CONSTRUCTION OF PLASMID VECTOR FOR EXPRESSION OF PRELIMINARY MICRORNA-367 IN MAMMALIAN CELL

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SCHOOL OF HEALTH SCIENCES

UNIVERSITI SAINS MALAYSIA

2025

CONSTRUCTION OF PLASMID VECTOR FOR EXPRESSION OF PRELIMINARY MICRORNA-367 IN MAMMALIAN CELL

by

ANGELYN LEE CHUI YEE

Dissertation submitted in partial fulfillment of the requirements for the degree of

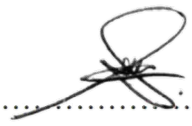
Bachelor of Health Science (Honours) (Biomedicine)

JANUARY 2025

CERTIFICATE

This is to certify that the dissertation entitled "Construction of plasmid vector for expression of preliminary microrna-367 in mammalian cell" is the bona fide record of research work done by Ms. Angelyn Lee Chui Yee during the period from September 2024 to January 2025 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfillment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

Main supervisor



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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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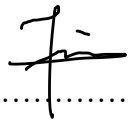

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Date: 27 January 2025

ACKNOWLEDGEMENT

I would like to take a moment to offer my heartfelt thanks to everyone who has been a part of this incredible journey, both in the lab and beyond, making my final year research project an experience I will always cherish. First and foremost, I extend my deepest gratitude to my esteemed supervisors, Assoc. Prof. Dr. See Too Wei Cun and Assoc. Prof. Dr. Few Ling Ling, for entrusting me with the project titled "Construction of plasmid vector for expression of preliminary microRNA-367 in mammalian cells." Their expert guidance, mentorship, and continual support were invaluable throughout this journey. I am truly fortunate to have had their direction, as their profound knowledge and insightful feedback shaped both my research and personal growth. I would also like to sincerely thank my seniors, Mr. Mhd Houssam Tounbakjy and Ms. Shaleniprieya A/P Muniandy, along with my junior, Ms. Lim Yin Wun, for their unwavering support and guidance throughout my research project. Their guidance in laboratory work and willingness to share their expertise have been invaluable in helping me overcome challenges and develop essential skills for my future pursuits. A special mention goes to my lab partner and friend, Ms. Nurul Alya binti Mazri, whose collaboration and support were indispensable throughout this project. Her assistance and teamwork have made this experience both productive and enjoyable. Lastly, I am deeply grateful to my family for their unwavering love, encouragement, and prayers, which have been a constant source of strength throughout this research journey. I wish to express my deepest gratitude to my other friends, Ms. Hasya Athirah binti Hanizam and Ms. Hanina Sofea binti Faizal, whose constant encouragement and uplifting words have been a pillar of comfort throughout this journey. Their unwavering presence has lightened the load and made this experience not only bearable but truly rewarding. I am incredibly lucky to have such inspiring and supportive friends in my life.

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
µg/mL	Microgram per Millilitre
µg/µL	Microgram per Microlitre
µL	Microlitre
µM	Micrometre
µm	Micrometre
A	Ampere
bp	base pair
CFU/µg	Colony Forming Unit per Microgram
g	Gram
g	Gravitational force
kb	Kilobase
kDa	Kilodalton
mg	Milligram
mg/mL	Milligram per Millilitre
mL	Millilitre
ng	Nanogram
ng/ µL	Nanogram per Microlitre
psi	Pound per Square Inch
rpm	Revolutions per Minute
s^{-1}	Turnover Rate
V	Volt

LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AGO	Argonaute
AKT	Protein Kinase B
AMPK	AMP-activated Protein Kinase
AP-1	Activating Protein 1
APAF1	Apoptotic Protease Activating Factor 1
ATF/CREB	Activating Transcription Factor/cAMP Response Element-binding Protein
ATP	Adenosine Triphosphate
brca	Breast Cancer Gene
C.elegans	*Caenorhabditis elegans*
CAF	Cancer Associated Fibroblast
cAMP	Cyclic Adenosine Monophosphate
CCR4-NOT	Carbon Catabolite Repression-Negative On TATA-less
CDP	Cytidine-5'-diphosphocholine
cdk	Cyclin-dependent Kinase
CHKA	Choline Kinase Alpha Protein
CHKB	Choline Kinase Beta Protein
chka	Choline Kinase Alpha Gene
chkb	Choline Kinase Beta Gene
ChoK	Choline Kinase
CLL	Chronic Lymphocytic Leukaemia

CMV	Cytomegalovirus
CSCC	Cutaneous Squamous Cell Carcinoma
DAG	Diacylglycerol
DGCR8	DiGeorge Syndrome Critical Region 8
DLBCL	Diffuse Large B Cell Lymphoma
DNA	Deoxyribonucleic Acid
E.coli	*Escherichia coli*
E2F1	E2F Transcription Factor 1
EB	Elution Buffer
ERK	Extracellular Signal-related Kinase
fasl	Tetrafunctional Fatty Acid Synthase Subunit Gene
fxr1	Fragile X-related Protein-1
GW182	Glycine-tryptophan Protein of 182 kDa
HIF-1α	Hypoxia-inducible factor 1-alpha
HMGA2	High Mobility Group AT-hook 2
HOXA9	Homeobox A9
Kan	Kanamycin
KDAC	Lysine Deacetylase
LPC	Lysophosphatidylcholine
m7G	7-methyl Guanosine
miRNA	MicroRNA
miRISC	MiRNA Induced Silencing Complex
mRNA	Messenger Ribonucleic Acid
MSC	Multiple Cloning Site

mTORC1	Mammalian Target of Rapamycin Complex 1
MYF	Myelocytoma
NF-κB/Rel	Nuclear Factor Kappa B/Rel
NIH 3T3	National Institutes of Health 3T3 Cells
OD	Optical Density
ori	Origin of Replication
p85α	Phosphatidylinositol 3-kinase Regulatory Subunit of Alpha Gene
P13K	Phosphoinositide 3-Kinase
PA	Phosphatidic Acid
PAN	Poly(A)-nuclease
PC	Phosphatidylcholine
pcd4	Programmed Cell Death Gene 4
PCho	Phosphocholine
PDAC	Pancreatic Ductal Adenocarcinoma
PE	Protein Extraction Buffer
PEG	Polyethylene Glycol
PI3K-AKT	Phosphatidylinositol 3-kinase/Protein Kinase B
PIP2	Phosphatidylinositol 4,5-biphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
pre-miR-367	Preliminary MicroRNA-367
pri-miRNA	Primary MicroRNA
PSA	Prostate-specific Antigen

pten	Phosphatase and Tensin Homolog Gene
RAB23	Ras-associated Binding Protein 23
RAR-RXR	Retinoic Acid Receptor Complex
RE	Restriction Enzyme
reck	Reversion-inducing Cysteine-rich with Kazal motifs Gene
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic Acid
shRNA	Short Hairpin Ribonucleic Acid
SP-1	Specificity Protein 1
TAE	Tris-acetate-EDTA
T-ALL	T-cell Acute Lymphoblastic Leukaemia
tp53	Tumour Protein 53
UTR	Untranslated Region
UV	Ultraviolet
UVB	Ultraviolet B
XRN1	Exoribonuclease 1

