

ClpP: A mitochondrial chaperone protease that helps cancer cells to escape ferroptosis through enhanced mitophagy

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STATEMENT OF ORIGINALITY

I, Enqiang Chang, hereby declare that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

The two MRes students that I had supervised during my PhD research, Miss Yiwen Chen and Dr Xinyi Li, had contributed to experiments in Chapter Four and Chapter Five of this thesis. Western blot data from Fig. 3-7 and Fig. 3-9 were acquired and analysed with the help of Miss Yiwen Chen, and parts of Fig. 3-10 and Fig. 3-11 had been presented as Fig.5A and Fig.5B in her MRes project report titled “ClpP promotes renal carcinoma cells migration and invasion by regulating mitochondrial function via mitophagy”, dated March 2020. Western blot data from Fig. 3-6 and Fig. 3-8 and flow cytometry data from Fig.4-5 and Fig.4-7 were obtained and analysed together with Dr Xinyi Li. Parts of Supplementary Fig.1 and Fig.3-17 had been presented as Fig.1A, Fig.1B, Fig.2A and Fig.2B in Dr Li’s MRes project report titled “Caseinolytic protease P (ClpP) and Ferroptosis in Renal Carcinoma Cells (RCC) exposed to Hypoxia”, dated March 2020.

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SUMMARY

The Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit (ClpP) is crucial in maintaining mitochondrial homeostasis in cancer cells. Here, I combined The Cancer Genome Atlas (TCGA) databases, and proteomic analyses (proteomics) to identify critical associations between ClpP and cellular signalling pathways and to explore the involvement of reactive oxygen species (ROS), mitochondrial autophagy (mitophagy), and ferroptosis in clear cell renal cell carcinoma (ccRCC) cells. I demonstrated that ClpP promoted HIF-1 α nuclear translocation and increased BCL2 Interacting Protein 3 (BNIP3), Microtubule Associated Protein 1 Light Chain 3 Alpha/Beta (LC3A/B), and Glutathione Peroxidase 4 (GPX4) expression but decreased Acyl-CoA Synthetase Long Chain Family Member 4 (ACSL4) expression. Consequently, ClpP enhanced RCC cell malignancy by promoting BNIP3-LC3A/B-dependent mitophagy and compromising GPX4-mediated ferroptosis in both in vitro and xenograft models. ClpP inhibition led to mitochondrial dysfunction and attenuated RCC growth and survival. In addition, analyses of 431 patients with RCC revealed a positive correlation between ClpP expression and an unfavourable prognosis of the disease. Collectively, my findings highlight ClpP as a novel mediator in enhancing renal cancer malignancy through mitophagy-mediated decreased ferroptosis. In summary, while the development of ClpP inhibitors shows great promise for kidney cancers and potentially other cancers, further research is needed to fully understand the tissue-specific roles of ClpP and to design inhibitors that can effectively target cancer cells while sparing normal tissues.

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ABBREVIATIONS

ClpP	caseinolytic mitochondrial matrix peptidase proteolytic subunit
ClpX	Clp proteolytic complex
ClpXP	Clp proteolytic complex P
AAA+	ATPase associated with various cellular activity
RCC	Renal cell carcinoma
ccRCC	Clear cell renal cell carcinoma
VHL	Von Hippel-Lindau
HIF-1 α	hypoxia-inducible factor-1-alpha
VEGF	vascular endothelial growth factor
BAP1	BRCA1-associated protein 1
ROS	reactive oxygen species
mtDNA	mitochondrial DNA
LDH	lactate dehydrogenase
TCA	tricarboxylic acid
PINK1	serine/threonine kinase
Drp-1	mitochondrial fission
BNIP3	BCL2 interacting protein 3
NIX	Bnip3L
PINK1	PTEN-induced putative kinase protein 1
BCL2	B-cell leukemia/lymphoma 2
LC3	Linteracting region
GPX4	glutathione peroxidase 4
ACSL4	Acyl-CoA Synthetase Long Chain Family Member 4
MDA	malondialdehyde
Lip-1	Lipoxstatin-1
Fer-1	Ferrostatin-1
TCGA	the Cancer Genome Atlas
GEO	gene-on-chip database
siRNA	Small interfering RNA
RNAi	RNA interference
mRNA	messenger RNA
shRNA	short-hairpin RNA

dsRNA	double-stranded RNA
PFA	paraformaldehyde
NDS	normal donkey serum
SDS	sodium dodecyl sulfate
BSA	bovine serum albumin
HRP	horseradish peroxidase
co-IP	Co-immunoprecipitation
RT-PCR	Real-time polymerase chain reaction
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
CCK8	Cell Viability by Cell Counting Kit-8
OS	overall survival
RNA-seq	RNA sequencing
PCA	Principal Component Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
PPI	Protein-protein interaction
TME	tumour microenvironment

CHAPTER ONE

Introduction

A. Renal cell carcinoma

A.1 Overview of renal cell carcinoma

Renal cell carcinoma (RCC) is a malignant tumour originating from the tubular epithelial cells of the kidney, accounting for approximately 3% of all adult malignancies and ranking among the ten most common cancers in adults.⁸ Globally, its incidence has significantly increased over the past few decades, with an annual increase of about 2% to 3%.⁹ In the past two decades, significant advancements in RCC's histopathological and molecular characterisation prompted major revisions to its classification.^{10,11} According to the latest histological classification of RCC by the World Health Organization (WHO) in 2022, there are over 15 recognised subtypes. However, three main subtypes are Clear cell renal cell carcinoma (ccRCC), which is the most common and represents about 75% of RCC cases; papillary renal cell carcinoma, comprising about 15%; and chromophobe RCC around 5%. The remaining subtypes are sporadic, each with 1% or less incidence. If a cancer does not meet the diagnostic criteria for any of the established subtypes, it is classified as unclassified RCC, which has an overall incidence of approximately 4%.⁸

A.2 Epidemiology

The incidence of renal cell carcinoma (RCC) increases significantly with age and is notably higher in men compared to women. In the United States, incidence rates vary by race, with the highest prevalence among Native Americans, Native Alaskans, and African Americans. In contrast, the lowest incidence is seen among Asian Americans and Pacific Islanders.⁸ Major risk factors for RCC include obesity, hypertension, and smoking, collectively accounting for approximately half of all diagnosed cases. Additionally, epidemiological studies have identified several other conditions associated with RCC, such as chronic kidney disease, haemodialysis, kidney transplantation, acquired renal cystic disease, and diabetes.¹² Furthermore, various lifestyle, dietary, occupational, and environmental factors have been somewhat linked to RCC. For instance, studies suggested that the RCC risk is linked to red meat consumption, moderate alcohol intake (≥ 11 g per day), and moderate physical activity.⁹

A.3 Pathogenesis of renal clear cell carcinoma

Renal cell carcinoma (RCC) can be sporadic or hereditary, with both forms often associated with structural changes in the short arm of chromosome 3. RCC is linked to mutations in several genes, and sequencing studies have identified driver genes involved in its pathogenesis, including Von Hippel-Lindau Tumor Suppressor (VHL), Polybromo 1 (PBRM1), BRCA1 Associated Deubiquitinase 1 (BAP1), SET Domain Containing 2 (SETD2) and Lysine Demethylase 5C (KDM5C). Somatic mutations or epigenetic changes in the tumour suppressor gene VHL are observed in more than 75% of clear cell RCC (ccRCC) cases. The VHL protein plays a crucial role in the ubiquitination and proteasomal degradation of hypoxia-inducible factors (HIF) in kidney tissues, and its inactivation leads to the accumulation of HIF expression.¹³

A.3.1 VHL-HIF signal pathway

The central genetic event in the pathogenesis of clear cell renal cell carcinoma (ccRCC) is the biallelic inactivation of the Von Hippel-Lindau (VHL) gene due to somatic mutation or promoter hypermethylation.¹⁴ In ccRCC cases, up to 90% of patients with sporadic ccRCC exhibit VHL gene inactivation. Of these, 50% are associated with mutations in the VHL gene, and 10-20% involve promoter hypermethylation. Although some cases lack VHL mutation and hypermethylation, they display an mRNA profile similar to VHL inactivation. This suggests that these tumours may harbour other genetic or epigenetic alterations that directly or indirectly impair the function of the VHL gene product (pVHL).¹⁵

The phosphorylated von hippel-lindau (pVHL) protein is a component of the E3-ubiquitin ligase multiprotein complex, facilitating targeted protein degradation. The pVHL contains alpha (α) and beta (β) domains, connected by two short polypeptide junctions and polar interfaces. The α domain binds to elongin C and elongin B proteins within the E3-ubiquitin ligase complex, forming a ternary complex known as VCB.^{16,17} The β domain mediates interactions between pVHL and the substrates of the E3 ubiquitin ligase.¹⁸ Additionally, two other proteins, Hs-Cul-2 (Homo sapiens-Cullin 2) and Rbx1 (RING box protein 1), are associated with this complex. While the interaction of Hs-Cul-2 with the VCB complex depends on the integrity of the complex, Rbx1 may be activated by ubiquitination through the E1/E2 ubiquitin ligase system.¹⁹

Alterations in these genes activate various downstream signalling pathways, resulting in angiogenesis, increased cell proliferation, and migration. The activation of hypoxia-inducible factors (HIF-1 α and HIF-2 α) due to VHL gene inactivation is the primary event influencing all

aspects of cellular metabolism. These factors regulate glycolysis, promote angiogenesis, inhibit apoptosis, enhance cell proliferation and migration, and affect other biological processes (see the specific mechanisms of HIF-1 α and HIF-2 α below).²⁰ Studies have also found that enhanced tyrosine kinase activity and the activation of MAPK, mTOR, and NF- κ B signalling pathways also contribute to increased tumour growth.⁷ Recently, the STAT3 and SHH signalling pathways have also been observed to play a role in the occurrence of clear cell renal cell carcinoma (ccRCC). It has been shown that the STAT3 pathway can synergistically enhance tumour progression with hypoxia-inducible factors (HIF). In contrast, tumours driven by SHH pathway activation do not rely on VHL alterations. Numerous studies indicate that the occurrence and progression of ccRCC is a highly complex process at the molecular level (**Figure. 1-1**).^{21,22} The limited understanding of the disease at the molecular level has resulted in a relative lack of molecular drug targets for ccRCC. Therefore, advancing knowledge of the disease's molecular basis could significantly contribute to drug development for ccRCC.

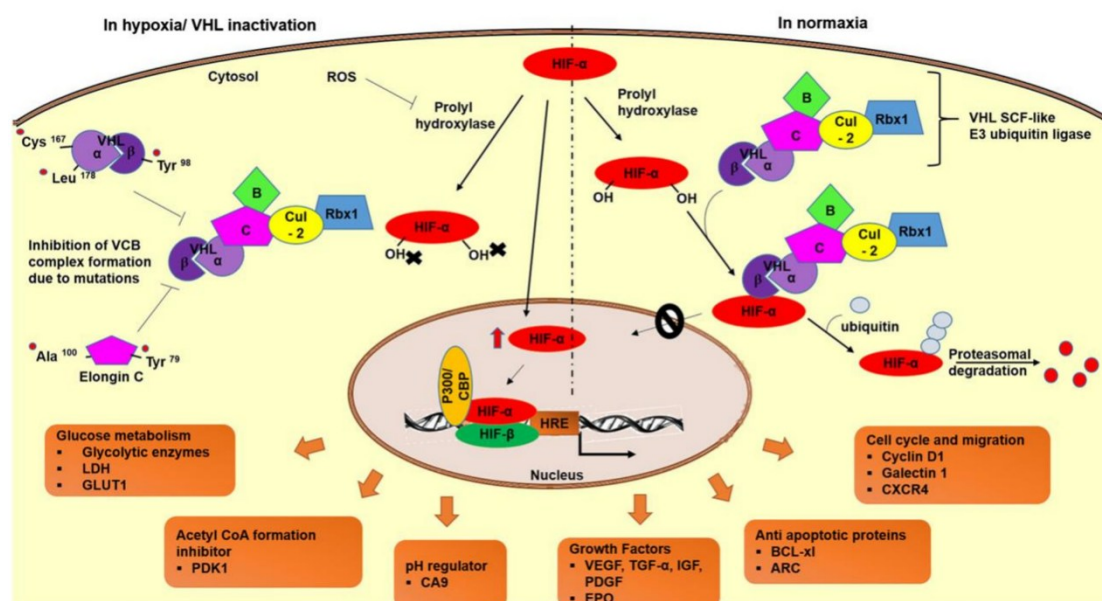


Figure 1-1. Role of VHL complex and HIF-1α in ccRCC.

Under normal oxygen conditions (normoxia), HIF-α undergoes hydroxylation at one of its two proline residues, a process catalyzed by prolyl hydroxylase in an oxygen-dependent manner. This hydroxylation allows the VHL complex, a SCF-like E3 ubiquitin ligase, to recognize HIF-α, leading to its ubiquitination and subsequent degradation by the proteasome. However, during low oxygen conditions (hypoxia), reactive oxygen species (ROS) inactivate prolyl hydroxylase, preventing the hydroxylation of HIF-α. As a result, HIF-α evades recognition and degradation by the VHL complex. VHL= von Hippel–Lindau, HIF= hypoxia inducible factor, B= elongin B, C= elongin C, Cul-2= cullin 2, Rbx1= RING box protein 1, ROS= reactive oxygen species, CBP= CREB binding protein, HRE= hypoxia responsive element, LDH= lactate dehydrogenase, GLUT1= glucose transporter 1, CA9= carbonic anhydrase 9, PDK1= pyruvate dehydrogenase kinase 1, VEGF= vascular endothelial growth factor, PDGF= platelet derived growth factor, TGF-α= transforming growth factor, IGF= insulin like growth factor, EPO= erythropoietin, ARC= apoptosis repressor with a CARD domain, CXCR4= chemokine receptor 4. (Figure modified from Kumar et al.⁷)

A.3 Treatment status of renal cell carcinoma

Despite advancements in the understanding of renal cell carcinoma (RCC) biology, surgical intervention continues to be the principal treatment for kidney cancer. Radical nephrectomy has traditionally been the standard intervention for kidney cancer. With the rising identification of small kidney lesions and accumulating evidence indicating that surgical intervention may elevate the risk of chronic kidney disease, a conservative treatment approach is increasingly recommended.⁵ For patients with surgically resectable renal cell carcinoma (RCC), the standard treatment consists of surgical excision via partial or radical nephrectomy. Conversely, individuals with inoperable or metastatic renal cell carcinoma (RCC) are generally managed through systemic treatment involving targeted therapies and/or immune checkpoint inhibitors.

