Identification of Novel Modulators of mTORC2 Activity

Nina Su
Half Hollow Hills High School West

Acknowledgements

I would like to thank the HOPP Summer Student Program, the entire Pavletich Lab and the Mass Spectrometry Core Facility. In particular, I would like to give a special thank you to Christopher Warren for his mentorship and support this summer. I would also like to thank the Lowe, Jiang, and Nikolov Labs for providing A549, HeLa, and U-2OS cells used in these experiments.

Table of Contents

Introduction

Overview of mTOR

The mammalian target of rapamycin (mTOR) is a conserved protein kinase in all eukaryotes that acts as a master regulator of homeostasis. mTOR uses environmental inputs, including growth factors, energy, and nutrients to regulate many cellular processes such as cell growth and metabolism. This kinase is found in two structurally and functionally distinct complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).

mTORC1 is composed of mTOR, Raptor, GβL, and PRAS40. It assembles at the surface of lysosomes in an inactive state and becomes activated when bound by the protein RHEB-GTP in response to available nutrients. The activity of mTORC1 is sensitive to inhibition by rapamycin; a drug that is currently in use as an immunosuppressant and being studied as a potential cancer therapeutic. mTORC1 plays a central role in increasing production of proteins, lipids, and nucleotides, while suppressing autophagy to allow a cell to grow and divide. While mTORC1 regulates cell growth and metabolism, mTORC2 controls proliferation and survival.

mTORC2 is composed of mTOR, Rictor, GβL, and Sin 1 and Mutations and abnormal amplifications of these core components are the main factors for hyperactivation, which is commonly seen in many types of human cancers. It is thought to assemble at or near the plasma membrane and responds mainly to growth factors, such as insulin which then phosphorylates AGC kinase proteins, including Akt. The most crucial role of mTORC2 is most likely the phosphorylation and activation of Akt which promotes cell survival, proliferation, and growth

therefore having an important role in cancer. However, the mechanism underlying this activation remains unknown.

Akt

The most characterized effector of mTORC2 is Akt, a family of three members (Akt 1, Akt 2, and Akt 3). In the absence of a stimuli Akt is found in the cytosol and then recruited to the plasma membrane by the interaction between its PH domain and Phosphatidylinositol (3,4,5)-

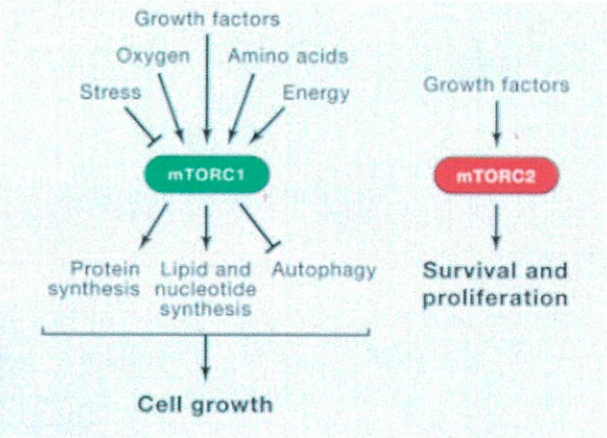


Figure 1: Comparison of the mTORC1 and mTORC2 main functions and pathway
Source:https://www.sciencedirect.com/science/article/pii/S0092867417301824

trisphosphate which is caused by PI3K activation. This results in Akt phosphorylation at two sites, T308 in the activation loop (A-loop) by PDKI and S473 in hydrophobic motif (HM) by mTORC2 which fully activates Akt. mTORC2 predominantly phosphorylated Akt-S473 although it can be phosphorylated by other kinases including DNA-dependent protein kinase (DNA-PK) and integrin linked kinase (ILK). However, deleting any core components of mTORC2 could drastically result in the abrogation of phosphorylating Akt-S473. Even though Akt-T308 and Akt-S473 phosphorylation is required for a full activation of Akt, and defective Akt-S473 impacts only a subset if Akt targets *in vivo*, while other Akt targets such as its downstream effector mTORC1 remains unaffected.

The kinase that phosphorylates Akt-T450 has also been identified to be mTORC2 in its

turn motif (TM) which allows the correct folding and maturation of the Akt protein. mTORC2 has shown to phosphorylate Akt-T450 differently than Akt-S473 by using different mechanisms. A canonical post translational mechanism is used to phosphorylate Akt-S473 which can be resembled by *in vitro* kinase assay with immunoprecipitated mTORC2; while, the phosphorylation of Akt-T450 is believed to be done by a cotranslational mechanism which includes ribosomes that cannot be achieved *in vitro* kinase assay.

