Analyzing the Interaction Between HRAS, STAT3, and LRPPRC in the Mitochondria

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Personal Section

Like so many others, my life has been impacted in countless ways by cancer. Two of my grandfathers and my first cousin are just a few of my family members who have battled with it. So when I was given the opportunity to work in a cancer research lab, I immediately seized it.

The way I began my research may be different from others. I did not come up with the idea, present it to a scientist, convince them to work with me, and then perform the research. I set out with the goal of working in any type of professional science research lab over the summer between my junior and senior year of high school. I was not picky; I simply wanted the experience that comes with working in a sophisticated environment and wanted to be able to engage in high-quality science research, of any kind. I emailed more than fifty doctors and scientists, and the lab that I ended up working at was solely because one of these doctors finally answered me. I worked at the Philips Cancer Research Lab at NYU Langone, the head of which is Dr. Mark Philips. He invited me to work in his lab and arranged for me to do my work with Dr. Cristina Nuevo Tapioles, a postdoctoral fellow in the lab.

The Philips Lab, among many other things, studies an oncogene called RAS that is implicated in about a third of all human cancers. There are four RAS genes in mammals: HRAS, NRAS, KRAS4A, and KRAS4B. Having an extensive background in researching the mitochondria, Dr. Nuevo Tapioles began an investigation into many different aspects of how the RAS genes interact with the mitochondria. Finding that HRAS was a possible binding partner of LRPPRC, a mitochondrial protein, Dr. Nuevo Tapioles began to study this interaction specifically and its possible consequences. Upon entering the lab, Dr. Nuevo Tapioles explained

all of this to me and proposed that I study the interaction between HRAS, LRPPRC, and another protein called STAT3 and how this interaction affects LRPPRC function and cell proliferation. I embarked on this journey with her help and had to learn many complex scientific techniques along the way, including protein transfection, immunoprecipitation, Western Blotting, Bradford Assays, and more.

Although I did not initially come up with this research idea, I became wholeheartedly invested in it and am so thankful that I had the opportunity to learn about it. Because of this, my advice to high school students attempting to engage in professional research is to not put too much pressure on yourself to be a pioneer! If you are someone who has an idea that you came up with all on your own and want to pursue, that is fantastic and by all means you should. But you should not feel like this is necessary for you to perform research and like if you do not have your own idea this will be a barrier in your research journey. There is just as much to be gained and learned from joining a professional scientist on their research and learning, from an expert in that field, about something you had previously known nothing about. All that you need to complete this type of research is a passion for science and/or mathematics and a willingness to work hard to further this passion. So if you have that, just take a chance and try to pursue research! And don't give up when the first twenty doctors don't answer you :)

Research Section

Abstract:

The RAS genes (KRAS, NRAS, HRAS) are the most frequently mutated oncogenes in human cancer, making up more than 20% of human cancers. HRAS specifically is implicated in cervical, prostate, salivary gland, skin, digestive tract, and urinary tract cancers. It was found to interact with mitochondrial protein LRPPRC, which regulates energy metabolism and

mitochondrial protein expression within the mitochondria, including the expression of mitochondrial subunits MTCO1 and MTCO2. STAT3, a transcription factor and suggested oncogene, has been found to facilitate RAS-driven transformation, aiding cells in becoming cancerous, and has been found to regulate LRPPRC gene expression. This study aims to assess the interactions between these three proteins as well as the consequences of these interactions regarding mitochondrial function and cellular proliferation. HEK293 (human embryonic kidney) shCRL (control) and shLRPPRC (induced LRPPRC knockdown) cells were transfected with GFP-EV, GFP-HRAS, GFP-STAT3, and FLAG-HRAS plasmids and then Co-Immunoprecipitation assays were performed to prove this interaction. Western Blots were then performed with GFP-EV, GFP-HRAS, GFP-STAT3, and GFP-HRAS + GFP-STAT3 conditions to study the levels of mitochondrial subunits MTCO1 and MTCO2, both in control cells and cells with an LRPPRC knockdown after 24 and 48 hours. Lastly, cellular proliferation assays were performed across these same conditions to study cell growth. The study found that HRAS, STAT3, and LRPPRC interact at the same time, proposing the possibility of a trimolecular complex between these proteins. The Western Blots found that the shLRPPRC HRAS conditions causes the levels of mitochondrial subunits to be lowest, indicating that this condition may cause a mitochondrial protein synthesis dysregulation. The proliferation assays found that this same condition after 24h in culture proliferated the most. It is clear that an HRAS overexpression combined with an LRPPRC knockdown is very damaging to cells and their mitochondria. These results can lead to a greater understanding of RAS's role in the mitochondria, possibly leading to anti-cancer therapies in the future.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for nearly one in six deaths, according to the World Health Organization (4). RAS genes (HRAS, NRAS, and KRAS in mammals) are the most frequently mutated oncogenes in human cancer and therefore, are considered one of the most important targets for anticancer therapy. (5). Acting as binary molecular switches, RAS genes alternate between a GDP (off) state and GTP (on) state. When in the GTP state, they transduce signals which regulate cell proliferation, differentiation, survival, and more (1). In the mutated oncogenic form of RAS, it remains solely in the GTP state, sending unwanted signals and causing unwanted processes (11,12). The dysregulation of these processes is considered a part of the hallmarks of cancer, which describe a set of functional capabilities acquired by normal human cells as they make their way to becoming malignant tumors (9). The various forms of RAS all have differing functions; HRAS has been found to be implicated most frequently in cervical, prostate, salivary gland, skin, digestive tract, and urinary tract cancers (12).