A.3.1 Surgical treatment

The surgical management of renal cell carcinoma (RCC) is contingent upon the clinical stage of the disease and the patient's overall health status. Surgery is generally designated for localised lesions; however, partial and radical nephrectomy can also be utilised for cytoreductive objectives in patients with metastatic disease. Randomised controlled trials from the 1990s, during which cytokine-based therapies were the standard for systemic treatment, demonstrated the advantages of this approach. Furthermore, while most patients in randomised controlled trials of targeted therapies also received cytoreductive nephrectomy, the advantage of excising the primary tumour in these individuals has yet to be established. Despite this, major international guidelines indicate that numerous medical centres still support cytoreductive nephrectomy at the primary tumour site in cases of large tumours with limited distant metastases.²³

Partial nephrectomy seeks to excise the primary tumour completely while maximising the preservation of healthy renal parenchyma. This procedure is primarily used in patients with typical T1 tumours in the contralateral kidney, as classified by the TNM staging system of the International Union for Cancer Control. Partial nephrectomy is strongly indicated for patients with renal cell carcinoma (RCC) who possess a single kidney (anatomically or functionally), bilateral synchronous RCC, or von Hippel-Lindau syndrome. Relative indications encompass conditions that compromise kidney function, including kidney stones, hypertension, diabetes, and pyelonephritis. Partial nephrectomy provides reduced renal impairment and similar patient survival outcomes when compared to radical nephrectomy for T1 tumours.^{24,25}

Radical nephrectomy typically entails the excision of the kidney, surrounding adipose tissue, adrenal glands, and adjacent lymph nodes. In patients with tumours measuring ≤ 5 cm situated in the lower pole, the adrenal gland may be preserved. Radical nephrectomy is generally conducted via the laparoscopic approach for the majority of patients with stage I and II tumours. In contrast, the open approach is considered the gold standard for more complex cases. When employed by skilled surgeons, robot-assisted techniques represent a feasible alternative to open surgery, especially in scenarios involving venous tumour thrombus. Evidence from the U.S. National Cancer Database endorses the application of cytoreductive nephrectomy in patients with metastatic disease, regardless of concurrent systemic targeted therapy. The median overall survival for patients who underwent cytoreductive nephrectomy was 17.1 months, in contrast to 7.7 months for those in the non-nephrectomy group.²⁶

A.3.2 Active Monitoring and Ablative Therapy

Active monitoring and ablation techniques, including cryotherapy and radiofrequency ablation, serve as alternative strategies for older patients and individuals with significant health risks or limited life expectancy, rendering them unsuitable for surgical intervention. A standardised program for active monitoring has yet to be established. The prevalent method consists of alternating ultrasound imaging with CT or MRI every three months in the first year, every six months in the second year, and annually after that. Intervention is warranted when the tumour size surpasses 3–4 cm or when annual growth exceeds 0.4–0.5 cm.²⁷

Ablation techniques eliminate all viable tumour tissue, ensuring no residual tumour areas remain. Cryotherapy and radiofrequency ablation can be executed via laparoscopic or percutaneous techniques, utilising CT or ultrasound guidance.²⁸ Statistical analysis indicates that the efficacy of cryoablation is 89%, while radiofrequency ablation's is 90%, with complication rates of 20% and 19%, respectively. Research suggests that ablation exhibits a higher local recurrence rate in comparison to partial nephrectomy.²⁹

A.3.3 Targeted therapy

Currently, the first-line and second-line tyrosine kinase inhibitors targeting the VEGF signalling pathway, approved in the United States and the European Union for the treatment of metastatic RCC, include sorafenib, sunitinib, pazopanib, axitinib, lenvatinib, and cabozantinib.^{30,31} These drugs leverage the highly vascularized nature of RCC by targeting VEGF

to inhibit angiogenesis and tumour growth. Except for lenvatinib in combination with everolimus, all approvals are for single-agent therapies.^{24,32-34} Additionally, the anti-VEGF monoclonal antibody bevacizumab has been approved for use with interferon-alpha. Sunitinib, pazopanib, and the combination of bevacizumab with interferon-alpha are approved as first-line options, while axitinib and cabozantinib are approved as second-line agents. mTOR inhibitors everolimus and temsirolimus are approved as single agents for second-line therapy.³⁵ Lanmark trials for first-line systemic treatment of metastatic RCC was the Phase 3 study of sunitinib versus interferon-alpha, reported in 2007,³⁶ which demonstrated advantages in response rate, progression-free survival, and overall survival. This trial established sunitinib as the standard treatment for metastatic RCC, and it remains the comparator in all Phase 3 studies of new drugs. Despite extensive research, no clinically effective biomarkers can predict a patient's response to a particular treatment. Consequently, the average duration of disease control with these drugs is approximately 8-9 months for first-line therapy and 5-6 months for second-line treatment **(Figure. 1-2)**.

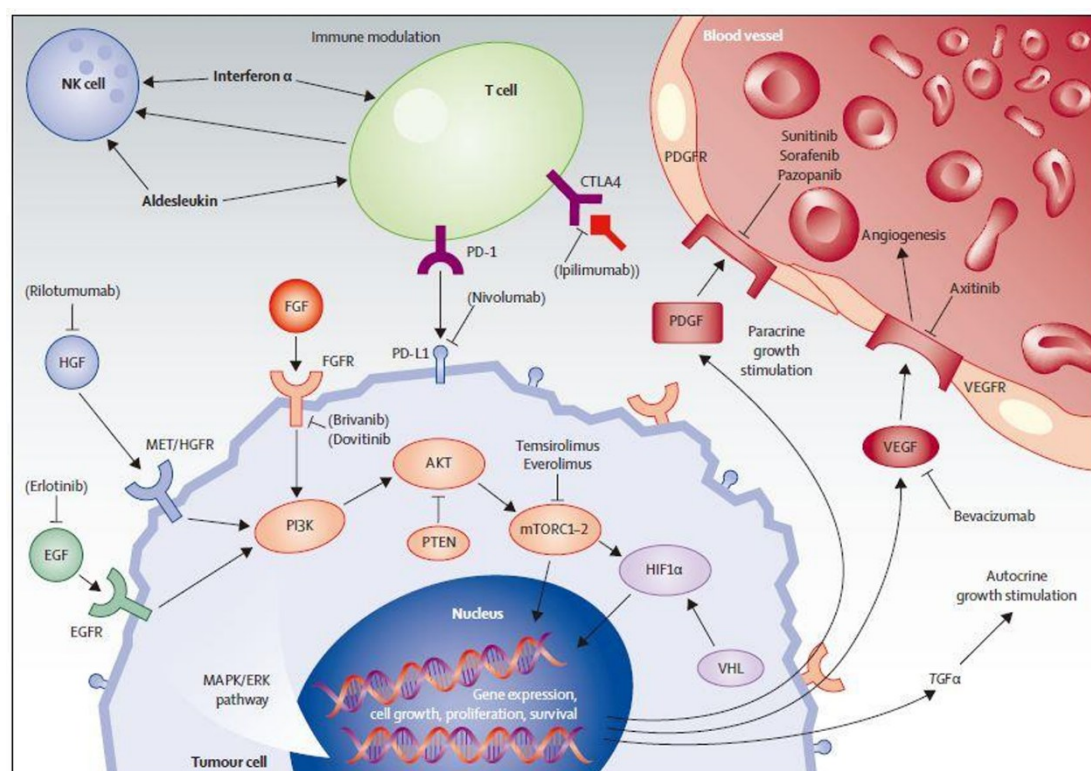


Figure 1-2. Signalling pathways associated with therapeutic targets for renal cell Carcinoma

In the last ten years, significant progress in managing metastatic renal cell carcinoma has centered on therapies designed to interact with particular biological pathways. Treatments focusing on the VEGF, PDGFR, and mTOR pathways remain the cornerstone of care. While greater target precision has reduced the likelihood of toxic side effects linked to these therapies, achieving sustained complete responses continues to be rare.

NK=natural killer, AKT=protein kinase B, VEGF= vascular endothelial growth factor, mTOR= mammalian target of rapamycin, PDGFR=Platelet-derived growth factor receptors. (Figure modified from Capitanio et al.⁵)

A.3.4 Immunotherapy

Cytokines that enhance anti-tumour immune activity, such as interferon-alpha and high-dose IL-2, have been used to treat metastatic RCC since the 1990s and were the standard treatment before the introduction of sunitinib.³⁷ However, both drugs are generally effective in only a tiny percentage of patients and are associated with significant toxicity. Numerous studies are currently investigating the combination of anti-VEGF therapy with a new generation of immunotherapy in the form of T-cell immune checkpoint inhibitors. These include antibodies

against programmed cell death ligand 1 (PD-L1), such as nivolumab and atezolizumab, and antibodies against programmed cell death protein 1 (PD-1), such as nivolumab and pembrolizumab. PD-1 negatively regulates T-cell function, and its ligand PD-L1 is highly expressed in cancer cells. Thus, blocking the PD-1/PD-L1 interaction can promote T-cell activation and the immune-mediated killing of cancer cells. Another combination under investigation is nivolumab with ipilimumab, an inhibitor of the T-cell checkpoints cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).

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A.3.5 Downstream pathways involved in disease manifestations

A.3.5.1 Tyrosine kinase pathway

The activation of vascular endothelial growth factor (VEGF) is responsible for the abundant capillaries' characteristic of ccRCC. Consequently, VEGF has been targeted for therapeutic intervention in ccRCC, using drugs such as bevacizumab, sunitinib, and sorafenib. VEGF binds to tyrosine kinase receptors (VEGFR-1/VEGFR-2) on endothelial cells. Upon binding to VEGFR-2, VEGF promotes cell proliferation and migration by activating downstream kinase pathways, specifically the MAPK and PI3K/AKT pathways. Additionally, VEGF binding to VEGFR-1 further contributes to angiogenesis.³⁹

Vascular Endothelial Growth Factor Receptor 2 (VEGF-R2) is also expressed on ccRCC cells and other growth factor receptors such as Epidermal Growth Factor Receptor (EGFR) and Insulin

Like Growth Factor (IGF) receptors. **Crosstalk with EGFR/IGF Receptors**, Synergistic Signaling: Activation of EGFR or IGF-1R may upregulate VEGF receptors or ligands, creating a feed-forward loop for tumor growth and angiogenesis. Resistance Mechanisms: Co-targeting VEGF and EGFR/IGF pathways (e.g., cabozantinib) is used to mitigate resistance in advanced ccRCC. Their interplay with EGFR/IGF receptors underscores the complexity of ccRCC biology, necessitating multi-targeted approaches to improve therapeutic outcomes. The overexpression of ligands, including VEGF, TGF- α , and IGF, results in the dimerisation and subsequent activation of these tyrosine kinase receptors. Upon activation, these receptors trigger the RAS/MEK/ERK or PI3K/AKT/mTOR pathways, ultimately leading to increased production of HIF- α , thereby promoting tumour progression.²⁰ Additionally, mTOR is activated to form the multiprotein complex mTORC1, which, through its downstream effectors such as P70S6K1, inhibits the apoptotic pathway and promotes cell cycle progression into the G1 phase. Moreover, PTEN, an inhibitor of PI3K, is often genetically absent in ccRCC.⁴⁰ A study by Dey et al. demonstrated that elevated levels of miR-21 can inhibit PTEN expression, thereby enhancing cell proliferation and migration (**Figure. 1-2**).⁴¹ In a study by White et al., Galectin-1, a downstream target of HIF-1 α , was overexpressed in ccRCC. Galectin-1 binds to integrins, extracellular matrix components, and various intracellular proteins involved in angiogenesis and metastasis. The study concluded that Galectin-1 promotes cell migration and invasion through the AKT/mTOR/p70S6K pathway.⁴² Mutations within the mTOR pathway in ccRCC have also been reported by Sato et al., who identified various carcinogenic mutations in PI3K.⁴³ Mutations in various mTOR suppressor genes, including PTEN and TSC1/2, have also been observed in ccRCC, highlighting the critical role of mTOR signalling in this cancer.⁴⁴ Therefore, numerous mTOR inhibitors, such as temsirolimus and everolimus, have been developed clinically as therapeutic agents for tumour treatment.⁸ Many tyrosine receptor inhibitors have demonstrated the ability to induce tumour regression, but response rates are often inconsistent due to drug resistance. The MET signalling pathway is one of the pathways implicated in ccRCC resistance to VEGFR inhibitors.⁴⁵ MET is a tyrosine kinase receptor activated by hepatocyte growth factor and involved in multiple pathways related to cell proliferation, differentiation, survival, and cytoskeletal rearrangement. Cabozantinib, a dual MET/VEGFR inhibitor, demonstrated a 28% response rate in a Phase I trial. This suggests that dual inhibitors may have more direct anti-tumour effects and greater efficacy than monotherapy. Therefore, further elucidation of the pathways involved in drug resistance is crucial in improving treatment outcomes for patients with ccRCC.

A.3.5.2 NF- κ B pathway

The upregulation of HIF also promotes the activation of the NF- κ B signalling pathway. TGF- α , released by ccRCC, binds to its receptor, EGFR, activating the downstream PI3K/AKT/IKK α /NF- κ B signalling cascade.³⁷ The activation of the NF- κ B transcription factor ultimately inhibits apoptosis while promoting cell proliferation, invasion, and angiogenesis. The promotion of cell proliferation by NF- κ B has been shown to occur through the induction of Cyclin D1 expression. Additionally, NF- κ B may regulate cell migration and invasion by modulating the mRNA binding protein 3 (IMP3) of IGFBP2 (**Figure. 1-3**). Furthermore, the inactivation of VHL has been reported to lead to the activation of the NF- κ B signalling pathway in both familial and sporadic cases of ccRCC.⁴⁶ Subsequent studies revealed that the inactivation of VHL can reduce the levels of vascular cell adhesion molecule 1 (VCAM-1) via the NF- κ B signalling pathway, thereby contributing to a worse prognosis in patients with ccRCC.⁴⁷ Therefore, targeting the NF- κ B pathway in ccRCC presents significant potential for future therapeutic strategy development.