PEMBINAAN VEKTOR PLASMID UNTUK EKSPRESI PRE-MIR-367 DALAM SEL MAMALIA

ABSTRAK

Tesis ini memberi tumpuan kepada pembinaan vektor plasmid untuk ekspresi pre-miRNA-367, dengan penekanan khusus pada hubungannya dengan miRNA-367-3p dan potensi peranannya dalam mengawal proses biologi utama. Pre-miRNA bertindak sebagai perantara penting dalam biogenesis miRNA matang, yang mengawal ekspresi gen dengan mengikat sasaran mRNA yang bersesuaian, menyebabkan degradasi atau penghambatan terjemahan mereka. MiRNA-367-3p, khususnya, telah dikaitkan dengan pelbagai fungsi selular seperti apoptosis, proliferasi sel, dan pembezaan, yang mencadangkan aplikasi terapeutiknya dalam penyakit yang berkaitan dengan disfungsi miRNA. Objektif utama kajian ini adalah untuk membina plasmid yang mampu mengekspresikan pre-miRNA 367 dalam sel mamalia, dengan itu memudahkan penyelidikan mengenai peranan fungsinya dan potensi terapeutiknya. Metodologi yang digunakan melibatkan mereka bentuk urutan pre-miRNA 367 sintetik dengan tapak pengehad yang diperlukan untuk pengklonan ke dalam vektor plasmid pCMV-MIR. Selepas pencernaan menggunakan *Xho*I dan *BamH*I, sisipan pre-miRNA disambungkan ke dalam vektor dan seterusnya dipindahkan ke dalam sel *E. coli* XL1-Blue. Walaupun usaha pengoptimuman dilakukan, termasuk pelarasan terhadap syarat ligasi dan nisbah molar sisipan-ke-vektor, tiada koloni yang diperolehi, menunjukkan bahawa isu semasa proses ligasi atau transformasi mungkin telah menyumbang kepada kegagalan penggabungan plasmid. Bahagian perbincangan menyediakan analisis terperinci tentang hasil eksperimen, menunjukkan langkah-langkah yang diambil untuk meningkatkan proses ligasi dan cabaran yang dihadapi. Walaupun pengklonan tidak berjaya, kajian ini memberikan pandangan yang berharga mengenai

cabaran yang berkaitan dengan pembinaan vektor ekspresi pre-miRNA dan menawarkan cadangan untuk mengatasi cabaran ini untuk mencapai ekspresi pre-miRNA 367 yang berjaya dalam sel mamalia.

CONSTRUCTION OF PLASMID VECTOR FOR EXPRESSION OF PRELIMINARY MICRORNA-367 IN MAMMALIAN CELL

ABSTRACT

This thesis focuses on the construction of plasmid vector for the expression of preliminary microRNA-367, with a particular emphasis on its connection to miRNA-367-3p and its potential role in regulating key biological processes. Pre-miRNAs serve as essential intermediates in the biogenesis of mature miRNAs, which regulate gene expression by binding to complementary mRNA targets, leading to their degradation or translational inhibition. MiRNA-367-3p, in particular, has been implicated in various cellular functions such as apoptosis, cell proliferation, and differentiation, suggesting its potential therapeutic applications in diseases linked to miRNA dysregulation. The primary aim of this study was to construct a plasmid capable of expressing pre-miRNA 367 in mammalian cells, thereby facilitating the investigation of its functional roles and therapeutic potential. The methodology employed involved designing a synthetic pre-miRNA 367 sequence with necessary restriction sites for cloning into the pCMV-MIR plasmid vector. Following digestion with *Xho*I and *BamH*I, the pre-miRNA insert was ligated into the vector and subsequently transformed into *E. coli* XL1-Blue cells. Despite optimisation efforts, including adjustments to the ligation conditions and insert-to-vector molar ratio, no colonies were obtained, suggesting that potential issues during the ligation or transformation process may have contributed to the lack of successful plasmid incorporation. The discussion provides a detailed analysis of the experimental results, highlighting the steps taken to enhance the ligation process and the challenges encountered. While the cloning was unsuccessful, this study provides valuable insights into the challenges associated with the construction of pre-miRNA expression vectors and

offers recommendations for overcoming these challenges to achieve successful expression of pre-miRNA 367 in mammalian cells.

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Cancer arises when certain cells in the body begin to grow uncontrollably and spread to other areas. It can originate in nearly any part of the body, which consists of trillions of cells. Normal cell growth and division result in the production of new cells as needed for development and repair. Old or damaged cells are taken out and replaced with new ones in a regulated process. However, this balance can be disrupted, leading to the uncontrolled growth of abnormal or damaged cells. These cells can aggregate into lumps called tumours, which can either be benign or malignant (National Cancer Institute, 2021). Metastasis is the term for the ability of malignant tumours to invade neighbouring tissues and spread to other areas of the body. Genetically, these tumours exhibit significant abnormalities. Their genes can be amplified, lost, or otherwise altered, and their chromosomes may be rearranged in ways that are uncommon in normal cells (Cooper, 2020).

Normal cells and cancer cells differ in several fundamental ways. While normal cells only divide when they receive specific signals, cancer cells can multiply without these signals and even ignore instructions to undergo programmed cell death, a process known as apoptosis. This resistance allows them to persist and keep growing. The way cancer cells obtain energy also differs from normal cells. They often rely on alternative nutrient sources and can use distinct metabolic pathways to convert those nutrients into energy, enabling them to grow rapidly (Chainitikun, 2022). For instance, to provide a greater supply of nutrients, cancerous tumours have the ability to induce angiogenesis, and the development of new blood vessels.

A complicated, multi-step process called carcinogenesis leads to the eventual transformation of healthy cells into tumour cells, usually progressing from a precancerous condition to a malignant tumour. This transformation occurs due to the interplay of an individual's genetic predispositions and three main categories of external factors.

There are three primary categories of genes involved in regulating cell growth, and their alterations can lead to the development of cancer. Oncogenes are altered genes responsible for promoting unchecked cell proliferation, which can result in cancer. Proto-oncogenes are the normal counterparts of these genes; they play a crucial role in regulating cell growth. However, when proto-oncogenes undergo mutations, they can become oncogenes (Cancer Research UK, 2019).

There are several ways oncogenes can be activated within cells. One mechanism is through genetic mutations. Some individuals may possess variations in their gene sequences that cause oncogenes to remain active continuously. These mutations can be inherited from one parent or can arise during an individual's lifetime due to errors in gene replication during cell division. Another important factor is epigenetic modifications. Cells possess mechanisms to regulate gene expression without altering the underlying DNA sequence. Various chemical groups can attach to DNA or RNA, influencing whether a gene is expressed (American Cancer Society, 2022). Oncogenes can occasionally become activated as a result of such epigenetic changes.