Researchers aim to learn as much about RAS as possible by investigating all of the cell processes that RAS is involved in or modulates and finding all of the proteins, enzymes, and other molecules that RAS interacts with because the more knowledge scientists have about it, the easier it may be to target. In a study done by the Philips Lab in 2019, researchers affinity purified proteins that interact with RAS. Among the metabolic proteins identified was mitochondrial leucine-rich PPR motif-containing protein (LRPPRC) (2). Following this experiment, co-immunoprecipitation of endogenous RAS and LRPPRC validated interaction between the two proteins. Additionally, using GFP-tagged RAS proteins and endogenous LRPPRC, Dr. Cristina

Nuevo Tapioles found that the interaction is isoform-specific (HRAS ≫ NRAS > KRAS4A = KRAS4B), strongly suggesting a potential role of HRAS in mitochondrial function (6).

LRPPRC is an RNA-binding protein that localizes to the mitochondrial matrix (7). Mitochondria are key organelles that play an essential role in cell biology and in particular, cell metabolism through their production of ATP (adenosine triphosphate) molecules. Among other characteristics, mitochondria have their own genome known as mitochondrial DNA (mtDNA) that encodes 13 mitochondrial proteins (3). LRPPRC regulates the stability of RNAs encoded by mitochondrial DNA, giving it a modulatory role in energy metabolism. LRPPRC is essential to the function of the mitochondria as prolonged knock-down of LRPPRC has been found to cause mitochondrial fragmentation and decreased ATP levels (13). It also regulates the expression of mitochondrial-encoded genes (7), such as MTCO1 and MTCO2, which are both subunits of the mitochondrial complex IV (13). Because of the mitochondria's role in energy production, over-active mitochondria can lead to extra molecules needed for cellular proliferation, thus aiding malignant transformation (16). Therefore, LRPPRC expression has been found to increase in several forms of human cancer. Similarly, the downregulation of LRPPRC has been linked to growth inhibition and increased apoptosis in cancer cells. It has been theorized to connect oncogenic gene expression with the production of energy, giving it a role in tumorigenesis (13).

The STAT family (signal transducers and activators of the transcription) modulates cellular proliferation, differentiation, survival, and immune function. STAT3, one of the STAT proteins, has been found to be highly involved in human cancer, being a suggested oncogene because its overexpression has led to fibroblast transformation (10). Mitochondrial STAT3 has been reported to facilitate HRAS(G12V)-driven transformation by promoting oxidative phosphorylation (8). Several STAT genes have also been found to regulate LRPPRC gene

expression (7). It was also found to have a metabolic function in the mitochondria, as decreased STAT3 was found to cause decreased ATP levels. Through this role, it increases electron transport chain activity (mitochondrial respiration) (15), and contributes to RAS-dependent cellular transformation — aids cells in their transition to becoming cancerous (8) .

Because of the interconnectedness of HRAS, LRPPRC, and STAT3, and because of their respective roles and prevalence in human cancer, all three of them should be explored further. The ways in which they interact with each other as well as the significance of these interactions is a critical question.

2. Aims

a. To assess the interaction between HRAS, STAT3, and LRPPRC

b. To investigate the effects of this interaction on mitochondrial protein levels

c. To investigate the effects of this interaction on cellular proliferation

3. Methodology

Cell culture

HEK293T cells (kidney carcinoma) were maintained in Dulbecco's Modified Eagle Medium (DMEM) in 37º C and 5% CO2 conditions. A Lentivirus system was used by Dr. Nuevo Tapioles to create shCRL and shLRPPRC cells, the shLRPPRC cells having a knock-down of LRPPRC. Cells were split once they reached 70-80% confluence using Trypsin.

Plasmid Purification

Plasmids were purified in Dr. Philips' Lab prior to experimentation. They were isolated from bacteria using QIAGEN Plasmid *Plus* Midi Kit. Empty Vector (EV) for control containing only GFP (Green Fluorescent Protein), GFP-HRAS, GFP-STAT3, and FLAG-HRAS plasmids were prepared.

Plasmid transfection

Transfection was performed using the indicated plasmids, OPTI-MEM media, P3000 Reagent, and Lipofectamine-3000. The materials were applied in a 1:2:3 ratio – 10 μg of DNA, 20 μL of P3000, and 30 μL of Lipofectamine (per condition). Transfection was performed in 24-well plates for the cellular proliferation assay, 6-well plates for the Western Blots, and 10 cm^2 plates for the co-immunoprecipitation assays. Cells were collected 24 and 48 hours after transfection.

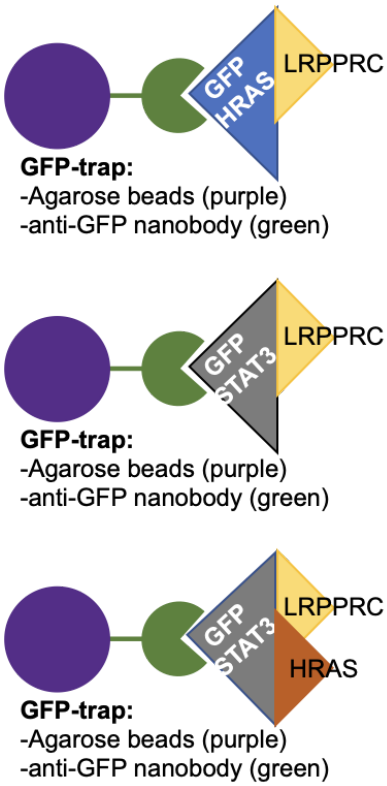
Western Blotting

Cells were incubated with a Lysis buffer containing protease and phosphatase inhibitors at 4ºC for 15 minutes and then centrifuged for 15 minutes at 4ºC. The supernatant was then collected and protein concentration was measured using a Bradford Assay (described in detail later in paper).