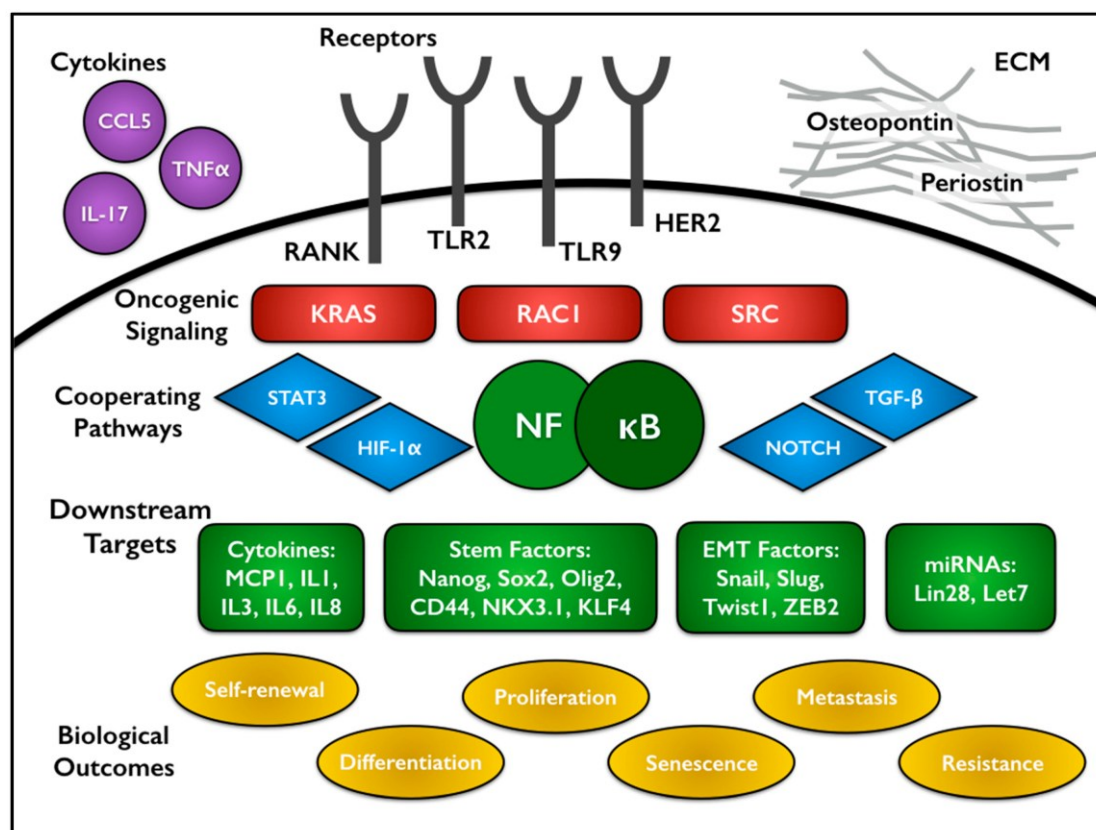


Figure 1-3. This diagram summarizes the various levels of NF-κB signaling in cancer stem cells. Both extracellular and intracellular sources of NF-κB activation are seen at the top. Either alone or in cooperation with other signaling pathways, NF-κB mediates a wide variety of transcriptional targets, which fall into several major categories such as cytokines and EMT factors. Ultimately, these targets mediate important aspects of CSC biology, including self-renewal, proliferation, and metastasis. (Figure modified from Amanda et al.⁵)

A.3.5.3 STAT3 pathway

In the study by Cuadros et al., the hepatitis A virus receptor/kidney injury molecule 1 (HAVCR/KIM-1) protein was found to be significantly overexpressed in ccRCC. KIM-1, a type 1 glycoprotein, features an extracellular immunoglobulin domain cleaved by metalloproteinases in the extracellular matrix. The authors demonstrated that the extracellular domain of KIM-1 activates the transcription of interleukin-6 (IL-6), which binds to the cell membrane receptor gp80. Subsequently, gp80 recruits the signal transducer gp130, which serves as a docking site for phosphorylated STAT3. Upon binding to gp130, STAT3 is phosphorylated and activated. Activated STAT3 induces the transcription of hypoxia-inducible factor 1-alpha (HIF-1α) and various other proteins involved in the cell cycle and apoptosis, such as BCL2 and Cyclin-D1, promoting tumour growth. Additionally, STAT3 has been reported to collaboratively activate

HIF-1 α target genes by binding to the HIF-1 α protein, initiating the transcription of these target genes.⁴⁸ In a study by Alchanati et al., GRIM19 was found to be significantly downregulated in ccRCC. It was also identified as an inhibitor of STAT3, with its downregulation further promoting tumour cell proliferation.⁴⁹ STAT3 is also linked to the prognosis of ccRCC and is associated with advanced tumour stages and poor patient survival. These findings underscore the potential of STAT3 inhibitors in the therapeutic targeting of ccRCC, as they inhibit signalling pathways that drive tumourigenesis (**Figure. 1-3**).^{39,50}

A.3.5.4 Sonic Hedgehog (SHH) pathway

Dormoy et al. reported the reactivation of the SHH signalling pathway in ccRCC, independent of VHL status. The authors found that increased Smo and Gli transcription factor expression promoted cell proliferation in ccRCC. These factors were also shown to interact with the PI3K/AKT pathway and activate the NF- κ B signalling pathway, thereby coordinating multiple signalling pathways to promote tumour growth. Additionally, they reported the long-term effects of the SHH signalling inhibitor cyclopamine on tumour growth in nude mice, suggesting that these inhibitors may have complementary roles in ccRCC therapy.²² SHH-Gli1 signalling has also been found to be overexpressed in ccRCC under conditions of oxygen deficiency, a process mediated by HIF-2 α , and is implicated in tumour radioresistance (**Figure. 1-4**).⁵¹

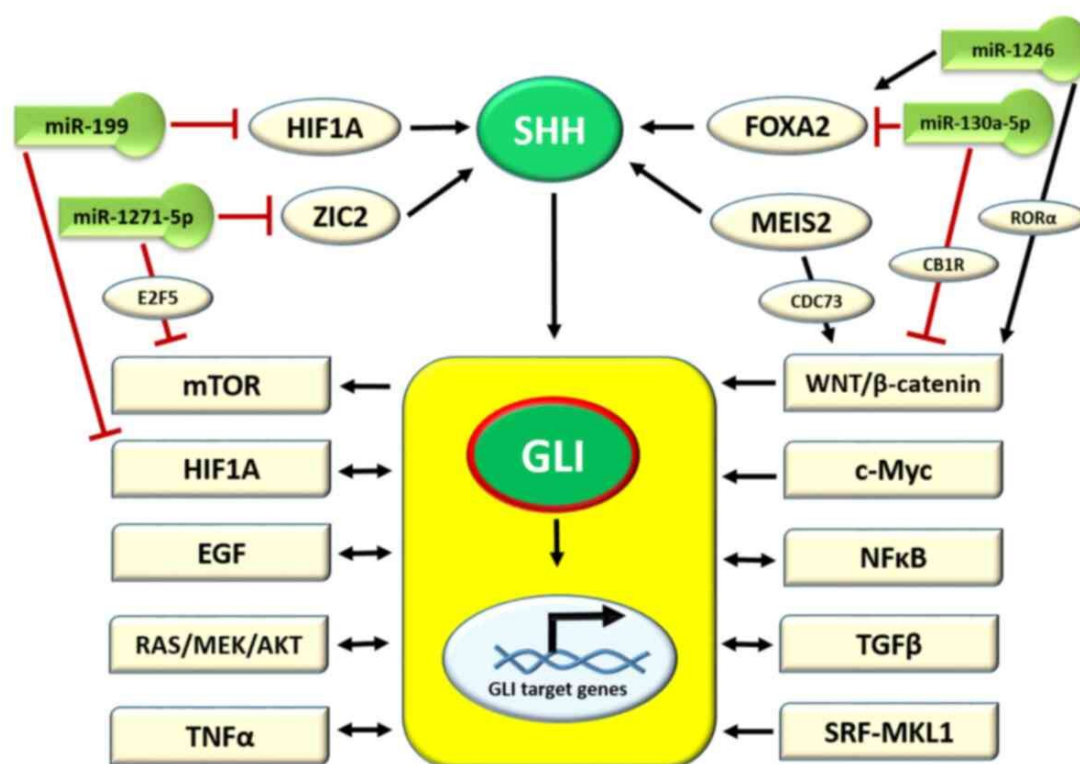


Figure 1-4. Schematic presentation of upstream regulation of the SHH signaling pathway and its associations with other cancer-related cellular pathways. Proteins are presented by oval shapes, microRNAs by hairpins and pathways by cropped rectangles. SHH, Sonic Hedgehog; mTOR, mammalian target of rapamycin; HIF, hypoxia-inducible factor; EGF, epidermal growth factor; TNF, tumor necrosis factor; NF- κ B, nuclear factor κ B; TNF, tumor necrosis factor. (Figure modified from Kotulak-Chrząszcz et al.)

A.3.6 Other genes involved in the pathogenesis of ccRCC

Although VHL is the central molecular mechanism involved in most cases of ccRCC, its inactivation alone is insufficient to induce tumour formation. Alterations in other molecules also play a significant role.¹⁴ The PBRM1 gene encodes BAF180, a subunit of the chromatin remodelling complex PBAF/SWI/SNF. The VHL gene is a tumor suppressor critical for regulating hypoxia-inducible factor (HIF) degradation. Its inactivation causes von Hippel-Lindau (VHL) disease, an autosomal dominant syndrome predisposing to multiple tumors. PBRM1 encodes a subunit of the PBAF/SWI/SNF chromatin remodeling complex, which regulates gene expression. This gene comprises six bromine domains that facilitate the binding of lysine residues in histone tails for acetylation, thereby regulating various cellular processes, including cell proliferation, DNA replication, repair, and transcription. Truncated mutants of PBRM1 have been identified in ccRCC cases, along with two in-box deletion mutations. Specifically, a six amino acid deletion (E1214delIMFYKKE) was found in the second BAH domain, which is involved in protein-protein interactions within the SWI/SNF complex. Additionally, the isoleucine codon (Ile57) deletion in the first bromine domain was observed.⁵² PBRM1 is localised to chromosome 3p21 and has the second-highest mutation rate in ccRCC after VHL. The inactivation of PBRM1 in ccRCC leads to increased cell proliferation and migration by regulating the p53/p21 signalling pathway, suggesting that it functions as a tumour suppressor gene involved in the development of ccRCC.⁵³

Another gene, BRCA1-associated protein 1 (BAP1), located on chromosome 3p between the VHL and PBRM1 genes, is mutated in 10-15% of ccRCC cases. BAP1 is a deubiquitinating enzyme associated with chromatin regulators, such as histone H2A. In large-scale studies of ccRCC (n = 609), BAP1 mutations were linked to higher tumour stages, grades, and poor cancer-specific survival, with most mutations being truncating or missense.^{54 43} Additionally, BAP1 mutations have been shown to activate mTORC1, and the poorer survival observed in patients with these mutations may be attributed to the activation of the mTOR pathway.⁵¹

Additionally, SETD2 mutations are found in 10-15% of ccRCC cases across all RCC subtypes. Located on chromosome 3p near the VHL, PBRM1, and BAP1 genes, SETD2 is a histone 3-lysine-36 methyltransferase that can activate the transcription of its downstream target genes (**Table 1-1**).⁵¹ In a study by Hakami et al., SETD2 was found to be mutated in 7.4% of cases (n = 609). These mutations are associated with patient-specific survival.⁵⁴ Since all of the above genes regulate chromatin structure, their mutations likely play a crucial role in the pathogenesis of ccRCC by altering the epigenetics of kidney cells.

Table 1-1 The key genes, mutations, cellular effects, and signaling pathways associated with therapeutic targets in renal cell carcinoma (RCC).

Gene	Mutation Type	General Cellular Effect	Effect on Epithelial Cells	Therapeutic Targets
VHL	Inactivation (loss-of-function)	Loss of HIF- α degradation	Enhanced proliferation, angiogenesis, metastasis	VEGF inhibitors (sunitinib, pazopanib), HIF-2 α inhibitors (belzutifan)
PBRM1	Truncating mutations/deletions	Disrupted chromatin remodeling	Epigenetic dysregulation, immune evasion	EZH2 inhibitors (tazemetostat), immunotherapy (anti-PD-1)
MET	Activating mutations/amplification	Hyperactivation of MET kinase	Proliferation, invasion, tubular epithelial dysplasia	MET inhibitors (cabozantinib, savolitinib)
PTEN	Deletion/mutation	Loss of PI3K/AKT regulation	Enhanced survival, resistance to apoptosis	mTOR inhibitors (everolimus, temsirolimus)

Gene	Mutation Type	General Cellular Effect	Effect on Epithelial Cells	Therapeutic Targets
TP53	Missense mutations/deletions	Loss of cell cycle control	Apoptosis evasion, epithelial-mesenchymal transition	MDM2 inhibitors (idasanutlin) – experimental
BAP1	Loss-of-function mutations	Altered histone H2A deubiquitination	Metastasis, poor prognosis	PARP inhibitors (olaparib) – under investigation
SETD2	Truncating mutations	Loss of H3K36 trimethylation	Genomic instability, resistance to therapy	ATR inhibitors (berzosertib) – in clinical trials
KDM5C	Deletions/mutations	Altered H3K4 demethylation	Impaired differentiation, metabolic reprogramming	HDAC inhibitors (panobinostat) – experimental
TFE3	Chromosomal translocation	TFE3 fusion proteins	Oncogenic transformation (in Xp11 translocation RCC)	mTOR inhibitors (everolimus), VEGF-targeted therapy

B. Mitochondrial ClpP serine protease-biological function and emerging target for cancer therapy

B.1 Caseinolytic peptidase P (ClpP)

The caseinolytic peptidase P (ClpP) proteolytic complex is a serine protease present in many forms located in the mitochondria of eukaryotic cells and chloroplasts, not present in the cytoplasm. In kidney epithelial cells, ClpP expression is elevated due to high mitochondrial

metabolic demands, where it maintains proteostasis by degrading misfolded proteins. Dysregulation of ClpP is linked to mitochondrial dysfunction in acute/chronic kidney injury and renal cancer. Therapeutic strategies targeting ClpP (e.g., activators like ONC201) are under investigation, though tissue specificity remains a challenge.⁵⁵ This peptidase complex has been extensively researched in bacteria, although its function in mammalian mitochondria needs to be more adequately comprehended. The inhibition and hyperactivation of ClpP in bacteria have emerged as novel strategies to combat drug-resistant infections.^{56,57} Additionally, recent findings suggest that targeting mitochondrial ClpP can be an effective anticancer strategy for malignancies such as acute myeloid leukaemia.

