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TITLE: Therapeutic Targeting of FLCN-Deficient Renal Cancers

PRINCIPAL INVESTIGATOR: Othon Iliopoulos, MD

CONTRACTING ORGANIZATION: Massachusetts General Hospita

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Fort Detrick, Maryland 21702-5012

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5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goals of this proposal are: 1) to identify the cellular phospho-proteome regulated by FLCN, 2) to discover the mechanisms by which FLCN suppresses the non-canonical translational initiation of a specific subset of mRNAs, and 3) to assign FLCN functions to specific protein domains.

So far we profiled the cellular proteome changes regulated by FLCN in vitro and we gained major insights into the mechanism by which FLCN regulates protein translation.

15. SUBJECT TERMS

Folliculin, GTPase activity, Rab7A, Phosphoproteome, Receptor Tyrosine Kinase activity, Tumor suppressor gene.

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
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TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Folliculin (FLCN) is a tumor suppressor gene linked to the development of renal cell carcinoma (RCC). My laboratory showed before that FLCN acts as a GAP protein for Rab7A and regulates trafficking and therefore activity of EGFR. Our proposal's goal is to show that (1) FLCN regulates not only EGFR, but a panel of cell surface receptor tyrosine kinases in a way similar to EGFR, because the "internalization" of these kinases is a general mechanism of regulation. (2) We showed that FLCN suppresses protein translation and it binds to two translation-promoting factors that are GTPases. We therefore propose to take a system biology approach in order to profile all the kinases that are regulated by FLCN and to evaluate which kinases can be used to target FLCN-driven, rare RCCs. We also propose a series of experiments that will uncover the biochemical details of how FLCN suppresses protein translation. (3) The third goal is to use mutants of FLCN to find out if all these functions depend on different parts of the protein and can be separated from each other. Such a "distribution" of functions (called "domains") will help understand which of these functions are important for tumor suppression.

KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Folliculin, GTPase activity, Rab7A, Phosphoproteome, Receptor Tyrosine Kinase activity, Tumor suppressor gene, Renal Cell Carcinoma

2. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: To determine the receptor tyrosine kinases (RTKs) and the cellular phospho-proteome changes regulated by FLCN.

Subtask 1: FLCN-dependent global phosphoproteome changes in cells stimulated with growth factors. Phospho-proteomic and proteomic analysis. Cell lines used: UOK257, FTC-133, infected with wild type FLCN, empty vector control or FLCN mutants.

Subtask 2: FLCN-dependent global phosphoproteome changes in cells stimulated with amino acids. Phospho-proteomic and proteomic analysis. Cell lines used: UOK257, FTC-133, infected with wild type FLCN, empty vector control or FLCN mutants

Specific Aim 2: To fully characterize the biochemical mechanism by which FLCN suppresses initiation of protein translation and identify the subclass of mRNAs regulated by FLCN.

Subtask 1: Establishment of in vitro GAP assay for eIF2gamma and eIF5B.

Subtask 2: Testing in vitro FLCN GAP activity for eIF2gamma and eIF5B.

Subtask 3: Testing tumor associated mutations and post translational FLCN modifications in the GAP assay

Specific Aim 3: To genetically dissect FLCN functions by using a panel of post-translational modification (PTM) and tumor-associated FLCN mutations.

Subtask 1: Generation of tumor-associated FLCN mutants that lead to expression of stable and detectable FLCN mutants

Subtask 2: Generation of single and compound phosphor-mimetic and phospho-inactivating FLCN mutants as well as scanning mutagenesis of FLCN protein.

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

SPECIFIC AIM 1: Our preliminary data indicated that FLCN regulates several receptor tyrosine kinases (RTKs) through its function as a Rab7A GAP and has a global effect on the cellular phosphoproteome. *We proposed to use proteomic approaches to map the FLCN-regulated phosphoproteomic changes in cells stimulated by growth factors and/or amino acids*. This knowledge will allow us to validate critical nodes of the phosphoproteomic changes as therapeutic targets.

In SOW we proposed to use the FLCN-null UOK257 and FTC-133 cell lines and create isogenic lines in which wild type FLCN was reintroduced. *We completed this goal* and we used FLCN -/- and +/+ cell lines for the experiments described in subtask 1 and subtask 2 of Aim 1. We stimulated the cells lines with amino acids or serum containing growth factors and obtained a phosphoproteome map of the cells. This is achieved through a new phospho-proteomics strategy developed in the Haas laboratory that combines two phospho-peptide fragmentation methods – collision-induced dissociation (CID) and higher-collision-induced dissociation – to increase the sensitivity of multiplexed phospho-proteomics measurements by two-fold (described in our proposal). *We therefore achieved the goal outlined in subtasks 1 and substask 2 in obtaining a map of phosphoproteins regulated by FLCN*.

The attached .xls files (FLCN_proteome and FLCN_phosphoproteome) are the raw data of the proteome changes and phsosphoproteome changes following serum stimulation of three isogenic cell lines: UOK257 FLCN-/- cells, UOK257 FLCN-replete cells, and UOK257 cells infected with the tumor causing FLCN-C9 mutant. The importance of these experiments consists in the discovery that FLCN regulates several receptor tyrosine kinases (RTKs) and their subsequent signaling pathways implicated in tumorigenesis. *In addition to these findings, that are consistent with the biochemical experiments we published before, we discovered that the phosphoproteomic changes regulated by FLCN affect strongly RNA translation, Golgi maturation and epigenetic regulation*. Future experiments based on this award will ask how to optimally block these pathways with existing drugs, a goal which is beyond the described scope of the award, but very important for development of medical therapies in FLCN-/- renal cell cancers.

To confirm these results in an orthogonal manner and test their conservation through evolution we collaborated with the laboratory of Mo Motamedi, in the MGH Cancer Center. We purified and characterized the Schizosaccharomyces pombe Bhd-Fnp Complex (BFC) and its cellular interactors. We showed that BFC complex physically interacts and regulates the highly conserved peptide transmembrane transporter Ptr2, the phosphoribosylformylglycinamidine synthase Ade3, and the V-ATPase complex. These are novel amino acid-dependent regulators of TORC1. BFC mutants exhibited a slower TORC1 repression and proliferate faster than wild-type cells following amino acid starvation. Therefore, we showed that the mammalian function of FLCN on mTORC1 through regulation of transmembrane RTKs is conserved evolutionarily. This work was published in *iScience* ***(Calvo et al. The fission yeast FLCN/FNIP complex augments TORC1 repression or activation in response to amino acid availability)***

SPECIFIC AIM 2: We showed that FLCN localizes to the polysomes, associates with factors regulating the initiation of protein translation and inhibits eIF2γ and EIF5B complex formation with tRNA-Met_i. The FLCN C-terminal domain, which is deleted by tumor-associated FLCN mutations, is necessary for the interaction of FLCN with eIF2γ and EIF5B. Reintroduction of wild type but not a C-terminus FLCN mutant into FLCN-/- cells results in suppression of serum or amino acid stimulated protein translation, independently of mTORC1 activity. Our data provide insights into a novel mechanism of cell growth restriction by FLCN (*We therefore achieved the goal outlined in Aim 2, subtasks 1, 2 and 3*).

We attach the manuscript "HUMAN FOLLICULIN TUMOR SUPPRESSOR PROTEIN BINDS TO TRANSLATION INITIATION FACTORS eIF2γ AND EIF5B AND SUPPRESSES PROTEIN SYNTHESIS" by Schneider et al, which describes the experiments corresponding to Aim 2A. This manuscript is currently under review.

In the second year of our work we tried hard to establish an in vitro GAP assay for the elongation factors eIF2γ and EIF5B, which are known to undergo secondary modification and be GAP targets. We were **not** able to establish such an assay, despite external collaborations with experts in the field. We will drop now this effort, because, despite being scientifically rigorous work, it may reflect unknown factors not present in the reactions and it detracts efforts from our main goal in analyzing FLCN targets.

*Work that remains to be done during N*on Cost Extension (NCE)*:*

We are now in the process of completing this work. In our proposal we describe how we will identify the RNAs regulated by FLCN (Aim 2B). We have isolated the polysome fractions shown in Figure 2 of the Schneider et al manuscript, and we extracted RNA. We now conduct RNA sequencing of polysomes and compare the RNA profile between FKCN-/- and FLCN reconstituted isogenic cell lines.

This will complete Aim 2B *(Aim 2, subtasks 4 and 5)* in order to discover these RNAs. We regard this necessary to complete the work for Aim 2 and we will report the final results to DOD at the end of the NCE period.

SPECIFIC AIM 3: The goal here is the dissection of FLCN functions by generating a panel of tumor-associated, truncation, phosphor-inactivating and phosphor-mimetic FLCN mutants (Subtasks 1 and 2).
We accomplished the first step of this goal. *We now have generated a panel of phospho-inactivating and phosphomimetic FLCN mutants. We also generated a panel of tumor associated point mutants*.

There raw data about these mutants, their stability and migration are shown in:
Manuscript by Schneider et al, Figures 1F and G (truncation mutants and tumor associated)
Laviolette et al, Figure 1a,1b, and 3a (Phosphomimetic, phosphor-inactivating and tumor associated mutants).

We are in the process of introducing these mutants in FLCN-/- cell lines and to test their ability to suppress the growth of the reconstituted cells as tumors in nude mice. We described this approach (using a patient derived mutant) in our publication: ***Schneider M, Dinkelborg K, Xiao X, Chan-Smutko G, Hruska K, Huang D, Sagar P, Harisinghani M, Iliopoulos O. Early onset renal cell carcinoma in an adolescent girl with germline FLCN exon 5 deletion. Fam Cancer. 2018 Jan;17(1):135-139. doi: 10.1007/s10689-017-0008-8. PMID: 28623476.***

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report." Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. Iliopoulos trained the following people that so far worked on the FLCN project generating the preliminary results of this proposal, the data included in the manuscripts currently submitted for publication and the data included in the current progress report.
Laura Laviolette, PhD. Obtained her PhD from University of Vancouver in Canada, completed her post-doctoral training with Dr. Iliopoulos, worked on FLCN and she is now an Associate Director, heading the Immuno-Oncology R&D, in Dragonfly Therapeutics, Inc.
Meike Schneider, MD completed her post-doctoral training with Dr. Iliopoulos after graduating from the Department of Urology, Medical Center Johannes Gutenberg University, Mainz, Germany and she is now a senior scientist at Bayer Oncology. She is the lead author in the paper describing the effect of FLCN on protein translation.
Katia Dinkelborg, MD completed her Diploma Thesis with Dr. Iliopoulos, graduated from University of Hannover Medical School in Germany and she is now working as a research fellow in the University of Hannover Department of Hepatology.
Ravi Sundaram, BS, obtained his Bachelor of Sciences from Northeastern University, Boston, MA and worked as a Research Technician for 3 years at the Iliopoulos Lab. He is now enrolled in the University of Sydney Medical School, in Sydney, Australia.
D**ongkook Min, PhD,** is a senior post-doctoral fellow recruited to the lab in January 2022, in order to complete the experiments remaining above and expand the FLCN project observations to deeper analysis.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The main avenue of data dissemination is publication of our data. In addition, we generated several collaborations, attracting colleagues to study the basic mechanism of tumor suppression by FLCN (as evidenced by our publication record) and presented the data in local national and international scientific meetings.

The following manuscripts are submitted for publication or published. These manuscripts were supported by the DOD award at hand.

1) **HUMAN FOLLICULIN TUMOR SUPPRESSOR PROTEIN BINDS TO TRANSLATION INITIATION FACTORS eIF2A AND EIF5B AND SUPPRESSES PROTEIN SYNTHESIS**
(Meike Schneider, Katja Dinkelbor, Syed I.A. Bukhari, Samuel S Truesdell, Vera A. Pisareva, Andrey V. Pisarev, Shobha Vasudevan and Othon Iliopoulos). *UNDER REVIEW*

2) **The fission yeast FLCN/FNIP complex augments TORC1 repression or activation in response to amino acid availability**
(Isabel A. Calvo, Shalini Sharma, Joao A. Paulo, Alexander Gulka, Andras Boeszoermenyi, Jingyu Zhang, Jose M. Lombana, Christina M. Palmieri1, Laura A. Laviolette, Haribabu Arthanari, Steven P. Gygi, Othon Iliopoulos and Mo Motamedi). iScience. 2021 Nov 19; 24(11): 103338.

3) **Genetic risk assessment for hereditary renal cell carcinoma: Clinical consensus statement.** Bratslavsky G, Mendhiratta N, Daneshvar M, Brugarolas J, Ball MW, Metwalli A, Nathanson KL, Pierorazio PM, Boris RS, Singer EA, Carlo MI, Daly MB, Henske EP, Hyatt C, Middleton L, Morris G, Jeong A, Narayan V, Rathmell WK, Vaishampayan U, Lee BH, Battle D, Hall MJ, Hafez K, Jewett MAS, Karamboulas C, Pal SK, Hakimi AA, Kutikov A, Iliopoulos O, Linehan WM, Jonasch E, Srinivasan R, Shuch B. Cancer. 2021 Nov 1;127(21):3957-3966. doi: 10.1002/cncr.33679. PMID: 34343338; PMCID: PMC8711633.

4) **Seventh BHD international symposium: recent scientific and clinical advancement.** Woodford MR, Andreou A, Baba M, van de Beek I, Di Malta C, Glykofridis I, Grimes H, Henske EP, Iliopoulos O, Kurihara M, Lazor R, Linehan WM, Matsumoto K, Marciniak SJ, Namba Y, Pause A, Rajan N, Ray A, Schmidt LS, Shi W, Steinlein OK, Thierauf J, Zoncu R, Webb A, Mollapour M.. Oncotarget. 2022;13:173-81. Epub 20220120. doi: 10.18632/oncotarget.28176. PubMed PMID: 35070081; PMCID: PMC8780807.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Within the remaining time of NCE we plan:

1) To identify the mRNAs regulated by FLCN, as described in our proposal (Aim 2, subtasks 4 and 5).
2) To complete testing the effect of FLCN secondary modifications in the ability of FLCN to suppress tumor formation in the xenogrtaft tumor suppressor assay (Aim 3, subtask 3).

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We are dissecting the function of FLCN tumor suppressor gene. Our goal is to discover what are the critical biochemical events that are deregulated in cells when FLCN is inactivated. To this end we are linking genetic analysis of FLCN to the biochemical events that are regulated by this tumor suppressor gene, namely changes in the cellular phosphoproteome and in protein translation.