2x Laemmli buffer was applied to cells, and then 30 μg of protein per cell line was loaded into 4-20% 10-well polyacrylamide gel. 5 μL of a protein ladder was loaded, and then SDS-PAGE was used to separate proteins according to their molecular weight. The proteins in the gel were then transferred onto a nitrocellulose membrane using a Bio-Rad Trans-Blot Turbo Transfer System and then incubated with a Blocking Buffer to avoid nonspecific interactions. They were then incubated with primary antibodies for LRPPRC, STAT3, HRAS, and mitochondrial subunits MTCO1 and MTCO2, and then with secondary antibodies (anti-mouse or anti-rabbit) containing fluorophores to bind to the primary antibodies.

The membrane was then scanned using a Li-Cor Odyssey infrared scanner and the bands for each protein were detected and quantified. The membrane was then scanned again after being incubated with Tubulin antibodies and each protein quantification was normalized.

Co-Immunoprecipitation



Cells for the immunoprecipitation were transfected with GFP-EV, GFP-HRAS, GFP-STAT3, and FLAG-HRAS and GFP-STAT3 plasmids. Transfection was confirmed using a microscope to ensure that the cells were green and had successfully been transfected with the GFP-tagged proteins. They were then incubated in lysis buffer and spun and centrifuged for 15 minutes each at 4ºC. 50 µL of each condition was separated for the input, and 950 µL was prepared for the immunoprecipitation. 10 µL of the GFP-TRAP, composed of agarose beads and an anti-GFP nanobody (See Figure), was applied to each IP condition. The IP conditions were then washed with lysis buffer several times and then left to incubate in 100 µL of lysis/condition overnight to immunocapture the GFP-overexpressing proteins.

The cells were then given 2x Laemmli buffer and boiled. Western Blot protocol was then followed, with the membrane being incubated with anti-GFP antibodies and then scanned to detect the interactions between the GFP-tagged proteins and LRPPRC.

Cellular proliferation assay

shCRL and shLRPPRC cells were collected 24h and 48h after transfection with GFP-EV, GFP-HRAS, GFP-STAT3, and GFP-HRAS GFP-STAT3 plasmids. DMEM media was removed and cells were washed with phosphate-buffered saline (PBS), and then removed using Trypsin. Cells were then put into 200 µL tubes and cell count was measured using Vi-CELL BLU Cell

Viability Analyzer. Three tubes for each condition were used to standardize the data and the mean and standard deviation for each group were then calculated.

4. Results

LRPPRC interacts with HRAS and STAT3 suggesting a trimolecular complex.

In order to validate the possible interaction between LRPPRC, HRAS and STAT3, I performed co-immunoprecipitation assays using FLAG- and GFP-tagged overexpressed proteins in HEK293 cells. The results of the immunoprecipitation are shown below (Figure 1), the left panel being the Input (cell lysates before they were immunoprecipitated with a GFP-trap), and the right panel being the IP (cell lysates after they were immunoprecipitated with the GFP-trap). Tubulin was used as a loading control on the bottom left (Figure 1). The input is used to confirm that the transfection has worked, as seen in the overexpression of the correct proteins in their respective conditions, and also to show that endogenous LRPPRC is present in the cells.

In the right panel, the LRPPRC found in columns 2 and 3 shows that LRPPRC interacts with HRAS and STAT3 (Figure 1). When the cell lysates were incubated with the GFP-trap, it pulled down the protein tagged with GFP (either HRAS or STAT3), as well as any proteins interacting with the immunocaptured protein. So for example, if LRPPRC was interacting with HRAS, it would be found in the HRAS-GFP cells. As shown in column 1, an empty vector expressing only GFP is used as a Co-IP control. Because LRPPRC is not present in column 1, it can be concluded that there are no nonspecific interactions (due to the errors in the methodology or the expression of GFP), thus validating the experiment. Because LRPPRC is present in columns 2 and 3 but not column 1, we can conclude that LRPPRC interacts with HRAS and STAT3, respectively (Figure 1).

Column 4 shows an important finding. This condition was transfected with both GFP-STAT3 and FLAG-HRAS plasmids. STAT3 and HRAS were tagged with different proteins so that both could be seen in the IP (if they were both tagged with GFP they would not be distinguishable from each other) (Figure 1). In column 4, after pulling down GFP-STAT3, both FLAG-HRAS and LRPPRC are visible, indicating the interaction between the proteins. The fact that all three of these proteins are present means that not only does LRPPRC interact with HRAS and STAT3, but all three of them might be interacting at the same time, suggesting the presence of a trimolecular complex. Whether this interaction is just STAT3 interacting with HRAS and also interacting with LRPPRC or if there is in fact a trimolecular complex present must be investigated with future research.