Cells possess double-membraned organelles known as mitochondria that utilise oxidative phosphorylation (OXPHOS) to synthesise ATP, the primary energy currency.^{58,59} Mitochondria are involved in more than energy production; they also contribute to other essential biological functions. This encompasses the generation of reactive oxygen species (ROS), modulation of calcium levels, and facilitation of the synthesis of macromolecules such as proteins and nucleic acids. Mitochondria also synthesise lipids, participate in apoptosis, and safeguard cells from oxidative stress.^{60,61}

The genetic material within mitochondria is referred to as mitochondrial DNA (mtDNA). The sequence is approximately 16.7 kb in length and encodes 13 proteins essential for the functionality of the mitochondrial respiratory chain. Mitochondrial DNA encodes all components of the respiratory chain complex proteins except for complex II. Mitochondria, between positions, synthesise 13 proteins.^{62,63} However, the majority of mitochondrial proteins are produced by nuclear genes. Proteins encoded by the nucleus are synthesised in the cytoplasm and subsequently transported into the mitochondria via particular sequences.⁶⁴ The quantity of mitochondrial proteins is influenced by transcription, RNA processing, translation rate, protein stability, and the efficacy of mitochondrial targeting.

Mitochondria employ various methods to maintain optimal protein structure and function. These encompass the accurate folding of new proteins and the elimination of damaged or misfolded proteins. Specialised molecular chaperones and proteases maintain protein equilibrium in mitochondria.⁶⁵

A crucial aspect of mitochondrial protein quality control is the degradation of malfunctioning

proteins. At least 45 proteases operate independently in various regions of human mitochondria, including the outer membrane, intermembrane space, inner membrane, and mitochondrial matrix. Among these proteases, 23 are exclusively located in the mitochondria. Other proteases translocate between the cytoplasm and mitochondria.^{66,67} Among the 23 enzymes present in mitochondria, five are classified as pseudomitoproteases. These enzymes lack catalytic activity yet remain crucial to proteolytic complexes. The 18 remaining innate mitochondrial proteases can be categorised into four groups: oligopeptidases, ATP-dependent peptidases, processing peptidases, and other mitochondrial peptidases (**Table 1-2**).

Table 1-2: Overview of intrinsic mitochondrial proteases and their roles⁶⁸⁻⁷²

Category	Protease	Localization	Regulatory functions
ATP-dependent proteases	AFG3L2 AFG3L2/SPG7	Matrix/IM	Ribosome assembly MCU assembly
	CLPP	Matrix	Transcription/translation Ribosome assembly
	LONP1	Matrix	mtDNA maintenance mtDNA replication Adaptation to hypoxia
	YME1L	IM/IMS	Protein import Lipid trafficking Mitochondrial dynamics
Processing peptidases	ATP23	IMS	Protein maturation F ₁ F ₀ -ATP synthase assembly
	IMMP	IM/IMS	Protein maturation Apoptosis/senescence
	METAP1D	Matrix	Protein maturation
	MIP	Matrix	Protein maturation Coenzyme Q biosynthesis Complex III and IV activity
	OMA1	IMS/IM	Mitochondrial dynamics
	PARL	IM	Apoptosis Coenzyme Q biosynthesis Complex III assembly Lipid trafficking Mitophagy
	PMPCB	Matrix	Protein maturation
	XPNPEP3	Matrix	Protein maturation and stability
Oligopeptidases	MEP	IMS	ND
	PITRM1	Matrix	ND
Other mitochondrial proteases	HTRA2	IMS	Stress signaling Apoptosis
	LACTB	IMS	PE metabolism

Abbreviations: IM, inner membrane; IMS, intermembrane space; MCU, mitochondrial Ca²⁺ uniporter; NA, not applicable; ND, no data; PE, phosphatidylethanolamine.

OMA1 is a processing enzyme found in mitochondria's inner membrane and intermembrane space. To change how mitochondria work, the inner mitochondrial protein OPA1 (a 120kDa protein that works like a scaffold) has to be cut. As the potential of the mitochondrial membrane drops, OMA1 cuts OPA1 with its enzyme.⁷³ It is called HTRA2, or OMI, and is found in the space between the mitochondrial membranes. To keep the shape of mitochondrial cristae, it is essential to work with and break down parts of the mitochondrial intermembrane space bridging (MIB) complex, such as the inner membrane mitochondrial protein (IMMT).⁷⁴ IAPs, which are also known as Baculoviral IAP Repeat Containing proteins, are released into the cytoplasm when cells go through apoptosis. There, HTRA2 binds to and stops these

proteins. These two things work together to make caspases work faster.^{75,76}

One kind of protease that can work in all parts of the mitochondria is an ATP-dependent protease. It is only possible for the mitochondrial proteolytic system to work with these proteases. They are vital parts that keep things in order and ensure good quality.⁶⁷ There are several types of proteases in this group. The Lon protease is in the mitochondrial matrix, the i-AAA and m-AAA proteases are in the inner mitochondrial membrane, and the ClpXP complex is in the mitochondrial matrix. ClpP and ClpX are two proteins that work together to form the ClpXP complex (**Fig. 1-5**).^{72,77,78} These proteases break down many proteins in mitochondria. Some of these are proteins in the inner membrane that are part of translocases and respiratory complexes. Besides that, they want proteins in the matrix, the gap between the membranes, and the outer membrane.

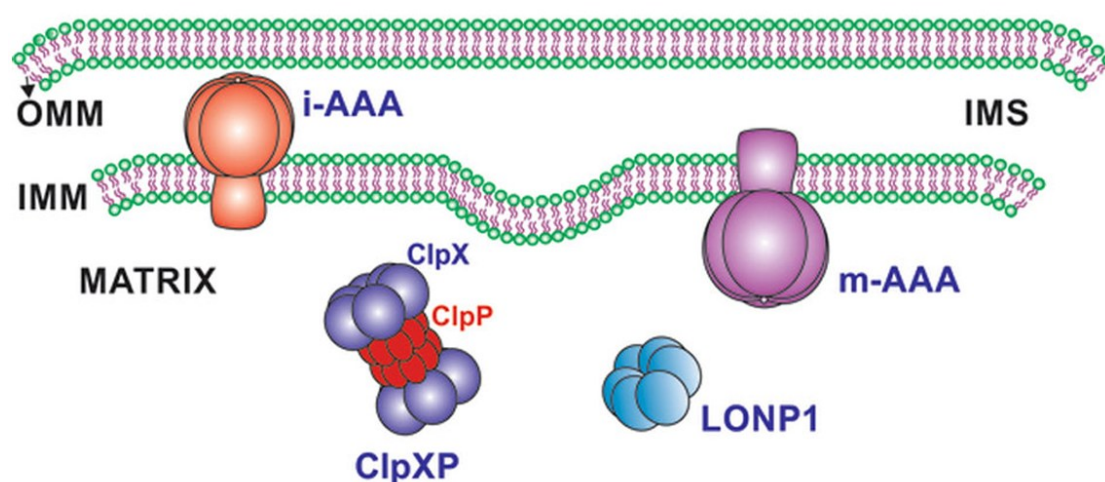


Figure 1-5. Schematic representation of ATP-dependent proteases.

Mitochondria in mammals harbor four proteases belonging to the AAA+ superfamily, which play a crucial role in maintaining protein quality control. Within the mitochondrial matrix, the Lon protease 1 and the ClpXP complex are active, while the inner mitochondrial membrane (IM) houses the i-AAA and m-AAA proteases. OMM outer mitochondrial membrane, IMS intermembrane space, IMM inner mitochondrial membrane. (Figure modified from Kazem et al.²)

This paper analyses the mitochondrial ClpP protease and its regulatory subunit ClpX,

collectively referred to as the ClpXP complex, situated in the mitochondrial matrix. For information regarding other mitochondrial proteases.⁷⁹ This research will investigate the molecular properties and biological roles of the mitochondrial ClpXP complex, along with prospective therapeutic approaches to target this protease for cancer treatment.

B.2 Mitochondrial ClpP

ClpP is present in the mitochondrial matrix of various organisms, including humans and *C. elegans*. Nevertheless, yeast lacks homologs of this protease. The ClpP gene is located on chromosome 19 in humans.^{75,80} ClpP is translocated to the mitochondrial matrix via a 56-residue segment at the N-terminus following translation in the cytoplasm. This area is subsequently cleaved during protein maturation in the matrix. The mature human ClpP (hClpP) consists of 277 amino acids and has a 71% sequence similarity and 56% identity with the ClpP from *E. coli*. Nevertheless, animal ClpP, including human ClpP, possesses an additional residue at the N-terminus of its polypeptide chain (**Fig. 1-6**).^{81,82} The C-terminal extension forms a loop that protrudes from the oligomer's surface. The loop lacks organisation and exhibits flexibility. The precise role of this region remains ambiguous; however, it is crucial for the stability of the protease, the assembly of the functional ClpP heptamer, and its capacity to interact with the chaperone ClpX.⁸¹

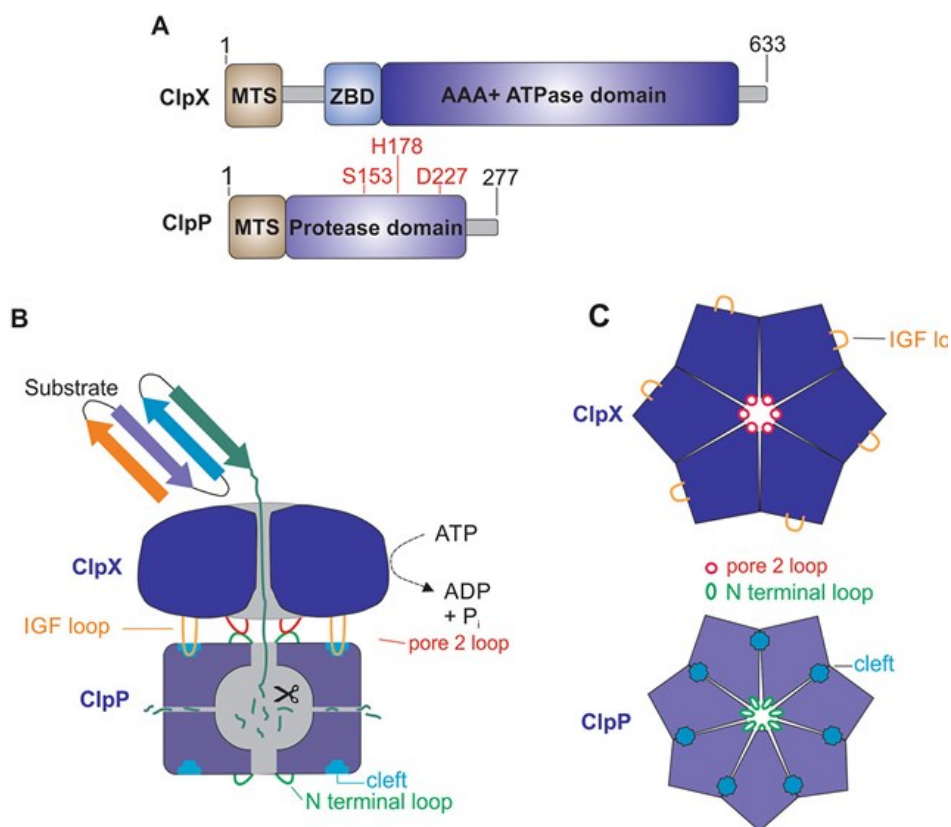


Figure 1-6. Structure and interaction of ClpP and ClpX.

A) Structural arrangement of ClpX (upper panel) and ClpP (lower panel), highlighting the catalytic triad residues Ser153, His178, and Asp227. Key features include the mitochondrial targeting sequence (MTS), zinc-binding domain (ZBD), and the AAA+ domain, which is associated with various cellular ATPase functions.

B) Diagrammatic overview illustrating the interaction between ClpX and ClpP and their proteolytic cycle. C. Top-down visualization of the ClpX hexamer (upper image) and the ClpP heptamer (lower image). ClpP=Caseinolytic peptidase P, ClpX=Caseinolytic peptidase X. (Figure modified from Kazem et al.²)

A significant portion of our comprehension of human ClpP's structure and function has been derived from investigations of its mitochondrial variant at a resolution of 2.1 Å (PDB: 1TG6). Like its bacterial equivalent, the functioning mitochondrial ClpP assembles into a massive, cylindrical tetradecamer, including two stable heptameric rings encapsulating a voluminous aqueous chamber. Each ClpP monomer consists of a compact "head region" and a unique extended α/β unit, referred to as the "handle region." The heads of seven monomers form the heptameric rings via predominantly hydrophobic interactions, while the handles establish transitory hydrogen bond interactions with the neighbouring heptameric ring. The protease contains 14 internal catalytic cleavage sites, with each subunit of the ClpP homo-tetradecamer featuring an active site composed of the catalytic residues Ser153, His178, and Asp227.⁸³

Mitochondrial ClpP, similar to its bacterial equivalent, displays three distinct structural states: stretched, compacted, and compressed. Among them, only the extended conformation exhibits catalytic activity. It can degrade substrates, while the compacted and compressed forms are thought to engage in a cycle that aids in opening the protease barrel.⁸⁴ In contrast to bacterial ClpP, which primarily assembles into a double-ring tetradecamer, human ClpP is found as a stable, although inactive, single heptamer ring under physiological settings and *in vitro*.⁸⁵

The ClpP double-ring tetradecameric protease exhibits flexible connections between its rings, with the N-termini of its subunits creating loops essential for the assembly of ClpXP and ClpAP complexes. Mitochondrial ClpP, in contrast to other proteases, lacks ATPase action. Each subunit of mitochondrial ClpP possesses a region specifically intended for the degradation of

short peptides, including six or fewer amino acids, and functions independently of ATP.⁸⁶ Human ClpP forms a tetradecamer to facilitate a processive proteolytic activity for the degradation of full-length proteins solely in the presence of its related ATPase, ClpX.

B.3 Mitochondrial ClpX associates with ClpP to create an active protease.

In mammalian cells, ClpP associates with its chaperone ClpX to form a heterodimeric proteolytic complex known as ClpXP. ClpX is a member of the AAA+ protein superfamily (ATPases Associated with Different Cellular Activities) and is the sole identified ATPase that interacts with mammalian ClpP. Bacterial ClpP can be associated with many ATPases, such as ClpX, ClpC, and ClpE.⁸⁷ Like ClpP, human ClpX possesses a 56-residue N-terminal mitochondrial targeting sequence (MTS). It exhibits 44% identity and 62% similarity with *E. coli* ClpX. ClpX assembles into a hexameric ring with six-fold symmetry, and its stability is augmented by ATP binding.