Essential genes known as tumour suppressors are involved in controlling cell division and development, repairing DNA damage, and initiating the typical process of programmed cell death, or apoptosis. They thereby serve as a cancer defense mechanism. Tumour suppressor genes are active when they are working properly and aid in preventing cells from dividing too rapidly. However, mutations can inactivate these genes, leading

to uncontrolled cell proliferation and potentially resulting in cancer. A notable example of a tumour suppressor gene is *tp53*. This gene encodes the P53 protein, which is vital for maintaining proper control over the cell cycle. Li-Fraumeni syndrome, a hereditary disorder that significantly increases the probability of acquiring multiple cancer types, can be caused by inherited mutations in the TP53 gene. Additionally, alterations in the TP53 gene are frequently observed in cancer cells among individuals without a hereditary cancer predisposition (American Cancer Society, 2022). These mutations are typically acquired throughout a person's life and can facilitate the growth of cancerous cells. Importantly, these mutations are specific to cancer cells and are not present in other body cells, meaning they cannot be inherited by the next generation.

Genes responsible for DNA repair are critical for correcting errors that may occur during DNA replication. Mutations in these repair genes impair their ability to correct errors in tumour suppressor and oncogene genes, which may lead to the development of cancer. *brca1* and *brca2* are two well-known genes involved in DNA repair. Those who inherit a detrimental variant (mutation) in any of these genes are more likely to develop some malignancies, particularly in women, breast and ovarian cancer (American Cancer Society, 2022).

In addition to compromised DNA repair mechanisms, dysregulated cellular metabolisms are important factors in tumour growth. An enzyme called choline kinase (ChoK), which converts choline into phosphocholine, has been implicated in cancer development. Choline kinase (ChoK) is frequently overexpressed and activated in cancers such as lung, breast, colon, prostate, and ovarian tumours, contributing to their development and progression. The alpha isoform (ChoKα) is particularly upregulated in tumour-derived cell lines and tissues, with increased expression observed in 40–60% of cases, driven by growth factors, hormones, and carcinogens. These cancers represent over

70% of cases in developed countries, highlighting ChoKα's significant role in cancer biology (Gallego et al., 2011).

Complementing these findings, microRNAs (miRNAs) have emerged as critical regulators of cellular processes, including those implicated in cancer progression. MicroRNAs (miRNAs) are small, non-coding RNA molecules approximately 22 nucleotides in length, found in all eukaryotic cells, where they play critical roles in regulating various biological processes. The discovery of their function began in 1993 when two separate studies identified the *Caenorhabditis elegans* heterochronic gene lin-4 as a small non-coding RNA. Seven years later, further research revealed that another heterochronic gene, let-7, along with lin-4, initiated a temporal regulatory cascade in *C. elegans* (Acunzo et al., 2015). These findings spurred extensive studies that confirmed the existence of a broad class of small non-coding RNAs with regulatory functions, now known as microRNAs.

Dysregulation of miRNA expression has been strongly associated with cancer progression, where they may act as either oncogenes or tumour suppressors. Abnormalities in their expression and function have been linked to tumour characteristics, including origin, invasiveness, stage, and response to treatment. The biogenesis of miRNAs involves several enzymes and cofactors which facilitate the formation of functional miRNA complexes. These regulatory molecules are pivotal in both normal physiological processes and pathological conditions, including cancer. Mutations or disruptions in miRNA-associated pathways can lead to oncogenesis (Babaei et al., 2020). By influencing cancer cell behaviour, miRNAs and their target interactions provide insights into the molecular underpinnings of cancer, enabling the development of novel diagnostic and therapeutic approaches.

The historical context of cancer research underscores the significance of understanding the genetic and molecular mechanisms that drive tumorigenesis. Targeted therapies have been made possible by advances in genetic research, underscoring the continuous need for novel approaches to cancer treatment. As research explores deeper into the molecular pathways involved in cancer, it becomes evident that microRNAs, such as microRNA-367, hold substantial potential for regulating gene expression and influencing cancer-related pathways.

The ongoing challenges in treating cancer serve as a reminder of the need for innovative therapeutic approaches. Developing a plasmid vector to express microRNA-367 in mammalian cells is a promising way to study its regulatory functions in tumour suppressor and oncogene genes. Understanding the roles played by microRNA-367 will help researchers create specific therapies that attempt to both suppress the growth of tumours and restore normal cellular processes.

1.2 Problem Statement

Despite the role of microRNAs (miRNAs) in the regulation of gene expression is widely recognised, the manner in which these short non-coding RNAs, in particular miR-367, affect the expression of choline kinase genes has been insufficiently focused on. Choline kinase alpha (CHKA) plays a vital role in cancer metabolism by converting choline into phosphatidylcholine, an essential element of cell membranes. However, the mechanisms that regulate CHKA expression, particularly through miRNAs, have not been thoroughly investigated (Yao et al., 2023). This lack of understanding is critical, as a more in-depth comprehension of the regulatory pathways controlled by miRNAs could provide new knowledge on how cancer develops, and potential implications for therapy.

Although microRNAs (miRNAs) have gained significant consideration as important aspects of gene regulation, there is still a significant gap in the techniques available for exploring the relationship between pre-miR-367 and choline kinase alpha (CHKA). Current methodologies used to examine these interactions often fall short, lacking the precision and sensitivity required to effectively resolve the complexities associated with cancer biology (Sayed et al., 2021). For instance, conventional techniques may fail to capture the diverse roles that pre-miR-367 plays under varying cellular conditions, resulting in a fragmented understanding of how this miRNA regulates CHKA expression and impacts essential cancer-related processes such as cellular growth, invasion, and the development of resistance to anticancer agents.

Current studies underscore a link between increased expression of choline kinase alpha (CHKA) and more aggressive tumour traits, indicating that reducing its levels may hinder cancer cell proliferation and their ability to spread. Moreover, accumulating evidence suggests that microRNAs, such as miR-367-3p, play a crucial role in shaping the tumour microenvironment, which could influence how tumours respond to chemotherapy (Sayed et al., 2021). However, the precise mechanisms through which miR-367-3p controls choline kinase alpha (CHKA) expression and how this alters cellular behaviour are poorly understood (Raikundalia et al., 2021). Additional studies are essential to understand the effects of miR-367-3p-induced downregulation of choline kinase alpha (CHKA) comprehensively. These investigations should include various cancer cell lines to evaluate how this regulatory mechanism impacts cell growth, invasion, and responsiveness to anticancer treatments. Gaining clarity on these dynamics could reveal important opportunities for therapeutic interventions aimed at miR-367-3p and CHKA, potentially improving treatment outcomes and tackling the issue of drug resistance.