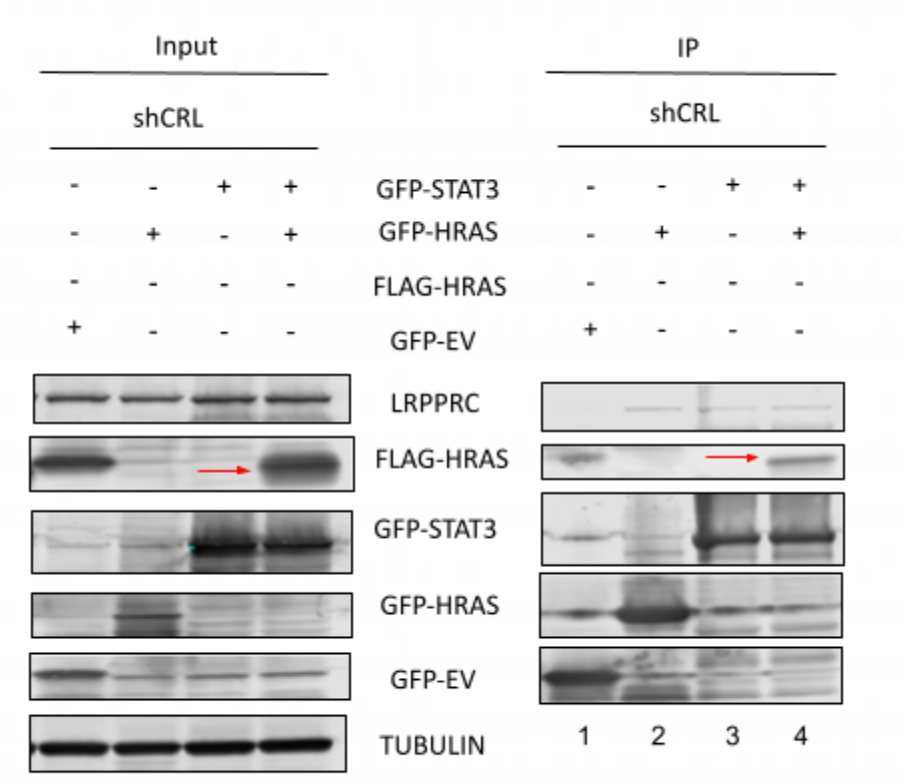


Figure 1: The indicated plasmids were transfected in HEK293 cells. Protein expression before performing the immunoprecipitation is shown in the input. Tubulin is shown as loading control (left panel). GFP-tagged proteins were immunoprecipitated showing the interaction between GFP-HRAS and LRPPRC and GFP-STAT3 and LRPPRC. In addition, the double interaction between GFP-STAT3 with LRPPRC and FLAG-HRAS (red arrows) suggest the presence of a trimolecular complex (right panel).

Using the Bradford protein assay to calculate protein concentration

When performing a Western Blot, knowing the protein concentration of the samples is an integral step to efficiently compare different conditions. In order to assess protein concentration, I implemented the Bradford protein assay, a dye-binding assay based on the differential color changes of a dye in response to various concentrations of protein. A standard curve using known increasing concentrations of a protein such as Bovine Serum Albumin (BSA) is used to convert the values of absorbance into protein concentration. The protein concentrations of different cell lines are different, so they must be calculated in order to ensure that the same amount of protein per condition is being loaded into the gel to perform the electrophoresis. To perform a Bradford Assay, increasing amounts of BSA are applied to 50 µL of Bradford Reagent combined with 200 µL of water to create a standard curve. The absorbance of each BSA concentration is measured and then a standard curve, like the one below, is created (Figure 2).

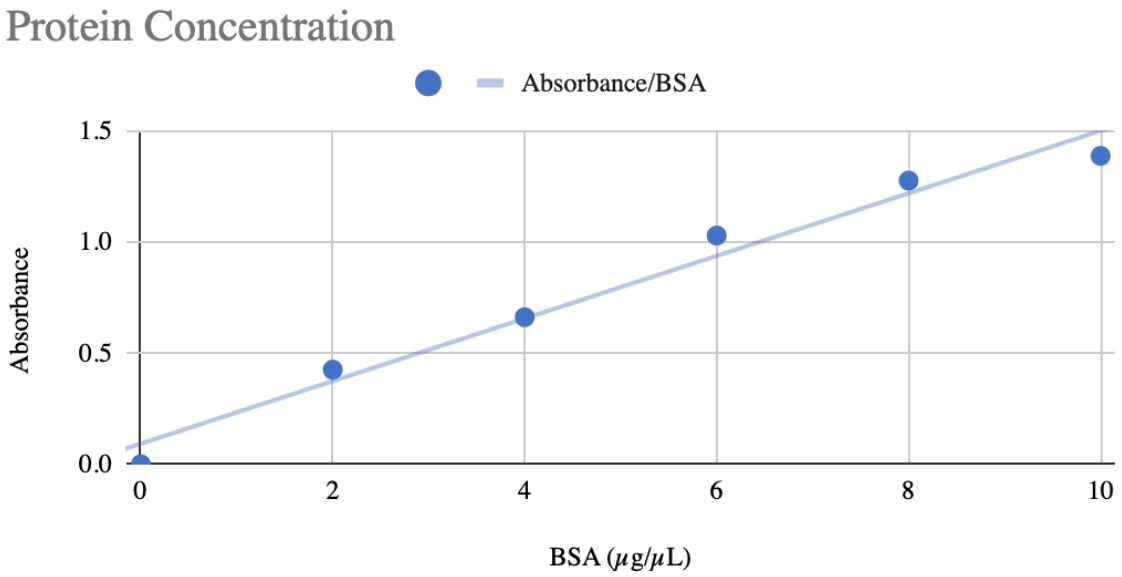


Figure 2. Example Bradford Assay standard curve to show process of calculating protein concentration. Known increasing concentrations of Bovine Serum Albumin (0, 2, 4, 6, 8, 10 µL) are used to create a standard curve.

On the X axis is the µg/µL of BSA added to the Reagent, and on the Y axis is the darkness — absorbance — of each amount combined with the Reagent. After the slope of this

trendline is calculated, using the slope formula, with y equal to the µg of that condition added and m equal to the slope, one can solve for the protein concentration (x) of any condition, after measuring the absorbance of the samples. Once the protein concentration is calculated, an amount of protein (for this experiment 30 µg) is chosen to be loaded into the gel. 30 is then divided by the protein concentration, and the resulting amount of µL of each condition is loaded into the gel. This method was solely used to perform Western blot analysis of protein expression.

Studying the role of HRAS and STAT3 on LRPPRC function

In order to further investigate whether the interaction between LRPPRC, HRAS, and STAT3 has a role in modulating LRPPRC function, I used the model of HEK293 cells with (shCRL) or without (shLRPPRC) LRPPRC to assess the expression of MTCO1 and MTCO2. The expression of MTCO1 and MTCO2 can be directly linked to LRPPRC function, as LRPPRC regulates the expression of mitochondrial subunits. Attempting to explore this, I transfected the cells with GFP-HRAS, GFP-STAT3 or a GFP-empty vector, collected the cells after 24 and 48h, and tested whether the expression of MTCO1 and MTCO2 had changed under the different conditions using Western Blots.