The formation of the human ClpXP protease complex entails capping each end of the barrel-shaped ClpP tetradecamer with a ClpX hexamer.⁸⁸ The assembly of the ClpXP complex in mitochondria still needs to be completed. Studies of bacterial ClpXP homologs suggest that a tripeptide IGF loop on ClpX reinforces the interaction between ClpP and ClpX. This loop dynamically integrates into a designated hydrophobic pocket on the apical surface of ClpP, situated between each pair of ClpP subunits at the ClpX–ClpP interface. The assembly of the human ClpXP complex is believed to be aided by contacts between the flexible N-terminal loop of ClpP and the pore-2 loop of ClpX (Fig. 4).^{89,90}

ClpX dictates the substrate specificity of ClpXP. Proteins designated for degradation by the ClpXP complex are recognised and unfolded by ClpX, which then transports them into the interior of ClpP's proteolytic chamber, where they are degraded into minute peptide fragments, presumably ejected through temporary side holes (Fig. 4). The unfolding and threading of proteins into ClpP by ClpX necessitates ATP, while the proteolysis of substrates by ClpP is independent of ATP hydrolysis.⁹¹

In bacteria, substrate recognition generally depends on specific linear sequence patterns, termed degrons, situated at the substrate's N-terminus or C-terminus.⁹² The specialised 11-residue SsrA peptide tagging mechanism can also introduce these degrons, which rescue stalled ribosomes by marking unfinished polypeptides for breakdown.⁹³ Alternatively, adaptor

proteins can increase substrate recruitment by binding to specific substrate proteins, so anchoring them to the ClpP proteolytic complex to augment their destruction. Although ClpXP can degrade substrates independently of adapters, the inclusion of adapter-like proteins, such as YjbH in *S. aureus*, markedly enhances the proteolytic efficacy of the complex.⁹⁴ The adaptor protein MecA similarly activates ClpC in *B. subtilis* by selectively targeting substrates and augmenting ClpC's ATPase activity, therefore promoting the degradation process.⁹⁵ The structural distinctions between bacterial ClpX and mitochondrial ClpX lead to species-specific changes in substrate specificities. The substrate recognition mechanisms of mitochondrial ClpXP and the putative functions of adaptor proteins are predominantly unclear and necessitate additional functional characterisation for comprehensive understanding.

B.4 Cellular function of mitochondrial ClpXP

The primary function of ClpXP in mitochondria is to maintain protein quality by degrading damaged or misfolded proteins.^{96,97} We have identified several targets for ClpXP thus far. These include proteins involved in metabolism, electron transport, and the tricarboxylic acid (TCA) cycle.⁹⁸ It maintains the integrity of the respiratory chain and ensures effective oxidative phosphorylation by degrading misfolded or damaged proteins. ClpXP maintains the integrity of the respiratory chain and facilitates oxidative phosphorylation by degrading misfolded or damaged proteins.

Additionally, ClpXP regulates the unfolded protein response (UPRmt), a stress signaling pathway that relays mitochondrial distress signals to the nucleus. This pathway diminishes mitochondrial translation, regulates cellular metabolism, and provides protection against illnesses. The majority of our understanding regarding ClpXP's role in UPRmt is derived from research on *C. elegans*. Nonetheless, a comparable pathway is probably present in more intricate organisms as well.⁹⁹

In *C. elegans*, ClpXP eliminates misfolded or unfolded proteins in the mitochondrial matrix during protein folding stress or cessation of oxidative phosphorylation. Small peptides are subsequently exported from the mitochondria to the cytoplasm through the HAF-1 transporter.¹⁰⁰ The release mechanism for these small peptides remains ambiguous; however, they initiate a transcriptional response that regulates the synthesis of mitochondrial chaperones and proteases. The activating transcription factor stress 1 (ATFS-1) regulates this process. It contains sequences that target both the nucleus and the mitochondria. The

peptides generated by ClpXP's enzymatic activity are proposed to be extricated from the mitochondria, hence preventing ATFS-1 from accessing the mitochondria. ATFS-1 translocates to the nucleus, where it collaborates with UBL-5 and DVE-1, a protein possessing a homeobox domain, to activate the expression of several UPRmt target genes. This regulates mitochondrial quality control and reinstates proteostasis.¹⁰¹ UPRmt is comprehensively understood in *C. elegans*; moreover, the determinants that induce it in mammals still need to be adequately elucidated. The animal counterpart of HAF-1 remains undiscovered; nonetheless, ATF5 is considered a probable homolog of ATFS-1. ClpXP is believed to be a crucial regulator of UPRmt in mammalian cells.¹⁰²

In bacteria, ClpX primarily facilitates the function of ClpP. ClpX has additional functions beyond only interacting with the ClpP protease in eukaryotes, such as human cells. In the absence of ClpP, ClpX autonomously regulates heme synthesis in the mitochondria. This job is consistent throughout species, from yeast to mammalian vertebrates. ClpX and its yeast counterpart, Mcx1, facilitate the synthesis of 5-aminolevulinic acid (ALA), the initial step in heme production. The ClpX protein associates with ALA synthase (Hem1 in yeast) and facilitates the incorporation of the nutrient pyridoxal phosphate (PLP) into the ALA synthase apoenzyme. This activates the enzyme and initiates the synthesis of heme.¹⁰³ Moreover, ClpX may act independently of ClpP as an auxiliary factor, potentially regulating the localization of mtDNA nucleoids by stabilizing mitochondrial transcription factor A (TFAM).¹⁰⁴ A study by Verhagen et al. demonstrated that ClpX operates in the cytoplasm, interacting with the BIR2 domain of XIAP (X-linked inhibitor of apoptosis) to promote cell apoptosis.^{55,105}

AML cells and stem cells, along with other malignancies such as chronic myeloid leukaemia (CML), pancreatic cancer, and breast cancer, have mitochondrial functionality that differs and relies more heavily on oxidative phosphorylation.¹⁰⁶ An increased influx of substrates into the TCA cycle results in a reduction of excess reserve capacity. This is one reason oxidative phosphorylation is gaining significance. Moreover, these cells are restricted to enhancing other metabolic pathways when oxidative phosphorylation is inhibited.^{107,108} These findings reveal a distinct metabolic vulnerability, suggesting that disrupting oxidative phosphorylation could eliminate these specific cancer cells. Building on this insight, a promising strategy to combat cancer involves targeting ClpXP, taking advantage of the reliance of these malignancies on oxidative phosphorylation.

To date, most cancer research has been on ClpP, but ClpX has received somewhat less scrutiny. ClpP is overexpressed in various cancer patients, including those with acute myeloid leukaemia, breast, lung, liver, ovarian, bladder, prostate, uterine, stomach, testicular, thyroid, and non-small cell lung cancer (NSCLC). The expression of UPRmt-associated genes positively correlates with ClpP synthesis. Nonetheless, the majority of us remain unaware of the specific mechanisms governing ClpP expression in mammals, such as transcription factors and epigenetic modifications that lead to dysregulation in cancer. Furthermore, less information exists on the expression of ClpX in cancer. Further investigation is required to comprehensively elucidate the function of ClpXP in the initial phases of oncogenesis.^{109,110}

ClpP is essential for certain malignancies to survive, proliferate, develop resistance, and metastasise to other body regions. Modifying genes or employing medications that inhibit ClpP can efficiently eradicate cancer cells exhibiting elevated ClpP levels. This observation is consistent with ClpP's critical role in preserving the integrity of respiratory chain complexes. ClpP inhibition elevates reactive oxygen species (ROS), disrupts respiratory chain complex function, and impairs oxidative phosphorylation, collectively promoting cell death. Limited research has been conducted on the function of ClpX in cancer cell survival; nonetheless, it is believed that the observed effects following ClpP inhibition may reflect the activity of the ClpXP holoenzyme. Inhibiting ClpX function in cancer may have analogous results. Experiments must be conducted to evaluate this theory.¹¹¹

Normal cells appear less impacted by the loss or inhibition of ClpP compared to cancer cells. ClpP is predominantly located in tissues rich in mitochondria, such as the heart, liver, and skeletal muscle. ClpP knockout (ClpP^{-/-}) mice exhibit elevated expression levels in these vital organs, yet they remain viable, though slightly smaller than their wild-type counterparts.^{112,113} Mice deficient in ClpP (ClpP^{-/-}) are infertile and experience hearing loss. Similarly, a few rare individuals from close-knit families with homozygous inactivating mutations in ClpP are alive but exhibit hearing impairments and infertility.¹¹⁴ The findings indicate that the development of ClpP inhibitors as a therapeutic approach may be beneficial in the treatment of specific cancer types. In contrast to other mitochondrial proteases utilised in cancer treatment, ClpP is distinctive in knockout mice, and individuals with alterations in ClpP exhibit no adverse effects. Cancer cells may undergo apoptosis when proteases such as LONP1 are inhibited. Nonetheless, embryos that are homozygously deficient in Lonp1 do not survive. Both humans and animals may survive without ClpP, raising significant enquiries regarding the mechanism

by which targeted inhibition of ClpP might eradicate specific cancer forms while leaving normal cells, which possess elevated ClpP levels, unaffected.¹¹⁵

B.5 Small molecule ClpP inhibitors—chemical probes to understand ClpP biology and leads for anticancer agents

The development of small molecules designed to inhibit ClpP-mediated protein degradation exemplifies the precision of modern research. These chemical probes were carefully made to look into the molecular roles of ClpP and to support the idea that it might be a good target for cancer treatments. The discovery that small molecule inhibitors, such as trans- β -lactones, can selectively target bacterial ClpP while sparing healthy cells is promising, indicating their potential safety. The crystal structure of *S. aureus* ClpP reveals that the hydrophobic R1 chain of β -lactones interacts with a deep pocket near the ClpP active site. This interaction positions the β -lactone core close to ClpP's active serine, enabling a nucleophilic attack. β -lactones inhibit ClpP function, effectively killing bacteria both in vitro and in vivo.

It has been found that an artificial β -lactone called A2-32-01 can react with the ClpP enzyme in mitochondria. It is possible for A2-32-01 to selectively kill AML cell lines and raw AML samples with high ClpP expression while leaving normal haemopoietic cells and AML cells with low ClpP expression alone (**Table 1-2**). Preclinical studies of A2-32-01 in animal models demonstrate its efficacy against cancer. A2-32-01 is a valuable chemical tool for studying ClpP, but it is not stable enough, which limits its promise for use in clinical trials. The chemical's cyclic ester structure, which is typical of β -lactones, breaks down quickly in water, breaking down over 90% of A2-32-01 in cell growth conditions in just one hour.⁹⁷

A study of over 137,000 chemicals showed that phenyl esters stop the bacterial ClpP peptidase from working. In a way similar to β -lactones, these phenyl esters stop ClpP by attacking the active serine residue with nucleophiles. This interaction causes the ester to split, which locks ClpP in an acyl-enzyme intermediate state. This sets off the deoligomerization of ClpP. Five newly found phenyl esters—AV126, AV168, AV127, AV167, and AV170—were more effective, reacted faster, and were more stable against bacterial ClpP than β -lactones. There is much similarity between bacterial and human ClpP, but only AV167 reacted with human ClpP (**Table 1-2**), showing that the active regions of human and bacterial ClpP are very different. The researchers made more potent and selective inhibitors of human mitochondrial ClpP by switching the naphthofuran part at position-2 of AV167.¹¹⁶ The changed counterparts, named

TG42, TG43, and TG53, mainly stop human ClpP from breaking down peptides and proteins. Still, they don't affect *S. aureus* ClpP (SaClpP) much. Interestingly, TG42 and TG53 have been shown to stop Huh7 liver cancer cells from migrating and cause them to die (**Table 1-2**). More research must be done to determine if the anticancer effects are directly caused by blocking ClpP or if interactions with other proteins cause them since these drugs have been shown to cross-react with several human proteases.¹¹⁷

Table 1-2 Mitochondrial ClpP inhibitors.

Inhibitors				
Class	Name	Cell lines	Biological effect	Reference(s)
β -lactones	A2-32	TEX	Acute	Induced cell death ⁹⁷
	01	OCI-AML2	myeloid leukemia	Cell death induction; R diminished functionality of respiratory chain complex II observed in SCID mouse xenografts
		K562	Chronic myeloid leukemia	Induced cell death ⁹⁷
Phenyl esters	AV167	N/A	N/A	N/A ⁷¹
	TG42	Huh7	Hepatocyte -derived carcinoma	Induced cell apoptosis
	TG53	Huh7	Hepatocyte -derived carcinoma	Induced cell apoptosis ⁶⁸

Peptide boronates have been recognised as inhibitors of ClpP. The α -aminoboronic acid compound 8a–c was identified as effective inhibitors of human ClpP, demonstrating efficacy similar to that of AV16781. Virtual modelling of α -aminoboronic acid with human ClpP indicates that these compounds interact with Ser97 and H122; however, the precise mechanism of inhibition necessitates validation through physical and structural analysis. Furthermore, it is essential to assess the capacity of these compounds to bind and inhibit ClpP within intact cells.¹¹⁸⁻¹²⁰

Currently, the majority of initiatives aimed at targeting ClpXP have focused on inhibiting the active site of ClpP. An alternative strategy may involve the development of compounds that interfere with the interaction between ClpP and ClpX, potentially resulting in novel inhibitors

with improved selectivity for the target. The conserved IGF motifs and pore-2 loops of ClpX, which are critical interaction sites with ClpP, may serve as potential targets for disrupting this interaction and inhibiting ClpXP activity. Molecules that inhibit the ATPase function of ClpX may serve as potential anticancer agents.

B.6 Concluding remarks and future directions

Recent research indicates that mitochondrial ClpXP plays a crucial role in the survival of certain blood cancers and solid tumours. Research has identified ClpXP as a novel target for cancer therapies, enhancing our understanding of the functional mechanisms of this mitochondrial protease and its role in mitochondrial metabolism. Simultaneously deactivating and activating ClpP disrupts oxidative phosphorylation, highlighting the unique role of this protease in cancer combat.

Recent research identifies multiple chemical groups that influence the activity of ClpP protease, exhibiting different levels of selectivity and specificity. These compounds may potentially target cancer cells specifically; however, further research is required to enhance their strength, stability, and selectivity. Additional research is needed to ascertain their efficacy and potential toxicity in living organisms. ClpP exists in both virtual and physical forms, which is advantageous for these projects.

Establishing biomarkers to identify patient groups most likely to respond to clinical-grade molecules targeting ClpP is essential for their development. In certain cancers, such as acute myeloid leukaemia (AML), ClpP levels correlate with the efficacy of ClpP-targeted therapies in laboratory settings. The expression of the ClpP protein may serve as a predictive measure for treatment efficacy and assist in patient selection for therapy. Investigating the effects of chemotherapy on ClpXP expression and assessing any alterations in ClpXP expression during recovery is essential.

Understanding the resistance mechanisms to ClpP inhibitors and activators is essential for developing strategies to address this resistance. A comprehensive understanding of ClpXP and related proteases is critical for successfully translating new therapies into clinical practice and advancing our knowledge of mitochondrial biology.