Our work so far provided important insights in the function of FLCN, that clearly influence the field.

The main achievement is that we showed that FLCN targets specific proteins (Rabs) and through them regulates the ACTIVITY of MANY transmembrane receptors that sense growth factors and extracellular nutrients. FLCN tempers the response of the cells to the environment. It provides a "break" for the growth signals to the cells by attenuating how the cells sense the "grow" extracellular signal. FLCN-negative cells "over-respond" to a given "grow" signal and turn malignant.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Our work highlighted the role of Rab7 as a regulator of growth factor and nutrient sensing transmembrane receptors. We therefore influenced the field and the work others.
As an example, colleagues in our immediate environment (Andi McClathey Lab) expanded this observation to their own work. (Chiasson-MacKenzie C, Morris ZS, Liu CH, Bradford WB, Koorman T, McClatchey AI. Merlin/ERM proteins regulate growth factor-induced macropinocytosis and receptor recycling by organizing the plasma membrane-cytoskeleton interface. Genes Dev. 32(17-18): 1201-14, 2018)

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*

- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As it is well known this has been a year of hardship for all. Our laboratory was closed for 4 months due to COVID pandemic. The PI was recruited to provide clinical care of COVID patients. In addition he was infected by COVID19 and had a prolonged course of illness. These issues were resolved and we therefore hope to move forward without any further obstacles. We recently recruited a senior post doctoral fellow (Dr. Min) to complete the remaining experiments described in the FLCN proposal.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

None expected

Significant changes in use or care of vertebrate animals

None expected

Significant changes in use of biohazards and/or select agents

None expected

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1) **HUMAN FOLLICULIN TUMOR SUPPRESSOR PROTEIN BINDS TO TRANSLATION INITIATION FACTORS eIF2A AND EIF5B AND SUPPRESSES PROTEIN SYNTHESIS**
(Meike Schneider, Katja Dinkelbor, Syed I.A. Bukhari, Samuel S Truesdell, Vera A. Pisareva, Andrey V. Pisarev, Shobha Vasudevan and Othon Iliopoulos). *UNDER REVIEW*

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Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

MYROVLITIS TRUST
https://myrovlytistrust.org/conference-report-from-the-7th-international-bhd-symposium-october-2021/
This is a Birt-Hogg-Dube (BHD) patient advocate organization. They update the list of basic scientific and clinical publications in the field of FLCN and BHD disease.
Our work was presented in the 7th International BHD Conference (2021) and included in the conference paper.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Othon Iliopoulos
PD/PI
1.8 CM

Tupa Basuroy
Post Doc
12 CM

Wilhelm Haas
Co-Investigator
1.2 CM

Shobha Vasudevan
Co-Investigator
0.6 CM

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

HUMAN FOLLICULIN TUMOR SUPPRESSOR PROTEIN BINDS TO TRANSLATION INITIATION FACTORS eIF2γ AND EIF5B AND SUPPRESSES PROTEIN SYNTHESIS

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ABSTRACT

Patients with Birt-Hogg-Dube (BHD) disease develop renal cancers, skin fibrofolliculomas and spontaneous pneumothoraces. The disease is caused by a germline mutation in the tumor suppressor gene Folliculin (*FLCN*). Many tumor associated *FLCN* mutations lead to message instability and/or deletion of the C-terminus FLCN domain. FLCN may act as a GTPase activating protein but the spectrum of its biological functions remains elusive. Here we report that FLCN localizes to the polysomes, associates with factors regulating the initiation of protein translation and inhibits eIF2γ and EIF5B complex formation with tRNA-Met_i. The FLCN C-terminal domain, which is deleted by tumor-associated FLCN mutations, is necessary for the interaction of FLCN with eIF2γ and EIF5B. Reintroduction of wild type but not a C-terminus FLCN mutant into FLCN-/- cells results in suppression of serum or amino acid stimulated protein translation, independently of mTORC1 activity. Our data provide insights into a novel mechanism of cell growth restriction by FLCN.

INTRODUCTION

Individuals with Birt-Hogg-Dube (BHD) disease have a high risk of developing multiple renal cell carcinomas (RCC), benign skin lesions called fibrofolliculomas, and lung cysts throughout their lifetime(1-3). BHD disease is caused by germline mutations in the folliculin (*FLCN*) gene. *FLCN* encodes an evolutionarily conserved 64kDa protein that is ubiquitously expressed in adult and embryonic tissues and is localized to both the nucleus and the cytoplasm(4-8). Tumors harbor a mutational inactivation of the second, wild type allele, indicating that *FLCN* is a typical tumor suppressor gene. Somatic inactivation of *FLCN* is also observed in sporadic renal cell carcinomas (9, 10).

Most germline and somatic mutations result in a truncated form of the FLCN protein lacking the C-terminus(2, 11), indicating that the C-terminus domain is required for tumor suppression. This C-terminus of FLCN (amino acids 341-579) has a crystal structure resembling a DENN domain (12). DENN domain proteins function as Guanine Nucleotide Exchange Factors (GEFs) that activate Rab GTPases by mediating the exchange of GDP for GTP (13). In addition, the FLCN C-terminal domain associates with folliculin interacting proteins 1 and 2 (FNIP1 and FNIP2) (7, 14).

Consistent with the crystal structure resolution of the C-terminus domain, the FLCN-FNIP complex was shown to interact with Rag GTPases at the lysosome(15, 16) and possess GTPase Activating Protein (GAP) activity for Rag C/D and GEF activity for RagA (15, 16). In these studies, FLCN was required for the recruitment and activation of mTORC1 in response to amino acids. The model proposed by these studies predicts that

loss-of-FLCN function would lead to suppression of mTORC1 function; such a model presents a conundrum with regards to the role of FLCN as a tumor suppressor. In vivo experiments provided conflicting results regarding FLCN's direct role on mTORC1 activation(7, 15-19).

To gain insight into the cellular functions of FLCN that it are linked to its tumor suppressor function, we affinity purified FLCN containing protein complexes and showed that wild type FLCN, but not tumor-associated mutants, interacts with and acts as a GAP protein for the small GTPase Rab7A. FLCN promotes Rab7A-mediated endocytosis and lysosomal inactivation of Epidermal Growth Factor receptor (EGFR), leading to suppression of TORC1 signaling in response to EGF (20). $FLCN^{-/-}$ tumors display enhanced EGFR activity and afatinib, an EGFR inhibitor, suppressed the growth of human $FLCN^{-/-}$ cells as xenografts in nude mice(20). Our work highlighted a function of FLCN, which is compatible with its role as tumor suppressor, whereby FLCN attenuates the activation of TORC1 by growth factors(20).

Here we analyzed additional FLCN containing protein complexes and we report that wild type FLCN binds to endogenous eukaryotic translation initiation factors eIF2γ and EIF5B and suppresses protein translation in asynchronously growing cells.

EIF2γ is one of the subunits of the eIF2 hetero-trimetic protein complex; the latter is essential for protein synthesis. The eIF2 complex binds to Met-tRNAiMet and recruits it to 40S ribosomal subunit to form the 43S preinitiation complex for translation (21). EIF5B stimulates recruitment of 60S into the preinitiation complex to form the 80S

ribosomal subunit and this step completes translation initiation (22).

Deregulation of translational control has been strongly implicated in tumorigenesis (23). "Driver" oncogenic mutations promote cellular transformation by regulating cap-dependent and cap-independent translation. Copy number changes of several subunits of the translational machinery have been detected in human malignancies (23). Experimentally, overexpression of eIF4E or eIF6 proved sufficient to transform immortalized human fibroblasts (24, 25).

Binding to eIF2γ and EIF5B appears necessary for the ability of FLCN to suppress protein synthesis, since the tumor-associated FLCN C9 mutant, which lacks the C-terminus of the protein, fails to bind to the initiation factors and consistently, lacks the ability to suppress protein synthesis. Lastly, we show here that FLCN restricts the increase in cellular protein synthesis as a response to the stimulation of mammalian cells with serum and amino acids, in a TORC1-independent manner. These findings complement our previous work and show how FLCN can attenuate the cellular growth response to growth factors and amino acids, providing additional insights into the mechanisms of tumor suppression by FLCN.

RESULTS

FLCN interacts with eIF2γ and EIF5B

To gain further insight into the mechanisms of tumor suppression by FLCN we sought to identify cellular proteins that stably interact with FLCN in mammalian cells. To this end

we used FLCN-/- UOK257 human RCC cells, stably transfected with plasmids expressing wild type FLAG-FLCN or empty vector as control. To confirm that the tagging of FLCN in the N-terminus does not interfere with the tumor suppressor function of the protein and therefore is not likely to disrupt the formation of protein complexes necessary for this function, we compared the ability of untagged and FLAG-tagged versions of FLCN to suppress the growth of FLCN-/- FTC133 cells that were xenografted in immunocompromised mice. We confirmed that N-terminally tagged FLCN suppresses the growth of FTC133 cells as xenografts in nude mice (Supplemental Figure 1). Furthermore, to increase the depth of protein complex identification we fractionated the cells into nuclear, cytoplasmic and membrane fractions and isolated FLCN containing complexes by anti-FLAG (M2) affinity purification. Several peptides (Table 1) indicated that FLCN is involved in protein translation. We decided to focus on the putative interaction between FLCN and the translation initiation factors EIF5B and EIF2G.

Mass spectrometry analysis of FLCN containing complexes indicated that FLCN binds to EIF5B and EIF2G, two factors involved in initiation of protein translation in mammalian cells. To confirm the mass spectrometry data, we immunoprecipitated endogenous FLCN from the lysates of 293T cells. Western blot analysis of the immunoprecipitated proteins confirmed that EIF5B was present in FLCN containing complexes (Figure 1A). We then investigated whether the presence of FLCN is required for the association of EIF5B with FNIP1 containing complexes and vice versa. FNIP1 co-immunoprecipitated with endogenous EIF5B in the presence or absence of FLCN (Figure 1B). In addition, EIF5B co-immunoprecipitated with FLCN from the lysates of U2OS in which FNIP1 expression

was significantly reduced by FNIP1-targeting shRNA (Figure 1C). Therefore, we concluded that FLCN or FNIP1 have the ability to associate with EIF5B independently of each other. To confirm that eIF2γ interacts with FLCN/FNIP1 we transiently transfected 293T cells with plasmids expressing FLAG-FLCN, FLAG-FNIP1 and HA- eIF2γ and showed that EIF2G co-immunoprecipitates specifically with exogenous FLCN and FNIP1 (Figure 1D). Using co-immunoprecipiation experiments we also showed that endogenous FLCN interacts with endogenous EIF2G (Figure 1E).

To identify the FLCN domain that interacts with eIF2γ we co-transfected into 293T cells FLAG-tagged FLCN mutants and HA-EIF2G. Wild type or mutant, FLAG tagged-FLCN was immunoprecipitated with M2 beads and the interaction with eIF2γ was detected with anti-HA Western Blot (Figure 1F). Despite differences in the expression levels of the mutant proteins the results indicate that FLCN 451-579 is sufficient for interaction with FLCN. In addition, we infected FLCN-/- UOK257 cells with plasmids expressing the FLCN WT protein or the tumor-associated FLCN 1-469 mutant and tested the interaction with endogenous EIF5B (Figure 1G). The results indicate that the C-terminus of FLCN (470-579) is necessary for its interaction with EIF5B.

FLCN localizes to polysomes

To show that FLCN co-localizes with the translation initiation factors EIF5B and EIF2G in the organelles of protein translation, we purified polysomes from 293T cells (Figure 2A) and the FLCN-/- and isogenic replete UOK257 cells (Figure 2B). FLCN and FNIP1 were present in the early polysome fractions, correlating with the presence of EIF5B and

eIF2γ. The polysome profile indicates that translation in FLCN replete cells was decreased compared to FLCN-/- cells.

FLCN suppresses protein synthesis

The presence of FLCN/FNIP1 in the polysomes and its interaction with EIF5B and EIF2G raises the hypothesis that FLCN/FNIP1 may interfere with protein translation. To directly test the effect of FLCN on protein translation we compared the fraction of newly synthesized proteins in FLCN-/- FTC-133 or UOK257 cells and their isogenic replete counterpart cell lines (Figure 3A and B). Cells were starved from methionine for an hour, followed by addition of L-Azidohomoalanine in the medium for the indicated time (Figure 3A,B), followed by cell lysis. Extracted proteins were resolved in SDS-PAGE and the newly synthesized fraction detected with streptavidin bound horseradish peroxidase. Re-introduction of wild type FLCN into the FLCN-/- parental cell lines (Figure 3A for FTC-133, Figure 3B for UOK257) significantly suppressed protein synthesis. Because FLCN is implicated in mTORC1 signaling and TORC1 regulates protein synthesis by phosphorylation of S6K and EIF4E-BP, we examined whether the effect of FLCN on protein synthesis is mTORC1-dependent. To this end we detected newly synthesized proteins in FLCN-/- UOK257 and isogenic replete cells in the presence or absence of the dual mTORC1/2 inhibitor AZD5088 (26). Protein synthesis was higher in the FLCN-/- cells, compared to their isogenic replete counterparts even in the presence of the inhibitor (Figure 3C).

Because the C-terminus of FLCN appeared necessary and sufficient for the interaction of FLCN with EIF5B and eIF2γ we tested whether the tumor-associated FLCN C9 mutant was able to suppress protein synthesis after stimulation with serum or amino acids. Since the C-terminus is necessary for the FLCN-EIF5B interaction, the FLCN C9 mutant does not interact with EIF5B (Figure 4A). In keeping with the hypothesis that the FLCN-EIF5B/eIF2γ interaction is required for protein synthesis regulation by FLCN, the C9 mutant does not repress protein synthesis after either serum or amino acid stimulation (Figure 4B).

We hypothesized that FLCN down regulates the activity of eIF2 complex and inhibits its interaction with the initiator tRNA, methionyl tRNA (tRNA-Met_i). To support this hypothesis we immuno-precipitated eIF2β, the component of the eIF2 complex that binds tRNA-Met_i along with eIF2γ from FLCN-null cells or cells FLCN-null cells which were replete with wild type FLCN or the tumor associated FLCN-C9 mutant (Figure 4D-E) and quantified the tRNA-Met_i bound to eIF2 complex by QRT-PCR. As shown in Figure 4D introduction of wild type FLCN but not the FLCN C9 mutant suppressed the binding of tRNA-Met_i to eIF2, consistent with the functional data showing that the presence of FLCN suppresses protein synthesis independently of TORC1.

