Validation of interaction between proteins and monoacylglycerol

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by

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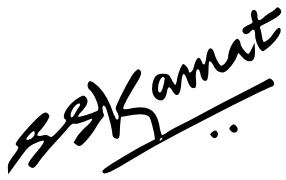
Certificate

This is to certify that this dissertation entitled **Validation of interaction between proteins and monoacylglycerol** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Anisha Rai (20181061)** at **Indian Institute of Science Education and Research** under the supervision of **Dr Siddhesh S. Kamat, Associate Professor, Department of Biology**, during the academic year **2022-2023**.



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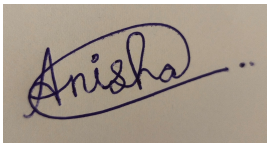
This thesis is dedicated to my five years at IISER Pune.

Declaration

I hereby declare that the matter embodied in the report entitled **Validation of interaction between proteins and monoacylglycerol** are the results of the work carried out by me at the **Department of Biology, Indian Institute of Science Education and Research, Pune**, under the supervision of **Dr Siddhesh S. Kamat** and the same has not been submitted elsewhere for any other degree.



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Contributions

Contributor name	Contributor role
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This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Abstract

Monoacylglycerols are lipids which are known to interact with different receptors highlighting their role in signalling pathways. These lipid-protein interactions provide an interesting insight into the possible role of monoacylglycerols in treating various diseases, as they have shown promising results in regulating insulin secretion and energy homeostasis. The undiscovered interaction of monoacylglycerols with different proteins provides a similar possibility and scope for further research. To find novel protein interactors of monoacylglycerols, proteomic experiments using bifunctional lipid probes were performed, which resulted in a set of putative protein interactors of these lipids. We initially conducted a literature survey of the proteins and performed preliminary experiments to validate these lipid-protein interactions. This involved preparing constructs of the proteins and expressing them in mammalian cells. The results of my study can serve as a starting point for developing biochemical assays to corroborate the interactions between proteins and monoacylglycerol. Further experiments can be designed using these protein constructs, which could highlight the exciting roles of monoacylglycerols in different biological pathways.

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Chapter 1: Introduction

Lipids are an important class of biological compounds primarily recognised for their function as structural elements of cell membranes. In addition, they serve as the body's energy reserves and are crucial for the insulation and protection of internal organs. Although lipids have various significant functions, their role as signalling molecules in biochemical pathways that affect many downstream processes is what attracts the majority of research attention. This is for a good reason, as lipids are unique among the four major classes of biomolecules; most of the lipid secondary messengers are formed due to the hydrolysis of various lipids in response to extracellular signals (Wymann and Schneiter 2008). Hence, they are not always present in the cells and are only synthesised on demand.

Examining some of the well-explored lipids performing this role can help us understand how they affect signalling pathways. Sphingosine-1-phosphate is a major signalling lipid discovered as an agonist of the G-protein coupled receptor and is mainly involved in vascular development and immune cell trafficking (Pyne and Pyne 2000). Today, it is being investigated for its possible use as a drug molecule in cancer treatment (Wang et al. 2008). Inositol trisphosphate is a known secondary messenger that rose to fame in the 1970s when it was discovered as a hydrolysis product of phosphatidylinositol 4,5 bisphosphate diacylglycerol (Michell 1975). With time scientists working on this compound proved that inositol trisphosphate was mainly involved in increasing intracellular calcium levels, which was necessary for various downstream signalling processes such as carbohydrate metabolism, membrane trafficking, cell division and synaptic transmission (Foskett et al. 2007). These are just a few illustrations of lipids' critical roles in signalling networks and how, over time, study on these molecules has helped us map crucial biological pathways. Several novel secondary messengers have emerged from the literature on lipids; their functions have yet to be thoroughly investigated, but identifying their roles in various signalling cascades has brought them to the forefront. One such class of lipids recently explored for controlling the insulin secretion pathway is monoacylglycerols.

Monoacylglycerols

Monoacylglycerols (MAGs) lipids consist of a glycerol molecule where one of the hydroxyl groups is replaced by a fatty acid via an ester linkage. When we ponder the functions of lipids, we usually associate them with energy storage and the formation of cell membranes. However, the above examples indicate how lipids are involved in other crucial biological functions, such as intracellular signalling and hormone regulation. The lipid in consideration, MAG, is no stranger to this role.

Research on MAGs gained widespread attention with the discovery of 2-arachidonoyl glycerol (2-AG) acting as an endocannabinoid, i.e. an agonist for the cannabinoid receptors (Hanus 2007). Cannabinoid receptors are widely investigated due to their roles in different physiological processes like regulation of appetite, impact on anxiety and depression, pain sensation and control of immune responses (Mackie 2008; Kendall and Yudowski 2016). Due to these reasons, research on cannabinoid receptors along with their agonists escalated, leading to a plethora of literature on the endocannabinoid signalling system. Though this provided valuable results, it also created a bottleneck for monoacylglycerol research, as most studies mainly focused on the role of 2-AG as an endocannabinoid. However, the past decade has seen researchers exploring other possible interactors of MAGs, which has led to the discovery of new receptors for this lipid.

The biosynthesis of MAGs is a classic example of lipolysis where hydrolysis of triacylglycerols (TAGs) is the primary route for its formation. During hydrolysis of TAG in cells, the enzyme adipose triglyceride lipase converts the TAG into 2,3-diacylglycerol or 1,3-diacylglycerol along with free fatty acid (Zimmermann et al. 2004). The formation of a specific isomer depends on the presence or absence of the adipose triglyceride lipase activator CG158; in the presence of the activator, 2,3 diacylglycerol is formed, while its absence leads to the formation of 1,3 diacylglycerol (Eichmann et al. 2012). Further hydrolysis of diacylglycerol is performed by hormone-sensitive lipase, which forms MAG and fatty acid (Schweiger et al. 2006). Though hydrolysis of TAG is the most common method for creating MAGs, other routes are also present for its biosynthesis, like hydrolysis of phospholipids by phospholipase C to form diacylglycerol.

Understanding the biosynthesis of MAGs allows one to appreciate the processes involved in forming this crucial lipid. Since the discovery of endocannabinoids, significant attention has been given to the enzymes involved in the degradation of MAG. This is necessary since regulating the levels of MAGs in cells can significantly impact several diseases due to its role as an agonist for various receptors. Following this trend, we will explore different enzymes in literature involved in MAG degradation.

The most popular and well-researched enzyme known for the degradation of MAGs is monoacylglycerol lipase (MAGL). MAGL is an intracellular serine hydrolase primarily localised in the brain, liver, adipose tissue, and intestine (Scalvini, Piomelli, and Mor 2016). The discovery of MAGL dates back to 1963 when researchers working on intestinal cells identified an enzyme involved in the hydrolysis of MAG (Senior and Isselbacher 1963). Though MAGL was discovered in the 1960s, it was only in the early 2000s that this enzyme gained widespread popularity due to its relation with endocannabinoids. In 2002, researchers investigating possible enzymes for the degradation of 2-AG recognised MAGL as an effective enzyme for this purpose (Dinh et al. 2002).

Experiments indicated that the inactivation of MAGL resulted in increased levels of 2-AG, thus decreasing the levels of arachidonic acid (AA), a hydrolysis product of 2-AG. AA is known for its interaction with different types of oxygenases; hence, reduced AA levels can decrease levels of inflammatory bioactive lipids required for neuroinflammation (Tallima and El Ridi 2018). In 2010, researchers showed the increased expression of MAGL in cancer cells where the entire signalling lipid network modulated by MAGL resulted in increased tumour growth and aggravated the disease conditions (Nomura et al. 2010). This led to inhibitor-based studies where experimental results implicated that changes in free fatty acids and other downstream lipid metabolites due to MAGL activity were associated with cancer in human cells (Deng and Li 2020). These results highlighted the role of MAGL in lipid signalling, leading to widespread research on these molecules.

Apart from MAGL, the lipases α-β-hydrolase domain 6 (ABHD6) and α-β-hydrolase domain 12 (ABHD12) are involved in the hydrolysis of MAGs (Blankman, Simon, and Cravatt 2007). Though the initial focus of ABHD6 hydrolysis activity was on 2-AG, in recent years, it has been established that ABHD6 can regulate a wide variety of lipids (Cao, Kaplan, and Stella 2019). It is involved in the hydrolysis of 1-AG, 2-AG, 2-lauroyl glycerol, and multichain lipids like lysophosphatidic acid and lysophosphatidyl choline (Lord, Thomas, and Brown 2013).

In contrast, 2-AG is the only recognised MAG substrate for ABHD12, linking it to the endocannabinoid system (Navia-Paldanius, Savinainen, and Laitinen 2012). An interesting point to note is that ABHD6 and ABHD12 are membrane-bound enzymes with an active site towards the intracellular surface, while MAGL is primarily present in the cytosol (Murillo-Rodriguez 2017). This indicates a possible distinction between the roles of these enzymes, with MAGL mainly hydrolysing MAGs present near the cytosol while ABHD6 and ABHD12 hydrolysing intracellular MAGs.

The above discussion provides insight into the biosynthesis and degradation of MAGs along with an introduction to their most well-known signalling molecule 2-AG whose discovery as an endocannabinoid resulted in its widespread research. However, there is more to the story of MAG than its role as an endocannabinoid. Initially, MAGs were only considered as byproducts of triacylglycerols with no significant signalling function. When this changed with the discovery of 2-AG, it also propelled MAG research in a different direction, where the past decade has seen novel signalling functions of MAGs being discovered, opening up exciting possibilities for research.

This started in 2011 when researchers working with GPR 119, a G-protein coupled receptor, performed a study on three different MAGs, 2-oleoyl glycerol (2-OG), 2-palmitoyl glycerol (2-PG) and 2-linoleoyl glycerol (2-LG), and established the interaction of these lipids with the GPR119 receptor (Hansen et al. 2011). The results indicated that 2-OG significantly activated the receptor compared to other MAGs, causing the intestinal cells to release glucagon-like peptide-1, a compound involved in maintaining glucose and insulin levels. This was the first time a study had indicated any role of MAGs in regulating blood sugar and insulin, highlighting a new signalling function of these lipids.

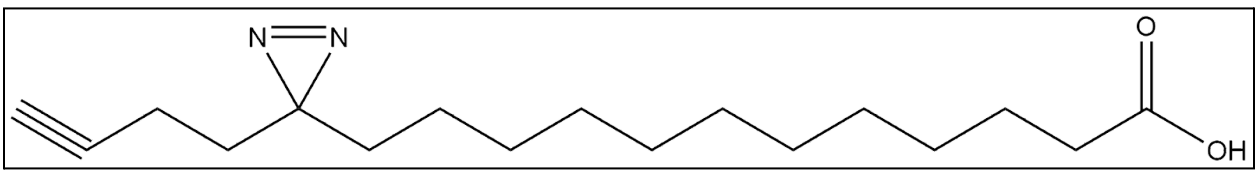
This was supported by another study which showed that high glucose levels promote activation of the Munc13-1 receptor by MAG, which results in the translocation of the receptor to the plasma membrane, where it modulates the priming of insulin granules for exocytosis (Zhao et al. 2014). These examples indicate that MAGs might have a role in regulating glucose-stimulated insulin secretion, and further research is required to gain a comprehensive understanding of this process.