Regulation of mTORC2

Even though much is known about mTORC1, the regulation of mTORC2 is very understudied and less understood than mTORC1. Nonetheless, increasing evidence has shown that a multitude of mechanisms can directly or indirectly affect the function of mTORC2. It has been known that growth factors bind to the membrane receptor, receptor tyrosine kinase (RTK). This activates Phosphoinositide 3-kinase (PI3K) to phosphorylate Phosphatidylinositol 4,5-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the plasma membrane. Then the protein phosphatase and tensin homolog (PTEN) is a key negative regulator of PI3K signaling and dephosphorylates PIP3. It is either PIP3 or other unknown factors that activate mTORC2 which promotes the phosphorylation of several AGC kinases including Akt, PKC, and SGK1. Akt is phosphorylated at the T308 and S473 sites by PDK1 which results in maximal activation. It has been shown that only the recruitment of Akt is required for the activation of PI3K. Finally, mTORC2, subsequently, and respectively stimulates mTORC1 activation, which phosphorylates several downstream effectors such as S6K, 4E-BP1, and ULK1.

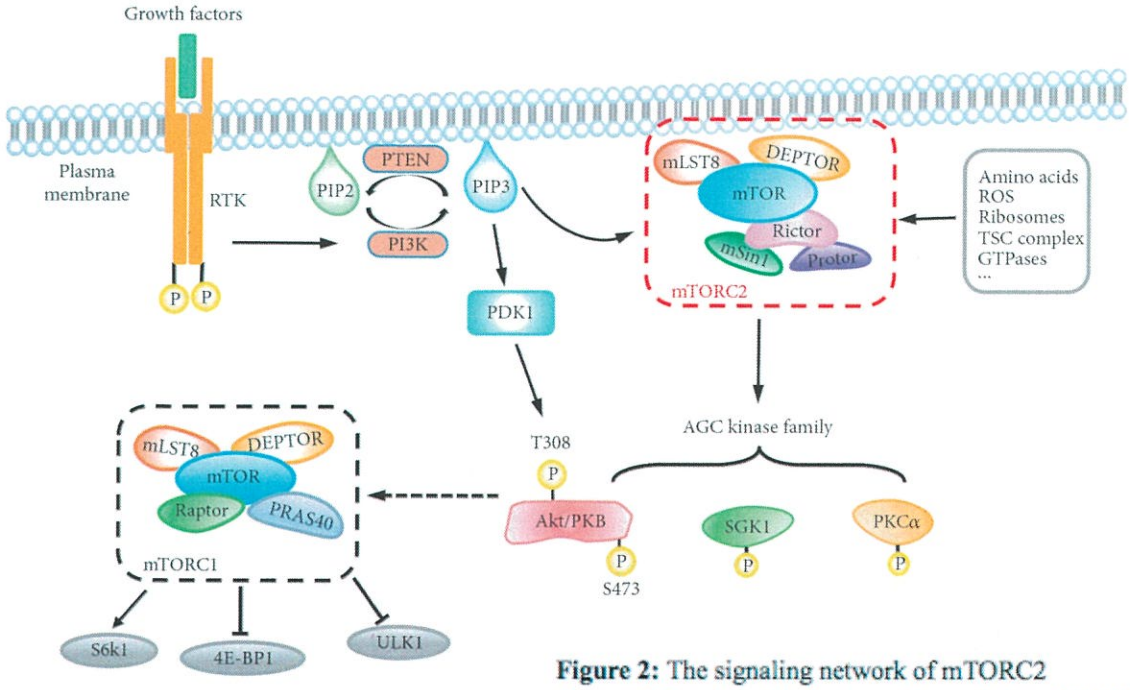


Figure 2: The signaling network of mTORC2
Source: https://new.hindawi.com/journals/omcl/2018/7838647/

Objective

The first aim of this project was to determine the optimal insulin treatments conditions required for robust mTORC2 activation in multiple cell lines. The cell lines being used include, HEK293 (human embryonic kidney), HeLa (cervical cancer), U20S (osteosarcoma), and A549 (lung cancer), these will all be treated with insulin for various times. We then followed mTORC2 activation by western blot of whole cell lysates using phosphorylated Akt as a marker of mTORC2 activity. Optimal treatment conditions were then used in Aim 2.