1.3 Rationale of Study

The prospect of targeting choline kinase alpha (CHKA) as a therapeutic approach in oncology is increasingly being acknowledged. Evidence suggests that diminishing choline kinase alpha (CHKA) expression can hinder tumour development and its ability to spread, positioning it as a significant target in various malignancies. This research intends to examine the role of microRNA-367 (miR-367-3p) in regulating choline kinase alpha (CHKA) expression, thereby presenting a unique strategy to inhibit tumour growth while reducing potential side effects associated with conventional treatments.

This study also highlights the cost-effectiveness of using miR-367-3p through its precursor, pre-miR-367, as a significant advantage in miRNA research. Unlike synthetic miRNAs, which are expensive and have transient effects, the expression of miR-367-3p using constructs such as pCMV-MIR-pre-miR-367 offers a more efficient and affordable alternative. This method reduces the dependency on costly synthetic miRNAs, providing a practical and scalable approach for research applications without compromising efficacy.

Furthermore, understanding the significance of pre-miR-367 synthesis within cells is vital for the successful implementation of this treatment method. As miR-367-3p is generated from pre-miR-367, it is essential for the desired modulation of choline kinase alpha (CHKA) expression that this precursor is produced in target cells. This highlights the need to thoroughly investigate the regulatory mechanisms governing the transition from pre-miR-367 to miR-367-3p and how they influence cancer cell dynamics. By clarifying this regulatory framework, the research aims to offer valuable insights that could improve the effectiveness of future cancer therapies, establishing miR-367-3p as a potentially critical component in cancer treatment protocols.

1.4 Objectives

The general objective of the project was to construct plasmid vector for expression of preliminary microRNA-367 in mammalian cell.

There were three specific objectives:

1. To design synthetic gene for pre-miR-367 containing suitable restriction enzyme for cloning into pCMV-MIR vector
2. To clone the synthetic pre-miR-367 into pCMV-MIR vector
3. To verify the pCMV-MIR-pre-miR-367 construct propagated in *E.coli* XL1-Blue

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of microRNAs

MicroRNAs were initially discovered in the 1990s within *Caenorhabditis elegans* and are believed to play an essential role in controlling gene expression in both animal and plant cells. They are small, non-protein-coding RNA molecules, about 22 nucleotides long. They are primarily transcribed from DNA into primary microRNAs (pri-miRNAs) and then sequentially processed into precursor forms (pre-miRNAs) and mature miRNAs. Most often, miRNAs bind to the 3′ untranslated regions (UTRs) of target messenger RNAs (mRNAs) to inhibit gene expression. Nevertheless, it has also been observed that miRNAs interact with other mRNA regions, including coding sequences, the 5′ UTR, and even gene promoters (O'Brien et al., 2018). Interestingly, miRNAs can also promote gene expression in specific contexts. Research has revealed that miRNAs can move between different subcellular areas, where they influence both translation and transcription rates. miRNAs play essential roles in normal development and regulate many biological processes. Their dysregulation has been linked to various human diseases. Furthermore, miRNAs are released into body fluids, where they are recognised as promising biomarkers for many diseases and function as signalling agents, facilitating communication between cells (Wang et al., 2019).

2.2 microRNAs biogenesis

The generation of miRNAs begins when RNA polymerase II or III transcripts undergo processing either after transcription or simultaneously with it. Half of known miRNAs are located within genes, often derived from introns and, to a lesser extent, from exons of protein-coding genes. The rest exist outside genes and are transcribed independently under the control of their promoters. Occasionally, miRNAs are expressed

as one lengthy transcript called clusters, which may share seed regions and are thus categorised as a family. The pathways for miRNA production are divided into canonical and non-canonical.

2.2.1 Canonical Pathway of miRNA Production

The canonical pathway is the primary route for miRNA processing. In this pathway, pri-miRNAs are transcribed and subsequently trimmed into pre-miRNAs by the microprocessor complex, composed of DGCR8, an RNA-binding protein, and Drosha, a ribonuclease III enzyme. DGCR8 identifies specific motifs in pri-miRNA sequences, while Drosha cleaves the hairpin structure at the base, creating a two-nucleotide overhang at the 3′ end of the pre-miRNA (Jorge et al., 2021).

Based on Figure 2.1, the process of canonical miRNA biogenesis begins with the generation of the pri-miRNA transcript. This primary transcript is then processed by the microprocessor complex, composed of Drosha and DGCR8, which cleaves it to form the precursor-miRNA (pre-miRNA). Once the pre-miRNA is transported to the cytoplasm with the assistance of Exportin5 and RanGTP, it undergoes further processing to produce the mature miRNA duplex. The strand direction within this duplex designates the mature miRNA: the 5p strand arises from the 5′ end, while the 3p strand is from the 3′ end (Bofill-De Ros & Vang Ørom, 2023). Both strands can be incorporated into Argonaute proteins (AGO1-4 in humans), and the strand that is loaded depends largely on the thermodynamic stability of each strand's 5′ end, with the less stable or 5′ uracil strand more likely to be loaded as the guide strand (Meijer et al., 2014). The other, or passenger strand, is released and typically degraded, especially if loaded into AGO2, facilitating its cleavage. Finally, one strand from the mature miRNA duplex, either the 5p or 3p strand, is incorporated into the miRNA-induced silencing complex (miRISC) by loading into the AGO protein family.

Alternatively, miRNA duplexes with central mismatches are unwound passively (O'Brien et al., 2018).

2.2.2 Non-Canonical Pathways of miRNA Production

Various non-canonical pathways for miRNA maturation also exist, employing distinct combinations of canonical proteins, including Drosha, Dicer, exportin 5, and AGO2. Non-canonical pathways are broadly categorized into Drosha/DGCR8-independent and Dicer-independent routes. Pre-miRNAs formed independently of Drosha/DGCR8, such as mirtrons produced from spliced introns, can enter the cytoplasm directly via exportin 1, bypassing Drosha cleavage. These pathways often exhibit a preference for the 3p strand, as modifications like the m7G cap hinder the loading of the 5p strand into AGO (Jorge et al., 2021).

Based on Figure 2.1, the microprocessor complex first processes small hairpin RNA (shRNA) by cleaving it. Following this, the Exportin5/RanGTP complex transports the shRNA to the cytoplasm. In the cytoplasm, shRNA undergoes further cleavage, which is dependent on AGO2 but not on Dicer. While both Mirtrons and 7-methylguanine-capped (m7G)-pre-miRNAs require Dicer for their final maturation in the cytoplasm, their nucleocytoplasmic transport mechanisms differ. Mirtrons are exported by Exportin5/RanGTP, whereas m7G-pre-miRNAs are transported by Exportin1. Regardless of the pathway, all lead to the formation of a functional miRISC complex. For translational repression, the miRISC typically binds to target mRNAs, likely disrupting the eIF4F complex (Hayder et al., 2018). This attracts poly(A)-deadenylases PAN2/3 and the CCR4-NOT complex, which are recruited by GW182 family proteins bound to Argonaute. The PAN2/3 complex initiates deadenylation, which is completed by the CCR4-NOT complex, subsequently leading to the removal of the m7G cap by the

decapping complex. Finally, the exoribonuclease XRN1 can degrade the decapped mRNA in a 5′−3′ direction (Hayder et al., 2018).