DISCUSSION

FLCN is a tumor suppressor protein mutated in the germline of BHD patients. Loss-of-FLCN function leads to RCC of various histologies, including chromophobe, oncocytoma, clear cell, and papillary as well as hybrid forms of the above histologies. FLCN inactivation is also encountered in the sporadic forms of these RCC. Lastly, patients with germline FLCN mutations may develop spontaneous pneumothoraxes and non-malignant skin lesions, such as fibrofolliculomas and trichodiscomas. Resolution of the crystal structure of the C-terminus of FLCN revealed that FLCN resembled DENN domain proteins and therefore indicated that FLCN may be implicated in the regulation of small GTPase proteins. FLCN has been shown to act as a GTPase activating protein for the small GTPases RagA, RagD/C and Rab7A [15, 16, 20]. In addition to its GAP/GEF function FLCN was reported to interact through its C-terminal domain with the substrate of the small GTPase Rab34 and promote Rab34-RILP interaction under amino acid starvation, leading to perinuclear accumulation of lysosomes (27). Facilitation of Rab34-RILP interaction by FLCN appears, at least under in vitro experimental conditions, to be independent of FLCN's GEF or GAP activity, raising the possibility that FLCN C-terminus may also participate in catalytically independent functions(27).

Here we report a novel function of FLCN, whereby FLCN is found in complexes containing the protein translation initiation factors of the EIF2 family (EIF2γ) and EIF5B. EIF2G/eIF2γ/EIF2S3 is an essential component of eIF2 that also includes eIF2α, which is the regulatory arm of the complex, and eIF2β. EIF2γ enables eIF2 GTPase activity and in

association with eIF2β, binds and recruits initiator tRNA-Met_i (21, 28, 29). EIF5B catalyzes the final step of initiation to bring in 60S subunits and it has been implicated in stabilizing tRNA-Met recruitment and in 40S subunit proofing (22). Interaction with these critical translation factors, therefore can affect initiation of translation, as observed previously (30, 31). The C-terminus of FLCN, which contains the DENN-like domain and is targeted in the majority of tumor-associated mutations, is necessary and sufficient for this interaction to occur. Lastly, we show that this interactions is not a mere biochemical observation, but has biological consequences; wild type FLCN, but not a prevalent tumor-associated mutation missing the C-terminus (mutant C9), regulates new protein synthesis in response to amino acid and serum stimulation.

Protein translation is an active and dynamic process, targeted by many oncogenic processes (i.e., PI3K-AKT-mTORC1 activation, c-myc amplification) to sustain tumor growth. It is becoming clear that deregulation of translational control is a common mechanism by which diverse oncogenic pathways promote cellular transformation and tumor growth and that coordination of translation with growth is critical for tumor maintenance (23). Classic experimental studies strongly indicate that changes in protein translation can be necessary and even sufficient to promote tumor growth. For example, overexpression eIF4E or eIF6 was suficiente for transformation of immortalized human fibroblasts (24, 25). In addition, expression of a dominant negative form of eIF4E in the prostate of Pten-/- mice led to decreased incidence of prostate intraepithelial neoplasia (32). Our work shows that human FLCN exerts its tumor suppressor role, at least in part, by regulating non-canonical (non-cap dependent) protein translation. Whether this FLCN

function regulates global changes in protein translation or, more likely, the translation of specific mRNAs is an important question for FLCN biology and the subject of current investigation.

Human FLCN forms complexes with FNIP1 and FNIP2, and both proteins were shown to interact with RagC/D. In addition, the C-terminus of FLCN, which is deleted in the overwhelming majority of the tumor-associated FLCN mutants, is required for the ability of FLCN to enter in complexes containing FNIP1(7). The data we present here show that the FLCN C-terminus is also required for FLCN's interaction with EIF2G and EIF5B. The stoichiometry of interactions between FLCN and EIF2γ or EIF5B, or for this matter other GTPases regulated by FLCN such as RagC/D, is not currently clear and is under investigation. In this report we provide evidence that EIF2γ and EIF5B GTPases can form complexes with FNIP1 in the absence of FLCN and vice versa. In addition, we show that FLCN and FNIP1 have the ability to localize to the polysomes, where mRNA translation occurs, independently of each other. Nevertheless, it is not clear if the interaction between FLCN and FNIP1 is required for the putative GAP/GEF activity of the complex or which exchange state (GDP or GTP bound) is mostly affected by the absence of FLCN or FNIP1.

We showed that the function of FLCN is unmasked when starved cells are stimulated with serum or amino acids. Despite the fact that FLCN has been implicated in mTORC1 regulation, the repression of protein synthesis by FLCN does not appear to be due to changes in mTORC1 activity. Phosphorylation of S6K, which serves as one reporter of

TORC1 activity, is not significantly altered by the presence of FLCN in our experiments. This supports the notion that FLCN regulates protein synthesis after serum and amino acid stimulation, at least in part, at the level of EIF2γ/EIF5B activity, in a cap-independent, IRES-type initiation of translation. We are currently investigating whether serum-induced changes in FLCN-EIF2γ/EIF5B interactions can be attributed to post-translational modifications of either partner.

The majority of FLCN mutations result in frame shift and deletion of the C-terminus of FLCN and/or significant destabilization of the mRNA message (3, 11, 33). Nevertheless, point mutants within or out of the C-terminal domain exist (33). The precise role of these mutations in relation to FLCN localization, message or protein stability as well as their effect on functional interaction(s) between FLCN-FNIP-GTPases are under investigation. Although the genotype-phenotype correlation between FLCN mutations and the resulting clinical manifestations of BHD disease is unclear, a complete biochemical understanding of the effect of stable FLCN mutants on FLCN function may help uncovering currently unrecognized genotype-phenotype correlations.

MATERIALS AND METHODS

Cell lines and cell culture

The UOK257 renal carcinoma cell line (a generous gift from Drs. Marston Linehan and Laura Schmidt, NCI/NIH) was originally derived from the clear cell renal tumor of a BHD patient(34). The UOK257 and FTC133 cells were infected with retroviruses encoding for the pBABE-puro vector, FLCN WT, Flag-FLCN WT, or the FLCN tumor-associated mutants(35). HEK293T and U2OS cells were purchased from ATCC. The UOK257, HEK293T and U20S cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and the FTC-133 cells in DMEM/Nutrient mixture F-12 (F12), both supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine (Invitrogen, Carlsbad, CA). Mycoplasma testing was performed regularly to ensure that the cells were mycoplasma negative. Infected cells were selected in 2 mg/ml (UOK257) or 3 mg/ml (FTC-133) of puromycin.

Cell fractionation and protein purification

We fractionated UOK257 vector only and UOK257 Flag-FLCN WT replete cells into nuclear, cytoplasmic, and membrane fractions. Briefly, cell pellets were washed twice in RBS buffer (10 mM HEPES, 10 mM NaCl, 1.5 mM $MgCl_2$) containing protease and phosphatase inhibitors. Pellets were resuspended in RBS buffer on ice for 10 min and then lysed with a Dounce homogenizer. When approximately 95% of the cells were disrupted, the nuclei were pelleted by centrifuging at 380 x g for 10 min. The membrane fraction was separated from cytosol by centrifugation at 150,000 x g for 1.5 hours. Nuclear and membrane fractions were washed with RBS twice and proteins were

extracted in EBC buffer (50 mM Tris pH 8, 120 mM NaCl, 1% Nonidet P-40) containing protease and phosphatase inhibitors. Flag-FLCN protein complexes were isolated using anti-flag M2-agarose beads (Sigma-Aldrich, St. Louis, MO). The beads were washed with NET-N buffer (100 mM NaCl, 20 mM Tris-HCl pH 8, 1 mM EDTA, 0.5% NP-40) and the protein complexes eluted in 80 mM glycine pH 2.5 + 2.5% SDS.

Mass spectrometry

Protein complexes were run in a SDS-PAGE gel and subjected to a modified in-gel trypsin digestion procedure, as previously described. Briefly, proteins in the gel were digested with 12.5 ng/µl modified sequencing-grade trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate at 37ºC overnight. Peptides were eluted in a nano-scale reverse-phase HPLC capillary column and subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). MS2 spectra were assigned using a SEQUEST proteomics analysis platform(36). All databases include a reversed version of all the sequences. Based on the target-decoy database search strategy(37) and employing linear discriminant analysis and posterior error histogram sorting, peptide and protein assignments were filtered to false discovery rate (FDR) of < 1 %(38).

Immunoprecipitations

U2OS, 293T and UOK257 cells were washed twice with cold PBS, centrifuged at 100xg for 10 min and lysed in lysis buffer containing 0.5% NP-40, 150mM NaCl, 40mM HEPES pH 7, 6mM MgCl, 2mM DTT and 10% Glycerol, on ice for 45 min with protease

and phosphatase inhibitors. Nuclei were removed by centrifugation at 400xg for 10 min. 1-2 mg of protein lysate was immunoprecipitated overnight with protein A magnetic Dynabeads (Invitrogen) coupled to the respective antibody according to the manufacturer's instructions. The beads were washed five times with wash buffer (0.5% NP-40, 150mM NaCl, 40mM Hepes pH 7, 6mM MgCl) and boiled for 5 min at 95°C in 1.5X protein loading buffer. The supernatant was analyzed by standard immunoblot techniques.

For co-immunoprecipitation experiments in U2OS cells with acute knockdown of FNIP1, U2OS cells were infected with FNIP1 shRNA (TGAGCTTATTCGACGAATATT) or PLKO empty vector using lentiviruses and Lipofectamine 2000 Reagent (Invitrogen, # 11668019). 48 hours after infection, cells were lysed and further processed as described above. For cotransfection experiments 293T cells were transiently transfected with DNA using Polyjet (SignaGen Laboratories, Rockville, MD) according to the manufacturer's instructions. Approximately 24-30 hours after transfection, cells were lysed in EBC lysis buffer containing proteinase and phosphatase inhibitors. Magnetic M2 beads (M8823, Sigma, St. Louis, MO) were used to immunoprecipitate FLAG-FLCN or FLAG-FNIP1. Immunoprecipitations were further processed as described above.

In vivo crosslinking coupled immunoprecipitation for tRNA association

Cells were harvested and cross-linked with 0.3% formaldehyde in culture media for 10 minutes at 37^0C as described previously(39). Cells were fractionated into nuclear and cytoplasmic fractions and the extracts from the two fractions were combined for pre-

clearing and immunoprecipitation. The combined fractions were pre-cleared with agarose beads and then clarified by low speed centrifugation to remove non-specific contaminants that bind agarose. Anti-eIF2β (Santa Cruz Biotech) was used for immunoprecipitation(40). The immunoprecipitates were subject to inactivation of the cross-links to separate RNA and protein for subsequent quantitative real time-PCR (qRT-PCR) and Western blot analyses(39).

RNA isolation and quantitative RT-PCR analysis

RNA was isolated from eIF2β cross-linked immunoprecipitation samples as described previously(41). Cross-linked immunoprecipitation samples were first subjected to inactivation of the cross-links, followed by RNA isolation. cDNA synthesis was performed using random hexamer primers and Superscript III enzyme (Invitrogen) as per the manufacturer's direction. qRT-PCR was performed using SYBR-green mix and Roche-480 light cycler with tRNA-Met_i primers as described earlier(40, 41).

Polysome analysis

HEK 293T and UOK257 cells were grown to 80% confluence. Cells were washed twice with cold PBS containing 100 μg/mL cycloheximide, pelleted and resuspended in 300μl polysome lysis buffer (5 mmol/L Tris pH 7.4, 2.4 mmol/L MgCl2, and 1.5 mmol/L KCl), freshly supplemented with 10 μg/mL cycloheximide, 2 μmol/L DTT, 0.5% Triton-X, and 0.5% sodium deoxycholate as well as protease and phosphatase inhibitors. Lysis buffer volume was adjusted for cell number. Lysates were cleared for 10 minutes at 15,000 × g at 4°C. Cleared lysates (270 μL) were loaded onto 12-mL 10% to 50% sucrose gradients

(prepared in 15 mmol/L Tris pH 7.4, 15 mmol/L MgCl2, and 150 mmol/L NaCl) and spun for 2 hours in a SW40Ti rotor (Beckman Coulter), at 40,000 rpm and 4°C. Immediately following centrifugation, 12 fractions were collected using a BioComp Gradient Master instrument. For Western blot analysis, 200 μL of each fraction were precipitated by methanol/chloroform extraction and loaded on a gel.

Western blots and antibodies

Proteins were detected by Western blotting, as previously described(42). Briefly, proteins were separated by SDS-PAGE electrophoresis, transferred to a PVDF membrane and detected with the cognate antibody. The following antibodies were used: anti-Pan Actin (MS1295P, Neomarkers, Fremont, CA); The anti-HA tag (C29F4, #3724), anti-FLAG tag (#F1804), anti-FLCN antibody (#3697), anti-pS6 S240/244 (#5364), anti-pS6 Ser235/236 (#4858), total S6 ribosomal protein (#2217), anti-EIF6 (#3833), Steptavidin-HRP (#3999) and IgG control (#3900) antibodies were from Cell Signaling Technology (Danvers, MA). Anti-EIF5B antibody was purchased from Bethyl (#A301-744A). FNIP1 antibodies were used from Abcam (#134969) and Origene (IQ351, for immunoprecipitations). Anti-EIF2G antibody was purchased from Proteintech (#11162-1-AP). Western blots were developed with the Bio-Rad ChemiDoc system and protein expression was quantified using BioRad Image Lab Software (Bio-Rad Laboratories, Hercules, CA).

Starvation and growth factor/amino acid treatment of cells

UOK257 FLCN-/- and replete cells were starved in either serum free (SF) DMEM media overnight (Invitrogen, Carlsbad, CA, #11995-065) or in amino acid depleted RPMI 1640

media for 2.5 hours (US Biological Life Sciences, Salem, MA # R9010-01), supplemented with 4% dialyzed FBS. The cells were then stimulated with 10% FBS for one hour (serum stimulation) or addition of 1X amino acids mixture (amino acid stimulation).