The ultimate goal of this project is to identify and characterize novel activators of mTORC2 which helps us understand how mTORC2 exactly becomes activated. Here, we used in-cell chemical cross-linking, immunoprecipitation and mass-spectrometry. Our cell lines were treated with and without insulin using the optimal conditions from Aim 1 and proteins were chemically cross-linked within cells. mTORC2 was then immunoprecipitated from cell lysates using an

antibody specific towards the Rictor subunit. Immunoprecipitated material was digested while on beads using trypsin and peptides from interacting proteins will be identified via mass-spectrometry (in collaboration with the MSK mass-spectrometry core). Statistical analysis was performed to identify proteins that are significantly enriched or depleted upon activation of mTORC2. Gene ontology and bioinformatic analyses were conducted and used to develop a list of candidate mTORC2 activators.

Materials and Methods

Cell Culture and Insulin Treatment:

HEK293, HeLa, U2OS, A549 cells were cultured in DMEM with 10% FBS and 1X Penicillin-Streptomycin. HEK 293 cells stably expressing FLAG-tagged mTORC2 were grown in suspension with Freestyle 293 media. For mTORC2 activation assays, cells were treated with 4μg/mL of human insulin for indicated time points.

Immunoblot Analysis:

Cells were detached from plates by scraping and pelleted by centrifugation. Cells were disrupted by sonication in lysis buffer composed of 25mM Tris pH 7.5, 150mM NaCl, 0.2% Triton- X100, protease and phosphatase inhibitor cocktails. Lysates were clarified by centrifugation protein concentrations were determined by Bradford assay. 10μg of protein was loaded per condition and proteins were separated by SDS-PAGE. Proteins were transferred to a PVDF membrane, blocked in 5% milk in TBST buffer for 30 minutes at room temperature, incubated with primary antibodies for 1 hour at room temperature, washed 3X for 10 minutes in TBST, incubated with HRP- conjugated secondary antibody for 1 hour at room temperature, and

washed again 3X for 10 minutes in TBST buffer. Blots were exposed to substrate and image on CCD imager. Bands intensities were quantified in ImageJ software.

Immunoprecipitation and Mass Spectrometry Analysis:

Lysates from ~10e6 cells were rotated with 10μL of anti-FLAG sepharose for 1 hour at 4℃. Beads were then transferred from Eppendorf tubes to a 96 well filter plate and pelleted by centrifugation. Beads were washed five times in 500μL of wash buffer (25mM HEPES pH 7.5 and 150mM NaCl) using a vacuum apparatus. Proteins were digested by incubating washed resin with MS-grade trypsin overnight at 37℃, and peptides were subject to tandem-mass tag labeling and identified by mass-spectrometry.

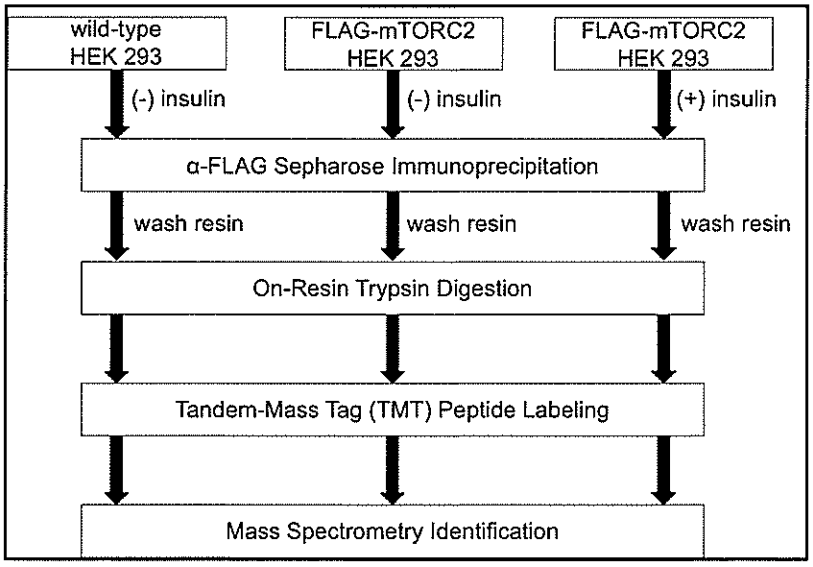


Figure 3: Workflow of affinity enrichment mass spectrometry (AE-MS) experiments. The wild-type HEK 293 cells were treated with no insulin, while half of the samples of FLAG-mTORC2 HEK 293 cells were treated with insulin and the other half were treated without insulin. Using α-FLAG Sepharose, mTORC2 was immunoprecipitated from cell lysates. This material was then digested on beads using trypsin and mass spectrometry performed TMT labeling and finally identified multiple proteins.