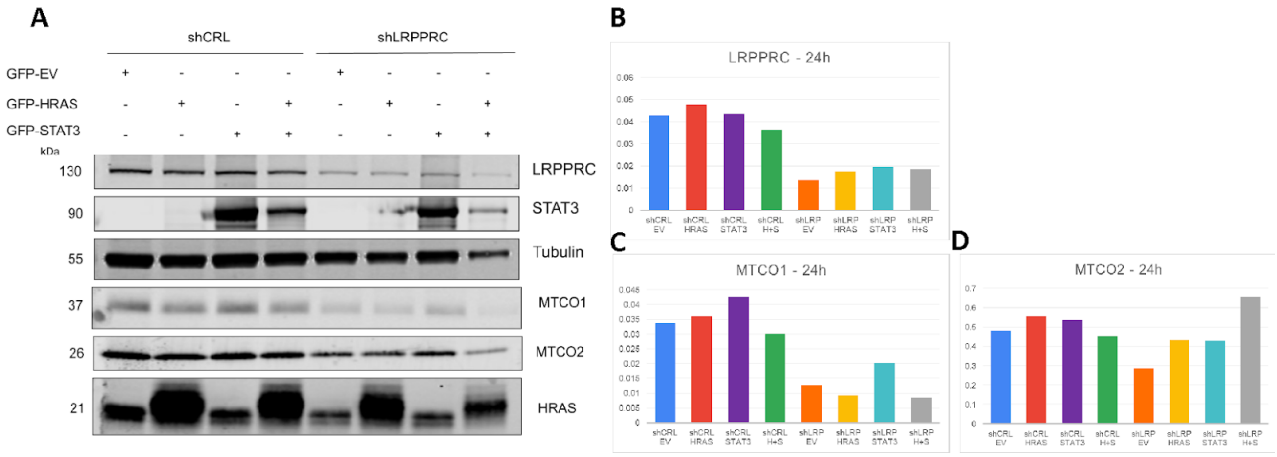


Figure 3A. Western Blot analysis of the expression of LRPPRC, MTCO1 and MTCO2 in HEK293 shCRL and shLRPPRC cells transfected with GFP-EV GFP-HRAS, GFP-STAT3, and GFP-HRAS GFP-STAT3 plasmids (left to right) after 24h. Tubulin used as a loading control (3rd row). **B-D.** Histograms showing the quantification of the expression of LRPPRC (B), MTCO1 (C) and MTCO2 (D) normalized by Tubulin expression.

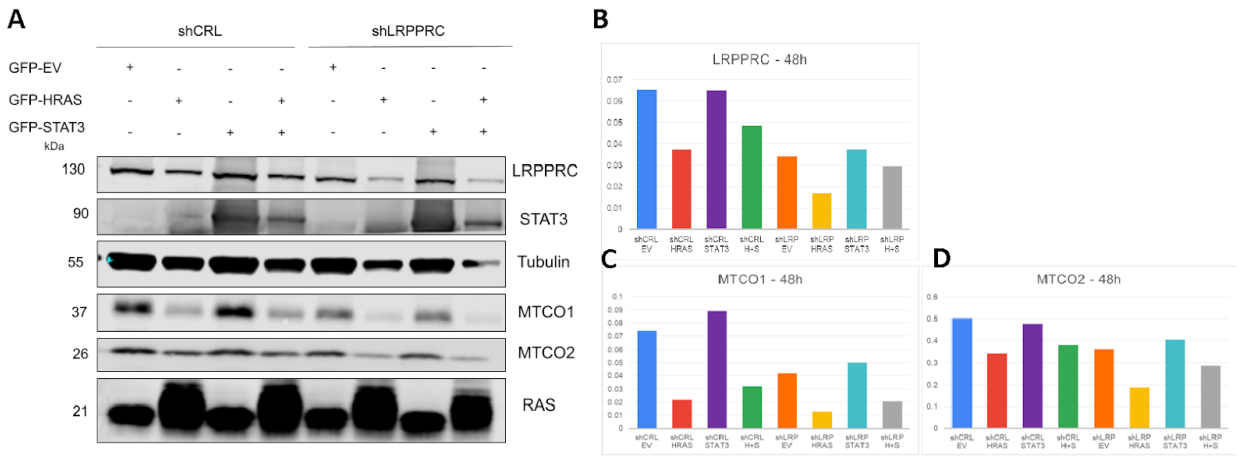


Figure 4. A. Western Blot analysis of the expression of LRPPRC, MTCO1 and MTCO2 in HEK293 shCRL and shLRPPRC cells transfected with GFP-EV GFP-HRAS, GFP-STAT3, and GFP-HRAS GFP-STAT3 plasmids (left to right) after 48h. Tubulin used as a loading control (3rd row). **B-D.** Histograms showing the quantification of the expression of LRPPRC (B), MTCO1 (C) and MTCO2 (D) normalized by tubulin expression.

The intensity of the bands in the Western Blots were quantified using a Li-Cor Odyssey infrared scanner; the darker the band the higher the protein expression is. Tubulin was used as a loading control — if there are unequal amounts of protein loaded into the gel, there will be unequal amounts of Tubulin shown in the Western Blot as it is considered a housekeeping gene (essential for the function of the cell, therefore it can be concluded it will be present in all cells) and widely used as a loading control. Therefore, by dividing each condition by the amount of Tubulin in that condition, the values can be normalized. From these results, it is first clear that for the most part, LRPPRC is regulating the expression of MTCO1 and MTCO2. This is shown through the expression of MTCO1 and MTCO2 being similar to the expression of LRPPRC in both blots. Although there are some variations in the expression pattern, when LRPPRC expression is higher, MTCO1 and MTCO2 are also generally higher and vice versa. These results validate our model demonstrating that the expression of both MTCO1 (Figure 3C,4C) and MTCO2 (Figure 3D,4D) is directly linked to the expression of LRPPRC (Figure 3B,4B), as previously shown in the literature (7).