Translation initiation assay

Translation assays were performed following the supplier's instructions from Click-IT Metabolic Labeling Reagents for Proteins (Life Technologies). Asynchronous cells were incubated in the presence of DMEM without cysteine and methionine (Invitrogen Inc, MA, #21013-024) for 1 hour, then grown in the presence of 50 μmol/L AHA (l-azidohomoalanine; Life Technologies #C10102) for the indicated time points, washed five times with cold PBS and collected in PBS. Cells were lysed in 1% SDS in 50 mmol/L Tris pH 8.0 (including protease inhibitors) and sonicated using a bath sonicator (QSonica Q700) for 15 minutes. The Click-IT reactions were performed following the supplier's instructions from Click-IT Protein Reaction Buffer Kit (Life Technologies). To assess the effect of FLCN on protein synthesis under serum stimulation, we serum starved FLCN -/- and replete UOK257 cells overnight in DMEM (#11995-065). Following overnight starvation the cells were additionally starved from methionine for 1 hour and then methionine labeled under 10% dialyzed FBS (dFBS) stimulation. To assess the effect of FLCN on protein synthesis under amino acid stimulation, cells were starved in amino acid free RPMI containing 4% dFBS, for 2 hours. Cells were then labeled with methionine as described for asynchronous cells. For all the labeling and stimulation experiments, approximately 100 μg of lysates were used per reaction with 40 μmol/L

Alkyne-Biotin (Life Technologies; #B10185), and 20 μg were assayed by Western blot analysis using a Streptavidin antibody conjugated to horseradish peroxidase (HRP). AZD5088 was purchased from Selleckchem and treatment of the cells was done for a total of 3 hours prior to cell lysis.

Data analysis and statistics

Statistical comparisons were done with a two-tail Student's T-test, unless otherwise indicated. All error bars represent SEM.

Supplemental information

Plasmids

The plasmids and oligonucleotides used to generate FLCN wild type (WT) and FLCN 1-469 mutant retroviruses were previously described(35). To create FLAG tagged-FLCN WT, we used the FLCN WT template and oligonucleotides 5'-GCGCGGATCCGCCACCATGGATTACAAAGATGATGATGATAAAAATGCCATC GTGGCTCTCTG-3' (forward) and 5'-GCGCGAATTCAGTTCCGAGACTCCGAGGC TGTG-3' (reverse). The PCR product was restricted with BamHI and EcoRI and ligated into the pBABE-puromycin plasmid.

FLAG-tagged FNIP1 was created by PCR of WT FNIP1 with oligonucleotides (5'-GGAATTCGCTAGCGCCACCATGGACTACAAAGACGATGACGACAAGGCCCCT ACGCTGTTCCAG-3' (forward) and 5'-CCGTACTCGAGTTAAAGGAGTATTTGTG

CAACATATGGAG-3’ (reverse), restricted with NheI and XhoI and ligated into pcDNA3.1 plasmid. The EIF2G plasmid was obtained from Dharmacon (Clone ID 4419438; pCMV-SPORT6 backbone). To clone HA-tagged EIF2G into pcDNA3.1 the following oligonucleotides were used: 5’-GGAATTCGCTAGCGCCACCATGTAC CCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTGC GGGCGGAGAAGCTG (forward) and 5’-CCGTACTCGAGTCAGTCATCATCTACTG TTGGCTTGATT-3’ (reverse). The PCR product was restricted with NheI and XhoI and ligated into pcDNA3.1.

For the truncated FLCN mutants a FLAG tag was first added into the pcDNA3.1 backbone. Oligos 5’-AGCTCGCCACCATGGATTACAAAGATGATGATGATAAAG-3’ (forward) and 5’-GATCCTTTATCATCATCATCTTTGTAATCCATGGTGGCG-3’ (reverse) were annealed, kinased and ligated into pcDNA3.1 which was restricted with BamH1 and EcoR1. Truncated FLCN proteins were amplified by PCR from FLCN WT pBABE backbone as described above. For C-terminal truncated mutant FLCN 1-300 the following primers were used: 5’-GCGCGGATCCAATGCCATCGTGGCTCTCTG-3’ (forward) and 5’-GCGC GAATTCGTCCCAGCTTTCTGATTC-3’ (reverse). Similarly N-terminally truncated mutants (FLCN 301-579 and FLCN 451-579) were created with respective forward oligos (5’-GCGCGGATCCAACTCTGAGGCTGAAGAG-3’ and 5’-GCGC GGATCCCCTGTGGGGTGTGAGGATG-3’) using the same reverse primer (3’-GCGC GAATTCAGTTCCGAGACTCCGAGGCTGTG-5’). PCR products of the truncated BHD proteins were ligated with EcoR1 and BamH1 into the pcDNA3.1 backbone. To create the FLCN C9 mutant we used the following oligos: 5’-

GCCCGCACGTGCAGATCCCCCCCCCACGTGCTCTCCTCAGAG-3' (forward) and 5'-CGGGCGTGCACGTCTAGGGGGGGGGTCGACGAGAGGAGTCTC-3' (reverse).

In vivo tumor growth assay

10E6 FLCN, or Flag-FLCN cells were injected subcutaneously in the right flank of female nude/nude mice (n=4). 10E6 cells infected with the empty vector control were injected as a control in the left flank in every mouse. The growth of the s. c. tumors was followed by means of caliper measurements and volume calculation.

$V=(Width^2*Length)/2$.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

FIGURE 1: FLCN and FNIP1 form complexes containing EIF5B and EIF2G.

(A) *EIF5B is present in complexes containing endogenous FLCN and FNIP1*: Endogenous FLCN or FNIP1 were immunoprecipitated (IP) from HEK293T cell lysates as indicated. IgG antibody from non-immunized animals was used as control. The lysates were resolved in SDS-PAGE gels and proteins detected by Western Blot (WB) as indicated. **(B)** *EIF5B co-immunoprecipitates with FNIP1 independently of FLCN*: *FLCN*-/- UOK257 human renal cell carcinoma cells were stably infected with FLCN-expressing plasmids or empty vector control. Endogenous EIF5B was immunoprecipitated from cell lysates, the IPs resolved in SDS-PAGE gel and the presence of FNIP1 and/or FLCN detected by WB. FNIP1 co-immunoprecipitated with EIF5B in FLCN-/- and FLCN replete cells. **(C)** *EIF5B co-immunoprecipitates with FLCN in FNIP1 depleted cells*: FLCN or FNIP1 were immunoprecipitated from U2OS cell lysates infected with lentiviruses encoding FNIP1 shRNA or empty vector control. Cells were lysed 48 hours (48h) after infection. EIF5B co-immunoprecipitates with FLCN independently of FNIP1 expression. (**D)** *FLCN and FNIP1 interact with eIF2γ*: 293T cells were transiently transfected with plasmids encoding FLAG-FLCN, FLAG-FNIP1, HA- *eIF2γ* or empty vector as control. Approximately 24h post transfection cells were lysed and the lysates immunoprecipitated with anti-FLAG antibody. Immunoprecipitated proteins were resolved in SDS-PAGE and detected by Western Blot with anti-FLAG or anti-HA antibodies. **(E)** *Endogenous FLCN interacts with eIF2γ*. Endogenous FLCN is immunoprecipitated (IP) from HEK293T cell lysates and the protein complexes resolved

in SDS-PAGE gels. Proteins detected by Western Blot (WB) as indicated. **(F)** *The tumor-associated FLCN 1-496 mutant does not bind EIF5B:* Lysates of FLCN-/- UOK257 and isogenic replete cells, stably infected with vectors expressing WT FLCN, FLCN 1-469 or empty vector as control, were used to immunoprecipitate FLCN or FNIP1. Protein complexes were resolved in SDS-PAGE and the interaction of FLCN and/or FNIP1 with EIF5B was detected by WB, as indicated. **(G)** *FLCN interacts with EIF2G through its C-terminal domain*: 293T cells were transiently transfected with plasmids expressing FLAG-tagged wild type (WT) FLCN, FLCN (amino acids) 1-300, FLCN 301-579, FLCN 451-579 and HA-EIF2G, as indicated. WT or mutant FLCN was immunoprecipitated with anti-FLAG antibodies and the complexes resolved in SDS-PAGE gels. The presence of EIF2G was detected by WB using anti-HA antibodies.

FIGURE 2: FLCN and FNIP1 localize to early polysome fractions.

(A) *Localization of endogenous FLCN to polysomes:* HEK293T cells were fractionated on a 10-50% sucrose gradient and absorbance was measured at 254nm. Proteins were extracted from polysome fractions and FLCN, FNIP1 as well as initiation factors EIF5B, EIF2G and EIF6 were detected by WB, as indicated. Detection of phosphoS6 Kinase (anti-pSer235/Ser236 antibody) served as a positive control for the polysome fractions. FLCN and FNIP1 localize predominantly in the early polysome fractions, in a pattern similar to the localization of EIF5B and EIF2G. **(B)** *Localization of reconstituted FLCN to polysomes:* FLCN-/- UOK257 cells were stably infected with wild type FLCN or an empty vector control. We analyzed the polysome localization of wild type FLCN, FNIP1, EIF5B and EIF2G by Western Blot. Similar to the endogenous FLCN, and as in the case

of 293T cells, exogenously introduced FLCN, FNIP1, EIF5B and EIF2G localize predominantly to early polysome fractions.

FIGURE 3: FLCN inhibits nascent protein synthesis in an mTOR independent way. (A, B) *Reintroduction of FLCN in FLCN-/- cells inhibits translation initiation***:** *FLCN*-/- FTC133 (A) or UOK257 (B) cells were stably infected with retroviruses expressing FLCN or empty vector as control. Cells were starved of methionine and cysteine for 1 hour and then grown in the presence of labeled methionine (L-Azidohomoalanine) for the indicated times. Newly synthesized proteins were labeled with Biotin and detected by Western Blot using Streptavidin-HRP. Quantification of newly synthesized proteins (NSP) in the FLCN replete cells (red columns) was normalized to FLCN-/- control cells labeled for the same period of time (gray columns). Figure 1A represents one characteristic experiment **(C)** *FLCN inhibits translation independently of mTOR:* FLCN-/- UOK257 and their isogenic FLCN replete cells were treated with 500nmol AZD5088 (mTOR1/2 inhibitor) for a total of 3 hours. Cells were also starved of methionine and cysteine for an hour, followed by labeling of NSP. Starvation and re-stimulation media were supplemented with mTORC1/2 inhibitor.

FIGURE 4: Wild type FLCN but not a tumor-associated mutant represses protein synthesis following stimulation with serum or amino acids. (A) *The tumor associated FLCN C9 mutant does not interact with EIF5B.* FLAG-tagged wild type FLCN or the prevalent tumor associated FLCN C9 mutant were immunoprecipitated from FLCN -/- UOK257 cells stably infected with plasmids expressing the corresponding proteins.

Protein complexes were analyzed by WB as indicated. **(B)** *Wild type FLCN but not the FLCN C9 mutant represses serum or amino acid stimulated protein synthesis*. FLCN-/- UOK257 cells were stably infected with wild type FLAG-FLCN or the tumor associated FLAG-FLCN C9 mutant. Cells were serum or amino acid starved, and stimulated with ether 10% FBS or amino acids correspondingly. WB analysis was used to detect newly synthesized proteins (NSP), as described in Figure 3. **(C)** Quantification of three independent experiments. Values of FLCN wild type (WT) or C9 mutant (C9) were normalized to vector only transfected FLCN -/- cells (vector). **(D)** *FLCN suppresses the formation of complexes between EIF2B and* tRNA-Met_i. Complexes containing tRNA-Met_i bound to EIF2B were immunoprecipitated from FLCN -/- cells (FLCN -/-) and isogenic cells replete with FLCN wild type (FLCN WT) or FLCN C9 mutant (FLCN C9) and the tRNA-Met_i was quantified with qRT-PCR. Quantitation of tRNA-Met_i co-immunoprecipitated with eIF2β in FLCN-/-, FLCN WT, and FLCN C9 mutant expressing cells, compared to that immunoprecipitated with control IgG after normalizing to Input. tRNA-Met_i shows decreased association or immunoprecipitation with eIF2β in FLCN WT expressing cells compared to FLCN-/- cells, consistent with decreased translation in FLCN WT cells. FLCN C9 mutant expressing cells do not show decreased association/immunoprecipitation with eIF2β, consistent with the inability of the C9 mutant to repress translation. (The graph shown in the figure is the representative from 3 biological replicates, Error bars indicate SEM). **(E)** Detection of eIF2β in immunoprecipitates from the cell lines used in the Figure 4D. Western blot showing eIF2β from cross-linked immunoprecipitation samples from FLCN-/-, FLCN WT, and FLCN C9 mutant expressing cells.

SUPPLEMENTAL FIGURES

Supplemental Figure 1: N-terminally FLAG-tagged FLCN suppresses tumor formation. **(A)** *FLCN*-/- FTC133 cells were stably reconstituted with untagged or FLAG-tagged WT FLCN. We confirmed protein expression by WB, using an anti-FLCN antibody **(B)** *FLCN*-/- FTC133 cells, stably infected with FLAG-tagged and non-tagged WT FLCN were injected subcutaneously in immunocompromised mice. The final size of tumor xenografts generated by each cell type is presented.

Supplemental Table 1: Number of specific peptides detected by mass spectrometry of protein complexes isolated by anti-FLAG affinity purification.