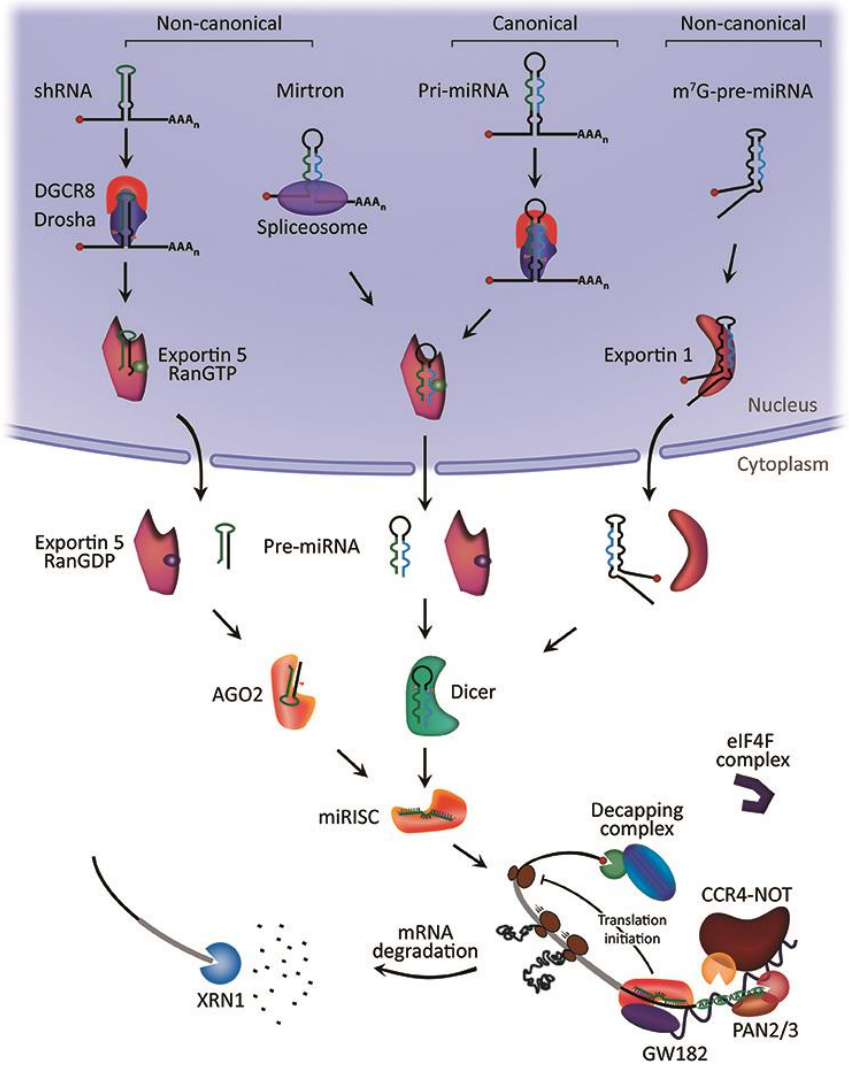

Figure 2.1: Summary of the canonical and non-canonical miRNA biogenesis pathway (O' Brien et al., 2018)

MicroRNAs (miRNAs) influence gene expression by binding to messenger RNAs (mRNAs) through sequence similarity, which alters the production of proteins encoded by those mRNAs. This binding can vary in strength, depending on how closely matched the miRNA and mRNA sequences are, with stronger matches leading to more lasting

effects. MiRNAs are recognized as key modulators of gene expression, operating through flexible mechanisms shaped by where they are located and how they interact with mRNAs.

The process by which miRNAs function is complex, involving multiple steps and protein partners. Typically, miRNAs attach to the 3' untranslated region (UTR) of target mRNAs but can also bind to other areas, such as the 5' UTR or even promoter regions. Where miRNAs bind on the mRNA influences the biological outcomes; in most cases, miRNAs inhibit gene expression by blocking the protein production of target mRNAs. However, when miRNAs bind to gene promoter regions, they can enhance gene expression instead of repressing it (Jorge et al., 2021).

In some interactions, miRNA binding can result in mRNA degradation through a cleavage process carried out by an enzyme within the RNA-induced silencing complex (RISC), which the miRNA associates with. Early studies suggested that this degradation required full sequence matching, and that partial matching led only to temporary inhibition. However, more recent findings reveal that in animal cells, most miRNAs only partially match with mRNAs, yet this level of binding is sufficient to consistently block protein translation from target mRNAs (Jorge et al., 2021).

Because miRNAs are small and can bind even without complete sequence alignment, they have the potential to interact with numerous mRNAs, leading to different biological outcomes depending on the cell type (Dexheimer & Cochella, 2020). As a result, the effects of a particular miRNA can vary widely across different cellular contexts.

2.3 Mechanisms of miRNA-Driven Gene Regulation

2.3.1 Silencing Mechanisms via miRISC

The miRNA-induced silencing complex (miRISC) is composed of a guide strand paired with an Argonaute protein (AGO), which directs it to mRNA targets based on sequence compatibility. When the miRNA fully complements the target mRNA's sequence, AGO2 cleaves the mRNA directly (Jungers & Djuranovic, 2022). In most cases, however, the miRNA only partially pairs with the target, which blocks the slicing function of AGO2 and shifts the role of AGO to translational repression and mRNA degradation instead. Binding typically relies on the "seed" sequence of the miRNA (nucleotides 2–8), while additional bonding at the 3′ end stabilizes this interaction. For miRISC-mediated silencing, the complex recruits GW182 proteins, which act as scaffolds, recruiting deadenylation complexes like PAN2-PAN3 and CCR4-NOT. These complexes remove the poly(A) tail from the mRNA, leading to decapping and 5′-3′ degradation by exonucleases, such as XRN1 (Duchaine & Fabian, 2019).

2.3.2 miRNA-Induced Gene Activation

While most miRNA research has centred on gene repression, some studies show miRNAs can also increase gene expression under certain conditions. For instance, in serum-deprived cells, miRNAs interact with AU-rich elements in the 3′ UTR, in association with *ago2* and *fxr1*, to promote translation (Vaschetto, 2018). This miRNA-driven activation has been observed in non-dividing cells, like oocytes, and can also occur during amino acid shortages where miRNAs bind to 5′ UTRs of mRNAs coding for ribosomal proteins, enabling selective translation (Jorge et al., 2021).