After overexpressing HRAS or STAT3 alone or in combination, we can observe that excluding Figure 3D, the remaining expression of the mitochondrial subunits show a similar pattern — with a knockdown of LRPPRC and an overexpression of HRAS, the expression of the proteins are the lowest (yellow bar, Figure 3C and 4C,D). Furthermore, the overexpression of both HRAS and STAT3 showed similar results (grey bar, 3C and 4C,D) indicating that a knockdown of LRPPRC and overexpression of HRAS (even when combined with an overexpression of STAT3) may be the most harmful of these conditions to mitochondrial protein levels. Additionally, the overexpression of STAT3 alone did not show any effect on the expression of MTCO1 and MTCO2 (light blue bar, Figure 3C,D and 4C,D) discarding any role of STAT3 in LRPPRC function. This suggests that this combination of low LRPPRC levels and high HRAS levels may cause a mitochondrial protein synthesis dysregulation, suggesting that HRAS is playing a detrimental role in mitochondrial function when LRPPRC is not completely functional. However, future experiments are needed to further validate these findings and understand the underlying mechanism of the role of HRAS in LRPRPC function.

Studying the role of HRAS and STAT3 in cellular proliferation

Mitochondria are the powerhouse of the cell and therefore, a key component of cellular growth. After assessing the role of HRAS in LRPPRC function and thus mitochondrial subunit expression, we questioned whether the differential expression of MTCO1 and MTCO2 caused by HRAS could modulate cellular proliferation. With this aim, I transfected shCRL and shLRPPRC HEK293 cells with an empty vector, HRAS, STAT3, and a combination of both. Cell number was counted using a Vi-CELL BLU Cell Viability Analyzer 24h and 48h after transfection. Figure 5A is an overall mean cell count after incubation with the plasmids for 24 hours. Figures 5B and 5C show cell numbers normalized to the Empty Vector cell number to better illustrate

differences in proliferation. The Empty Vector cell count for both the shCRL and shLRPPRC cells was set to 1, and corresponding cell numbers in each condition were divided by the Empty Vector cell count (standard deviation was normalized to the standard deviation of the EV as well). An Analysis of Variance (ANOVA) was performed using this data by comparing each condition with its respective control (ex: shCRL HRAS was compared to shCRL EV and shLRPPRC HRAS was compared to shLRPPRC EV). This analysis showed that only shLRPPRC HRAS and shLRPPRC HRAS+STAT3 proliferation was significantly increased when compared to the control ($p=0.0141$; yellow bar, $p=0.02322$; grey bar). Interestingly, the shCRL HRAS+STAT3 ($p=0.05991$), as well as the shLRPPRC STAT3 proliferation ($p=0.06024$) did not reach significance, but were trending towards it. Based on this data, it seems that the conditions with a knockdown of LRPPRC and an overexpression of HRAS (or a combination of HRAS and STAT3) have the highest cell counts, therefore the highest proliferation rate (yellow and grey bars). Again, no differences were observed when overexpressing STAT3 alone, suggesting that only HRAS can modulate cellular proliferation in the absence of LRPPRC.

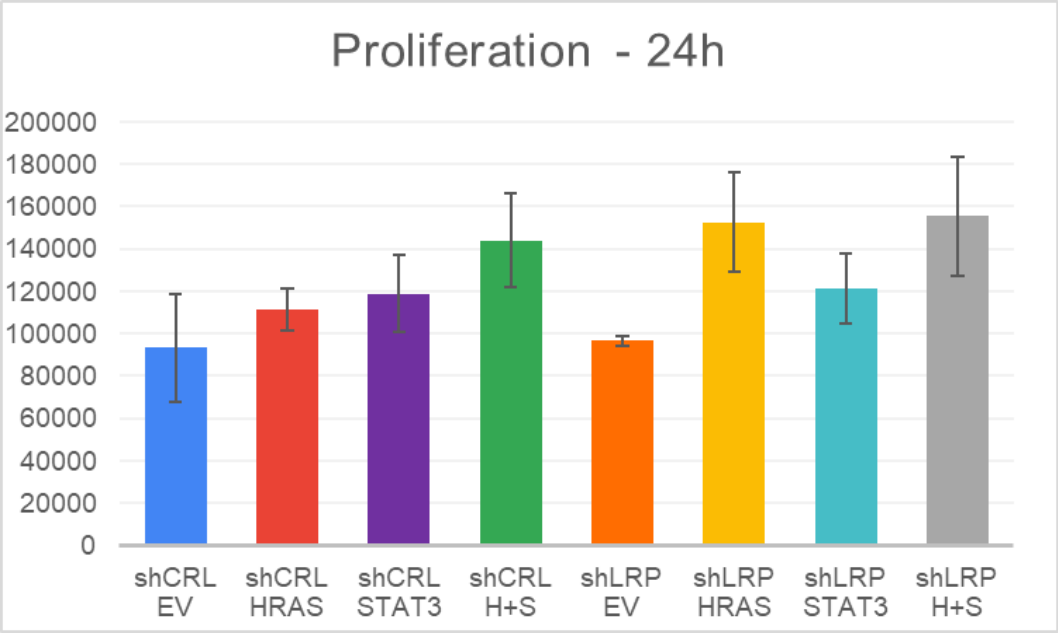


Figure 5A. Cellular proliferation increases with application of HRAS most in shLRPPRC cells. shCRL and shLRPPRC cells transfected with indicated plasmids and incubated with plasmids for 24h.