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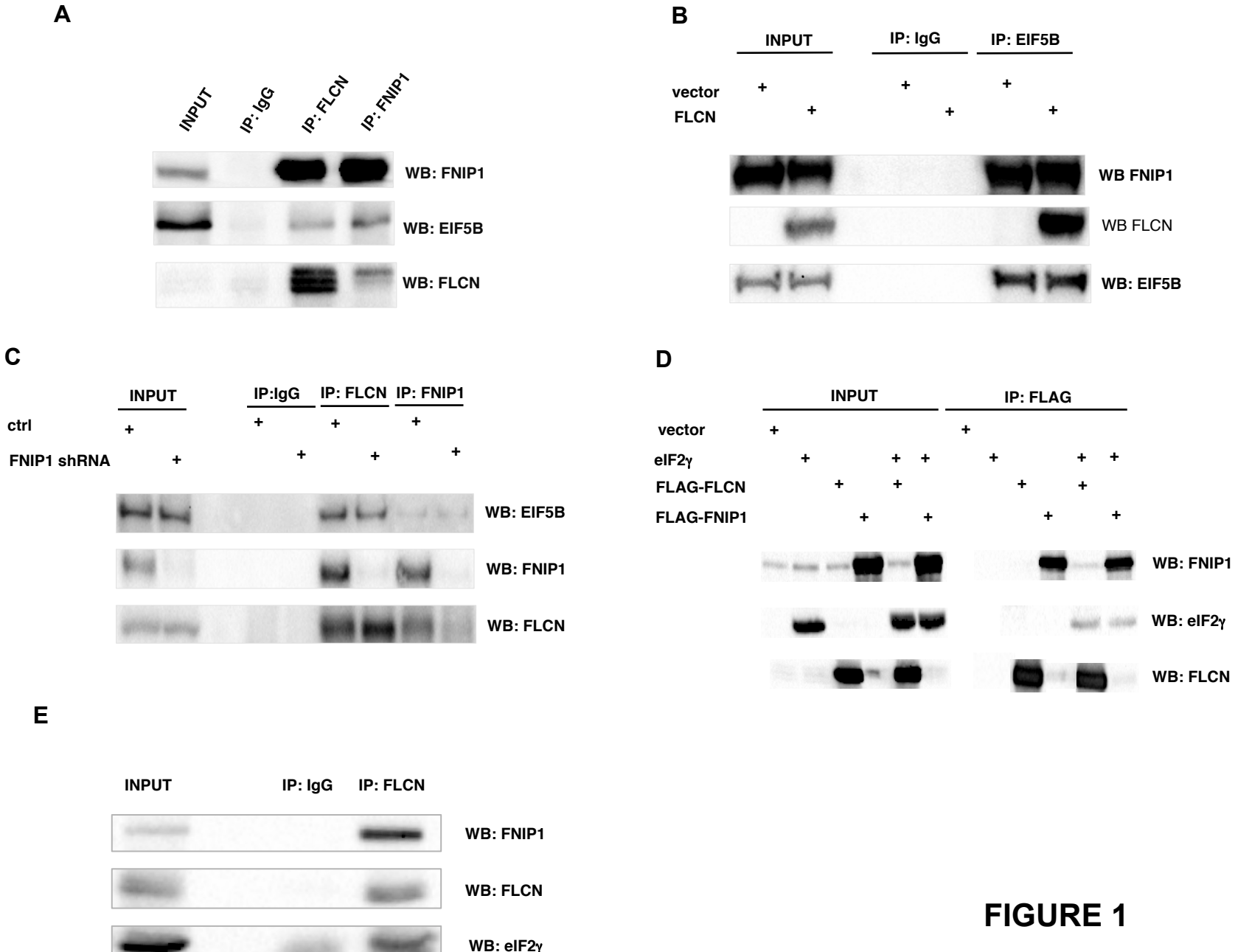
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vector
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WB FNIP1
WB FLCN
WB: EIF5B
C
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ctrl
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WB: FLCN
D
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eIF2γ
FLAG-FLCN
FLAG-FNIP1
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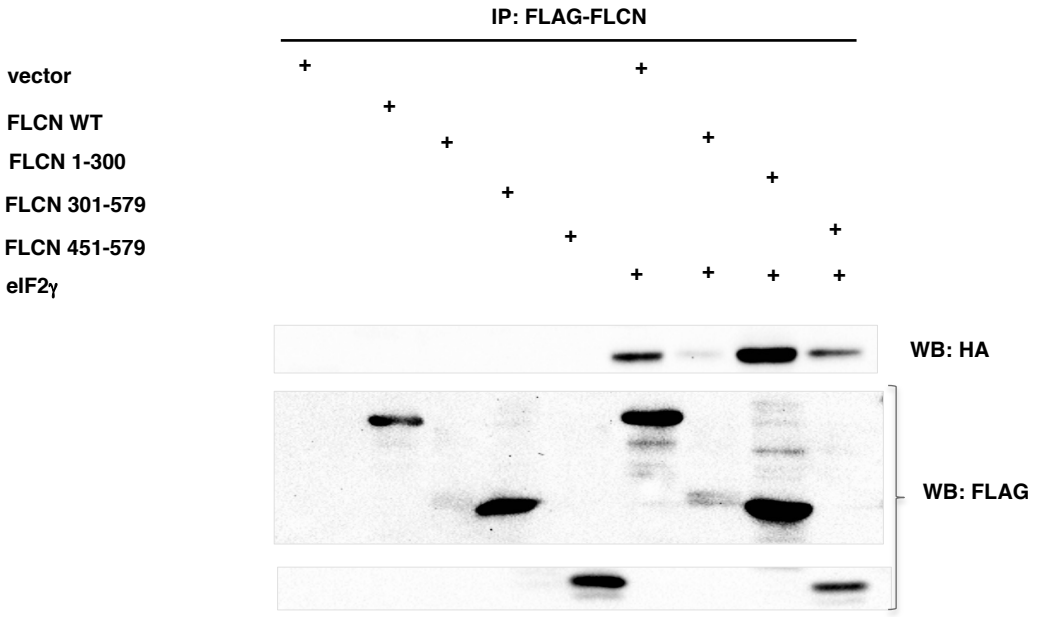
FIGURE 1

F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G

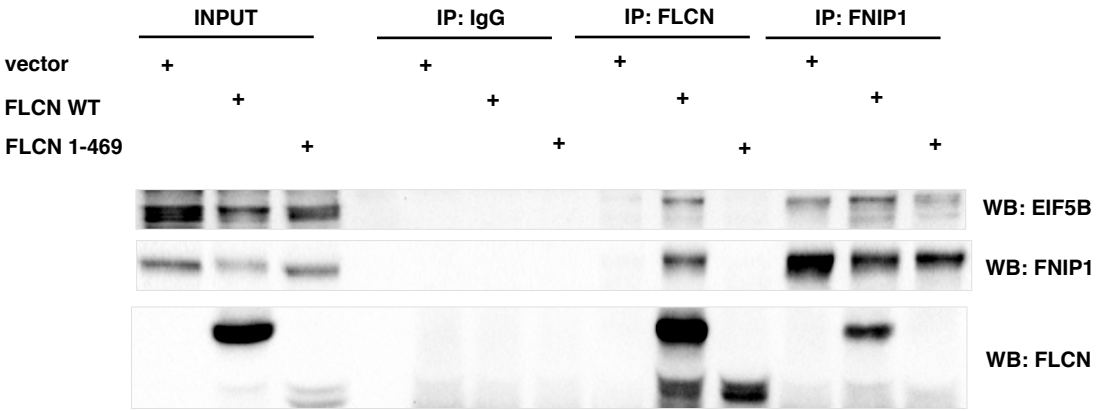
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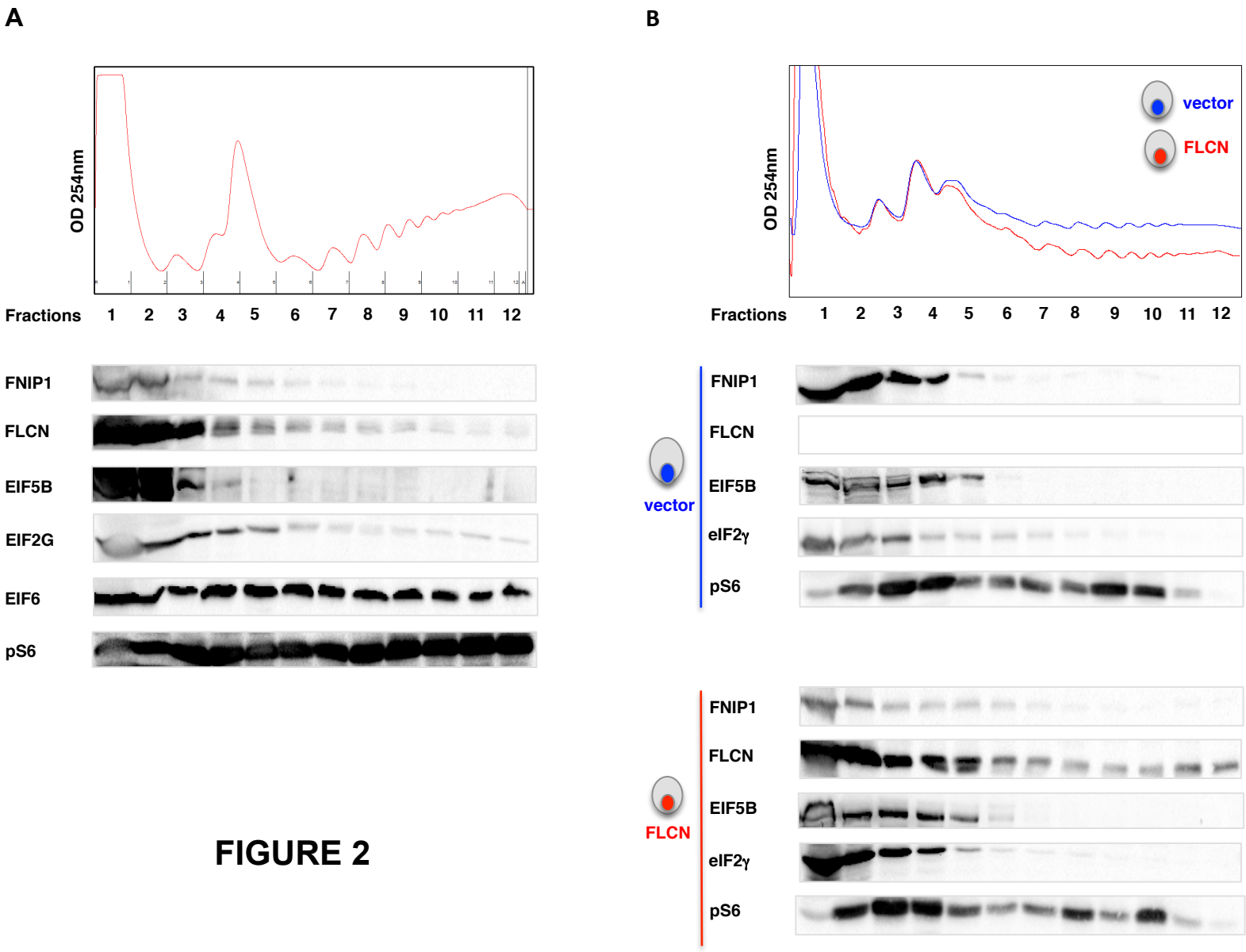
FIGURE 1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vector
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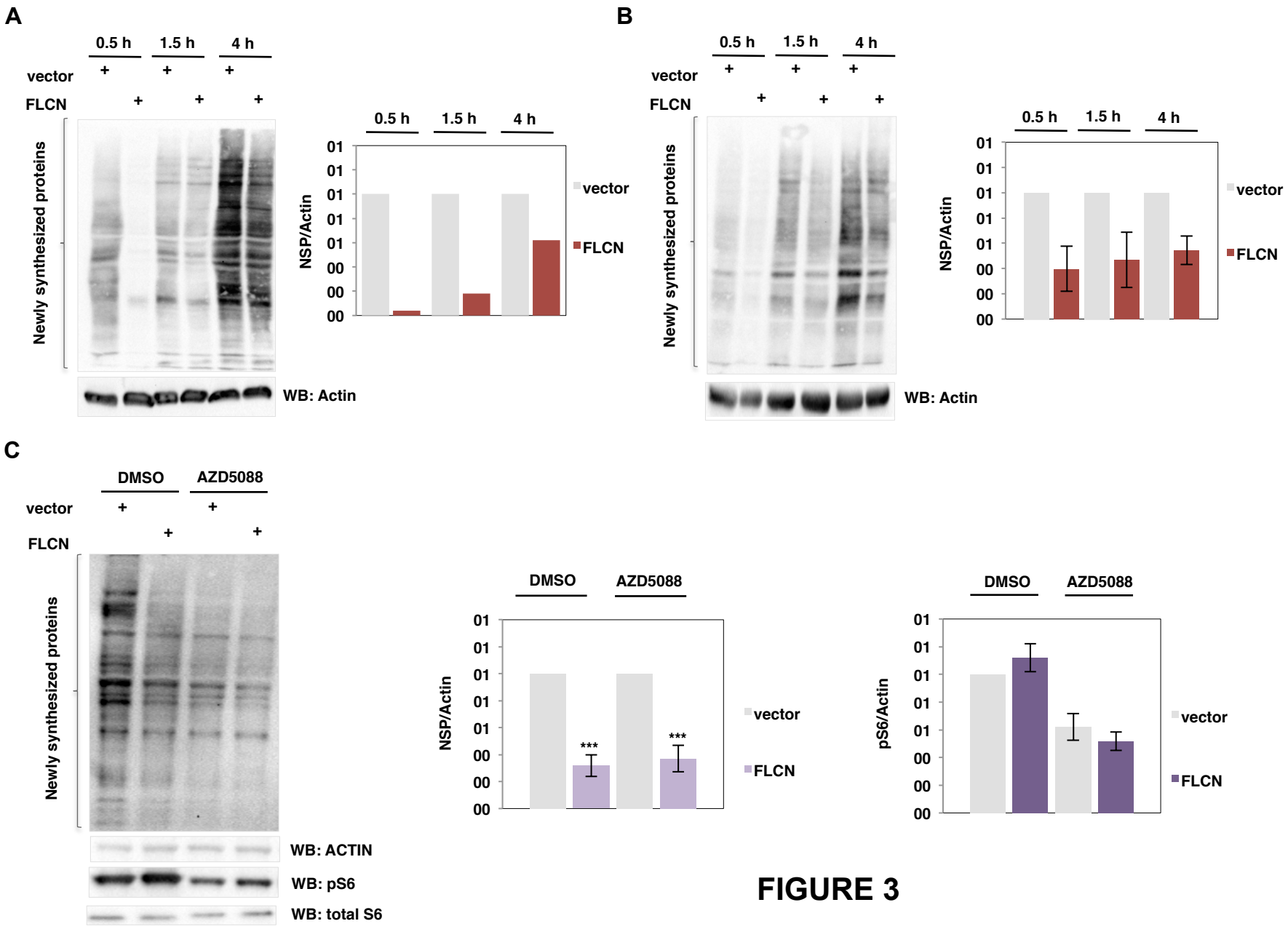
FIGURE 2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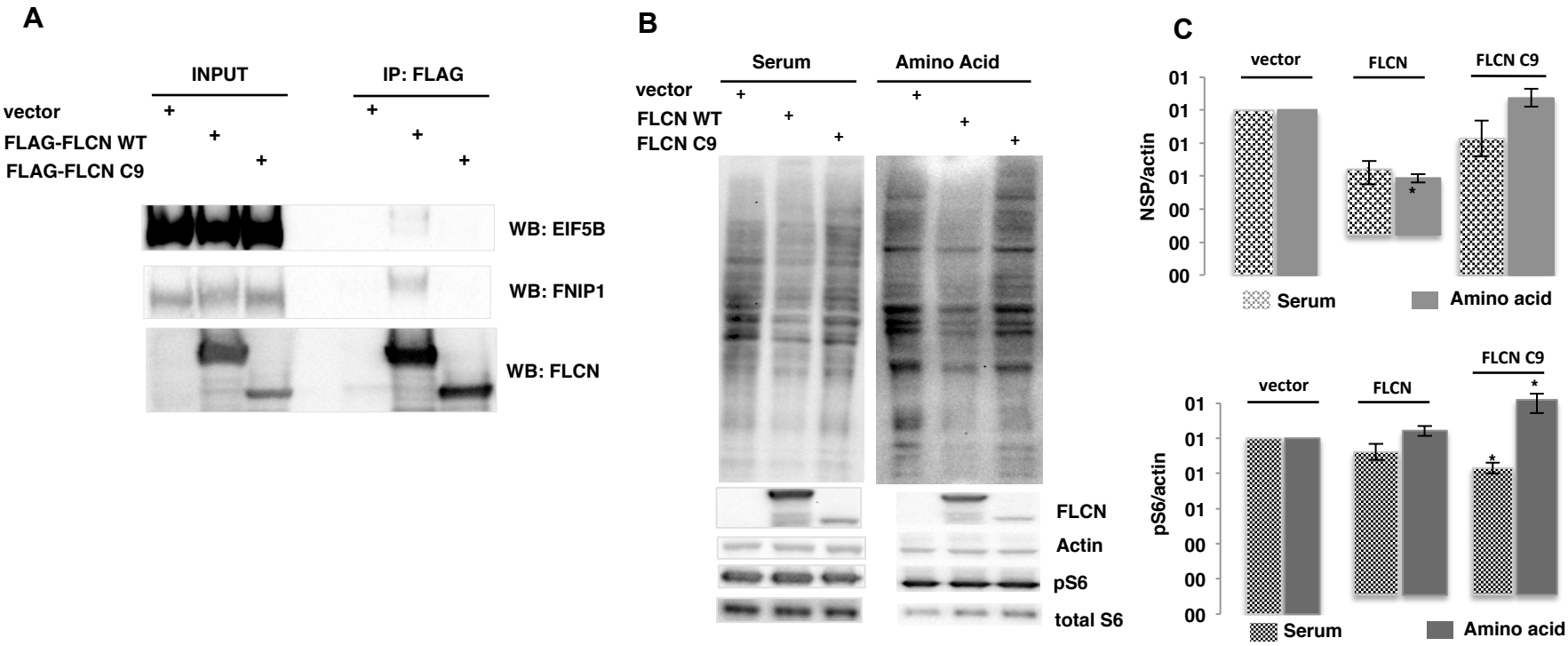
pS6/Actin