2.3.3 miRNA Role in Nuclear Gene Regulation

Through interactions with import and export proteins, AGO2 shuttles between the nucleus and cytoplasm. Inside the nucleus, miRISC has been linked to transcriptional regulation and post-transcriptional mRNA modulation. Nuclear miRNAs may degrade mRNA or modify transcription at active gene regions (Billi et al., 2024). Additionally, AGO2 has been implicated in suppressing genes promoting cell growth in senescent cells by binding to specific gene promoters. miRISC may also associate with enhancer regions, activating nearby genes and impacting chromatin structure, suggesting that miRNAs have broader transcriptional functions resembling transcription factors (O'Brien et al., 2018). Some evidence suggests miRISC might even contribute to chromatin condensation, gene methylation, and genomic restructuring (O'Brien et al., 2018).

2.4 microRNA Deregulation and Cancer

The connection between microRNA dysregulation and cancer was first identified in a study by Croce and colleagues, which revealed that the miR-15a/16-1 cluster is frequently deleted in chronic lymphocytic leukaemia (CLL). It was discovered that these microRNAs had tumour-suppressive properties (Balatti & Croce, 2020). Since then, many microRNAs that show either decreased or increased expression in comparison to normal tissues have been linked to cancer. For example, miR-21 promotes tumour formation through multiple mechanisms, including the upregulation of Bcl-2, suppression of *pten*, *reck*, *fasl*, *p85α*, and *pcd4*, as well as enhancement of the PI3K-AKT signalling pathway, which drives pancreatic ductal adenocarcinoma (PDAC) cells to proliferate, invade, and migrate (Baradaran et al., 2019). It also contributes to creating a tumour-supportive environment by engaging with cancer-associated fibroblasts (CAFs), facilitating cancer cell invasion and metastasis (Baradaran et al., 2019).

The study of the processes that underlie microRNA dysregulation in cancer has accelerated dramatically. MicroRNAs such as miR-365 acts as a tumour suppressor in some cancers, while in others, it is critical for tumour growth. Its effects depend on the cancer type and the surrounding cellular environment, influencing processes such as invasion, migration, cell death, and proliferation both in laboratory studies and in living organisms. Several genes targeted by miR-365 include *nf-i/b*, *bax*, *cyclin d1*, *bcl-2*, *fos*, *ezh2*, *mcl-1*, and *pik3r3*, reflecting its broad regulatory impact (Zhu et al., 2017). Research on NIH 3T3 cells exposed to UVB light, a major contributor to skin cancer, identified miR-365 as highly responsive to UV damage. Overexpression of pre-miR-365-2 in HaCaT skin cells leads to cancerous transformations, subcutaneous tumour development in BALB/c-nude mice, and increased cell proliferation, migration, and invasion *in vitro* (Garcia et al., 2019). Conversely, blocking miR-365 with anti-miR-365 oligonucleotides in A-431 cells induces G1 phase arrest and increases apoptosis. In cutaneous squamous cell carcinoma (CSCC), miR-365 downregulates HOXA9, a tumour-suppressing protein that promotes apoptosis and limits cell growth. Loss of HOXA9 results in the upregulation of HIF-1α, which drives hypoxia adaptation, glucose metabolism, and tumour progression (Zhou et al., 2018).

Recent studies on another microRNA, miR-186, suggest its association with various diseases, including vascular conditions, solid tumours, haematological malignancies, and bone-related disorders (Wu et al., 2018). High levels of miR-186 have been shown to suppress cell growth and reduce metastasis, while its reduced expression is associated with poor outcomes in lung adenocarcinoma. Additionally, miR-186 acts as a tumour suppressor in prostate and bladder cancers but exhibits carcinogenic properties when promoting the growth of pancreatic and endometrial cancers. Changes in drug sensitivity have also been linked to this microRNA (Li et al., 2019). Several target genes

of miR-186 are thought to influence key cellular processes, such as the cell cycle, epithelial-to-mesenchymal transition (EMT), and cell migration. These include *hIf1α*, *map3k2*, *cyclin d1*, *cdk2*, *cdk6*, *twist1*, *foxo1*, and *rock1* (Zhu et al., 2016). In A-431 cutaneous squamous cell carcinoma (CSCC) cells, miR-186 directly targets the apoptosis protease activating factor-1 (APAF1) gene (Tian et al., 2018). APAF1 plays a pivotal role in the intrinsic apoptosis pathway by forming the apoptosome in response to cytochrome c release. However, when miR-186 levels rise, APAF1 expression is suppressed, leading to reduced cell death and increased cell invasion, migration, and proliferation (Shakeri et al., 2017).

MicroRNA expression is also significantly influenced by transcriptional regulation. One well-known example is the MYC oncogene's stimulation of the miR-17/92 cluster, which strengthens E2F1's anti-apoptotic properties and encourages MYC-driven cell division. Furthermore, the control of the miR-34 family, which encourages apoptosis and senescence, has been connected to the tumour suppressor p53. A considerable percentage of ovarian cancer cases with p53 mutations exhibit the downregulation of miR-34, which is caused by the loss of p53 activity (Acunzo et al., 2015).

2.5 Role of miR-367 in Disease Progression

2.5.1 miR-367 in Cancer Development

MicroRNA-367 (miR-367) is part of the miR-302/367 cluster, which is crucial for processes like cell growth and division. Disruption in the regulation of this cluster has been associated with several conditions, including cancer and inflammation (Li et al., 2023). Elevated miR-367 levels in cancers such as breast, gastric, and prostate cancers have been linked to better survival rates and improved outcomes. Moreover, miR-367

shows promise as a diagnostic marker, particularly in breast and gastric cancers (Muniandy et al., 2023).

Breast cancer, a leading cause of cancer-related mortality in women, is influenced by changes in miR-367 levels, which impact tumour growth, metastasis, and cell death. Research shows that breast cancer patients with lymph node metastases tend to have lower miR-367-3p levels in their blood (Yang et al., 2023). Reduced miR-367-3p expression is also associated with larger tumours, more advanced disease stages, and worse prognoses (Liu et al., 2021). In laboratory studies using MCF-7 breast cancer cells, miR-367-3p was shown to reduce tumour cell migration and promote cell death by suppressing choline kinase alpha, pointing to its potential as a therapeutic target (Raikundalia et al., 2021).

In gastric cancer, one of the most common and fatal cancers worldwide, miR-367-3p acts as a tumour suppressor. Lower miR-367 levels in gastric tissues are correlated with disease progression. The increased miR-367 expression can inhibit tumour growth by targeting and blocking proteins such as RAB23, which contribute to tumour invasion. Additionally, miR-367-3p has been found to suppress the activity of HMGA2, an oncogene frequently implicated in gastric tumours, further underscoring its tumour-inhibiting properties (Tao et al., 2020).

Prostate cancer, one of the most frequently diagnosed cancers in men, often relies on prostate-specific antigen (PSA) levels for detection, but the limited accuracy of PSA tests necessitates alternative biomarkers. miR-367-3p has been shown to suppress tumour growth in prostate cancer by inhibiting the Hedgehog and Rab23 pathways, both of which are known to drive cancer progression when overactive. Experiments suggest that miR-367-3p can reduce cell proliferation, migration, and spread, indicating its potential as a therapeutic agent (Du et al., 2021).