Results and Discussion

Overall mTORC2 activation

The core components of mTORC2, mTOR and Sin1, as well as Akt-S473 and pan Akt, were present in all the four common cell lines. The HeLa and A549 cells had the least amount of mTORC2 and Akt, while the HEK 293 and U-2OS cells showed the presence of more mTOR, Sin1, and Akt-S473.

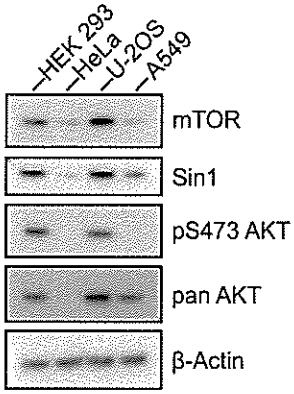


Figure 4: Steady-State Levels of mTORC2 and AKT in Four Common Cell Lines

The insulin treatments for each cell line were in 30-minute intervals. By treating these four human cell lines with insulin and by monitoring AKT phosphorylation we found that mTORC2 is rapidly activated in both HEK 293 and A549 cells, however no measurable activation was observed in HeLa or U-2OS cells. The insulin treatments were in 30-minute intervals, where the first sample were treated with 4μg/mL of human insulin for 30 minutes and the last sample was treated for 180 minutes. By comparing all the samples by the sample treated without insulin and by monitoring Akt phosphorylation we found that mTORC2 is rapidly activated in both HEK 293 and A549 cells, however no measurable activation was observed in HeLa or U-2OS cells. The A549 cells were shown to have the most significant and an optimal activation at around 60 minutes compared to HEK 293 cells which had a lot less activation of mTORC2.