FIGURE 3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01
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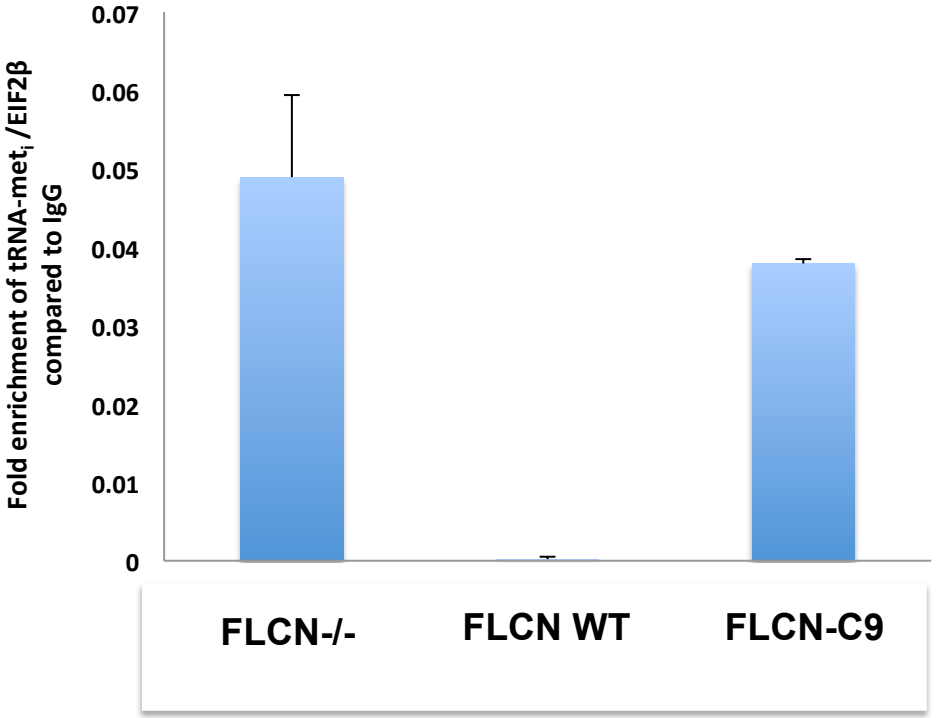
Serum
Amino acid

FIGURE 4

D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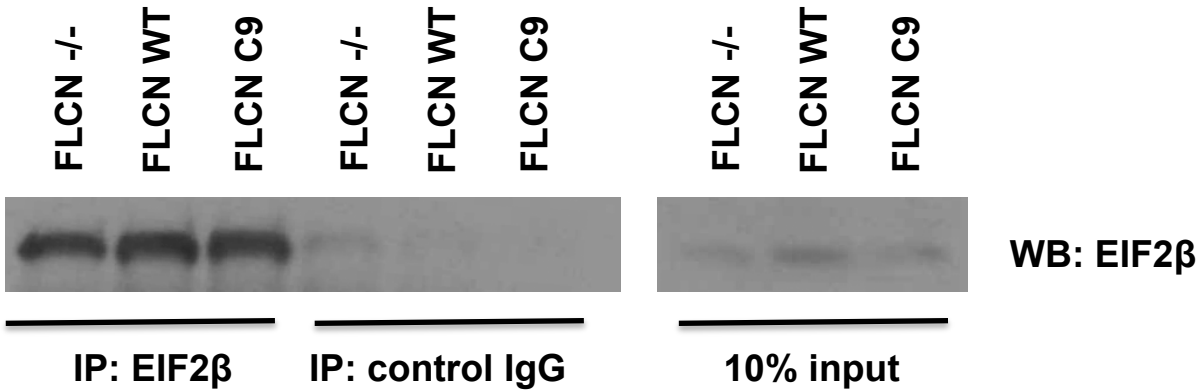
FLCN-C9

E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FLCN C9
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IP: control IgG
10% input

FIGURE 4

SUPPLEMENTAL FIGURE 1

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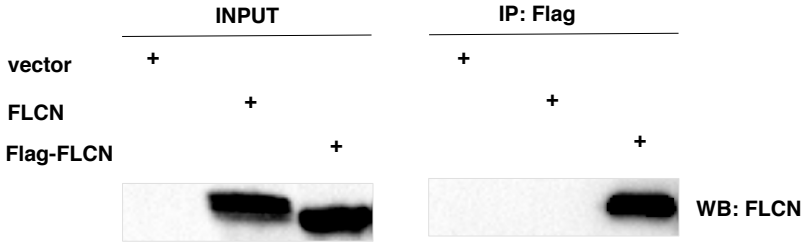
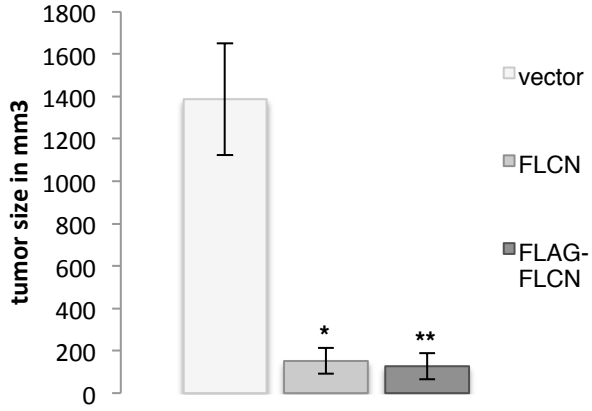


B



SUPPLEMENTAL TABLE 1: Mass Spect Spectral counts

Entry	Gene Name	Protein Description	starvation condition	Spectral Counts					
				FLCN-/- Nucleus	FLCN-/- Cytoplasm	FLCN-/- Membrane	Flag FLCN Nucleus	Flag FLCN Cytoplasm	Flag FLCN Membrane
PREVIOUSLY DESCRIBED FLCN INTERACTING PROTEINS									
Q8NFG4	FLCN_HUMAN	Folliculin	serum	0	0	0	14	24	4
Q8NFG4	FLCN_HUMAN	Folliculin	amino acid	0	0	0	20	38	9
Q8TF40-3	FNIP1_HUMAN	Isoform 3 of Folliculin-interacting protein 1	serum	0	0	0	0	2	0
Q8TF40-3	FNIP1_HUMAN	Isoform 3 of Folliculin-interacting protein 1	amino acid	0	0	0	2	3	0
Q9P278-2	FNIP2_HUMAN	Isoform 2 of Folliculin-interacting protein 2	serum	0	0	0	0	1	0
Q9P278-2	FNIP2_HUMAN	Isoform 2 of Folliculin-interacting protein 2	amino acid	0	0	0	0	0	0
Q8TBX8	PI42C_HUMAN	GABARAP	serum	1	0	0	1	0	0
Q8TBX8	PI42C_HUMAN	GABARAP	amino acid	0	1	1	1	0	0
Translation initiation factors									
Q9BY44	EIF2A_HUMAN	eukaryotic translation initiation factor 2A	serum	0	0	0	2	0	0
Q9BY44	EIF2A_HUMAN	eukaryotic translation initiation factor 2A	amino acid	3	0	0	7	0	0
Q9UI10	EI2BD_HUMAN	eukaryotic translation initiation factor 2B4	serum	0	0	0	1	0	0
Q9UI10	EI2BD_HUMAN	eukaryotic translation initiation factor 2B4	amino acid	0	0	0	0	0	0
P05198	IF2A_HUMAN	eukaryotic translation initiation factor 2S1	serum	9	0	0	17	0	0
P05198	IF2A_HUMAN	eukaryotic translation initiation factor 2S1	amino acid	10	0	2	15	2	0
P20042	IF2B_HUMAN	eukaryotic translation initiation factor 2S2	serum	9	0	0	12	0	2
P20042	IF2B_HUMAN	eukaryotic translation initiation factor 2S2	amino acid	13	2	0	10	2	2
Q2VIR3	IF2GL_HUMAN	eukaryotic translation initiation factor 2S3L	serum	6	2	1	7	2	1
Q2VIR3	IF2GL_HUMAN	eukaryotic translation initiation factor 2S3L	amino acid	7	2	2	7	2	1
O75822	EIF3J_HUMAN	eukaryotic translation initiation factor 3J	serum	0	0	0	2	0	0
O75822	EIF3J_HUMAN	eukaryotic translation initiation factor 3J	amino acid	1	0	0	0	0	0
F8VP89	F8VP89_HUMAN	eukaryotic translation initiation factor 4B	serum	0	0	0	0	1	0
F8VP89	F8VP89_HUMAN	eukaryotic translation initiation factor 4B	amino acid	0	0	0	1	0	0
O60573	IF4E2_HUMAN	eukaryotic translation initiation factor 4E2	serum	0	0	0	1	0	0
O60573	IF4E2_HUMAN	eukaryotic translation initiation factor 4E2	amino acid	0	0	0	2	0	0
P78344	IF4G2_HUMAN	eukaryotic translation initiation factor 4G2	serum	0	0	0	0	0	0
P78344	IF4G2_HUMAN	eukaryotic translation initiation factor 4G2	amino acid	0	0	0	1	0	0
P63241	IF5A1_HUMAN	eukaryotic translation initiation factor 5A	serum	0	0	0	0	0	0
P63241	IF5A1_HUMAN	eukaryotic translation initiation factor 5A	amino acid	0	0	0	4	0	0
O60841	IF2P_HUMAN	eukaryotic translation initiation factor 5B	serum	0	0	0	4	0	0
O60841	IF2P_HUMAN	eukaryotic translation initiation factor 5B	amino acid	5	0	1	9	0	0
P56537	IF6_HUMAN	eukaryotic translation initiation factor 6	serum	3	0	0	4	2	0
P56537	IF6_HUMAN	eukaryotic translation initiation factor 6	amino acid	4	3	2	4	2	0