This raises the question of the other signalling pathways in which MAGs might be involved and how we can explore such interactions. One way to achieve this is to map out the interactions between MAGs and different proteins and determine which proteins act as possible interactors of these lipids. The protein's function in a particular biochemical pathway will indicate the potential role of MAG and its place in the scheme of things.

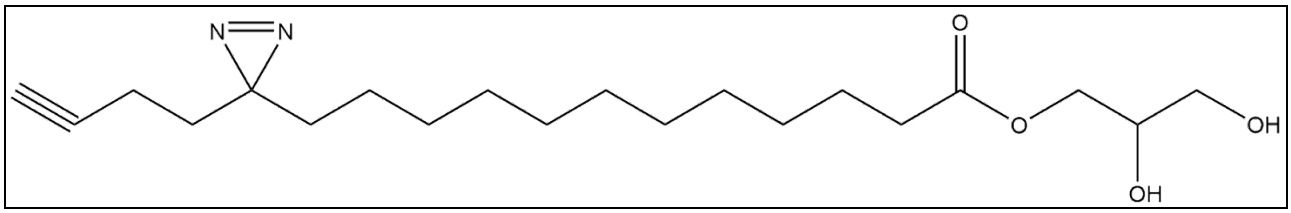
Chapter 2: Literature Review

The previous chapter provided a brief overview of MAGs and their possible role in signalling pathways. Building on this, we wanted to explore novel interactions of MAGs and proteins in the hopes that we might uncover a significant lipid-protein interaction involved in signalling functions. To achieve this goal, proteomic screening using bifunctional lipid probes of MAG was performed previously in the lab, which resulted in a set of putative protein interactors of MAGs. These proteins now had to be validated for their interactions with MAGs, forming the basis of this project. Before we move ahead and discuss these proteins in detail, a brief understanding of the bifunctional lipid probes and the experiments performed using these probes are required.

Bifunctional lipid probes have two major modifications introduced in the general structure of the lipid - a photoreactive group for crosslinking and a bioorthogonal handle attached to a reporter tag. The probes used for the initial experiments had a diazirine group acting as a photoreactive group and an alkyne handle which reacted with the azide group present on the reporter tag via CLICK chemistry. For in-gel fluorescence, rhodamine-azide was used as a reporter tag, while for mass spectrometry, biotin-azide was used. Two different probes were used for the experiments - a palmitic acid diazirine probe (PA-DA) and a palmitoyl glycerol diazirine probe (PG-DA). The main difference between these two probes was the lipid backbone, where the PA-DA probe is just fatty acid, while the PG-DA probe had a glycerol headgroup attached to the PA-DA probe backbone.



(A) PA-DA probe



(B) PG-DA probe

Fig 2.1: Parts (A) and (B) of the figure represent the two bifunctional lipid probes used for the experiments mentioned in the text.

Proteomics pull-down experiments were carried out using bifunctional lipid probes. Initial work with both probes focused on performing experiments in the presence and absence of UV light. This was followed by competition-based experiments between the two probes, where filtering criteria were applied to narrow down proteins showing preferential binding to the MAG probe.

The filtering criteria used were as follows:

(1) The H/L ratio of peptides should be greater than or equal to two
(2) The experiment was performed in triplicates; hence at least two of the replicates should have three or more peptides
(3) After these two conditions were applied, only those proteins were considered which were present in at least two of the five different types of cell lysates used

For proteomic screening, five different lysates were used - BV2, RAW and N2A cell lysates along with mouse brain soluble and membrane fractions. BV2 is a microglial cell line representing the nervous system's immune cells, RAW is a macrophage cell line, and N2A is a neuronal cell line. The reason behind using these cell lines, in particular, was that the well-characterized MAG, 2-AG, interacts with the cannabinoid receptors, which are mainly present in the brain and the peripheral nervous system. Since the role of one MAG molecule was already well established in the nervous system, the aim was to study cell lines which could explore the signalling functions of other MAGs in the brain.
After the initial set of experiments, the filtering criteria were applied, which resulted in a list of putative protein interactors of MAGs.

NUCB1	Nucleobindin 1
PCYOX1L	Prenylcysteine oxidase 1like
VIM	Vimentin
HPCA	Hippocalcin
SAMM50	Sorting and assembly machinery component 50
TOMM22	Translocase of outer mitochondrial membrane 22
NAP1L1	Nucleosome assembly protein 1 like 1

Table 2.1: This table represents the putative protein interactors of MAGs which were obtained after the initial set of experiments. NUCB1 is a known lipid interactor which will be used as a positive control for all the experiments, while all the other proteins will be validated for their interaction with MAGs.

We will now explore each of these proteins in detail and understand their essential functions and known lipid interactions.

NUCB1

Nucleobindin 1 is a close homolog of the protein nucleobindin 2, a well-characterised protein with multiple functions mainly associated with the central nervous system (Tulke et al. 2016). NUCB1 is a multidomain protein which has both DNA and calcium-binding domains along with other functional domains. The N terminus of the protein is mainly involved in DNA binding, while the well-known EF-Hand domains are responsible for binding to calcium (Kapoor et al. 2010). The literature available on NUCB1 suggests that it is involved in multiple functions like endosomal sorting, autoimmunity, and apoptosis and is also associated with certain types of tumours (Valencia et al. 2008). Apart from this, it also interacts with various subunits of the G-protein coupled receptor implying its role in crucial downstream signalling pathways (Lin et al. 1998).

NUCB1 was recognised as a lipid-binding protein when a study using chemo proteomic techniques identified this protein as a significant interactor of the fatty acid amide-based probe, which is an endocannabinoid (Niphakis et al. 2015). Results indicated that adding calcium chloride to the cell lysates increases the preferential binding of NUCB1

to the fatty acid amide-based probe, suggesting its role as a lipid-binding protein. Due to the findings of this study, we have used NUCB1 as a control for our experiments since it is already established as a lipid-interacting protein.

PCYOX1L

Prenylcysteine oxidase-1-like protein belongs to a class of enzymes known as oxidoreductases which catalyse the transfer of electrons between two molecules (Lu et al. 2020). Though little is known about this protein, some information about its close homolog, prenyl cysteine oxidase-1 protein, is available. These proteins are involved in the catalytic conversion of prenylcysteine, a compound released during the degradation of prenylated proteins (Tschantz, Zhang, and Casey 1999). The prenylcysteine is converted to prenal and cysteine, along with the release of hydrogen peroxide (L. Zhang, Tschantz, and Casey 1997). Apart from this, no significant functions of PCYOX1L have been documented in the literature so far. Also, no known lipid interaction exists for this protein making the investigation of the interaction between PCYOX1L and MAGs even more exciting.

VIM

Vimentin is a structural protein which belongs to the family of intermediate filaments and is ubiquitously expressed among all major tissues of the cell. Vimentin is a multifunctional protein, and its alterations are known to cause changes in cell morphology and stiffness, increased chances of cell invasion, problems in cellular repair and reduction in cell adhesion and motility, to name a few (Ostrowska-Podhorodecka and McCulloch 2021). Apart from maintaining the structural integrity of the cell, vimentin is also linked to various diseases in humans, which has led to widespread research on this protein in recent years (Danielsson et al. 2018). Vimentin overexpression is a general marker observed in a majority of cancer cells, where its increased expression is usually linked to the aggressiveness of the disease (Ridge et al. 2022). Apart from its role in cancer, vimentin is also associated with various other disorders like cataracts, arthritis, HIV, atherosclerosis and reduced wound healing. Though vimentin is not a direct interactor of any known lipid molecule, evidence in the literature suggests that its

lower expression might result in reduced storage and transport of cholesterol in cells (Sarria, Panini, and Evans 1992). Since vimentin is involved in regulating several diseases, an interaction with a lipid molecule like MAG might contribute to its signalling function in these cases.

HPCA

Hippocalcin is a calcium-binding protein mainly expressed in the neurons of the brain (Burgoyne 2007). Like all other calcium-binding proteins, it has the EF-hand domain responsible for binding to calcium. In response to variations in intracellular calcium levels, HPCA gets localised in the cellular membranes, where it binds downstream targets and initiates the signalling cascade (O'Callaghan, Tepikin, and Burgoyne 2003). Some of the essential functions of HPCA include the regulation of apoptosis in neurons, modulating the activity of adenylate and guanylyl cyclases, control of neurite outgrowth due to overexpression of the protein, and reduced gene expression in signalling pathways in the absence of the protein (Helassa et al. 2017). Interestingly, HPCA has been shown to bind to the signalling lipid phosphatidylinositol 4,5-bisphosphate with high affinity (O'Callaghan, Haynes, and Burgoyne 2005). The binding of HPCA to this lipid is attributed to the presence of a myristoyl group at the N-terminus and basic residues for interaction with the lipid head groups. This is an exciting find as it puts HPCA at a high probability of interacting with MAGs due to its interaction with another signalling lipid and EF-hand domains in NUCB1, a known lipid-binding protein.

SAMM50

Sorting and assembly machinery component 50 is a part of the SAM complex, which is involved in the folding and insertion of beta-barrel proteins into the outer mitochondrial membrane (Diederichs et al. 2020). SAMM50 is known for two main functions: regulating mitochondrial cristae and eliminating mitochondria by mitophagy (Jian et al. 2018). Overexpression of SAMM50 leads to mitochondrial fragmentation in cells, while its depletion protects mitochondrial DNA from mitophagy. Though no lipid-protein interactions have been reported for SAMM50, a study focusing on fatty acid oxidation in mitochondria indicated a possible role of the protein in controlling the expression of

genes involved in the oxidation process (Li et al. 2021). The results showed that knocking out SAMM50 led to lipid accumulation due to decreased fatty acid oxidation, while overexpression of SAMM50 reduced the levels of lipids in the cells. Whilst SAMM50 suggests a potential involvement in controlling the lipid levels in mitochondria, more research is necessary before drawing any conclusions.

TOMM22

The translocase of outer mitochondrial membrane subunit 22 is similar to SAMM50 since it is also part of a bigger TOM-TIM complex in the mitochondria. The TOM complex is a major regulator of the import of mitochondrial proteins from the cytosol into the mitochondria (Bauer et al. 2000). TOMM22 is an essential part of the complex as it is involved in recognition of proteins for mitochondrial import and is the primary component responsible for assembling the different subunits of the TOM complex (Yano et al. 2000). The interaction of TOMM22 with various proteins and small molecules results in its role in the regulation of apoptosis, mitochondrial protein quality control and fragmentation of mitochondria in case of lower protein levels (Pitt and Buchanan 2021). A study exploring the role of mitochondrial proteins in steroid synthesis discovered that TOMM22 interacts with the enzyme 3-hydroxysteroid dehydrogenase 2 (3HSD2), which converts pregnenolone to progesterone (Rajapaksha et al. 2016). This interaction is crucial as it results in the metabolic regulation of steroidogenic cells. The study indicates the possibility of a scenario where the interaction of TOMM22 with another protein could be responsible for modulating MAG levels in cells.