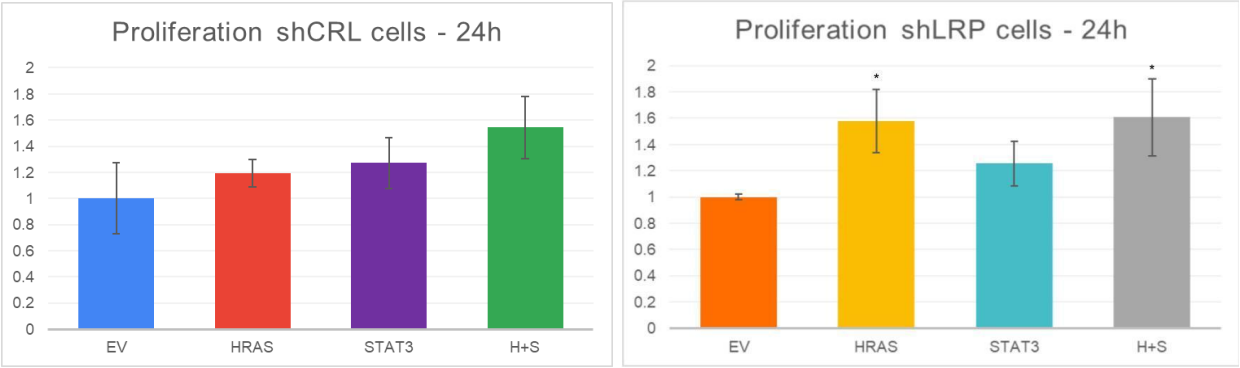


Figure 5B. Mean cell count of shCRL cells (24h) normalized to control (EV) proliferation normalized to control (EV) proliferation

Figure 5C. Mean cell count of shLRPPRC cells (24h) control (EV) proliferation normalized to control (EV) proliferation

Effects of HRAS, STAT3, and HRAS + STAT3 on cellular proliferation after 48h incubation with plasmids

Figure 6A is a total cell count with the same conditions after a 48-hour incubation period with the plasmids. Figures 6B and 6C have been normalized in the same way as Figures 5B and 5C (the 24h proliferation results). An Analysis of Variance (ANOVA) was performed for these results as well, and all six conditions were significant (p=0.00061; red bar, p=0.03707; purple bar, p=0.00083; green bar, p=0.00187; yellow bar, p=0.0073; blue bar, and p=0.00079; grey bar). These results show the same pattern as the 24h results, that HRAS and HRAS+STAT3 conditions have the highest proliferation rates. This finding, however, was not specific to an LRPPRC knockdown after 48h in culture — the shCRL HRAS and shCRL HRAS+STAT3 bars (red and green bars) are equally as high as the shLRPPRC HRAS and shLRPPRC HRAS+STAT3 bars (yellow and grey bars), meaning that for the 48h cells, the shCRL and shLRPPRC cells showed little difference in proliferation. No conclusions can be definitively drawn at this point regarding the effects of an LRPPRC knockdown combined with overexpression of HRAS or STAT3 because for the 24h cells, the shCRL conditions were not significant, and for the 48h cells, the

shCRL and shLRPPRC conditions showed relatively equal proliferation. Nevertheless, we hypothesize that the effect observed in proliferation is specific to LRPPRC knockdown cells after 24h and this might be lost when proliferation is assessed after 24h. Future experiments repeating the conditions with these and other cell lines are needed to validate and better understand this hypothesis.

