **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:	2 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:	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 projec**t:
        - **Collaboration**: Assisted with in vitro kinase assay and Raptor overlay assays.

8. **SPECIAL REPORTING REQUIREMENTS**

Not applicable.

9. APPENDICES