NAP1L1

Nucleosome assembly protein-1 like-1 belongs to the family of nucleosome assembly proteins, known for their roles as histone chaperones and mediators of chromatin assembly (Park and Luger 2006). Though NAP1L1 is mainly established as a histone chaperone involved in the formation of nucleosomes, it also performs other functions like transcription regulation by interacting with transcription factors and controlling the affiliation and interchange of proteins at the entry site of nucleosomes (Zlatanova, Seebart, and Tomschik 2007). Apart from these functions, NAP1L1 is also present in different types of tumour cells where its mechanistic interaction with the cells and

relation to the growth of tumours is largely unexplored (Zhu et al. 2022). Due to its association with various cancers, it is often used as a biomarker to detect these diseases. Since not much is known about this protein apart from its role in regulating nucleosome assembly and disassembly, any possible interaction with lipids has yet to be explored.

We have covered all the putative protein interactors of MAGs obtained from the initial proteomics analysis. From the literature review, we can conclude that the proteins in consideration are quite different in function and structure; most of them have not been characterised extensively. This makes the analysis of these proteins even more interesting, as novel lipid-protein interactions might provide more insight into the biochemical functions of the proteins. After analysing these proteins in detail, we move on to the next chapter, which will focus on the experimental aspects of the project, mainly the materials and methods used for conducting experiments.

Chapter 3: Materials and Methods

3.1 Preparation of protein constructs

Polymerase chain reaction (PCR): PCRs were done in Eppendorf Mastercycler X50s, where the reactions were performed in 200 µL PCR tubes. Initially, a gradient PCR was set up for each gene, where five different temperatures were used for the reaction. Each gene's standard temperature was chosen depending on the results, and an amplification reaction was performed. Each PCR was a 100 µL reaction where the components of the reaction included: Milli Q (volume was calculated such that the total reaction volume was 100 µL), PROMEGA Pfu 10X buffer (1X was used), NEB deoxynucleoside triphosphates (200 µM), SIGMA primers (1 µM each), DNA template (200 ng) and PROMEGA Pfu polymerase (1µL).

PCR amplifications were performed at the following conditions: 95°C for 2 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing temperature (depending on the primer pair) for 1 minute, and extension at 72°C for 2 minutes, ending with another extension at 72°C for 6 minutes.

Agarose gel electrophoresis: A 0.8% agarose gel was prepared in 1X TAE buffer and ethidium bromide to visualise the PCR product. A 6X loading dye was added to the PCR tubes, and the samples were loaded onto the agarose gel. NEB 1kb ladders were used to estimate the size of the PCR product. These gels were run at 100V for approximately 30-40 minutes, depending on the resolution required. The gels were then visualised in a Gel Doc system (Syngene G-box-Chemi 16), and the images were saved for further use.

DNA extraction from agarose gel: A Qiagen gel extraction kit was used to extract DNA from the agarose gel. The bands were visualised under ultraviolet light on the UV transilluminator. The desired bands were cut out using a clean blade, and the gel piece was stored in a 2 ml microcentrifuge tube. The weight of the gel piece was determined,

and the Qiagen QG buffer equivalent to 3 times the volume of the gel was added. This was then incubated at 50°C for 10 minutes to dissolve the gel piece. The next step was the addition of isopropanol equal to the volume of the gel. This solution was transferred to a gel extraction column and centrifuged for 30 seconds at 17,900 G in a tabletop centrifuge. The flow-through was discarded, and 500 µL QG buffer was added to the column. This was again spun in the centrifuge, and the flow-through was discarded. 750 µL PE wash buffer was used to wash the column and centrifuged at similar conditions. The flow-through was discarded, and the column was again spun for 30 seconds to remove any residual wash buffer. The column was transferred to a 1.5 ml microcentrifuge tube, and 30 µL of Milli Q was added to elute the DNA. This was then incubated at room temperature for 5 minutes, and after another centrifugation reaction, the purified DNA fragment was obtained.

Restriction digestion: The pcDNA3.1(+)/myc-His vector (C terminal histidine tag) and the pCMV-Sport 6 vector (N terminal FLAG tag) were digested using NEB restriction enzymes (enzymes used depended on the primer pair). The components for the double digestion of the vector included - plasmid (1 µg), NEB 10X rCutSmart Buffer (1X was added), NEB restriction enzymes (20 units of each enzyme), milli Q (volume added was such that the total reaction volume was 50 µL). This reaction was set up in a 1.5 ml microcentrifuge tube and incubated at 37°C for 3 hours. The digested vector was then run on an agarose gel, and gel extraction was performed to obtain the vector. This was done according to the protocol mentioned above.

Transformation: For transformation, 50 µL of PPY-competent cells were used. 100 ng of digested vector and 300 ng of the purified PCR product (molar ratio of 1:3 for the backbone to insert) were added to the competent cells. The total volume of the mixture was not allowed to exceed 5 µL to maintain transformation efficiency. The tube was then gently tapped, and the solution was allowed to mix. The cells were then incubated on ice for 20 minutes, after which a heat shock was given at 42°C for 1 minute. After the heat shock, the cells were kept on ice for 5 minutes. 1ml of Luria broth was added to the cells and incubated at 37°C for 1 hour. Luria agar plates with ampicillin antibiotic were used to plate these cells, and the plates were kept at 37°C for approximately 14-16 hours.

Colony PCR: The colonies obtained after transformation were screened for positive transformants by PCR. The protocol for PCR amplification was as described above, the only difference being colonies from the plate acted as template DNA. A small amount of the colony was picked up using a pipette and added to the PCR reaction mixture in each tube. The PCR product was then run on 0.8% agarose gel to check for the band at the appropriate size. Only the colonies showing positive results were considered for further analysis.

Primary culture and plasmid extraction: The colonies that showed positive transformants were used to set up a primary culture. 10 ml of Luria broth and 10 µL of ampicillin were added to a freshly autoclaved 50 ml falcon tube. The positive colonies were picked up from the plate and added to the tube. They were incubated at 37°C for approximately 14-16 hours for plasmid extraction. A Qiagen Miniprep kit was used for this purpose. After incubation, the culture was pelleted at 6000 G for 5 minutes at 4°C. The pellet was resuspended in 250 µL buffer P1 and transferred to a 1.5 ml microcentrifuge tube. The cells were lysed using 250 µL buffer P2 and mixed thoroughly. 350 µL buffer N3 was added to the tube to neutralise the solution. The solution was centrifuged at 17,900 G for 10 minutes in a tabletop centrifuge. After the spin, the supernatant was removed carefully and transferred to a DNA-binding column. The solution was spun at similar conditions for 30 seconds, and the flow-through was discarded. Washing was done for the spin column by adding 750 µL PE wash buffer and centrifuging it for 30 seconds. The flow-through was discarded, and the column was centrifuged again to remove any residual buffer. The column was transferred to a 1.5 ml microcentrifuge tube, and 30 µL of Milli Q was added. This was then incubated at room temperature for 5 minutes and spun for another 30 seconds. After the final centrifuge, the purified plasmid was obtained and sent for sequencing to validate successful cloning.

Maxi prep for plasmid DNA: To get transfection grade DNA, maxi prep was performed for positive clones verified by sequencing results. A 10 ml primary culture was initially set up using the above protocol and incubated at 37°C for 12 hours, after which a secondary culture was set up. 5 ml of the primary culture was added to a beaker along

with 300 ml of Luria broth and 300 µl of ampicillin. This was then incubated at 37°C for 12-14 hours. The bacterial cells were harvested by centrifugation at 6000 G for 20 minutes at 4°C. The cells were resuspended in 10 ml of buffer P1 and transferred to an Oakridge centrifuge tube. 10 ml of buffer P2 was added to lyse the cells and incubated at room temperature for 5 minutes. 10 ml of pre-chilled buffer N3 was added for neutralisation, and the tube was kept on ice for 20 minutes. The solution was then centrifuged at 20,000 G for 45 minutes at 4°C. A QIAGEN-tip 500 was equilibrated by applying 10 ml buffer QBT, and the column was emptied by gravity flow. The supernatant from centrifugation was allowed to enter the resin by gravity flow. The tube was then washed twice with 30 ml of buffer QC. After washing,15 ml buffer QF was used to elute the DNA in another Oakridge centrifuge tube. 10.5 ml of isopropanol was added to the eluted DNA to precipitate it. After mixing, the solution was centrifuged at 15,000 G for 45 minutes at 4°C. After centrifugation, the supernatant was decanted, and the DNA pellet was washed with 5 ml of 70% ethanol. After washing, the solution was centrifuged at 15,000 G for 15 minutes at 4°C. The pellet was air-dried overnight and resuspended in 200 µL of Milli Q the next day.

3.2 Cell culture and transfection

HEK cells were cultured in Roswell Park Memorial Institute (RPMI) media supplemented by 10% (v/v) Fetal bovine serum (FBS) and 1X penicillin-streptomycin at 37°C with 5% (v/v) carbon dioxide. 10 cm plates were used for culturing the cells. Two different transfecting reagents were used - polycation polyethyleneimine (PEI) and lipofectamine 2000. In the case of PEI, cells were allowed to grow to a confluency of 20-30% for transfection, while when lipofectamine was used, cells were grown to a confluency of about 50%. For transfection, the following components were used: plasmid DNA (20 µg), transfecting reagent (PEI or lipofectamine), and media (serum-free media in case of PEI and Opti-MEM media for lipofectamine). The ratio of the plasmid to transfecting reagent was 1:3 in most cases. These compounds were added to a 1.5 ml microcentrifuge tube and incubated at room temperature for 45 minutes - 1 hour. After incubation, the transfection mixture was added to the 10 cm plate at the desired cell

confluency. While using lipofectamine as a transfection reagent, the media was changed after 12 hours to prevent cell death due to the toxic nature of the reagent.

Mock control cells with an empty vector were also transfected using this protocol. The transfected cells were allowed to grow to 100% confluency and harvested. An ice-cold solution of sterile 1X Phosphate buffer saline (PBS) was used for harvesting. The media was aspirated from the plates, and 1 ml of PBS was added to each plate. Cells were scrapped from the plate and added to a 1.5 ml microcentrifuge tube. These were spun at 500 G for 3 minutes at 4°C in a tabletop centrifuge. The supernatant was removed, and the pellet was resuspended in 1 ml PBS. The solution was again spun at the same conditions, and after removing the supernatant, the pellet was stored at -80°C.

3.3 Protein estimation

The pellet was removed from -80°C and kept on ice for thawing. After thawing, the pellet was resuspended in 300 µL of PBS. The cells were lysed by probe sonication, where the sonication conditions were: 2 sec on and 2 sec off for 10 seconds at 60% amplitude. The solution was diluted 20 times in PBS (3 µL sample in 57 µL PBS), and this diluted sample was prepared in another microcentrifuge tube. Bradford reagent was used for protein estimation of these samples. Protein standards made using BSA were used for this purpose. In a 96-well plate, 10 µL of protein standards along with 10 µL of samples (in triplicates) were added. To all these wells, 200 µL of Bradford reagent was added. The absorbance of samples was measured using varioskan flash at 595 nm. After estimation, the concentrations of protein in each sample were noted.