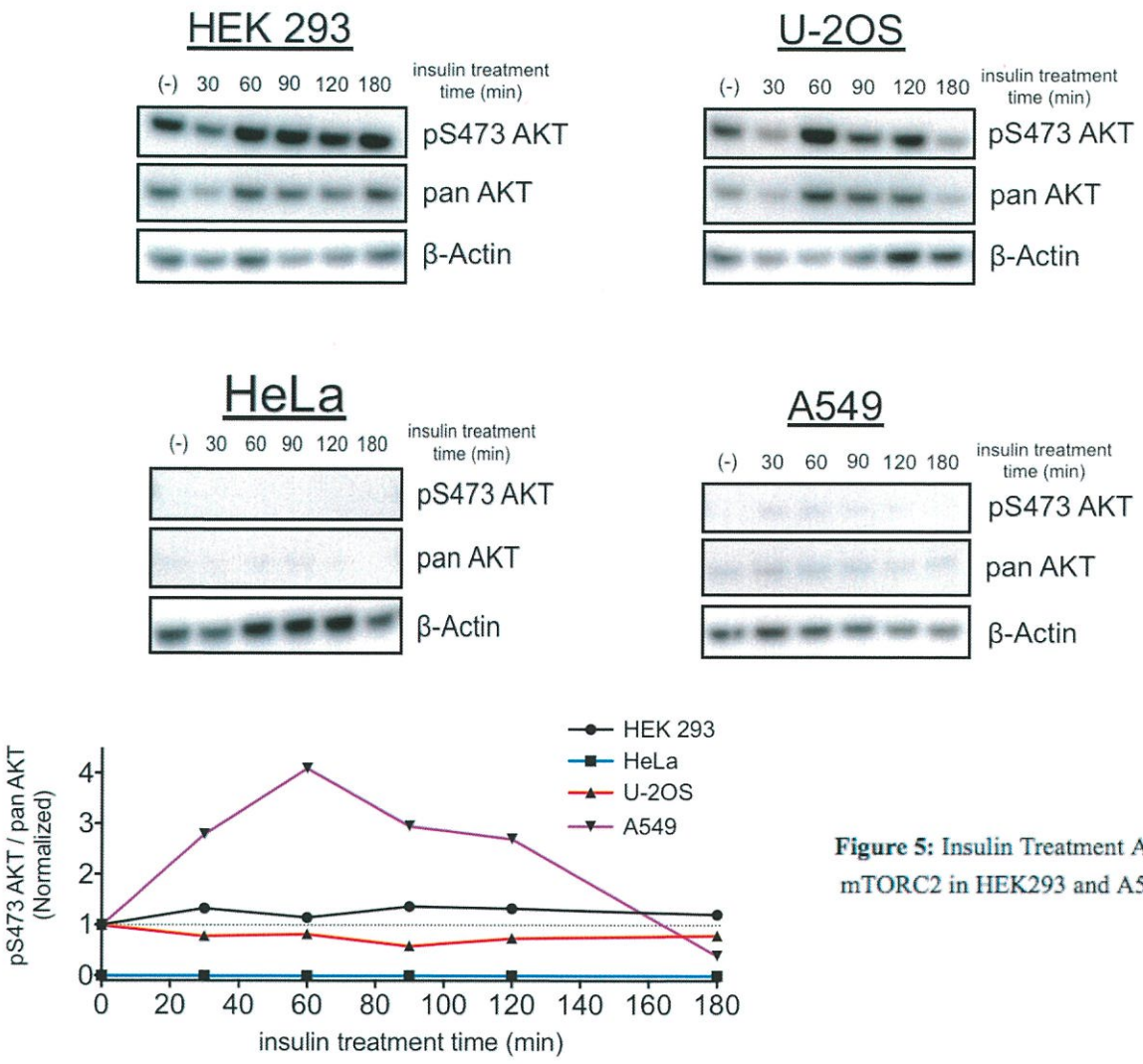


Figure 5: Insulin Treatment Activates mTORC2 in HEK293 and A549 Cells

These optimal treatment conditions were then used to activate mTORC2 in a cell line stably expressing FLAG-tagged mTOR, RICTOR, Sin1, and GβL. When mTORC2 was immunoprecipitated from the FLAG-mTORC2 cells the results showed all the bands that represent where mTOR, Rictor, Sin1, and GβL are. The FLAG-mTORC2 cells without insulin expressed less Akt compared to the cells treated with insulin. FLAG-tagged mTORC2 was pulled down from

a HEK293 stable cell line grown in suspension, which showed all the components of mTORC2 and many unidentified proteins.

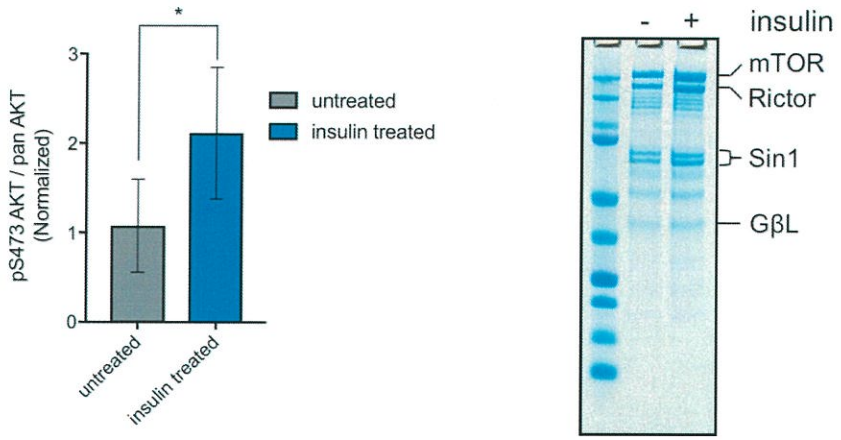
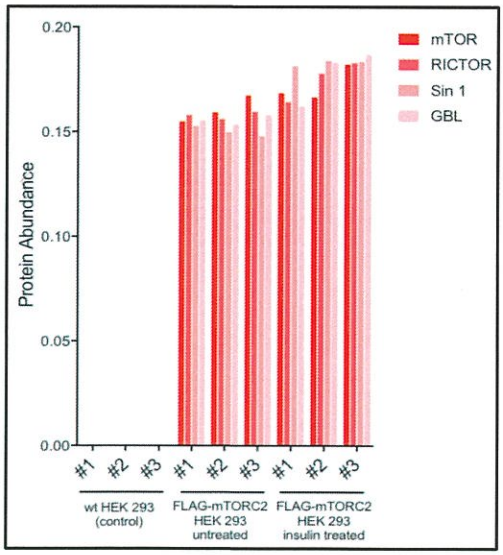


Figure 6: Activation and Immunoprecipitation of mTORC2 from FLAG-tagged Stable Cell Line Grown in Suspension

AE-MS Identification of mTORC2



The wild-type HEK 293 cells without insulin showed the expected results of having no components of mTORC2 because it was the control, while the FLAG-mTORC2 HEK 293 cells untreated has less mTORC2 components compared to the FLAG-mTORC2 HEK 293 cells treated with insulin.