Beyond these specific cancers, miR-367 holds promise as a diagnostic and therapeutic tool in aggressive malignancies. In embryonic central nervous system cancers, it may help in early detection, while in leukaemia, circulating miR-367 differentiates patients from healthy individuals and offers therapeutic potential. Elevated miR-367-3p expression in endometrial cancer has been linked to reduced metastasis and improved survival through HMGA2 suppression (Ma et al., 2018). Meanwhile, in melanoma, low miR-367 levels are associated with decreased tumour growth and spread, suggesting its role in disease progression (Long et al., 2018). In non-small cell lung cancer, overexpression of miR-367-3p has been found to block tumour growth and migration, indicating its relevance as a therapeutic target (Guo et al., 2020). These findings underscore miR-367's significant contributions to cancer research, highlighting its diagnostic and therapeutic potential across diverse cancer types.

2.6 The Human ChoK Enzyme Family

Choline kinase (ChoK) catalyses the phosphorylation of choline into phosphocholine (PCho) by utilising ATP as a phosphate donor (Raikundalia et al., 2021). Based on Figure 2.2, this reaction is a crucial step in the CDP-choline pathway, first described by Eugene Kennedy in 1956, which represents the primary mechanism for synthesizing phosphatidylcholine (PC) in mammalian cells. Phosphatidylcholine plays a vital role in forming cell membranes and producing lipid-derived signalling molecules. Alongside this pathway is the CDP-ethanolamine pathway, responsible for synthesizing phosphatidylethanolamine (PE), another essential phospholipid. Together, these two branches form the Kennedy pathway, which underpins the biosynthesis of key lipid components of cell membranes (WikiPathways, 2023). By facilitating membrane production, ChoK is integral to the growth and proliferation of eukaryotic cells, making

it a promising therapeutic target for treating cancers and parasitic infections (Zimmerman et al., 2019).

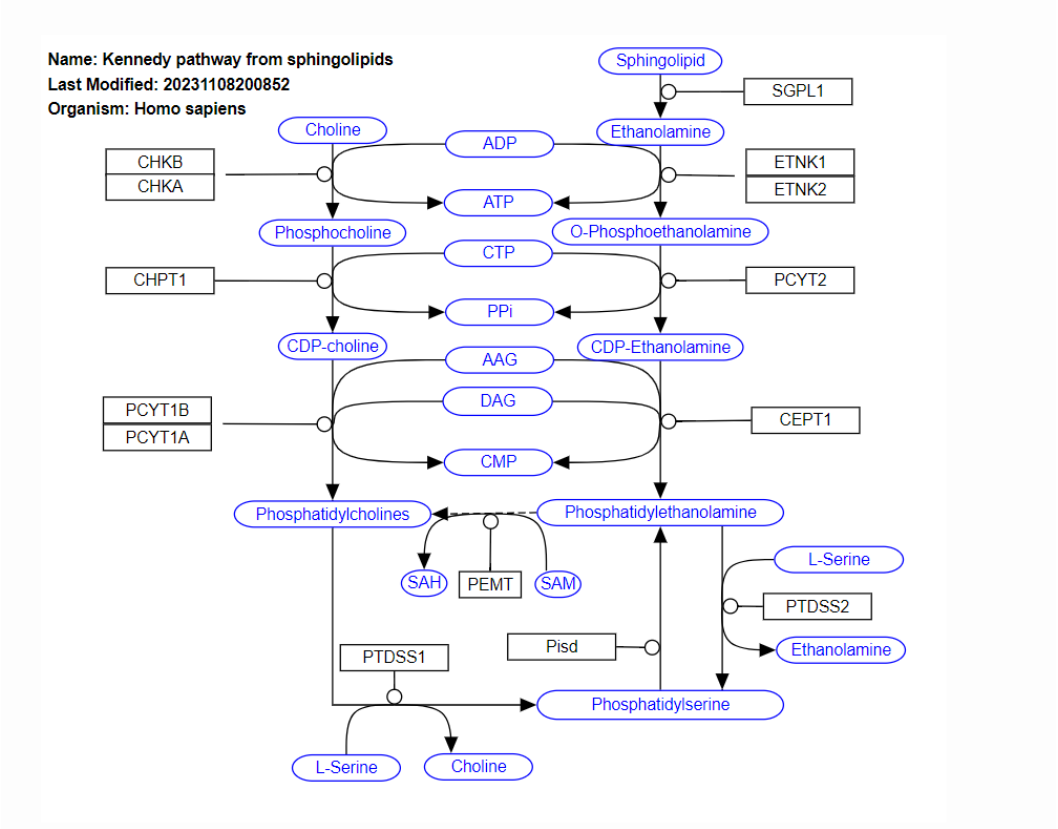

Figure 2.2: CDP-choline pathway

(WikiPathways, 2023)

In humans, the production of phosphorylcholine (PCho) is driven by two genes, *chka* and *chkb*. According to Table 2.1, both genes are located on chromosomes 11q13.1 and 22q13.33, respectively. These genes encode enzymes with molecular weights of approximately 50 kDa for ChoKα and 45 kDa for ChoKβ, the latter comprising 395 amino acids. The *chka* gene gives rise to two isoforms, ChoKα1 (52 kDa, 457 amino acids) and ChoKα2 (50 kDa, 439 amino acids), through alternative splicing (Lacal et al., 2021). While ChoKα and ChoKβ share about 56% sequence similarity, their roles in physiology and metabolism appear to differ significantly.

Table 2.1: Human Choline Kinases

(Lacal et al., 2021)

Subtypes	ChoKα1	ChoKα2	ChoKβ
Molecular weight	52 kDa	50 kDa	43 kDa
Size (amino acids)	457 aa	439 aa	395 aa
Intracellular location	Cytosol	Cytosol	Cytosol
Chromosomal locus	11q13.1	11q13.1	22q13.33

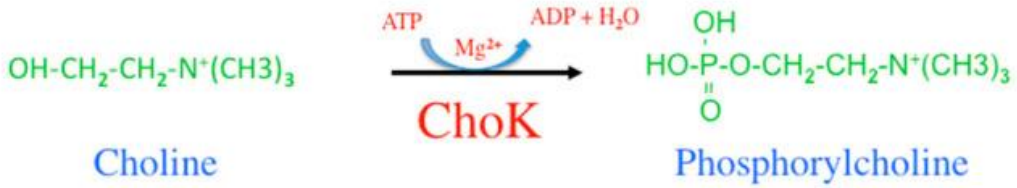

Figure 2.3: Human Choline Kinases

(Lacal et al., 2021)

Based on Figure 2.3, choline is phosphorylated in the presence of ATP and Mg^{2+} to form phosphorylcholine. The process involves the transfer of a phosphate group from ATP to choline, producing ADP and water as by-products. The chemical structures of the substrate (choline) and product (phosphorylcholine) are depicted.