3.4 Expression check

For checking the overexpression of proteins, the samples were run on a 12.5% SDS-PAGE gel. 50 µg of cell lysate was loaded in each well. The total volume of the solution was made to 40 µL by adding PBS for equal loading volumes. 4x SDS loading

dye was added to each sample. 3 µL of Abcam pre-stained protein ladder was loaded on the gel to visualise the size of the protein bands observed. The gel was run at 120-150 V till the desired resolution was reached.

After running the gel, it was kept in an ice-cold 1X transfer buffer used for the western blot. The following components were used to prepare 2 litres of 1X transfer buffer: 200 µL of 10X transfer buffer, 200 µL of methanol and the remaining volume was made up of distilled water. The western blot apparatus was set up in the following order:

(A) Sponge
(B) 2 Filter papers
(C) Membrane
(D) Gel
(E) 2 Filter papers
(F) Sponge

The arrangement was done such that the gel was towards the negative electrode while the membrane was facing the positive electrode to ensure transfer of proteins from the gel to the membrane. Nitrocellulose membrane was used for the Western blot experiments. This setup was then run at 90 V for 90 minutes at 4°C for the transfer.

After completing the transfer, the membrane was stained with ponceau to confirm the transfer of proteins and use it as a loading control. The ponceau stain was removed by washing the membrane in PBST (Phosphate buffer saline with tween 20 protein). After washing the membrane, it was blocked with 5% (w/v) milk in PBST (2.5 gm milk powder in 50 ml of PBST) for around an hour. After blocking, the addition of a primary antibody was done. An anti-FLAG antibody (from Sigma) was used for proteins expressed with an N-FLAG tag. Proteins with a His-myc tag were probed using an anti-myc antibody (Myc.A7 from Invitrogen). The primary antibody was used in a dilution of 1:1000. The membrane was held between a laminated sheet and probed with the primary antibody. This setup was kept overnight at 4°C.

After overnight incubation with the primary antibody, the next day, the membrane was washed three times with PBST at intervals of 5 minutes. The membrane was then

incubated in a secondary antibody prepared at a dilution of 1:10,000 at room temperature. An anti-rabbit secondary antibody was used for the proteins with an N-FLAG tag, while an anti-mice secondary antibody was used for proteins with a His-myc tag. After an hour, the membrane was washed thrice with PBST at intervals of 5 minutes.

Chemiluminescent substrates for the activity of the Horseradish peroxidase enzyme were mixed in a 1:1 ratio. The blot was developed using the mixture of these substrates, which was added dropwise onto the membrane. A Syngene G-box was used for imaging the blot.

Chapter 4: Cloning and Transfection

The previous chapters have provided a brief overview of the motivation behind the project and the preliminary data available to build upon further experiments. Before we discuss the experimental techniques in detail, an understanding of the experimental design is essential.

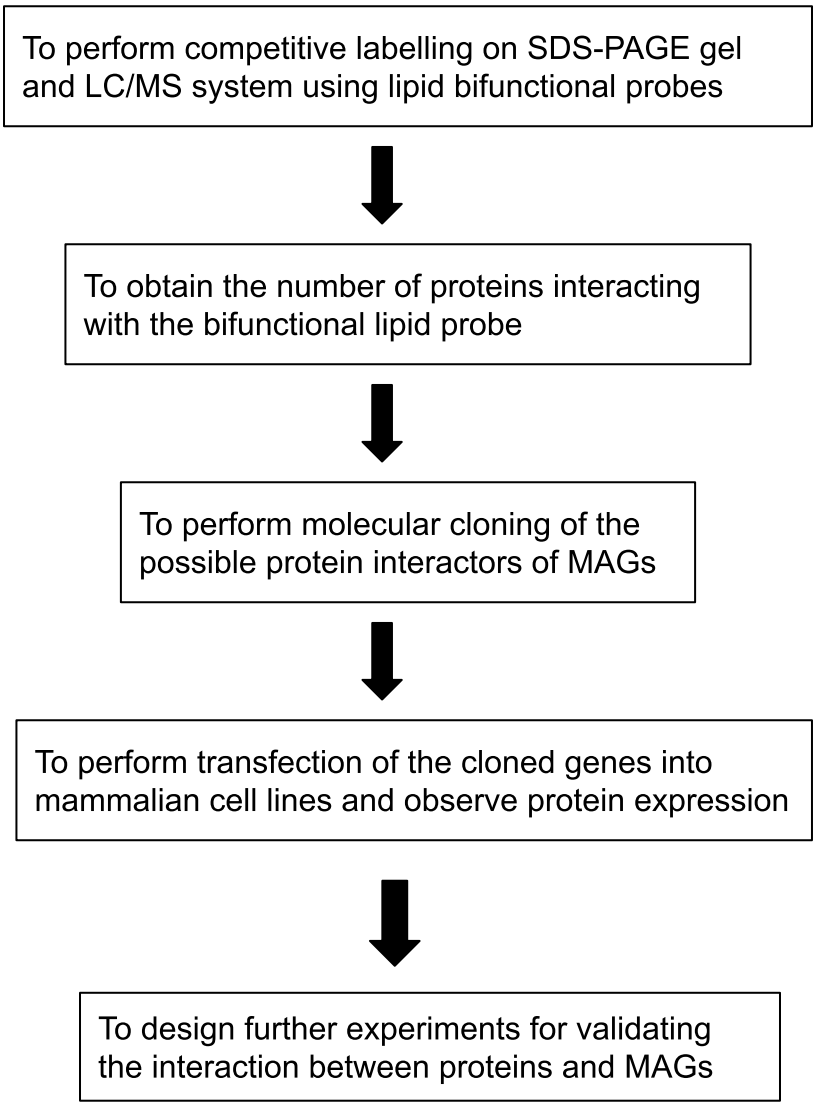


Fig 4.1: This figure represents the experimental design followed for the project. The details of each step are explained in the text.

The first goal was to perform competitive labelling assays using bifunctional lipid probes to come up with a set of putative protein interactors, which were narrowed down further by applying different filtering criteria. These proteins had to be validated for their interaction with the monoacylglycerol probe, and the first plan of action was to clone these genes into different expression vectors. After preparing these constructs, they had to be transfected into mammalian cells and analysed for their overexpression by Western blot. The final step includes designing experiments to validate these lipid-protein experiments. Among the steps shown in the figure, the project mainly focused on the last three parts, where the initial few months were mostly spent on cloning the genes into their expression vectors, followed by their overexpression. All the steps before this were preliminary experiments which were already completed, and their data was available for further analysis.

4.1 Cloning

(1) Literature survey

The first step while planning cloning experiments is to decide the tag used in the expression vector and then design primers accordingly. For selecting the tags to be used and the terminus at which they should be inserted, an initial literature review was performed for all the genes where their domain architecture was explored, along with previous examples in the literature where the gene had been cloned. For determining the domain architecture, data from UniProt was used. The information available for each gene has been summarised in the table below, along with the final tag chosen. The tags for use in cloning were: N terminal FLAG and C terminal Histidine tags.

The decision to choose a tag at either N or C terminus depends on the following criteria: Many proteins have a signal peptide at their N terminus hence a tag here could lead to the wrong signal causing mislocalisation of the protein. Also, some proteins have a propeptide signal which is removed after maturation, and if a tag is present at this terminus, then the tag might also get removed. Apart from this, regions important for protein function should also be considered.

GENE	LITERATURE ANALYSIS	TAG FOR CLONING
1) NUCB1	Signal peptide at N-terminus Literature has examples of tag at C-terminus	C-terminus tag
2) PCYOX1L	Signal peptide at N-terminus	C-terminus tag
3) VIM	Literature shows that tag at N-terminus makes the protein unstable for forming filaments and destabilises it	C-terminus tag
4) HPCA	Did not find any evidence in the literature for preferential use of tag at a particular terminus	Both C and N terminus tag
5) SAMM50	C-terminus contains beta sheets involved in forming beta-barrels Evidence of cloning using a tag at N-terminus	N-terminus tag
6) TOMM22	C-terminus is used to anchor the protein to mitochondria	N-terminus tag
7) NAP1L1	C-terminus has a propeptide which is removed in mature form	N-terminus tag

Table 4.1: This table summarises the literature analysis done to determine the use of a particular terminus tag for cloning. A detailed explanation for each protein is provided in the text.

The first protein in consideration, NUCB1, is our control for the experiment and has already been shown as a lipid-binding protein. From previous data in the literature, the cloning for NUCB1 was done with a tag at C-terminus, which worked effectively in the experiments. Also, UniProt's domain analysis shows that the protein's N-terminus has a signalling peptide (regions 1-26) which is essential for its export from the endoplasmic reticulum to the Golgi apparatus (Tsukumo et al. 2009). Hence, a C-terminus tag was chosen for NUCB1.

Though not much is known about PCYOX1L in literature, analysis of domain architecture and information available from databases shows the presence of a signalling peptide at the N-terminus, due to which the tag was at C-terminus (Z. Zhang and Henzel 2004).

An interesting study was done for vimentin, where an N-terminus tag was used to determine the efficiency of filament assembly (Usman et al. 2022). Results indicated that an N-terminus tag caused protein aggregation, and the filament network formed by vimentin was highly unstable. Based on this evidence, we used a C-terminus tag for vimentin.
In the case of HPCA, literature analysis did not provide any detailed insight into which tags are the best for cloning the gene; hence we decided to use tags at both C and N terminus.
For SAMM50, domain analysis revealed that the C-terminal part might contain beta sheets that have the potential to form a beta-barrel structure, indicating that a tag at this terminus might hinder the protein function. Also, there were examples in literature with SAMM50 clones with an N-terminus tag.
TOMM22 is a mitochondrial protein where the C-terminal tail holds the protein to the outer mitochondrial membrane, which is essential for its function (Rajapaksha et al. 2016). Hence, we used an N-terminus tag for this protein to prevent any functional inhibition.
Domain architecture analysis of NAP1L1 revealed that its C-terminus has a propeptide removed in the mature form of the protein. Hence, an N-terminus tag was used for this protein.

Thus, after deciding the tag for each protein, the cDNA clones were ordered, and primers were designed accordingly. We will now discuss the primer design process in detail.

(2) Primer design

The method used for cloning the genes in our experiments was SLiCE cloning, where regions homologous to the expression vector are introduced in the gene after PCR amplification. Hence, primers were designed such that this requirement was fulfilled. The first step was identifying any two restriction enzymes in the vector's multiple cloning sequence (MCS). The gene sequence was inserted between these two enzymes, and primers were designed such that 20 bases were chosen from the gene sequence while the remaining 20 bases were selected from the vector sequence.

In this way, the primer incorporates sequences from the expression vector, which are inserted into the gene after PCR. In the case of reverse primer, the complement sequence was used for designing primers.
The primers were then checked for the following conditions to ensure the efficiency of PCR: the difference in melting temperature of both primers should not be greater than 5°C, the primers should not form dimers easily, and the GC content of both primers should be comparable. Different enzyme combinations were used to determine the best set of primers for cloning.

(3) Cloning strategy

As mentioned above, the technique used for our experiments is the SliCE (Seamless Ligation Cloning Extract) method. This technique relies on in-vivo homologous recombination between similar regions (around 10-20 bases) in the amplified gene and the expression vector. This is an efficient cloning strategy as it is a restriction site-independent technique that does not require the presence of appropriate enzymes in the vector and the gene. It has a major benefit from the traditional restriction enzyme-based cloning method as no unwanted sequences are present at the junction sites, which is often the case with cloning based on joining sticky ends. Also, traditional methods usually require enzymes like ligases to join the DNA fragments after recombination, which is eliminated in the case of SliCE cloning.