Figure 7: The abundance of mTOR, RICTOR, Sin 1, and GβL identified in the different samples of FLAG-mTORC2 HEK 293 cells

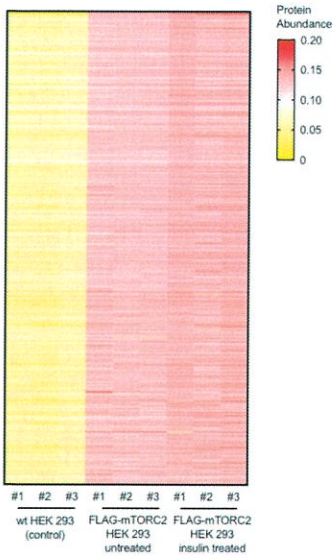
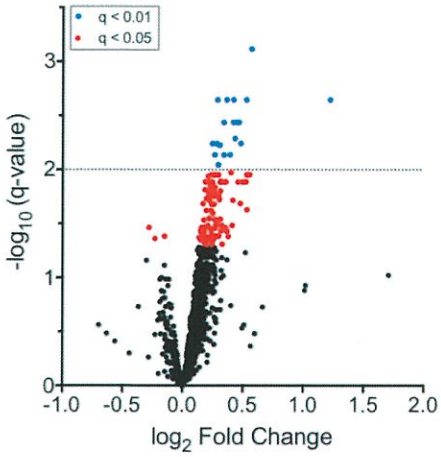


Figure 8: A total of 1638 proteins were identified. 1372 proteins were used in analyses and showed enrichment in FLAG-mTORC2 IP compared to wild type cell controls.

Like figure 7 shown above, figure 8 also shows that there is a higher protein abundance of mTORC2 components in the insulin treated samples compared to the untreated and the wild-type HEK 293 cells. However, unlike figure 7, figure 8 has little boxes that represent all the 1638 proteins that were identified by mass spectrometry.

Raw data was processed, and protein identification was filtered using a target-decoy approach in which the amino acid sequence is ordered randomized. Decoy hits were then used to estimate the false positive rate of 1%. After, raw data was processed by mass spectrometry, they found that out of the 1372 proteins 20 proteins were shown with high statistical value as a potential activator of mTORC2. 6 out of the 20 proteins were from the 14-3-3 protein family. Gene ontology and bioinformatic analyses were conducted and the expected results of unfolded protein binding and protein folding chaperon were shown to be the highest.



Protein Name	Log2 Fold Increase	q-value
Tetratricopeptide repeat protein 7B	0.566329119	0.000764853
DnaJ homolog subfamily A member 2	0.417868305	0.002259524
NADPH--cytochrome P450 reductase	0.36061612	0.002259524
14-3-3 protein eta	0.524615065	0.002259524
Target of rapamycin complex 2 subunit MAPKAP1	0.285172935	0.002259524
Paraneoplastic antigen Ma2	1.218562089	0.002259524
Programmed cell death protein 5	0.448222439	0.003660983
14-3-3 protein gamma	0.416759616	0.003660983
14-3-3 protein epsilon	0.461321787	0.003660983
Prefoldin subunit 5	0.337960002	0.003660983
14-3-3 protein zeta/delta	0.429454041	0.005149274
14-3-3 protein beta/alpha	0.477690711	0.005728556
Stress-70 protein, mitochondrial	0.243862817	0.005728556
Prefoldin subunit 3	0.279340709	0.005728556
Prefoldin subunit 6	0.302385569	0.005939894
Protein SGT1 homolog	0.302369405	0.005939894
Prefoldin subunit 1	0.262463708	0.007283498
14-3-3 protein theta	0.387903005	0.007283498
Dehydrogenase/reductase SDR family member 7	0.33842465	0.007314254
Prenylcysteine oxidase-like	0.292226282	0.00901675
Serine/threonine-protein phosphatase 2A catalytic subunit beta	0.398388783	0.01057576