C. HIF family and its research status

C.1 HIF Biology

Although VHL has various cellular functions, its primary role is regulating transcription factors such as HIF-1 α and HIF-2 α to control the cellular response to oxygen levels. HIF, a transcription factor, consists of a HIF- α subunit and a HIF- β subunit (also known as aryl hydrocarbon receptor nuclear translocator protein or ARNT).¹²¹ The HIF- β subunit is relatively stable, whereas the HIF- α subunit is less so. The HIF- α subunit includes three members: HIF-1 α , HIF-2 α , and HIF-3 α , with HIF-1 α and HIF-2 α sharing high protein sequence and structural homology. Both HIF-1 α and HIF-2 α possess a bHLH domain (basic Helix-Loop-Helix), an ODDD domain (Oxygen-Dependent Degradation Domain), two PAS (Per/Amt/Sim) domains, and the TAD-N (Transactivation Domain-N) and TAD-C domains at the N-terminal and C-terminal, respectively.¹⁴ In contrast, HIF-3 α can generate multiple protein isoforms; however, due to the lack of ODDD and CATD domains, its transcriptional activation functions remain unidentified. As a result, most research has concentrated on HIF-1 α and HIF-2 α .

C.2 Regulation of HIF-1 α

The stability of the HIF- α subunit is regulated by intracellular oxygen levels, along with prolyl 2-oxoglutarate-dependent Fe²⁺ dioxygenase enzymes, specifically PHD1, PHD2, and PHD3.^{122,123} Under normal oxygen levels, PHD enzymes utilise oxygen to hydroxylate two conserved proline residues on the HIF-1 α subunit. This hydroxylation allows the VHL/E3 ubiquitin-protein ligase complex to recognise and tag the HIF- α subunit for degradation by the proteasome.¹²⁴ However, under hypoxic conditions, the activity of PHD enzymes is reduced, leading to insufficient hydroxylation of HIF- α subunits. As a result, these subunits are not effectively recognised by VHL, preventing their degradation by the proteasome. Consequently, HIF- α accumulates in the cell and translocates to the nucleus, which binds to the HIF- β subunit to form an active HIF complex. This complex, along with co-activators such as p300 and CBP (CREB-binding protein), binds to hypoxic response elements (HREs), thereby activating a cascade of downstream genes.¹²⁵ Therefore, in ccRCC, the inactivation or deletion of VHL prevents the degradation of HIF- α by the proteasome, even under normal oxygen conditions. This leads to the accumulation of HIF- α and the persistent activation of its transcriptional program, which plays a crucial role in the progression of ccRCC.

In addition to the VHL and oxygen-dependent degradation pathways, the degradation of HIF-1/2 α is also regulated independently of the O₂/PHD/VHL pathway by heat shock protein 90 (HSP90). HSP90, a molecular chaperone, protects these proteins from misfolding and

degradation through its ATPase activity.¹²³ It has been found that HSP90 binds to the PAS domain of HIF-1/2 α , thereby increasing the stability of these proteins. Additionally, the receptor for activated C kinase 1 (RACK1) has been identified as a protein that promotes the degradation of HIF-1 α through a proteasome-dependent but O₂/PHD/VHL-independent mechanism. RACK1 competes with HSP90 for binding to the PAS-A domain of HIF-1 α . Once bound, RACK1 recruits Elongin-C and subsequently Elongin-B, along with other components of the E3 ubiquitin ligase complex, to HIF-1 α . This recruitment facilitates the ubiquitination and proteasomal degradation of HIF-1 α . Thus, RACK1 plays a crucial role in the O₂/PHD/VHL-independent pathway by regulating the stability of HIF-1/2 α through its competition with HSP90 and the recruitment of the Elongin-C/B complex.^{4,126} Furthermore, studies have shown that HSP90 inhibitors can competitively bind to HSP90 with ATP in VHL-deficient cells. This binding disrupts the interaction between HSP90 and HIF-1/2 α , leading to the degradation of HIF- α (**Figure. 1-5**).^{127,128}

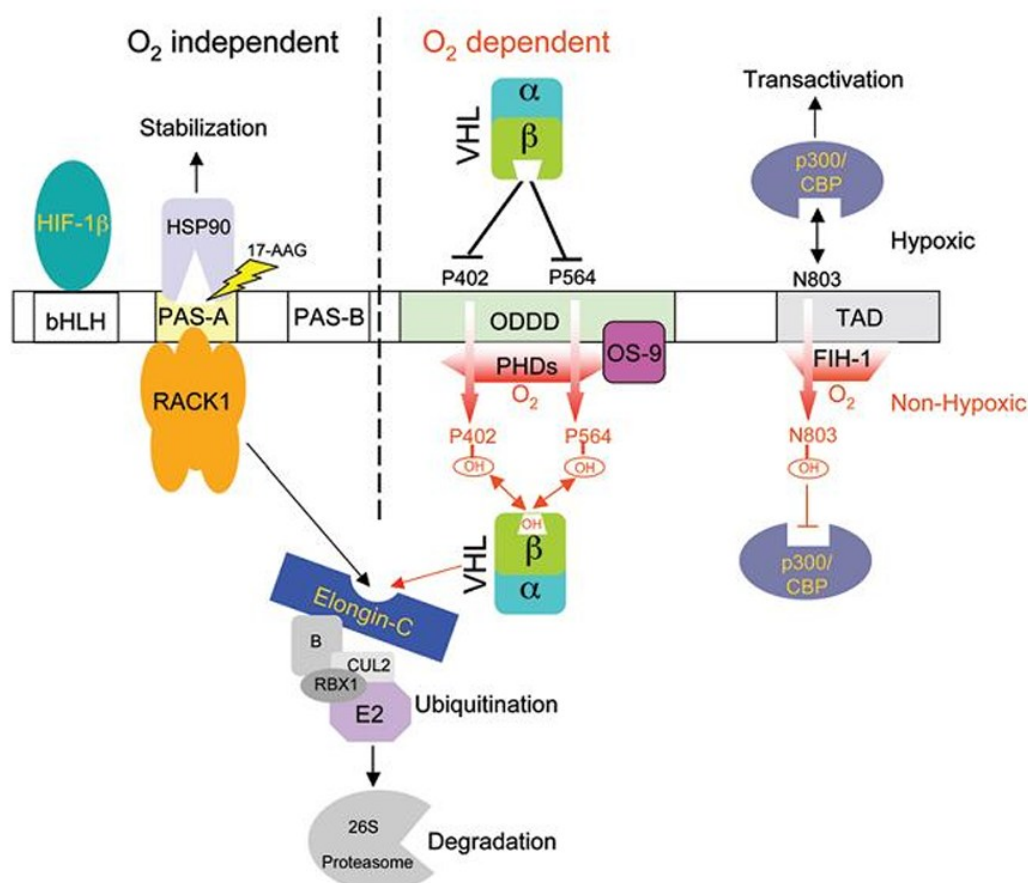


Figure 1-5. Regulation mechanism of HIF-1α.

The stability of HIF-1α protein is controlled through oxygen-dependent and oxygen-independent degradation mechanisms. In the oxygen-dependent pathway, prolyl hydroxylases (PHDs) hydroxylate proline residues 402 and/or 564 within the oxygen-dependent degradation (ODD) domain of HIF-1α in the presence of oxygen. OS-9 facilitates this process by simultaneously recruiting both PHDs and HIF-1α. Hydroxylated HIF-1α is then recognized by the VHL complex, which interacts with Elongin-C. In the oxygen-independent pathway, the binding of HSP90 and RACK1 to the PAS-A domain of HIF-1α plays a regulatory role. HSP90 stabilizes HIF-1α by preventing RACK1 from binding. When RACK1 does bind, it recruits Elongin-C and other components of the E3 ubiquitin ligase complex, leading to HIF-1α ubiquitination and degradation by the 26S proteasome. HIF-1 transcriptional activity is also modulated in an oxygen-dependent manner. Factor inhibiting HIF-1 (FIH-1) hydroxylates asparagine residue 803 within the carboxyl-terminal transactivation domain (TAD) of HIF-1α in the presence of oxygen. This hydroxylation prevents HIF-1α from interacting with its coactivators, p300/CBP, thereby suppressing its transcriptional function. (Figure modified from Liu et al.⁴)

C.3 Tumour mechanism of HIF-1 α

HIF-1 α and HIF-2 α play crucial roles in tumourigenesis and progression by regulating the expression of a lots of downstream target genes. Although there is substantial overlap in the genes regulated by HIF-1 α and HIF-2 α , each factor also governs distinct target genes. The tumour type and microenvironment closely influence the expression and biological impact of HIF-1 α and HIF-2 α on tumours. HIF target genes are involved in regulating numerous biological processes critical to tumour development and progression, including angiogenesis, glycolysis, cell migration and metastasis, lipid storage, cell cycle regulation, inflammation, and histone demethylation.

The role of HIF in tumours is primarily mediated through the regulation of various target genes. These genes are believed to be crucial for tumour development and progression.¹²⁹ Key target genes include those involved in glucose metabolism, such as lactate dehydrogenase (LDH) and glucose transporter 1 (GLUT1), as well as inhibitors of acetyl-CoA formation like pyruvate dehydrogenase kinase 1 (PDK1). Additionally, HIF regulates pH control through carbonic anhydrase 9 (CA9) and influences the expression of growth factors and angiogenic proteins, including VEGF, PDGF, IGF, TGF- α , and CCN1. HIF also promotes the expression of anti-apoptotic proteins like BCL-xl, as well as Cyclin D1 and erythropoietin (EPO), all of which contribute to tumour growth and survival.^{31,124}

As proposed by Warburg¹³⁰, the increased expression of glucose-metabolizing proteins, such as LDH and the lactate transporter MCT4, along with the decreased production of acetyl-CoA, facilitates the shift in tumour energy metabolism from oxidative phosphorylation to aerobic glycolysis. The export of lactate from the cell acidifies the tumour microenvironment, a process maintained by the upregulation of carbonic anhydrase 9, a transmembrane protein. Additionally, in ccRCC, a downregulation of specific electron transport chain proteins, including cytochrome C oxidase and NADH-ubiquinone oxidoreductase, have been observed, further diminishing the efficiency of oxidative phosphorylation.¹³⁰

HIF has also been shown to directly inhibit tumour cell apoptosis by increasing the expression of anti-apoptotic proteins. Razorenova et al. found that in 65% of ccRCC, HIF can promote the expression of the apoptosis suppressor protein ARC, which contributes to the early survival of tumour cells.^{39,48} Neoplastic polycythemia, sometimes observed in kidney cancer, is also linked to an increase in HIF-induced erythropoietin (EPO). Additionally, CXCR4, a chemokine receptor

that is another target of HIF, is present on tumour cells and, when activated by HIF, contributes to tumour cell metastasis.^{122,123,131}

C.4 Functional difference between HIF-1 α and HIF-2 α in renal clear cell carcinoma

HIF-1 α and HIF-2 α can be activated by hypoxia and VHL loss, and they share significant structural and biochemical similarities. However, emerging evidence suggests that they also exhibit distinct and even opposing roles in various biological processes, including embryonic development, the activation of hypoxia-related genes, and tumourigenesis.^{122,132,133} For instance, in PVHL-deficient clear cell renal cell carcinoma (ccRCC), HIF-2 α tends to function as an oncogene, whereas HIF-1 α acts as a tumour suppressor.¹³⁴ Various lines of evidence support the contrasting roles of HIF-1 α and HIF-2 α in ccRCC. On the one hand, HIF-1 α expression is significantly reduced in 30-40% of ccRCC cases, as it can inhibit tumour development by suppressing the proliferation of VHL-deficient tumour cells during the progression of ccRCC. In contrast, HIF-2 α consistently promotes tumour proliferation, contributing to the further development and progression of ccRCC.^{132,135} Furthermore, studies on pretumour kidney lesions in patients with VHL disease also underscore the pivotal role of HIF-2 α in the transformation of dysplastic cells. HIF-2 α expression increases with the severity of dysplasia, whereas HIF-1 α expression is diminished.¹²⁸ On the other hand, studies have demonstrated that HIF-2 α , rather than HIF-1 α , can counteract the tumour-suppressive activity of pVHL. Additionally, in preclinical models, the elimination of HIF-2 α using siRNA is sufficient to inhibit tumour formation in PVHL-deficient renal carcinoma cells.^{123,127} More importantly, a recent genome-wide association study investigated the risk associated with HIF-2 α polymorphisms in kidney cancer, identifying HIF-2 α , rather than HIF-1 α , as the underlying cause.^{134,136,137} It has been suggested that differences in DNA binding patterns between the two HIF-1 α subtypes may partly account for this, with HIF-2 α , rather than HIF-1 α , being identified as the primary cause.^{136,137}

C.5 Therapeutic application based on HIF

C.5.1 Antiangiogenic therapy

Hanahan and Weinberg outlined the fundamental principles necessary for the uncontrolled proliferation of cells that lead to tumour formation. Among the first six hallmarks of cancer, they identified angiogenesis, the formation of new blood vessels, as crucial. While small populations of tumour cells can initially survive through simple diffusion of nutrients, data suggest that sustained tumour growth eventually necessitates new blood vessel formation.