Since the entire cloning process depends on homologous recombination, the bacterial cells used for transformation should have a robust recombination system. Hence, PPY cells are used for this purpose which are genetically engineered to have efficient machinery for homologous recombination (Y. Zhang, Werling, and Edelmann 2014). Therefore, in our experiments, we used PPY cells for bacterial transformation.

Apart from the conditions mentioned above, this cloning method can also be used when multiple DNA fragments have to be cloned in a single step. Due to its multiple advantages, SLiCE cloning is now being used frequently in many research projects.

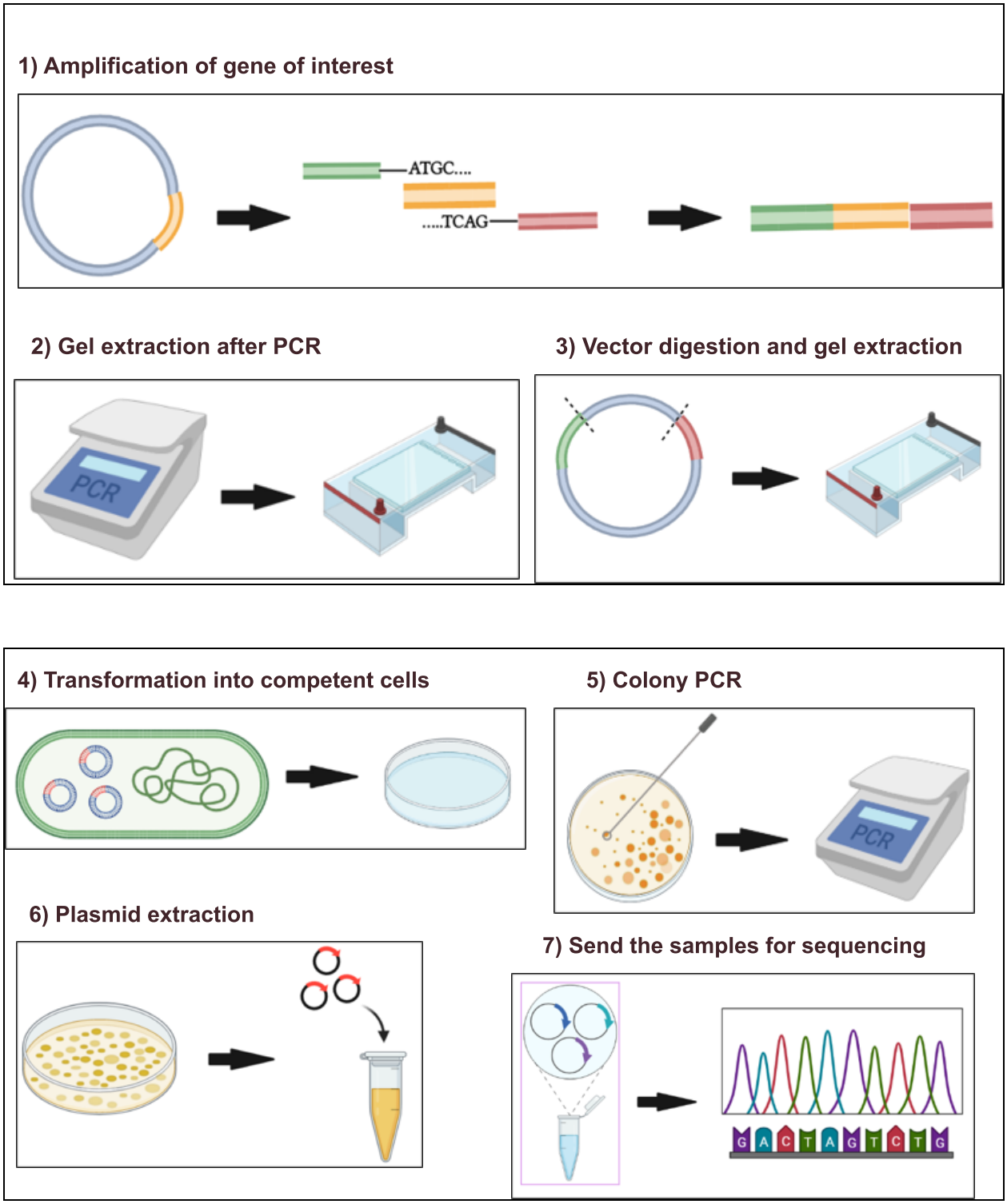


Fig 4.2: This figure provides a schematic representation of the SLiCE cloning protocol used for the experiments. A detailed explanation of each step is provided in the text.

The initial step of SLiCE cloning involves PCR, resulting in gene amplification and insertion of homologous regions. This was followed by vector digestion with two different restriction enzymes. The advantage of double digestion is the prevention of self-ligation between the two ends of the digested vector. After running it on an agarose gel, a gel extraction was performed to obtain the amplified product and the digested vector.

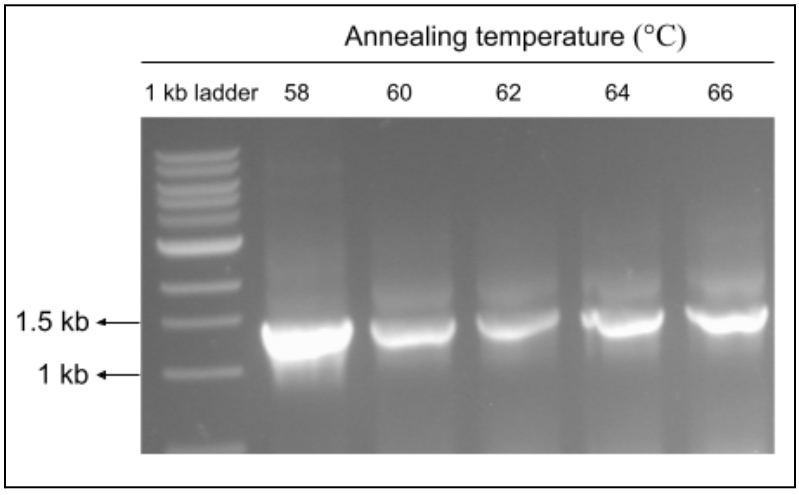
The PCR product and the digested vector were then added to PPY-competent cells, where homologous recombination allows the gene of interest to be inserted into the vector. Colony PCR enabled us to screen for positive colonies containing the insert, which were then subjected to plasmid extraction. These samples were then sent for sequencing, and the results confirmed successful cloning.

Results

The initial step of cloning involved PCR, where each gene was subjected to a gradient PCR. Five different temperatures were used for the reaction, and the temperature which gave the best results was chosen for further experiments.

1) NUCB1

Size of gene: 1379 bp



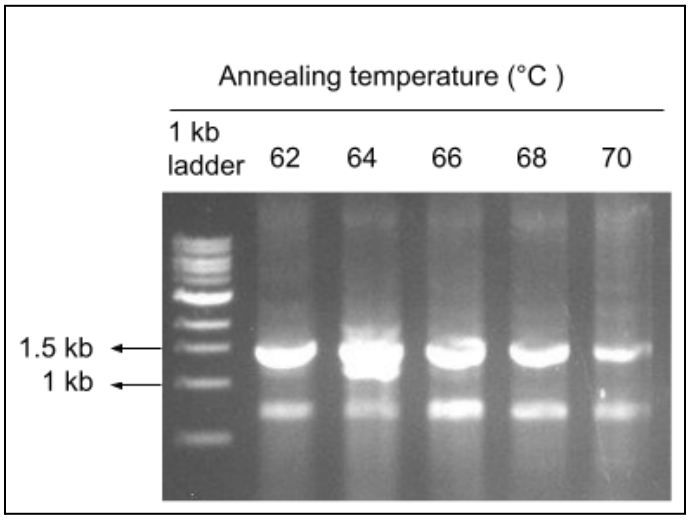
Results indicate that PCR amplification is maximum at 58°C. Hence, this temperature was chosen for the experiments.

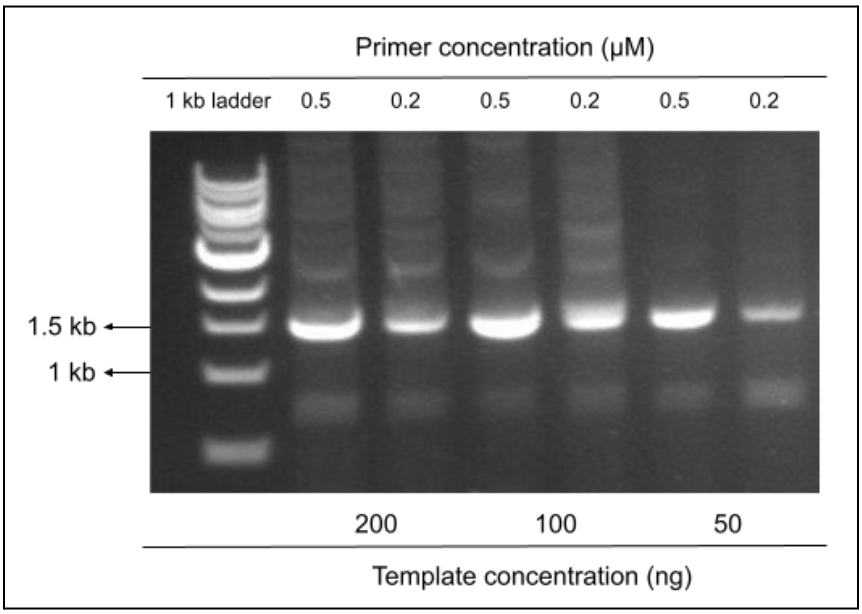
Fig 4.3: Results of gradient PCR for NUCB1

2) PCYOX1L

Size of gene: 1487 bp

From the initial PCR, it was found that multiple bands were observed at all five different temperatures used. Hence, troubleshooting techniques were used to improve the PCR results. Two main changes were made in the initial reaction conditions for troubleshooting PCR: Lower primer and template concentrations were used. The logic behind using these methods was that lower primer concentrations might prevent the formation of primer dimers and give cleaner results. Lower template concentrations help in dealing with any non-specific binding with the template and prevent the formation of multiple bands.