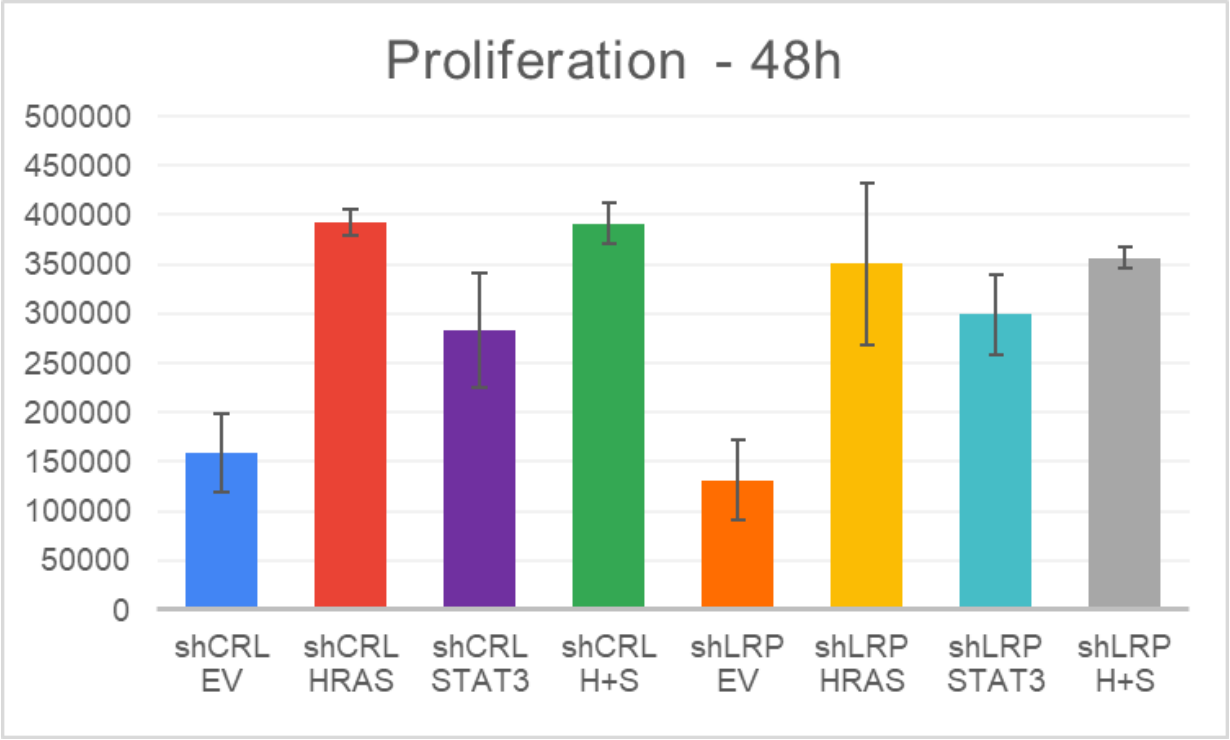


Figure 6A. Cellular proliferation increases with application of HRAS in both shCRL and shLRPPRC cells. shCRL and shLRPPRC cells transfected with indicated plasmids and incubated with plasmids for 48h.

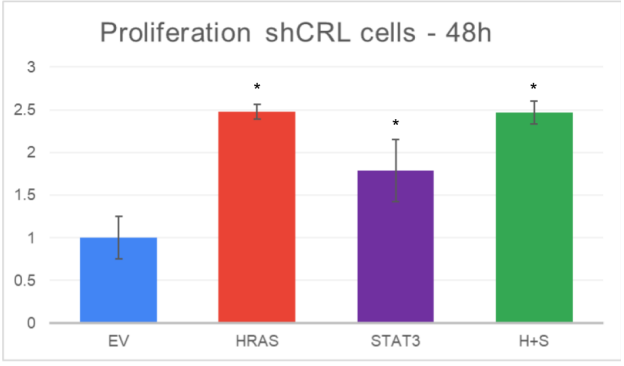


Figure 6B. Mean cell count of shCRL cells (48h) normalized to control (EV) condition

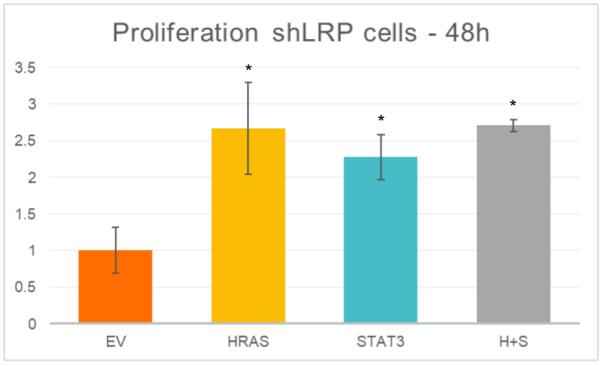


Figure 6C. Mean cell count of shLRPPRC cells (48h) normalized to control (EV) condition

5. Conclusion

Although many of these findings require future research for confirmation, several conclusions can be drawn from this study. The double interaction between STAT3, HRAS, and LRPPRC shown by the Co-Immunoprecipitation represents the most clear findings of this study, and is a novel finding in this field. A better understanding of this interaction may reveal new modes of anti-cancer therapy. It is also clear from the Western Blots that an overexpression of HRAS and a knock-down of LRPPRC are the most harmful conditions for the mitochondria. First validating this finding with future experiments and then exploring it may lead to a better understanding of how the interaction between HRAS and LRPPRC specifically impacts the mitochondria, and the specific mechanisms by which it does so. With this known, cancer researchers may be able to target this interaction therapeutically. The results regarding cellular proliferation must be explored further, but understanding why the overexpressed HRAS cells proliferated the most in the absence of LRPPRC after 24h in culture can also lead to new therapeutic approaches, as increased cellular proliferation is clearly a marking of cancer.

The results showing that an overexpression of HRAS combined with an LRPPRC knockdown cause both decreased mitochondrial function, shown through decreased expression of MTCO1 and 2, as well as increased proliferation, pose several questions. It would normally be assumed that if mitochondrial function is decreased, because of the mitochondria's role in energy production, proliferation would as well be decreased. A possible explanation for this occurrence could be the emergence of glycolytic flux, an energy production process that is often triggered when cellular homeostasis is broken (which it would be by the lost function of the mitochondria). Glycolytic flux would explain increased proliferation as it is a metabolic pathway that causes increased energy production. Stimulation of RAS has been found to enhance glycolytic flux, so it

is probable that the shLRPPRC HRAS cells would be experiencing it (14). An investigation into whether glycolytic flux is occurring and if so, in which cell conditions, could also in the future lead to new modes of therapy

6. Future Research

Although the set of experiments presented in this study should be repeated and further validated to confirm the results, future experiments can also be suggested to completely understand the biological consequences of the interaction between HRAS and the mitochondrial protein LRPPRC. Following the repetition of these experiments, the consequences of this interaction on other mitochondrial processes, such as ATP production and mitophagy, should be tested to gain a better understanding of how this interaction affects the mitochondria. The effects that this interaction has on other proteins coded by the mitochondrial genome, as well as whether HRAS or STAT3 interacts with any of these proteins, should be also addressed to understand whether this interaction can modulate the expression of other mitochondrial complexes. The different forms of RAS (i.e. wild type and G12V mutated) can also be compared as to how they interact with LRPPRC and STAT3 to better understand their role in cancer biology. Lastly, how STAT3 and HRAS interact in the absence of LRPPRC should be studied, as this was shown in the Co-Immunoprecipitation as well as the double interaction. This would validate our hypothesis of LRPPRC-STAT3-HRAS forming a trimolecular complex. To pursue this aim, Co-Immunoprecipitation assays should be repeated using the cells that do not express LRPPRC (shLRPPRC). All of these experiments could lead to a better understanding of the impacts RAS has on the mitochondria, potentially opening up the door for many modes of therapy in the future not only in cancer, but also in mitochondrial related diseases.

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