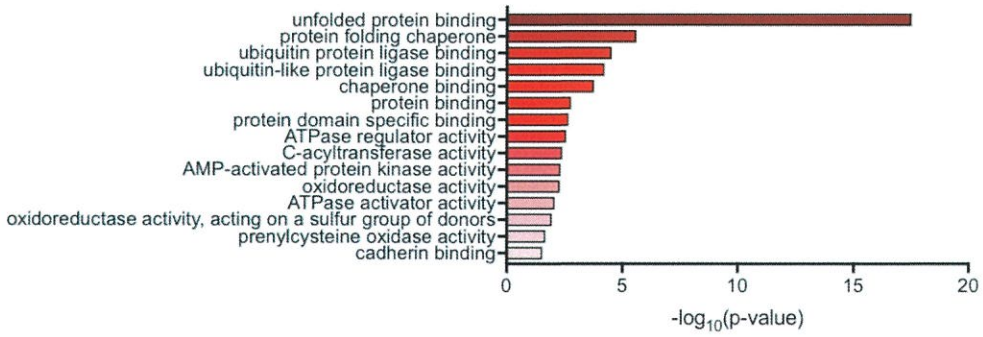


Figure 9:
Top: Identification of Candidate mTORC2 Activators by AE-MS
Bottom: Gene Ontology Analysis of Top Hits

Conclusion

In this study, mTORC2 is expressed at higher levels in HEK293 and U-2OS cells compared to HeLa and A549 cells. During the insulin time course treatment, activation of mTORC2 was observed in A549 and HEK293 cells, whereas HeLa and U-20S cells has no measurable activation. The A549 cells had the most activation at an optimal time of 60 minutes.

FLAG-tagged mTORC2 was pulled down from a HEK293 stable cell line grown in suspension, which illustrated all the core components of mTORC2 and many unidentified proteins.

These unidentified proteins were then processed by Affinity Enrichment Mass Spectrometry (AE-MS). AE-MS identified over 100 proteins as mTORC2 interactors and 20 of those proteins were shown with high statistical value as potential activators of mTORC2.

6 out of the 20 proteins shown with high statistical value were from the 14-3-3 protein family which plays an important role in diverse signaling processes including neuronal development and cell growth. In a previous study, 14-3-3 was said to inhibit the activity of proteins, such as AKT, involved in the signaling pathway, this results in reduced cell proliferation.

Future Works

For future directions, mass spectrometry can be used to identify and compare sites of phosphorylation on mTORC2 in non-stimulated and insulin-stimulated states. Additionally, potential activators will be cloned, expressed, purified, and titrated in *in vitro* kinase assays to quantitatively determine activators potential to activate mTORC2. These potential activators will be characterized and used to assemble an activated complex of mTORC2 for cryo-EM structure determination.

References

1. Sabatini et al. (1994). RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell, Volume 78, Issue 1, Pg 35-43.

2. Oh et al. (2011). mTOR complex 2 signaling and functions. Cell Cycle, Volume 10, Issue 14, Pg 2305-2316.

3. Lou et al. (2018). Weighing In on mTOR Complex 2 Signaling: The Expanding Role in Cell Metabolism. Oxidative Medicine and Cellular Longevity. Article ID 7838647.
4.
5. Gan et al. (2011). Evidence for Direct Activation of mTORC2 Kinase Activity by Phosphatidylinositol 3,4,5 - Trisphosphate. Program in Vascular Biology and Therapeutics.

6. Mohammed et. al (2016). Rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) for analysis of chromatin complexes. Cancer Research UK Cambridge.

7. Saxton et al. (2017). mTOR Signaling in Growth, Metabolism, and Disease. Whitehead Institute for Biomedical Research.

8. Yoon (2017). The Role of Mammalian Target of Rapamycin (mTOR) in Insulin Signaling. Department of Molecular Medicine.

9. Keilhauer et al. (2015). Accurate Protein Complex Retrieval by Affinity Enrichment Mass Spectrometry (AE-MS) Rather than Affinity Purification Mass Spectrometry (AP-MS). The American Society for Biochemistry and Molecular Biology.

10. Jeon et al. (2013). Inhibition of 14-3-3 binding to Rictor of mTORC2 for Akt phosphorylation at Ser473 is regulated by selenoprotein W. Laboratory of Cellular and Molecular Biochemistry.