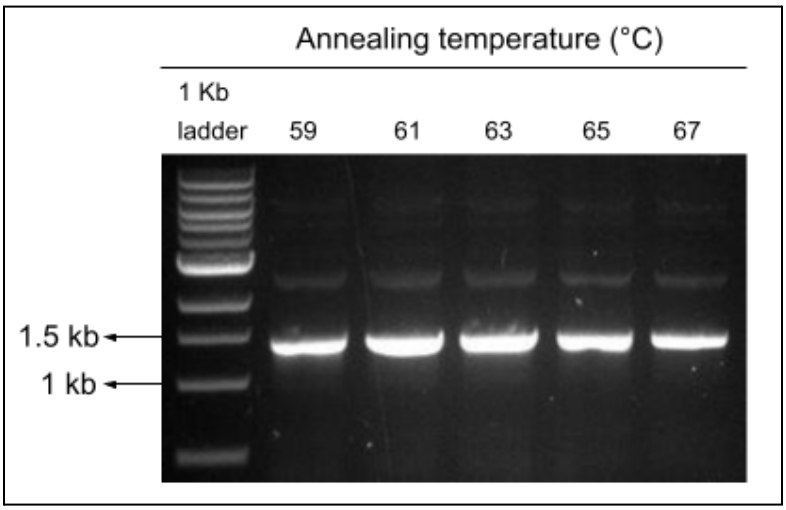


The initial results showed that the amplification was maximum at 64°C. Hence, the experiments were carried out at this temperature. The final results indicated that the best conditions for PCR were: 100 ng template and 0.5 μM primer concentration. These conditions were followed for further experiments.

Fig 4.4: The first image represents the results of initial gradient PCR. The second image represents the results at different primer and template concentrations.

3) VIM

Size of gene: 1400 bp

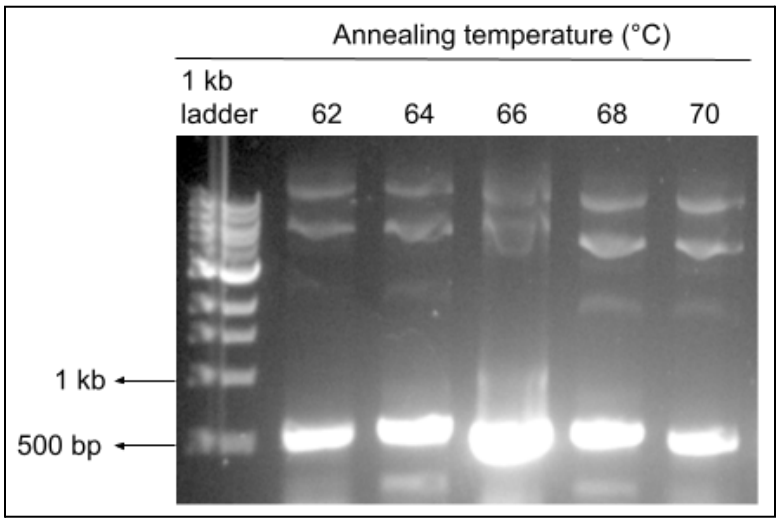


Results indicate that PCR amplification shows good results at all five temperatures. Hence, further experiments could be performed at any temperature. To maintain uniformity, 61°C was chosen for further amplification experiments.

Fig 4.5: Results of gradient PCR for vimentin

4) HPCA (tag at C-terminus)

Size of gene: 581 bp

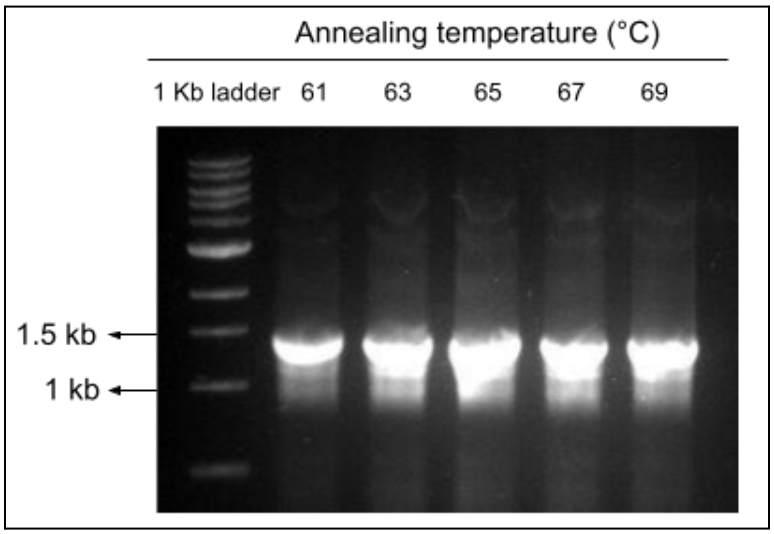


Results indicate that all temperatures show a good amount of amplification of the HPCA even though some bands are present in the background. For experimental purposes, 62°C for chosen for further amplification.

Fig 4.6: Results of gradient PCR for HPCA (C-His tag)

5) SAMM50

Size of gene: 1409 bp

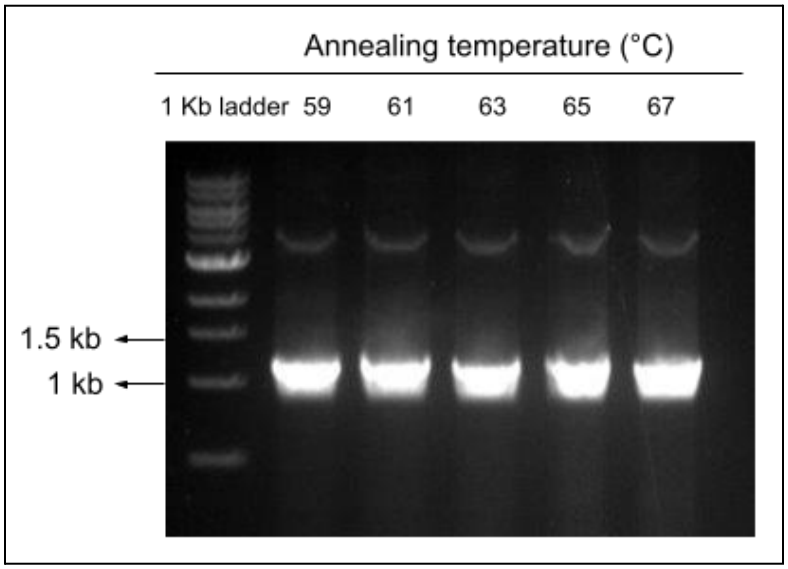


The results of gradient PCR show gene amplification at all five temperatures. For conducting further experiments to amplify the gene, 61°C was chosen as it gives a crisp band compared to all other temperatures.

Fig 4.7: Results of gradient PCR for SAMM50

6) NAP1L1

Size of gene: 1176 bp

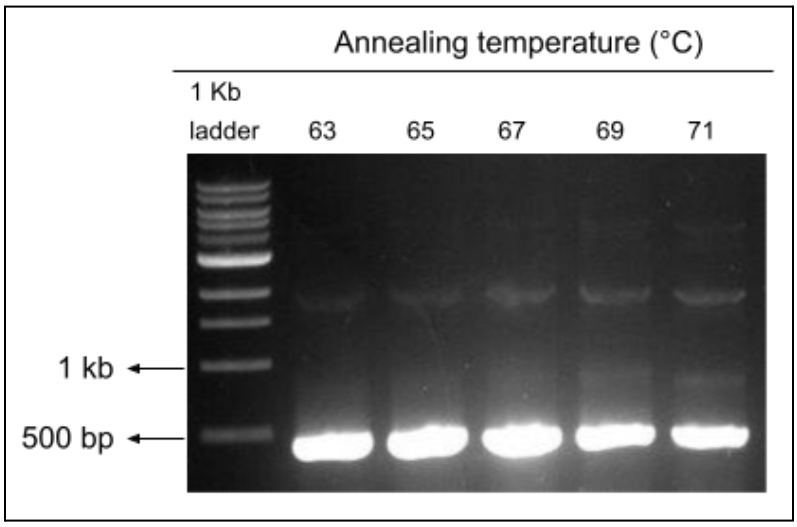


Results show that gene amplification occurs at all five temperatures. 59°C is the best choice among all the temperatures as it gives a sharp band compared to other cases where the bands are a bit more spread out.

Fig 4.8: Results of gradient PCR for NAP1L1

7) TOMM22

Size of gene: 428 bp

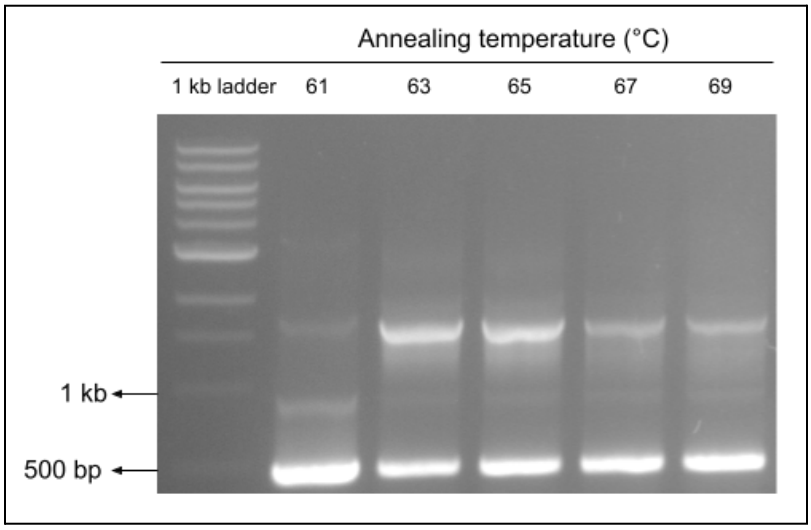


The gradient PCR results indicate that gene amplification occurs equally well at all five temperatures. To maintain uniformity in further experiments, 63°C was chosen.

Fig 4.9: Results of gradient PCR for TOMM22

8) HPCA (tag at N-terminus)

Size of gene: 581 bp



The results show that good PCR amplification was observed at all temperatures. We decided to use 61°C since both amplification observed was higher and non-specific bands were lesser.

Fig 4.10: Results of gradient PCR for HPCA (N-FLAG tag)

Troubleshooting cloning

The entire cloning strategy involved a lot of troubleshooting, and some of those cases are discussed below:

(A) The initial PCR products and digested vector did not undergo SLiCE reaction efficiently, and no colonies were obtained after transformation. To solve this problem, a new batch of PPY-competent cells was made, after which the transformation worked well.

(B) The experiments were carried out in an N-histidine vector in the beginning. However, sequencing results indicated that the vector might not have a histidine tag at the N terminus; hence we used an N-flag vector for all our experiments.

(C) The genes which had to be cloned into the vector having a C-terminus histidine tag did not give positive clones even with the new batch of competent cells. Hence an alternative cloning strategy, similar to SLiCE cloning, was used for these genes. This strategy showed positive results, and the clones were obtained.

We will now discuss the alternative cloning strategy in detail. The cloning method used was restriction-free cloning. The initial step of gene amplification was similar to SLiCE cloning. This was one of the major reasons for choosing this technique, as we could use the same primers used in SLiCE cloning. Extension PCR was the next step after amplification, where the expression vector was used as a template. The initial PCR product acted as the primer that amplified the expression vector.

After amplification, two products were present in the reaction mixture - the newly synthesised expression vector with the gene of interest and the initial expression vector used, which did not contain the gene. To eliminate the initial expression vector, Dpn1 digestion was used. This worked only on the expression vector as it had methyl tags due to its bacterial source, while the newly synthesised cloned product did not have these tags. After digestion, the product was transformed using competent cells, and the positive colonies were used for plasmid extraction. The samples were sent for sequencing, and the results confirmed the presence of positive clones.