ChoKα is critical for embryo development in both mice and plants, as shown by studies where the absence of ChoKα could not be compensated by ChoKβ. On the other hand, mice lacking ChoKβ survive but display muscular dystrophy and limb deformities due to reduced phosphatidylcholine (PC) levels, although this condition can be mitigated by ChoKα. Moreover, ChoKβ has been implicated in bone maintenance, aligning with

studies that show ChoK inhibition impairs mineralization in human bone cells. Mutations in *chkb* in humans cause similar muscle-related disorders (Chen et al., 2017).

Structural studies show that human ChoK proteins form dimers, with different levels of enzymatic activity among homo- and heterodimer formations, where the ChoKα homodimer is the most active, and the ChoKβ homodimer the least. This dimerization impacts cell cycle regulation, as varying the expression balance between ChoKα and ChoKβ isoforms may influence this process. The precise regulatory implications of these dimerization dynamics require further exploration to fully understand their role in enzyme function (Lacal et al., 2021).

2.7 Mechanism of Action of Choline Kinase (ChoK)

Choline kinase (ChoK) shares a structural similarity with other kinases through its ATP-binding site formed by the interaction of N- and C-terminal lobes, yet it stands out as an "atypical kinase" due to the presence of a flexible ATP-binding loop rather than the glycine-rich P-loop common in typical eukaryotic kinases. It has been known that the phosphorylation of choline by ATP requires magnesium ions for coordination. Recent studies by Hudson et al. (2013) reveal that ChoK operates via a double-displacement mechanism, meaning it does not form a ternary complex with both ATP and choline at once. Instead, ATP donates a phosphate group directly to ChoK at an aspartate residue (Asp306), which, once protonated, facilitates ADP release. The phosphorylated Asp306 then attracts choline, causing a structural change in ChoK that leads to PC release and restores the enzyme's original conformation. Interestingly, ChoK has been observed to exhibit ATPase activity even without choline, supporting this ping-pong mechanism (Hudson et al., 2013). Based on Figure 2.4, ChoKα follows this ping-pong mechanism, with Asp306 binding a phosphate group in a Mg^{2+}-facilitated reaction. This results in ADP

release and proton acquisition, followed by interaction with choline and subsequent PC release. The enzyme then resets to its initial state, ready for another catalytic cycle.

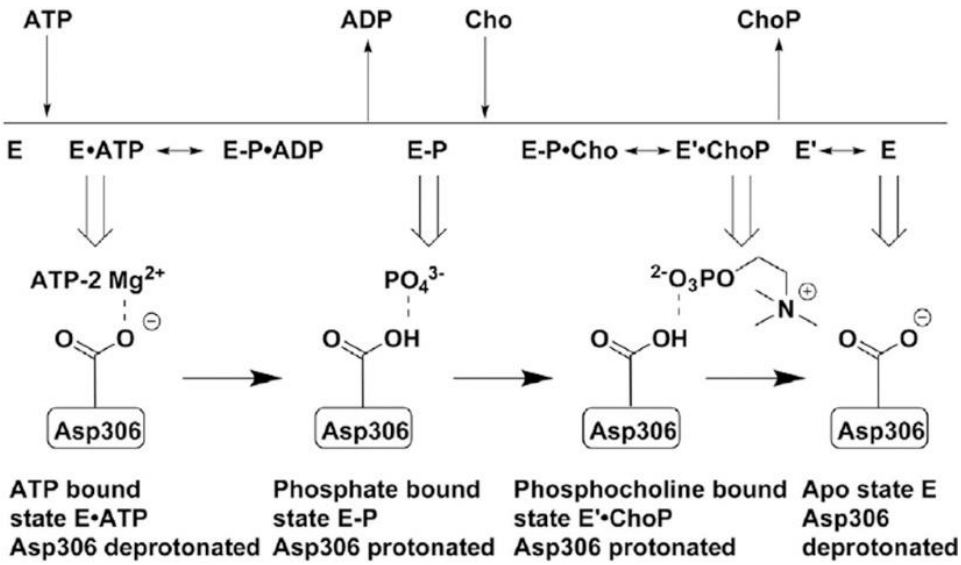

Figure 2.4: Phosphorylation of choline in an atypical ping-pong mechanism

(Hudson et al., 2013)

ChoK's active site differs from most protein kinases, with choline binding in a tunnel-like pocket of hydrophobic residues, which is surrounded by negatively charged amino acids. The enzyme's kinetic properties include K_m values for choline between 100 and 180 µM, K_m values for ATP from 410 to 760 µM, and a turnover rate (k_{cat}) of 69–83 s^{-1} (Arlauckas et al., 2021).

2.8 Role of ChoKα in Cancer

The activation of ChoK, specifically ChoKα, is linked to cancer progression, underscoring its role as a key player in tumour development. Elevated levels of ChoKα alone can initiate tumor formation, unlike ChoKβ, which does not have the same transformative potential (Kall et al., 2019). The association of increased ChoKα expression with several cancers was first noted in breast carcinoma, where 39% of patient tumour samples showed high ChoK activity. Since then, overactive ChoKα has been

documented in other cancers: 47% of colon, 56% of lung, 48% of prostate, as well as ovary, endometrial, and pancreatic cancers (Arlauckas et al., 2021). In non-small cell lung cancer, a retrospective study found that patients with ChoKα levels nearly twice as high in tumours as in normal tissue had significantly lower four-year survival rates (49%) compared to those with lower ChoKα expression (71%) (Arlauckas et al., 2021).

Overexpression of CHKα drives pathways such as PI3K-mTOR-AKT, Ras-ERK-AKT-MYC, and AMPK-mTORC1 in B and T cell malignancies, similar to its effects in solid tumours (Lin et al., 2017). The PI3K/Akt/mTOR signalling pathway is activated in response to nutrient availability, hormonal signals, and growth factor stimulation. At its core is the class IA PI3K heterodimer, which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP3). This reaction activates Akt, a serine/threonine kinase that regulates processes critical to cancer cell growth, survival, and division. Downstream of Akt lies mTOR, another serine/threonine kinase, which functions within two distinct complexes, mTORC1 and mTORC2, each with unique roles in cellular regulation (Paplomata & O'Regan, 2014). The overexpression of choline kinase (ChoK) leads to an increase in phosphatidylcholine (PC) production, driving the accumulation of phosphocholine (PCho). This process contributes to tumour development and progression by enhancing the PI3K pathway and amplifying AKT signalling, both of which are critical for regulating cell growth, survival, and proliferation (Lacal & Campos, 2015).

Elevated CHKα levels boost phosphocholine (PC) synthesis, and the breakdown of PC produces lipid messengers like DAG, phosphatidic acid (PA), LPC, and arachidonic acid, which activate various signalling cascades. In diffuse large B-cell lymphoma (DLBCL), upregulation of CHKα by the KDAC inhibitor panobinostat enhances the PI3K-AKT pathway (Pera et al., 2018). Silencing CHKα or inhibiting it with CK37