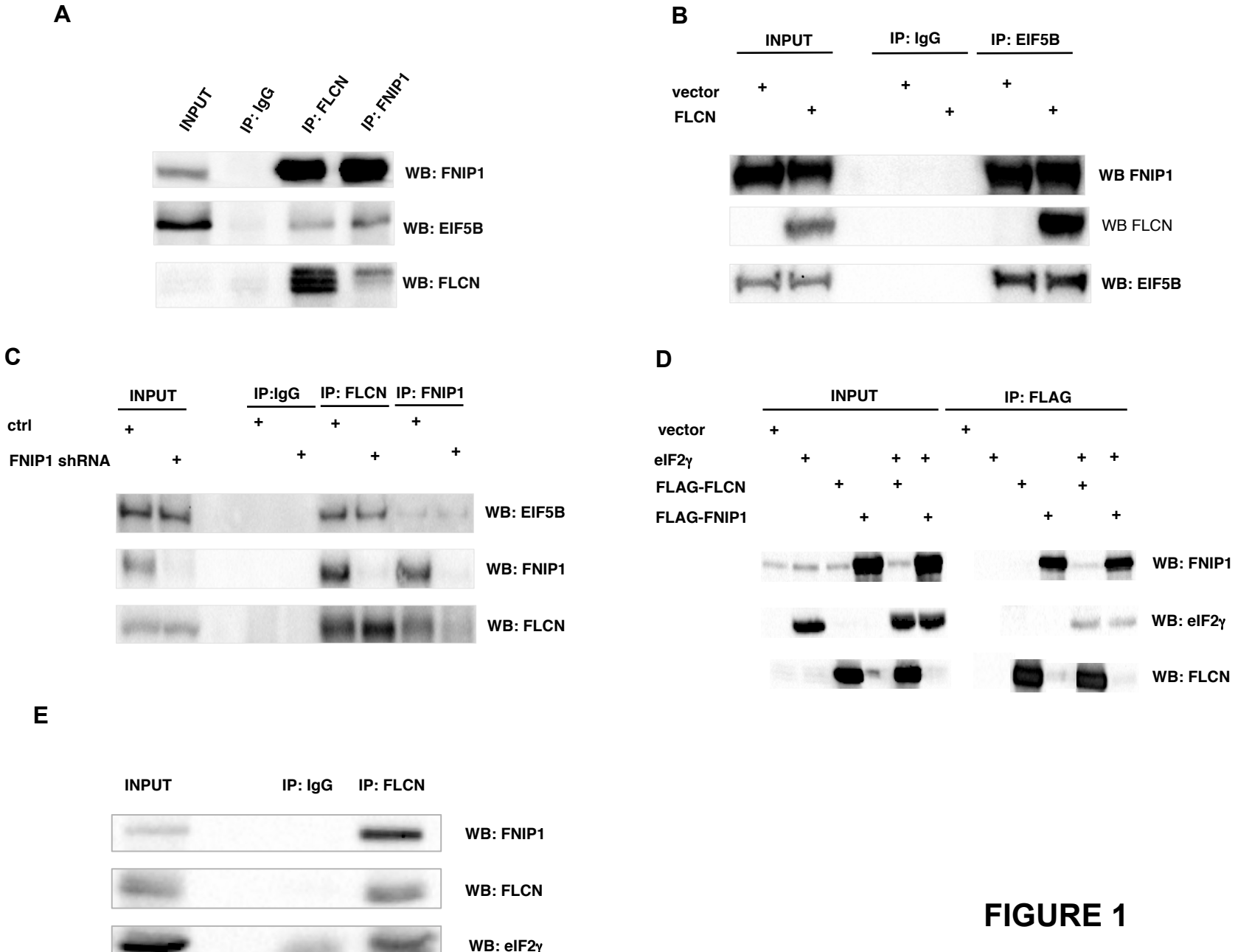
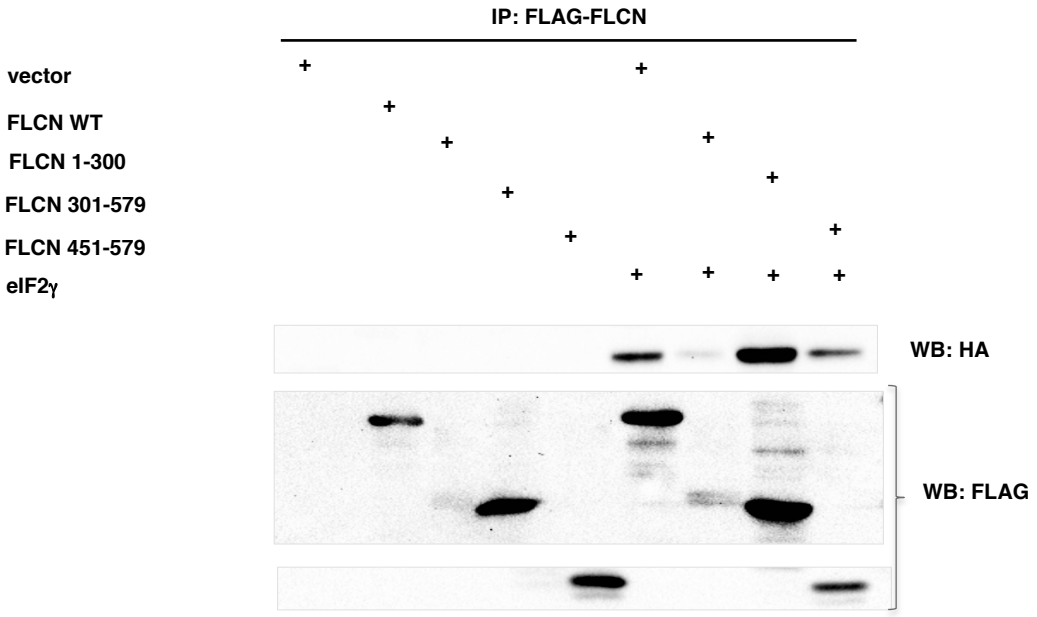

A
INPUT
IP: IgG
IP: FLCN
IP: FNIP1
WB: FNIP1
WB: EIF5B
WB: FLCN
B
INPUT
IP: IgG
IP: EIF5B
vector
FLCN
WB FNIP1
WB FLCN
WB: EIF5B
C
INPUT
IP:IgG
IP: FLCN
IP: FNIP1
ctrl
FNIP1 shRNA
WB: EIF5B
WB: FNIP1
WB: FLCN
D
INPUT
IP: FLAG
vector
eIF2γ
FLAG-FLCN
FLAG-FNIP1
WB: FNIP1
WB: eIF2γ
WB: FLCN
E
INPUT
IP: IgG
IP: FLCN
WB: FNIP1
WB: FLCN
WB: eIF2γ

FIGURE 1

F


IP: FLAG-FLCN
vector
FLCN WT
FLCN 1-300
FLCN 301-579
FLCN 451-579
eIF2γ
WB: HA
WB: FLAG

G

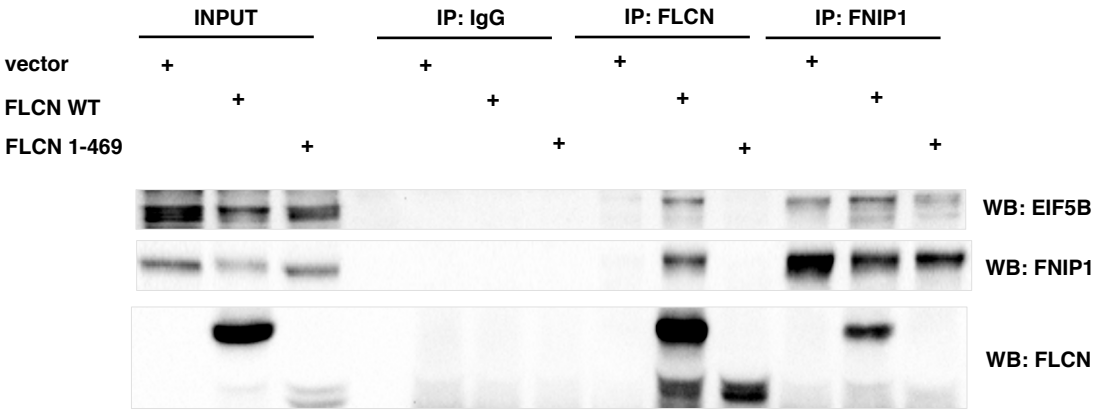

INPUT
IP: IgG
IP: FLCN
IP: FNIP1
vector
FLCN WT
FLCN 1-469
WB: EIF5B
WB: FNIP1
WB: FLCN

FIGURE 1

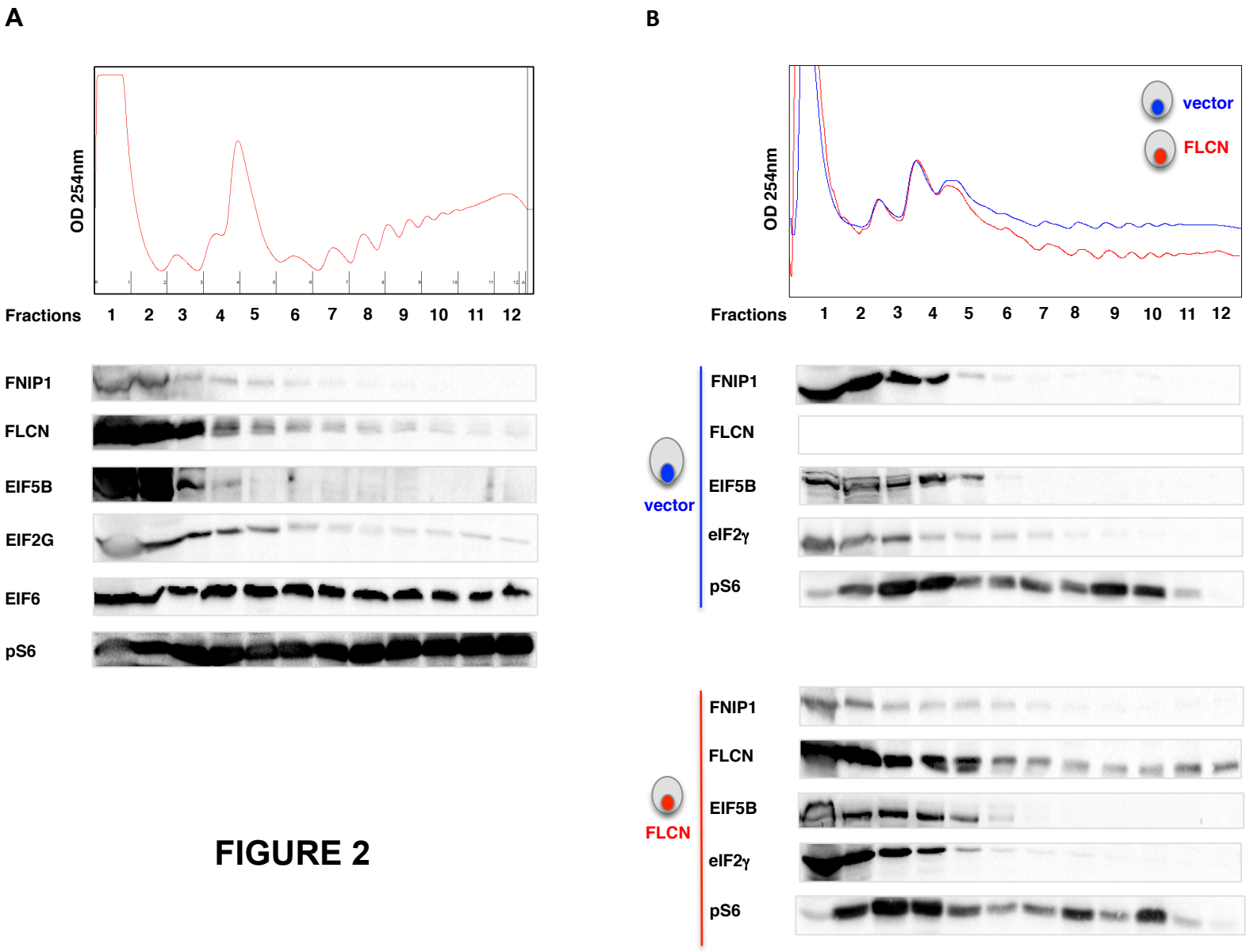

A
OD 254nm
Fractions 1 2 3 4 5 6 7 8 9 10 11 12
FNIP1
FLCN
EIF5B
EIF2G
EIF6
pS6
B
vector
FLCN
OD 254nm
Fractions 1 2 3 4 5 6 7 8 9 10 11 12
vector
FNIP1
FLCN
EIF5B
eIF2γ
pS6
FLCN
FNIP1
FLCN
EIF5B
eIF2γ
pS6

FIGURE 2

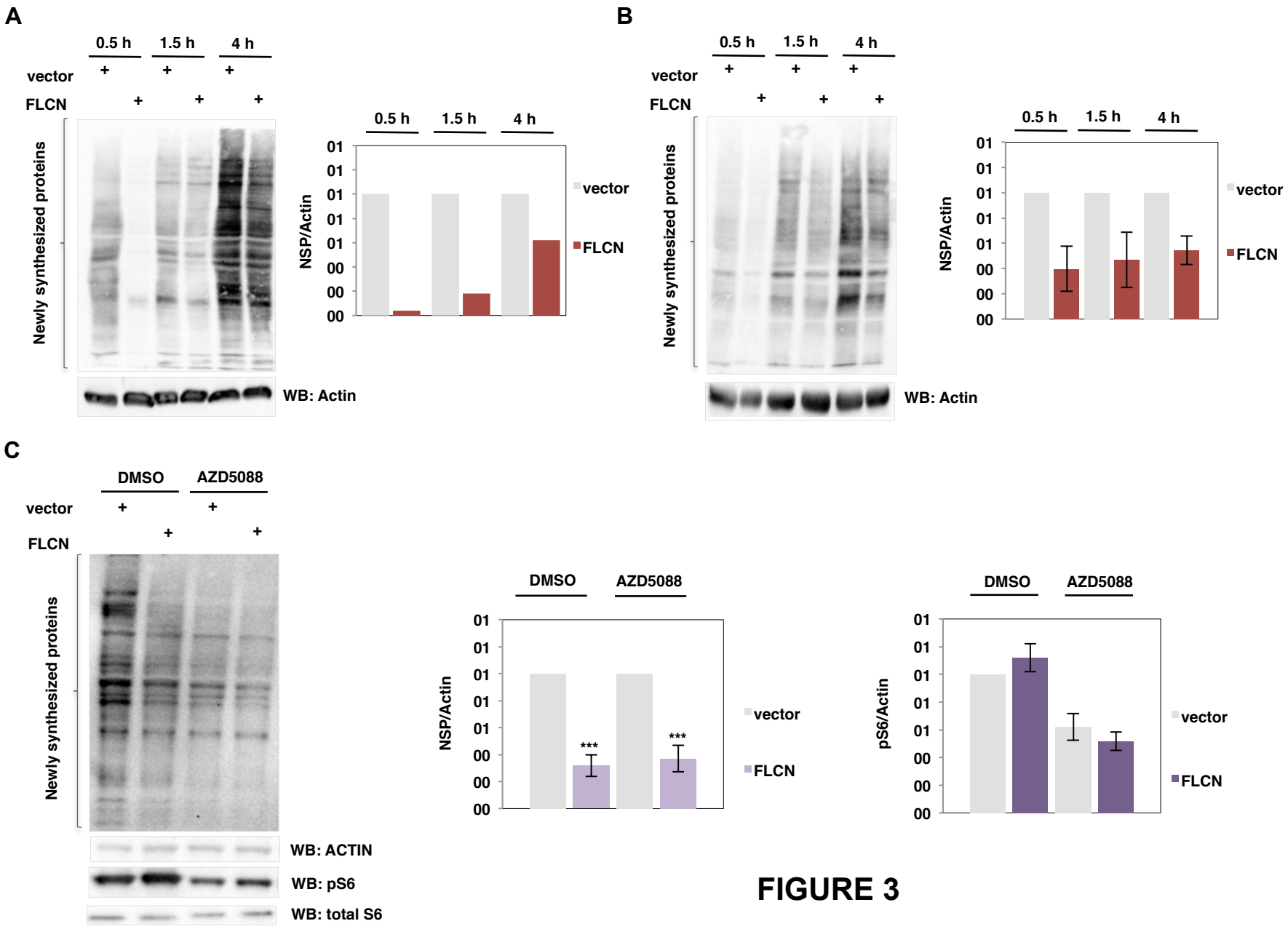

A
0.5 h
1.5 h
4 h
vector
FLCN
Newly synthesized proteins
WB: Actin
NSP/Actin
vector
FLCN
B
C
DMSO
AZD5088
WB: ACTIN
WB: pS6
WB: total S6
***
pS6/Actin