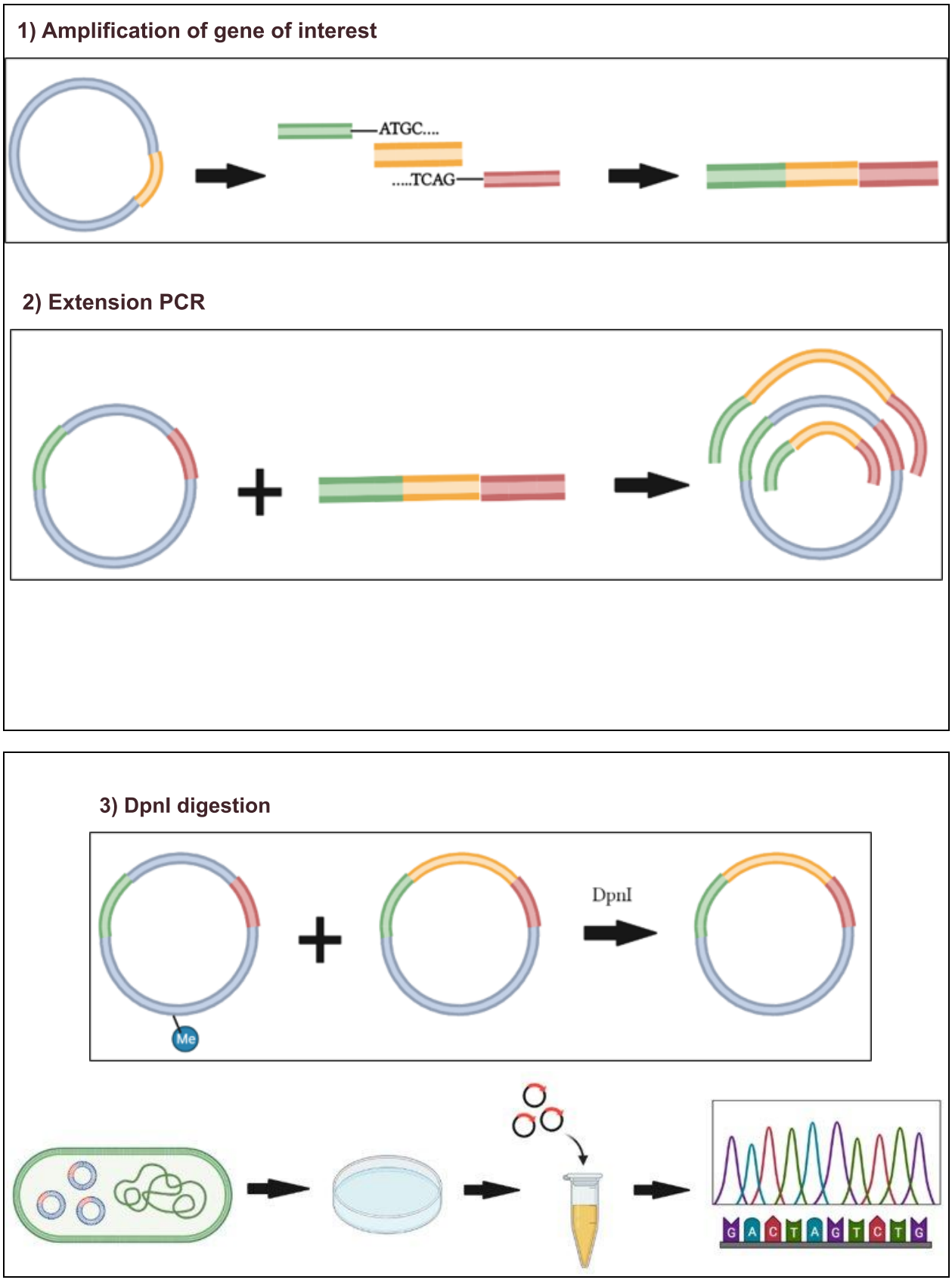


Fig 4.11: Schematic representation of restriction-free cloning, explained in detail in the text.

Conclusions

In-depth descriptions of the cloning procedure and troubleshooting methods have been provided. The results of PCR standardisation by conducting gradient reactions at various temperatures have been demonstrated. After plasmid extraction, the samples of all genes were sent for sequencing. We have successfully cloned seven genes among the eight, while the cloning of TOMM22 is still in progress.

4.2 Transfection

After successfully cloning the genes into expression vectors, the next step was transfecting them into mammalian cells and analysing their expression. HEK cells were used for transfection as, according to the literature, they are the most efficient cell lines for expressing recombinant proteins (Tan et al. 2021).

The initial step was to culture the cells and allow them to reach at least the 3rd or 4th passage, as transfection efficiency is good at this stage of cell culture. When the cells achieved the desired confluency, the transfection mixture was prepared. After incubating it for an hour at room temperature, the mixture was added to the cells, and they were grown to maximum confluency. After harvesting the cells, western blot analysis confirmed the expression of the proteins.

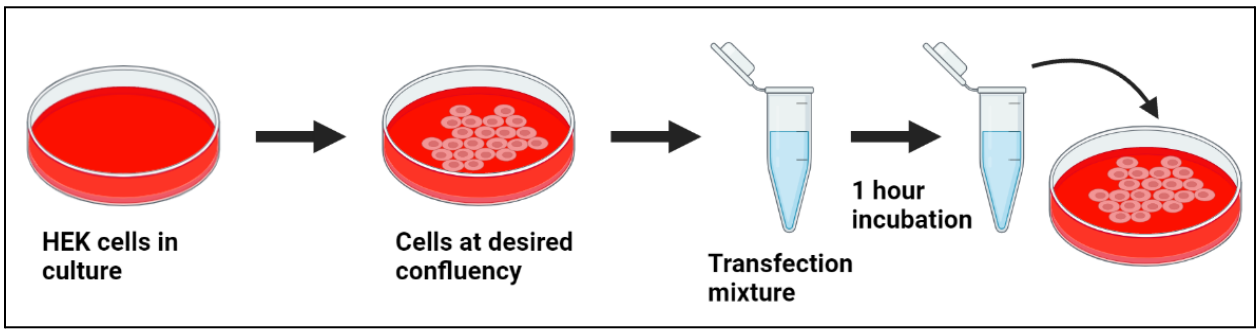


Fig 4.12: This figure shows the transfection protocol followed for the experiments. HEK cells were cultured, which were transfected and allowed to grow.

Standardising transfection

Initially, the transfecting reagent used was polycation polyethyleneimine (PEI). This reagent acts as a cationic polymer and binds to the DNA being transfected such that the DNA molecules act as small cationic nanoparticles (Raymond et al. 2011). This increases the uptake of DNA by the cells due to their interaction with negatively charged cell surface proteins. The PEI-DNA complexes are formed when the transfection mixture is prepared, and incubating the mixture allows these complexes to grow to a suitable extent before their addition to the cells. Hence, the transfection efficiency in this protocol depends on factors like incubation time for the transfection mixture, the ratio of DNA to the transfecting reagent and the confluency of cells at transfection.

For our experiments, a confluency of 20% at the time of transfection, along with a DNA-to-reagent ratio of 1:3, was found ideal for the highest transfection efficiency. For the transfection experiments, serum-free media was used. The logic behind this is that the serum contains proteins with a high affinity for binding with the cell surface proteins. If transfection is done in the presence of serum-containing media, these proteins will compete with the PEI-DNA complex to enter the cells, thus decreasing the transfection efficiency.

The protocol described above worked for most of the proteins; however, PCYOX1L and NUCB1 did not have good transfection results with this method. Hence, standardisation of transfection was done for these two proteins. Lipofectamine was used as the transfection reagent for these proteins as it is known to have better transfection efficiency than PEI.

Lipofectamine also follows the principle of cationic lipid-mediated transfection, where it forms liposomes in the media and DNA is enclosed within these liposomes. The liposome then fuses with the negatively charged cell membrane and enters the cells. Lipofectamine was not initially considered for our experiments, as examples in the literature suggest that it is toxic for cells and might result in reduced cell growth. Therefore, initial transfection experiments were done using PEI.

The protocol followed for transfection using lipofectamine was mostly the same as in the case of PEI. The only significant difference was that due to the cytotoxic nature of lipofectamine, the media was changed 12 hours after transfecting the cells. Also, from our optimisation experiments, 40-50% was the optimal confluency for transfecting the cells using lipofectamine. The efficacy of transfection for the cells was decreased by very high or low confluency.

Results

The results of transfection were evaluated by running the cell lysates on an SDS-PAGE gel and then performing western blot analysis. Ponceau stain was used for protein level normalisation to ensure equal loading in all wells.

(1) Genes with a FLAG tag at N-terminus (anti-FLAG antibody was used)

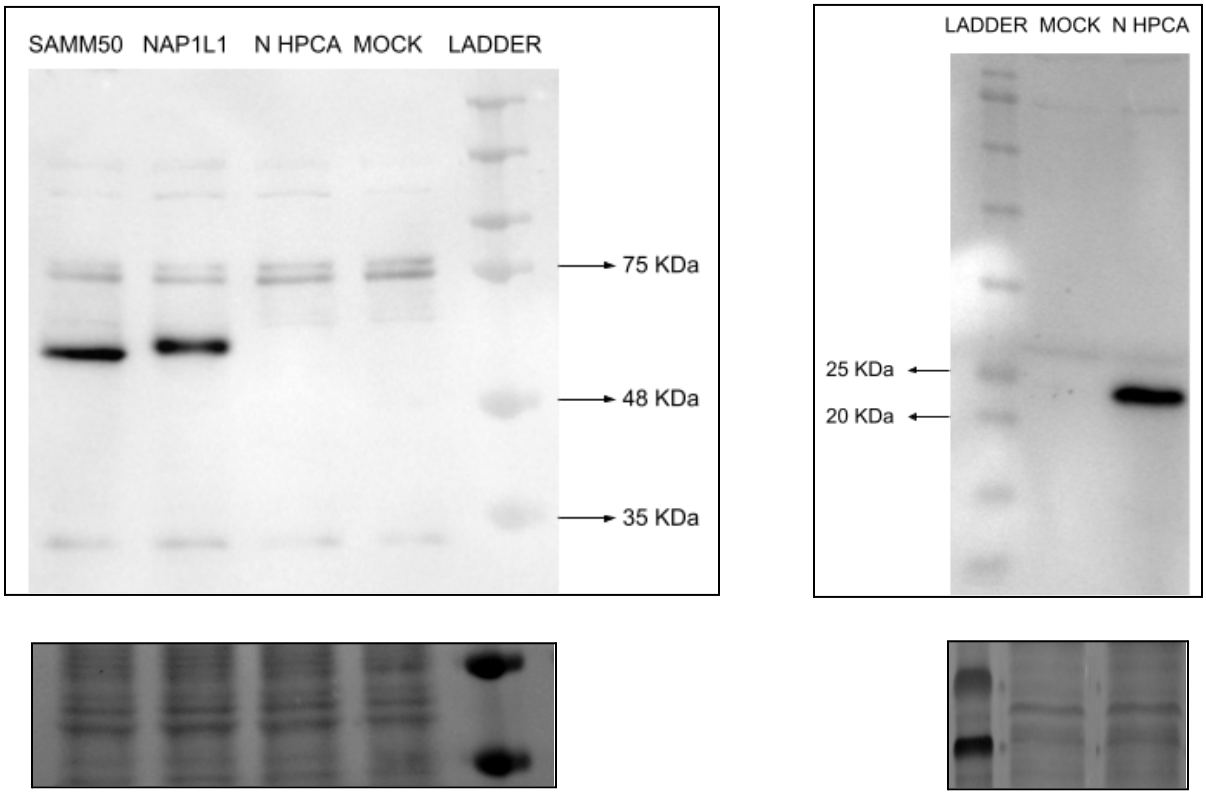


Fig 4.13: This figure represents the western blot results for proteins with an N-FLAG tag. The ponceau stain to indicate loading control is shown below each blot. Further details are explained in the text.