Clear cell renal cell carcinoma (ccRCC) is a highly vascularised tumour, primarily due to the intense hypoxia and pro-angiogenic response mediated by HIF within the tumour.¹³⁸ Therefore, the current preferred treatment for metastatic clear cell renal cell carcinoma (ccRCC) involves using tyrosine kinase inhibitors that target downstream genes of HIF, such as EGFR and FGFR, to inhibit angiogenesis (as previously detailed). While most patients initially exhibit significant disease control, approximately 10% demonstrate primary drug resistance. Furthermore, with prolonged treatment, many patients develop secondary resistance to Sunitinib and similar anti-angiogenic drugs, ultimately leading to a poor prognosis.^{130,139}

D. Mitochondrial autophagy and cancer development

D.1 Overview of mitochondria

Mitochondria are double-membrane organelles located in the cytoplasm and play a crucial role in various cellular processes, including ATP synthesis, calcium homeostasis, metabolic regulation, apoptosis, and the generation of reactive oxygen species (ROS).¹⁴⁰ Maintaining normal mitochondrial morphology and quantity is crucial for mitochondrial quality, which is regulated by four fundamental mechanisms: fission, fusion, biogenesis, and mitophagy. Mitochondria possess their own genome, a trait reflecting their bacterial evolutionary origin. Although nuclear genes encode most mitochondrial proteins, the mitochondrial genome also directs the synthesis of specific proteins.¹⁴¹ Mitochondrial homeostasis is crucial to human health, and its disruption can lead to various diseases, including cancer.¹⁴² The maintenance of mitochondria is a dynamic process, constantly balanced through mitochondrial biogenesis, fusion, fission, and the clearance of damaged particles. As a result, the mitochondrial network is continuously reshaped and repaired.¹⁴³

Mitochondrial fusion enables two mitochondria to merge, while mitochondrial fission splits a mitochondrion into two. Loss of mitochondrial fusion leads to mitochondrial fragmentation, and cells with impaired mitochondrial fusion exhibit significantly slower growth rates than wild-type cells.¹⁴⁴ Mitochondrial fission reduces the size of mitochondria, facilitating mitochondrial autophagy. When mitochondrial fission is disrupted, mitochondrial autophagy becomes abnormal. Therefore, maintaining a dynamic balance between mitochondrial fusion and fission is crucial for optimal mitochondrial physiology.^{145,146}

Mitochondria utilise 98% of the total oxygen intake to produce ATP for cellular activity through oxidative phosphorylation, which also involves producing reactive oxygen species (ROS). While

ROS can be generated in various cellular locations, mitochondria are responsible for approximately 90% of the total ROS production.^{147,148}

As reactive oxygen species (ROS) accumulate, oxidative stress intensifies, eventually leading to genomic instability and mutations. Mitochondrial DNA (mtDNA) is particularly vulnerable compared to nuclear DNA, primarily due to the absence of introns and histones in the mitochondrial genome. Additionally, mtDNA is located at the primary site of ROS production, and its replication process involves asymmetric division, further contributing to its instability.¹⁴⁹

D.2 Mitochondria and cancer

Unlike normal cells, which primarily rely on oxidative phosphorylation for energy production, cancer cells mainly obtain energy through aerobic glycolysis, a phenomenon known as the 'Warburg effect'.¹⁵⁰ Consequently, mitochondrial metabolism was long considered irrelevant to cancer cell metabolism, leading to the recognition of mitochondrial dysfunction and aerobic glycolysis as defining characteristics of cancer.¹⁵¹⁻¹⁵³ In earlier studies, Crabtree demonstrated that glucose inhibits respiration and oxidative phosphorylation in cancer cells, a phenomenon known as the "Crabtree effect."¹⁵⁴ Chance and Hess later discovered that glucose initially stimulates respiration for a brief period before the Crabtree effect occurs. This phenomenon, known as the 'Chance-Hess effect,' is attributed to the fact that ADP produced during the early steps of glycolysis—specifically in the synthesis of glucose-6-phosphate (G6P) and fructose diphosphate (FBP)—first stimulates respiration and subsequently inhibits it when ADP is converted to ATP.¹⁵⁵ Although most tumours primarily rely on glycolysis as their main metabolic pathway, functional mitochondria and mitochondrial ROS remain crucial for tumourigenesis and the proliferation of cancer cells.¹⁵⁶

In the early stages of glycolysis, cells convert glucose into pyruvate. Under limited oxygen conditions, lactate dehydrogenase (LDH) directly converts pyruvate to lactic acid. When sufficient oxygen is available, pyruvate undergoes dehydrogenation in the mitochondria to form acetyl-CoA. Acetyl-CoA then enters the tricarboxylic acid (TCA) cycle for further oxidation. Recent studies have shown that mutations in several enzymes involved in the TCA cycle, such as succinate dehydrogenase and fumarate hydratase, can lead to various human cancers.¹⁵⁷

Mitochondrial dysfunction has been identified in many common cancers, including liver cancer,

breast cancer, and ccRCC. Mitochondrial ROS is a crucial mediator of signal transduction within mitochondria. ROS cannot be effectively cleared when mitochondria are damaged, leading to continuous accumulation and further exacerbating mitochondrial dysfunction. Additionally, sustained ROS production is necessary for K-ras oncogene-driven cancer cell proliferation.^{158,159} Mitochondrial mutations that disrupt the TCA cycle and electron transport chain lead to an overproduction of reactive oxygen species (ROS), which activates tumour-related signalling pathways such as PI3K and MAP kinase. Notably, the transcription factor NF- κ B was the first identified to respond to ROS.¹⁶⁰ Other studies have found that patients with lower mtDNA copy numbers exhibit more advanced TNM staging and reduced sensitivity to 5-FU.¹⁶¹ Although mitochondrial dysfunction underlies the carcinogenicity of ccRCC and reducing mitochondrial damage is a crucial anti-cancer strategy, the loss of specific essential mitochondrial proteins may paradoxically increase the chemotherapy sensitivity of ccRCC.^{6,162} Mitochondria are regarded as a significant target for cancer therapy, and ROS-mediated mitochondrial dysfunction can inhibit both the growth and apoptosis of cancer cells.¹⁶³

D.3 Overview of mitochondrial autophagy

Autophagy is a process by which eukaryotic cells maintain metabolic balance through the degradation of unnecessary intracellular components, and it is primarily categorised into microautophagy, chaperone-mediated autophagy, and macroautophagy.¹⁶⁴ Mitochondrial autophagy, a targeted form of macroautophagy, involves autophagosomes recognising and enveloping mitochondria for degradation.⁶ Mitochondrial autophagy is essential for eliminating dysfunctional mitochondria, which is critical in preserving mitochondrial function and limiting the production of harmful reactive oxygen species (ROS). Additionally, mitochondrial autophagy can remove healthy mitochondria and reduce their overall number as an adaptive response to stress, such as hypoxia and nutrient deprivation. This ensures the efficient use of limited metabolites and oxygen, further curbing excess ROS production. Therefore, mitochondrial autophagy is crucial for maintaining normal cellular function.¹

Defective mitochondrial autophagy can lead to cardiovascular, nervous, and muscular diseases.^{140,160,165} Additionally, various indications suggest that mitochondrial autophagy plays a significant role in the onset and progression of cancer. Current research has identified several essential proteins involved in mitochondrial autophagy.¹⁶⁶

PINK1, a serine/threonine kinase, is pivotal in mitochondrial autophagy. In healthy

mitochondria, PINK1 utilises the mitochondrial membrane potential to access the inner mitochondrial membrane, which is subsequently degraded before exerting its effects. However, the plot thickens when PINK1 encounters damaged and depolarised mitochondria. Instead of accessing the inner membrane, it accumulates on the outer mitochondrial membrane, where it recruits Parkin, a crucial step in initiating mitochondrial autophagy. This intriguing process has captivated the scientific community, sparking further research and exploration.¹⁶⁷⁻¹⁶⁹

Moreover, PINK1 can induce mitochondrial autophagy upon accumulation in the outer mitochondrial membrane, independent of mitochondrial depolarization. DJ-1, a 19.9 kDa multifunctional protein encoded by the PARK7 gene, is an antioxidant, participates in metabolic regulation, and possesses enzymatic functions. The regular expression of DJ-1 is crucial for maintaining nervous system health, and its mutation is a recognised cause of Parkinson's disease.^{170,171} Subsequent studies demonstrated that DJ-1 mediates the recruitment of optineurin during mitochondrial autophagy. While the loss of DJ-1 does not affect the activation of PINK1/Parkin, it does block mitochondrial autophagy. However, when PINK1 or Parkin is epigenetically impaired, the overexpression of DJ-1 cannot compensate for the decreased level of mitochondrial autophagy. This finding suggests that PINK1/Parkin is an essential downstream mediator in DJ-1-dependent mitochondrial autophagy.¹⁷²

BNIP3, a crucial player in the process of mitochondrial autophagy, serves as an autophagy receptor. Its role is to link autophagy substrates to autophagosomes by binding to ubiquitin and LC3. When mitochondrial autophagy is initiated, ubiquitinated mitochondrial proteins interact with autophagy-related proteins to recruit autophagosomes for phagocytosis. BNIP3's function is vital, as it can promote mitochondrial ubiquitination independently of the PINK1/Parkin pathway. After mitochondrial ubiquitination, BNIP3 is recruited to bind to the mitochondria and is subsequently degraded by lysosomes and the mitochondria, highlighting the importance of its role in the process (**Figure 1-6**).¹⁷³

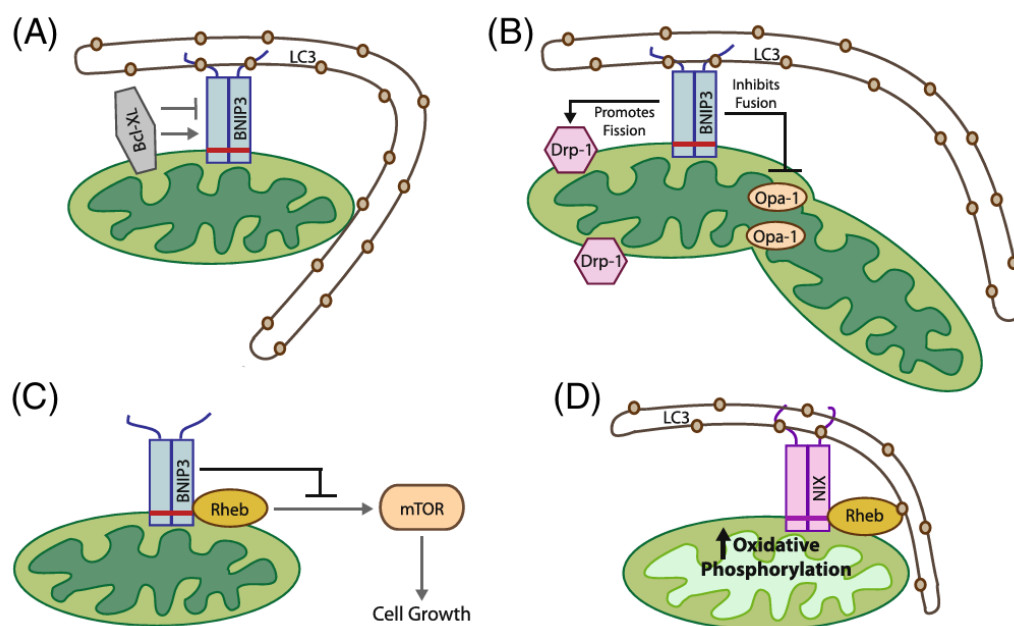


Figure 1-6 BNIP/NIX promotes mitophagy through direct interaction with LC3 at the phagophore.

- A) BNIP3 and NIX are hypoxia-inducible genes that encode molecular adaptors that promote mitophagy through interaction with processed LC3-related molecules at nascent phagophores (A). BNIP3 and NIX interact with Bcl-2 and Bcl-XL through their amino-terminal ends, and Bcl-2/Bcl-XL has been postulated to play both positive and negative regulatory effects on BNIP3 function.
 - B) BNIP3 has also been shown to interact with regulators of mitochondrial fission (Drp-1) and mitochondrial fusion (Opa-1). These interactions are positive and negative, respectively, resulting in a role for BNIP3 in promoting fission while inhibiting fusion.
 - C) BNIP3 has also been shown to interact with the small GTPase, Rheb, resulting in reduced Rheb activity, mTOR activity, and cell growth. This function for BNIP3 in modulating Rheb contrasts with the proposed functional interaction of NIX with Rheb.
 - D) That elicits a mTOR-independent effect on mitophagy by promoting LC3 processing and increased mitochondrial turnover in cells grown on oxidative substrates. NIX is required to recruit Rheb to mitochondria and its activating effect on mitophagy.
- (Figure modified from Aparajita et al.³)

The mitochondrial outer membrane protein NIX contains an atypical BH3 domain associated with the apoptosis-suppressing protein Bcl2. NIX resides in mitochondria and can directly bind to LC3. It plays a crucial role in erythrocyte maturation and is essential for the clearance of mitochondria during reticulocyte development.¹⁷⁴ The mitochondrial autophagy inducer CCCP enhances the interaction between NIX and LC3, indicating that NIX is closely associated with mitochondrial autophagy triggered by the loss of mitochondrial membrane potential.¹⁷⁵ Additionally, mitochondrial autophagy, activated in response to oxygen deficiency, is inhibited when NIX and BNIP3 are depleted.¹⁷⁶

In conclusion, mitochondrial autophagy is not just a critical mechanism for cells to regulate mitochondrial quality but also a vital process in maintaining cellular function. Its role in clearing out damaged or excess mitochondria is crucial for the cell's overall health. When mitochondrial autophagy is impaired, as in the case of defective NIX and BNIP3, mitochondrial homeostasis is disrupted, adversely affecting human health. Identifying and characterising several proteins involved in mitochondrial autophagy is a testament to the significance of this process in cellular function.

D.4: The Role of Mitochondrial Autophagy in Cancer

Mitochondrial autophagy plays a critical role in eliminating abnormal mitochondria and removing surplus mitochondria under stress conditions like hypoxia and nutrient deficiency. By reducing ROS production and conserving nutrients, this process supports cell survival in challenging environments.¹⁷⁷ The role of mitochondrial autophagy in tumourigenesis and progression remains unclear. However, current evidence suggests that the failure of mitochondrial autophagy may contribute to carcinogenic stress and tumourigenesis.¹⁷⁸ This may be due to defective mitochondria triggering ROS accumulation in the early stages of cancer. During this time, abnormal mitochondrial autophagy disrupts mitochondrial homeostasis, exacerbating oxidative stress and contributing to cancer development. However, as cancer progresses, mitochondrial autophagy shifts to a cancer-promoting phenotype, enabling cancer cells to sustain proliferation and evade apoptosis.

Mitochondrial autophagy enhances the metabolic plasticity and stemness of pancreatic cancer stem cells. Studies have shown that the mitochondrial autophagy-associated protein DJ-1 can serve as a biomarker for ccRCC and promote its progression through the activation of mitochondrial autophagy. In metastatic colorectal adenocarcinoma cells, DJ-1 knockdown

impairs the removal of damaged mitochondria, thereby increasing cancer cell apoptosis.^{179,180}

The role of mitochondrial autophagy in cancer resistance is controversial. Initially, it was believed that mitochondrial autophagy could lower oxidative stress levels, thereby maintaining cell genetic stability, reducing mutations, and inhibiting cancer development. However, subsequent studies revealed that mitochondrial autophagy promotes cancer cell survival under cytotoxic stress, particularly oxidative stress.¹⁸¹ Doxorubicin is a drug that induces apoptosis by causing mitochondrial dysfunction. Following treatment with doxorubicin, the expression level of the mitochondrial autophagy regulator NIX significantly increases in ccRCC stem cells, leading to the development of doxorubicin resistance. However, silencing NIX substantially enhances the sensitivity of ccRCC cells to doxorubicin, demonstrating that mitochondrial autophagy significantly contributes to chemotherapy resistance in ccRCC (**Figure. 1-7**).¹⁸²

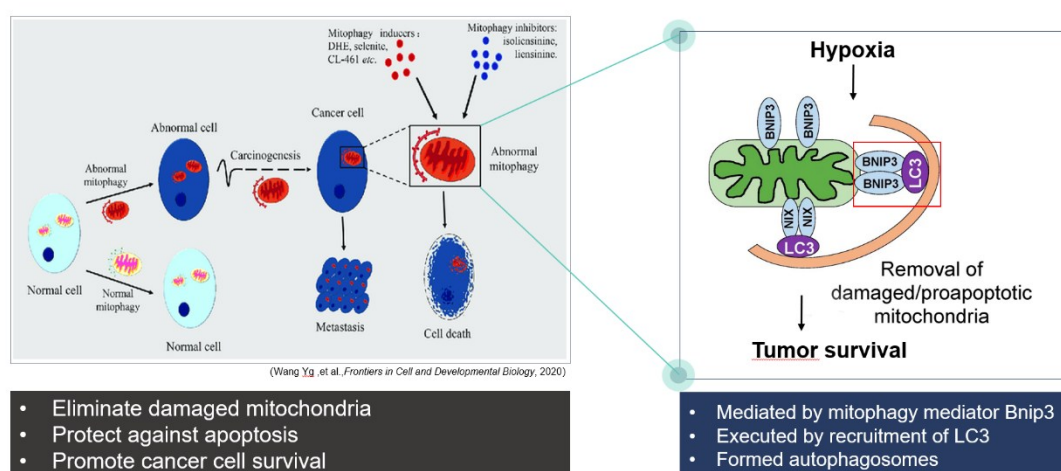


Figure 1-7 The role of mitochondrial autophagy in cancer.