FIGURE 3

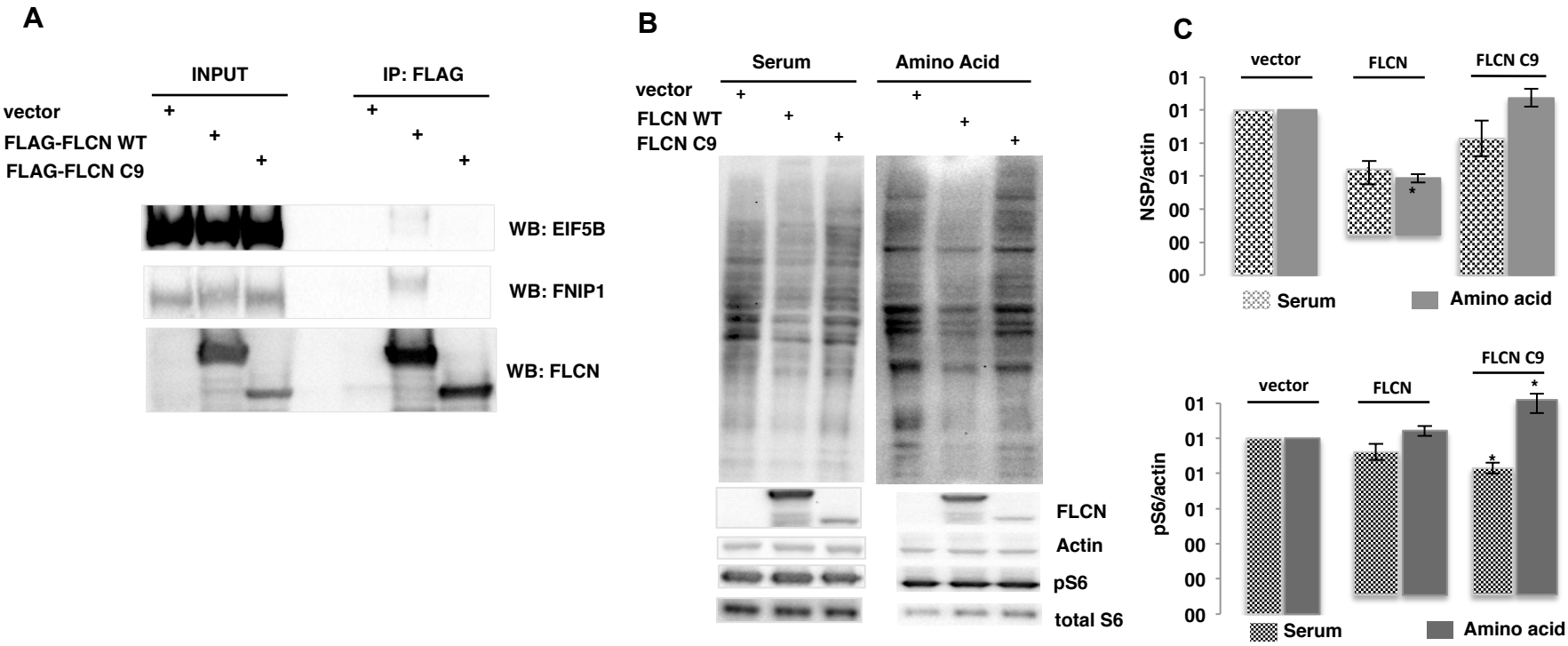
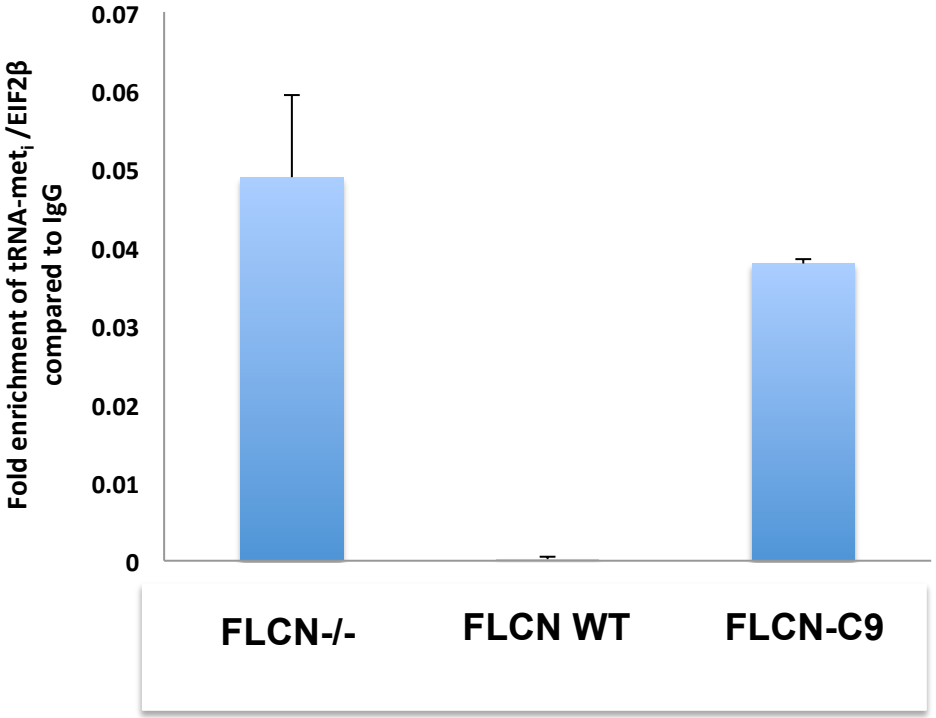

A
INPUT
IP: FLAG
vector
FLAG-FLCN WT
FLAG-FLCN C9
WB: EIF5B
WB: FNIP1
WB: FLCN
B
Serum
Amino Acid
vector
FLCN WT
FLCN C9
FLCN
Actin
pS6
total S6
C
vector
FLCN
FLCN C9
NSP/actin
01
01
01
01
00
00
00
Serum
Amino acid
pS6/actin
01
01
01
01
00
00
00
Serum
Amino acid

FIGURE 4

D


Fold enrichment of tRNA-met$_i$ /EIF2β compared to IgG
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
FLCN-/-
FLCN WT
FLCN-C9

E

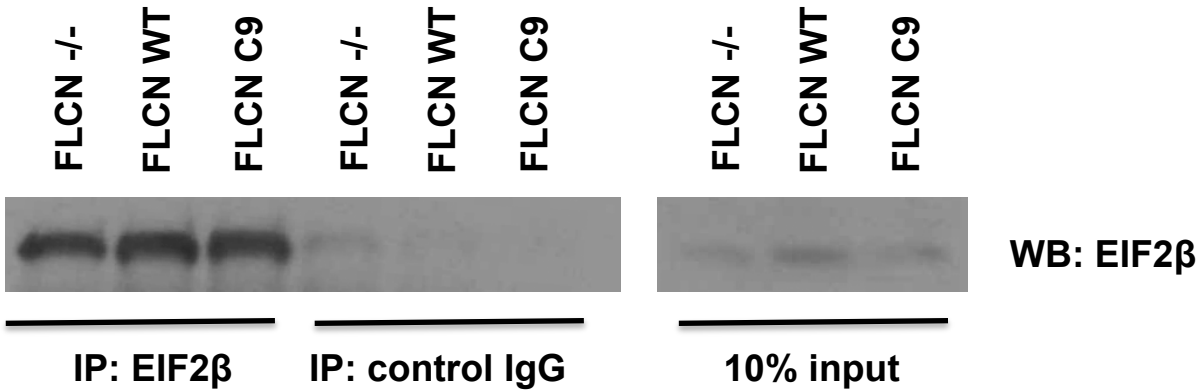
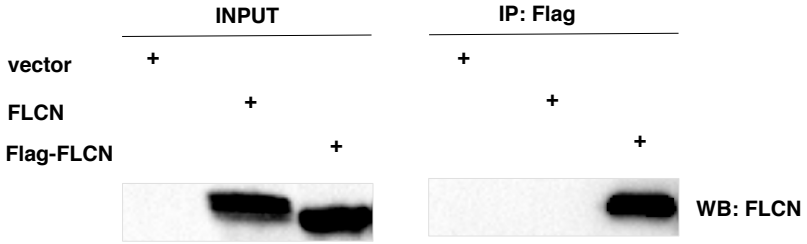

FLCN -/-
FLCN WT
FLCN C9
FLCN -/-
FLCN WT
FLCN C9
FLCN -/-
FLCN WT
FLCN C9
WB: EIF2β
IP: EIF2β
IP: control IgG
10% input

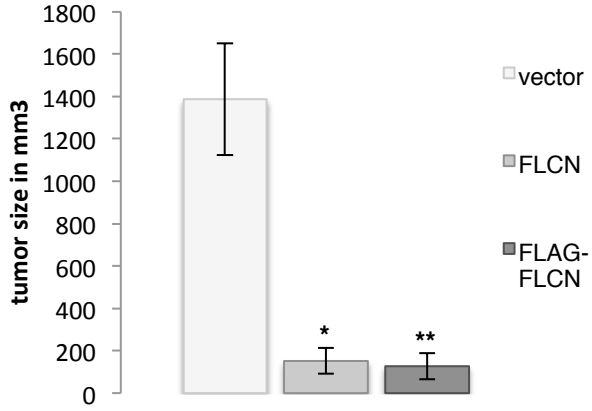
FIGURE 4

SUPPLEMENTAL FIGURE 1

A



B



SUPPLEMENTAL TABLE 1: Mass Spect Spectral counts

Entry	Gene Name	Protein Description	starvation condition	Spectral Counts					
				FLCN-/- Nucleus	FLCN-/- Cytoplasm	FLCN-/- Membrane	Flag FLCN Nucleus	Flag FLCN Cytoplasm	Flag FLCN Membrane
PREVIOUSLY DESCRIBED FLCN INTERACTING PROTEINS									
Q8NFG4	FLCN_HUMAN	Folliculin	serum	0	0	0	14	24	4
Q8NFG4	FLCN_HUMAN	Folliculin	amino acid	0	0	0	20	38	9
Q8TF40-3	FNIP1_HUMAN	Isoform 3 of Folliculin-interacting protein 1	serum	0	0	0	0	2	0
Q8TF40-3	FNIP1_HUMAN	Isoform 3 of Folliculin-interacting protein 1	amino acid	0	0	0	2	3	0
Q9P278-2	FNIP2_HUMAN	Isoform 2 of Folliculin-interacting protein 2	serum	0	0	0	0	1	0
Q9P278-2	FNIP2_HUMAN	Isoform 2 of Folliculin-interacting protein 2	amino acid	0	0	0	0	0	0
Q8TBX8	PI42C_HUMAN	GABARAP	serum	1	0	0	1	0	0
Q8TBX8	PI42C_HUMAN	GABARAP	amino acid	0	1	1	1	0	0
Translation initiation factors									
Q9BY44	EIF2A_HUMAN	eukaryotic translation initiation factor 2A	serum	0	0	0	2	0	0
Q9BY44	EIF2A_HUMAN	eukaryotic translation initiation factor 2A	amino acid	3	0	0	7	0	0
Q9UI10	EI2BD_HUMAN	eukaryotic translation initiation factor 2B4	serum	0	0	0	1	0	0
Q9UI10	EI2BD_HUMAN	eukaryotic translation initiation factor 2B4	amino acid	0	0	0	0	0	0
P05198	IF2A_HUMAN	eukaryotic translation initiation factor 2S1	serum	9	0	0	17	0	0
P05198	IF2A_HUMAN	eukaryotic translation initiation factor 2S1	amino acid	10	0	2	15	2	0
P20042	IF2B_HUMAN	eukaryotic translation initiation factor 2S2	serum	9	0	0	12	0	2
P20042	IF2B_HUMAN	eukaryotic translation initiation factor 2S2	amino acid	13	2	0	10	2	2
Q2VIR3	IF2GL_HUMAN	eukaryotic translation initiation factor 2S3L	serum	6	2	1	7	2	1
Q2VIR3	IF2GL_HUMAN	eukaryotic translation initiation factor 2S3L	amino acid	7	2	2	7	2	1
O75822	EIF3J_HUMAN	eukaryotic translation initiation factor 3J	serum	0	0	0	2	0	0
O75822	EIF3J_HUMAN	eukaryotic translation initiation factor 3J	amino acid	1	0	0	0	0	0
F8VP89	F8VP89_HUMAN	eukaryotic translation initiation factor 4B	serum	0	0	0	0	1	0
F8VP89	F8VP89_HUMAN	eukaryotic translation initiation factor 4B	amino acid	0	0	0	1	0	0
O60573	IF4E2_HUMAN	eukaryotic translation initiation factor 4E2	serum	0	0	0	1	0	0
O60573	IF4E2_HUMAN	eukaryotic translation initiation factor 4E2	amino acid	0	0	0	2	0	0
P78344	IF4G2_HUMAN	eukaryotic translation initiation factor 4G2	serum	0	0	0	0	0	0
P78344	IF4G2_HUMAN	eukaryotic translation initiation factor 4G2	amino acid	0	0	0	1	0	0
P63241	IF5A1_HUMAN	eukaryotic translation initiation factor 5A	serum	0	0	0	0	0	0
P63241	IF5A1_HUMAN	eukaryotic translation initiation factor 5A	amino acid	0	0	0	4	0	0
O60841	IF2P_HUMAN	eukaryotic translation initiation factor 5B	serum	0	0	0	4	0	0
O60841	IF2P_HUMAN	eukaryotic translation initiation factor 5B	amino acid	5	0	1	9	0	0
P56537	IF6_HUMAN	eukaryotic translation initiation factor 6	serum	3	0	0	4	2	0
P56537	IF6_HUMAN	eukaryotic translation initiation factor 6	amino acid	4	3	2	4	2	0