The results of the western blot have been displayed in the figure above. The first image shows the overexpression of SAMM50 and NAP1L1, along with a mock as a control. Due to HPCA's smaller size, it ran out of gel and was not visible in the first picture. As a result, another gel was prepared, and the western blot analysis demonstrated HPCA overexpression. We will now discuss these observations in detail.

(a) HPCA

Mass: 22.427 kDa

The observed band size for HPCA is between 20 and 25 kDa. This is at the expected position since its molecular weight is around 22.5 kDa confirming the expression of HPCA in the cells.

(b) NAP1L1

Mass: 45.374 kDa

The band for NAP1L1 is between 48 kDa and 75 kDa, much higher than its expected size of 45 kDa. This increase in size could be attributed to a large number of post-translational modifications in the protein. The post-translational changes observed are - mono glycated on glutamate residues, poly glutamylated on glutamate residues, phosphorylation, lipidation to form S-farnesyl cysteine, acetylation and methylation.

(c) SAMM50

Mass: 51.976 kDa

The protein band for SAMM50 is between 48 kDa and 75 kDa, which is higher than the expected size. This could be due to post-translational modifications in the protein, though literature analysis does not reveal many modifications. Some of the post-translational changes observed are - methylation and phosphorylation.

We have investigated the observed results in detail. Apart from HPCA, the other two proteins are at a larger size than expected, indicating the presence of post-translational modifications in the protein, which is confirmed by literature analysis. We will now explore the results of proteins tagged at the C-terminus.

(2) Genes with a his-myc tag at C-terminus (anti-myc antibody was used)

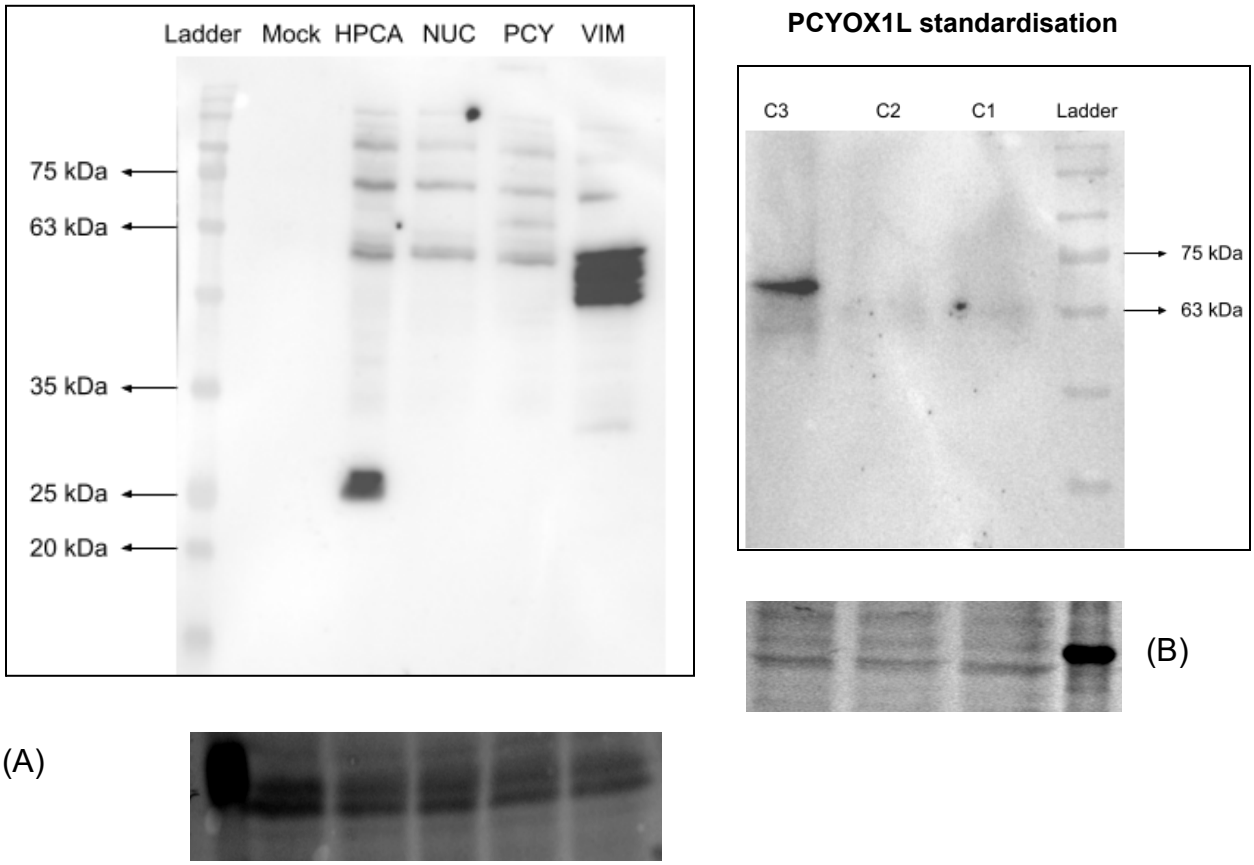


Fig 4.14: This figure shows the results of the western blot for genes with a tag at the C-terminus. (A) represents the initial western blot analysis where NUC stands for NUCB1 and PCY for PCYOX1L proteins, respectively. Here, bands are observed for HPCA and VIM but not for NUCB1 and PCYOX1L. (B) represents the different conditions in which the transfection of PCYOX1L was done to increase its transfection efficiency. The ponceau stains for all the proteins have been shown below the images. Further details of these images are explained in the text.

We will now discuss the observations of the western blot results in detail.

(a) HPCA

Mass: 22.427 kDa

The observed band size for HPCA is around 25 kDa which is approximately the expected size of the band. A slight increase in the size might be due to the presence of histidine and myc tags in the plasmid.

(b) NUCB1

Mass: 53.879 kDa

No band is observed in the case of NUCB1, indicating that transfection did not occur properly and its efficiency has to be increased. For this purpose, lipofectamine was used as a transfecting reagent, and its optimisation is still in progress.

(c) PCYOX1L

Mass: 54.646 kDa

Initially, the western blot results indicated that transfection for PCYOX1L required optimisation as no band was observed in the blot. For this purpose, lipofectamine was used as a transfecting reagent. Three conditions were followed for this: C1 - this was the control for the experiment where no DNA was added and only lipofectamine, and the media were present in the transfection mixture, C2- the ratio of the plasmid to reagent was 1:3 and C3 - the ratio of the plasmid to reagent was 1:4.

Part (B) of the figure shows the results of these conditions, where a band is observed in case of C3 indicating that it has the highest transfection efficiency. The observed band is between 63 and 75 kDa, which is higher than the expected size. According to the literature analysis, glycosylation is a common modification observed in this protein, explaining the band's presence at a larger size.

(d) VIM

Mass: 53.652 kDa

For vimentin, multiple bands are observed between 50 and 60 kDa on the western blot. This indicates the presence of many post-translational modifications

in the protein. This is confirmed by literature analysis, which revealed that vimentin is well known for several post-translational modifications like phosphorylation (around 25 phosphorylated residues), glycosylation, nitrosylation and sumoylation. These modifications are necessary for the role of vimentin as an intermediate filament. Hence, the presence of multiple bands confirms the expression of vimentin.

Conclusions

The transfection results of different proteins have been shown and explained in detail. Most proteins are observed at a larger size than expected due to post-translational modifications. The optimisation of the transfection of NUCB1 is in progress.

Chapter 5: Conclusions

Monoacylgylcerols have been the subject of interest for a long time due to one single molecule, 2-arachidonoyl glycerol. Its role as an endocannabinoid has led to extensive research over the past few decades, enabling us to know a great deal about this lipid. A few recent examples in the literature linking MAGs to insulin secretion highlighted a possible role of these lipids in regulating the signalling pathway involved in glucose-stimulated insulin secretion. Considering these examples, we aimed to uncover other such novel interactions between MAGs and proteins, which could provide insight into the role of these lipids as signalling molecules.

To achieve this, we used bifunctional lipid probes to find putative protein interactors of MAGs. After applying rigorous filtering criteria, we narrowed the list to six different proteins, which had to be explored further for possible interactions with MAGs. A detailed literature review of all these proteins revealed that they were all membrane proteins with different functions. While some were well-characterised, others needed more data to draw definite conclusions about them.

Among the list of proteins, PCYOX1L is the least characterised protein, and though its function as an oxidoreductase enzyme might not indicate a direct link with MAG, there is a possibility of discovering some other role of this protein which might make it a significant interactor of MAG. HPCA is the most promising protein on the list, as it has the characteristic EF-hand domains, also present in NUCB1, a known lipid-binding protein, and it is known to interact with another signalling lipid. Though the EF-hand domain has not been shown as a lipid-binding domain and is mainly associated with calcium-binding, the presence of this domain in both proteins makes HPCA a strong candidate as an interactor of MAGs.

Two different mitochondrial proteins with related functions have been indicated as possible interactors of MAGs. This is interesting as both TOMM22 and SAMM50 are involved in the transport of mitochondrial proteins, and they have been shown to regulate lipid levels by interacting with different proteins. This increases the likelihood that these proteins are connected to MAGs, either through direct interaction or via interactions with other proteins that control MAG levels.

The remaining two proteins, PCYOX1L and NAP1L1, have been shown as essential biomarkers in different types of cancer, with vimentin being a strong indicator of the disease. This raises the question of whether MAGs are involved in cancer signalling pathways and if the interaction of MAGs with any of these proteins has any role, something worth investigating.

The literature review established a brief background of all the proteins indicating their possible roles in interacting with MAGs. Coming to the experimental plan of the project, we were able to prepare protein constructs of seven genes and express them successfully in HEK cells. We now have the overexpressed protein, and further experiments can be carried out to validate the interaction between proteins and MAGs. Preliminary investigations using activity-based protein profiling could be performed where the proteins are incubated with the MAG and the fatty acid probe. A true interactor of MAG would show preferential binding to the MAG probe. This would allow us to select and use those proteins for validation experiments.

Incubation of cell lysates with various lipids and MAGs is also practical as it proves whether the protein is a general lipid interacting protein or binds explicitly to MAGs. Different classes of lipids can be used for this purpose. The proteins which show preferential binding to MAGs in the preliminary experiments can then be selected for further studies. Depending on the protein's function, experiments can be designed to examine the interaction between the protein and MAG.

These suggestions can prove helpful in making future plans for the project. Currently, this project is at an exciting stage with numerous possibilities for making novel discoveries.

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