BNIP3 located near the key tryptophan residue LC3, enhancing its association with LC3B-II. The kinase mediating this phosphorylation remains unidentified. Similarly, BNIP3L is phosphorylated and close to the tryptophan within its LIR motif, which facilitates its interaction with LC3B and NIX. NIX= BNIP3L (Figure modified from Poole et al.¹)

With advancing research, mitochondria and mitochondrial autophagy (mitophagy) have been increasingly recognised as critical factors in the occurrence and progression of ccRCC. Mitochondrial dysfunctions are more frequently observed in cancer, and evidence suggests that mitophagy may not only promote ccRCC progression but also enhance the stemness and

drug resistance of cancer cells. Consequently, an increasing number of studies have identified mitochondria and mitophagy as potential therapeutic targets for ccRCC, presenting a promising new direction for research in ccRCC treatment.

E. Research progress of Ferroptosis in malignant tumours

E.1 Overview of Ferroptosis

Ferroptosis is an iron-dependent form of cell death characterised by the accumulation of lipid peroxides.¹⁸³ This process involves iron accumulation, reactive oxygen species (ROS) activation, reduced cysteine uptake, glutathione (GSH) depletion, and the activation of the mitogen-activated protein kinase (MAPK) pathway.¹⁸⁴ Unlike other types of programmed cell death, ferroptosis uniquely relies on the accumulation of ferrous ions and the significant build-up of phospholipid hydroperoxides on the cell membrane. It represents a recently redefined mode of programmed cell death.^{185,186} Moreover, the morphological characteristics and bioenergetics of cells undergoing ferroptosis differ from those of other programmed cell death pathways, including mitochondrial atrophy, increased mitochondrial membrane density, compromised membrane integrity, and internal NADH depletion. Notably, ferroptosis cannot be inhibited by apoptosis, necrosis, or autophagy inhibitors, such as Z-VAD-FMK, but ferroptosis inhibitors, including iron chelators and antioxidants, can suppress it.¹⁸⁷ Increasing evidence suggests that ferroptosis is not only driven by oxidation induced by cystine/glutamate antiporters and GSH synthesis but also by the metabolism of iron ions and unsaturated fatty acids.¹⁸⁸ Recent studies have shown that ferroptosis is closely linked to the progression of various diseases. For example, in cancers such as gastric, liver, pancreatic, prostate, and breast cancer, ferroptosis can inhibit cancer cell growth. Conversely, the activation of ferroptosis may accelerate neurodegenerative diseases like Alzheimer's disease. An in-depth understanding of the mechanisms of ferroptosis may lead to significant advances in addressing critical clinical challenges.

E. 2 The Mechanism of Ferroptosis

Intracellular Fe^{2+} overload can induce ferroptosis through the Fenton reaction, mediated by reactive oxygen species.¹⁸⁹ Since various metabolic reactions can produce phospholipid hydroperoxides within cells, growing evidence suggests that metabolism plays a crucial role in ferroptosis.¹⁹⁰ Compared to normal tissues, tumour cells depend more on iron ions. Iron is essential for the human body and also acts as a significant regulator in the ferroptosis process of tumour cells.¹⁹¹

Mitochondria are critical sites for cellular metabolism, and their metabolites are vital inducers of ferroptosis. Substances like glucose and amino acids are metabolised in mitochondria, serving as the primary raw materials for the tricarboxylic acid (TCA) cycle. Abnormal metabolism of glucose and amino acids may ultimately lead to ferroptosis.¹⁹² The regulation of ferroptosis involves various physiological processes, including iron metabolism, lipid metabolism, and amino acid metabolism. Additionally, mitochondrial metabolism, which underpins ferroptosis, plays a dual role: it releases specific lipid precursors that promote ferroptosis (as intermediates of the TCA cycle can participate in lipid formation) and activates autophagy by generating reactive oxygen species.¹⁹³ Mitochondrial activity and glutamine metabolism are crucial for ferroptosis induced by cysteine inhibition and play a significant role in ferroptosis triggered by glutathione peroxidase 4 (GPX4) inhibition. The discovery of this process has enriched the theoretical understanding of ferroptosis.^{187,194}

Unrestricted lipid peroxidation occurs following iron-induced cell death, inactivating specific lipoproteins in the cell membrane, which leads to membrane rupture and subsequent cell death. Lipoxygenase, ACSL4, and lysophospholipid acyltransferase 3 (LPCAT3) are involved in the enzymatic reactions of lipid peroxidation. Polyunsaturated fatty acid coenzyme A (PUFA-CoA) is first synthesised, and LPCAT3 promotes its esterification. PUFA-CoA is then highly oxidised through the combined action of reactive oxygen species (ROS) and excess oxygen free radicals, impairing the normal physiological functions of lipoproteins on the cell membrane, disrupting membrane integrity, and ultimately causing ferroptosis.^{185,195}

Iron in lipid peroxides may simultaneously damage cells in several ways: 1. Lipid peroxides are further decomposed into ROS through the Fenton reaction, generating many oxygen radicals that amplify the lipid peroxidation process;¹⁹⁶ 2. Iron-related proteins can disrupt intracellular metabolism by altering physical structures of the membrane, such as thickness, curvature, and pore formation;^{197,198} 3. By-products of lipid peroxidation, such as malondialdehyde (MDA) and 4-HNE, can cause cell damage, and the Fenton reaction accelerates this process.¹⁹⁹ However, this outcome requires further validation through additional studies, as the specific mechanisms by which lipid peroxides induce ferroptosis still need to be elucidated. Nevertheless, it is evident that when cells fail to eliminate excess oxygen-free radicals through their antioxidant mechanisms effectively, the accumulation of lipid peroxides will ultimately induce ferroptosis.²⁰⁰

The process of ferroptosis in cells is marked by a disruption in the dynamic balance between lipid peroxidation and the cell's antioxidant defence system. When the antioxidant defence system fails to control lipid peroxidation in specific cells, it accumulates excessive ROS and oxygen free radicals, compromising cellular stability. This imbalance results in a series of events, including autophagy, cell membrane rupture, and necrosis.^{201,202} In the current study, several antioxidant mechanisms related to ferroptosis may be involved, including the DHODH-CoQH₂ pathway, the FSP1-CoQ-NAD(P)H pathway, the GSH-GPX4 antioxidant system, and the GCH1 pathway.²⁰³ Among these pathways, the GCH1, FSP1-CoQ NAD(P)H, and DHODH-CoQH₂ pathways operate independently of the GSH-GPX4 antioxidant system. Together with the GSH-GPX4 system, they collectively inhibit lipid peroxidation, thereby preventing the onset of ferroptosis (**Figure. 1-8**).²⁰⁴

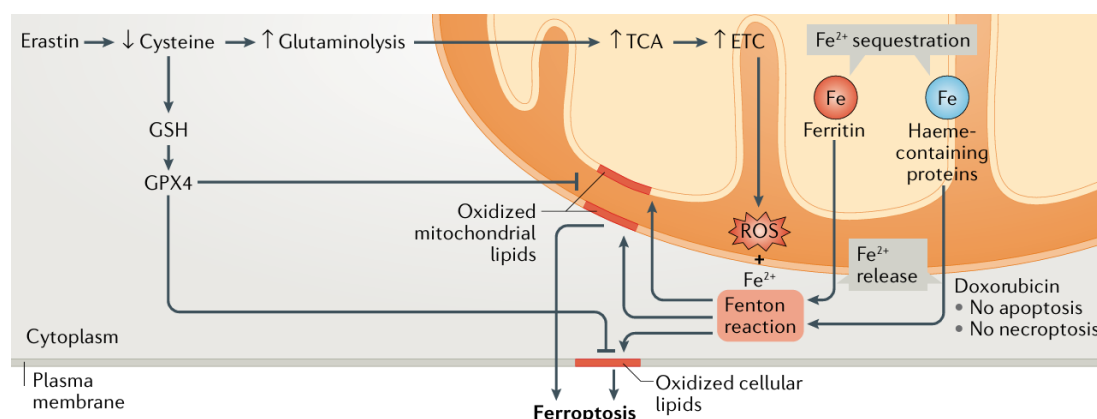


Figure 1-8. Mitochondria and Ferroptosis

Ferroptosis is initiated by the oxidation of lipids through reactions facilitated by iron and reactive oxygen species (ROS), such as the Fenton reaction. Protection against this process is provided by glutathione peroxidase 4 (GPX4), which neutralizes harmful lipid peroxides. Ferroptosis can be induced through treatment with erastin, which inhibits cysteine import and disrupts GPX4 function, or by depriving cells of cysteine altogether. In addition to impairing GPX4, cysteine deprivation leads to enhanced glutaminolysis, fueling the mitochondrial tricarboxylic acid (TCA) cycle, increasing mitochondrial respiration, and consequently elevating mitochondrial ROS levels. Iron, stored in proteins such as ferritin, heme-containing molecules, and mitochondria-associated proteins, is released when these iron-binding structures are degraded under certain cell death-inducing conditions. The close proximity of mitochondrial membranes to sources of free iron and ROS renders them highly susceptible to lipid peroxidation, a hallmark of ferroptosis. ETC, electron transport chain; GSH, glutathione. (Figure modified from Florian et al.⁶)

E. 3 An inhibitor of Ferroptosis

E. 3.1 Inhibitors by reducing lipid peroxides

Ferrostatin-1, first identified as an inhibitor of ferroptosis in studies proposed by Dixon in 2012, was initially found to prevent the death of HT-1080 cells induced by Erastin and RSL3. Ferrostatin-1 binds with aromatic amines, explicitly inhibiting the production of reactive oxygen species (ROS), which reduces lipid peroxidation and ultimately prevents ferroptosis.²⁰⁵ Lipoxstatin-1 (Lip-1) is a widely used lipid peroxidation inhibitor that, similar to Ferrostatin-1 (Fer-1), inhibits various ferroptosis activators, including Erastin. Additionally, Lip-1 protects cells from ferroptosis even in the absence of GPX4. Lip-1 has demonstrated effectiveness in

inhibiting ferroptosis across multiple disease models.²⁰⁶ Vitamin E (alpha-tocopherol) exhibits a similar effect to Ferrostatin-1 (Fer-1) and Liproxstatin-1 (Lip-1) in slowing the accumulation of lipid peroxides. However, the reaction rate of alpha-tocopherol with peroxy radicals is significantly faster than that of Fer-1 and Lip-1.²⁰⁷ Recent studies have found that FSP1 performs a function similar to GPX4 and is a significant factor influencing ferroptosis. It has been shown to effectively reduce vitamin K to hydroquinone, which acts as a (phosphate) lipid peroxidation inhibitor with high viability. Consequently, FSP1-dependent unconventional vitamin K cycling protects cells from harmful lipid overoxidation, thereby inhibiting ferroptosis.^{185,208}

E. 3.2 Ferroptosis inhibitors by affecting the GSH/GPX4 axis

In pharmacological terms, selenium (Se) exerts a protective effect on the nervous system by activating the transcription factors TFAP2c and Sp1. Additionally, it enhances the expression of GPX4 and other related genes during this transcription process.²⁰⁹ 2-Ammonia-5-chloro-N, 3-dimethylbenzamide (CDDO) specifically targets the inhibition of heat shock protein 90 (HSP90), thereby inhibiting not only necrotic apoptosis but also cell ferroptosis. Erastin-induced ferroptosis has been shown to increase levels of lysosome-associated membrane protein 2a (LAMP-2A), which promotes CDDO-mediated autophagy (CMA) and, in turn, facilitates GPX4 degradation. As previously mentioned, Ferrostatin-1 not only inhibits lipid peroxidation but also ferroptosis. Further studies have demonstrated that Ferrostatin-1 can significantly counteract the effects of glutamate on HT-22 cells by increasing GPX4 gene expression and reducing the expression of prostaglandin-endoperoxide synthase 2 (PTGS2). These findings indicate that ferroptosis is induced by glutamate toxicity. Moreover, Ferrostatin-1 protects HT-22 cells from glutamate-induced oxidative toxicity by inhibiting oxidative stress.^{202,210,211}

Ferroptosis, first proposed in 2012, has since become a focal point of extensive scientific research. As research in this area expands, the biological mechanisms underlying ferroptosis in various diseases are becoming increasingly understood. A growing body of evidence indicates that ferroptosis, a newly recognised form of programmed cell death, can be beneficial in tumour suppression. Additionally, some clinically approved drugs have been found to influence ferroptosis. For instance, the FDA has approved Altretamine, Sorafenib, and artemisinin and its derivatives as adjuvant therapies that induce tumour ferroptosis, suggesting that ferroptosis-based tumour therapies hold considerable promise. However, many aspects of ferroptosis in cancer remain to be explored, including its relationship with the

tumour microenvironment and immune-related clinical applications.

F. My PhD study: Aims, Hypothesis and Objectives

Renal cell carcinoma (RCC) is a global health issue, affecting over 400,000 individuals annually and causing approximately 179,000 deaths. This pressing problem underscores the critical need for innovative therapeutic strategies. Targeting mitochondria to suppress cancer cell survival and potentially eliminate cancer cells is a compelling approach.

Mitochondrial respiration is accompanied by the generation of reactive oxygen species (ROS); these may damage cellular structures and compromise their functionality. For example, ROS-induced lipid peroxidation can trigger ferroptosis, an iron-dependent form of regulated cell death.^{187,210} To mitigate these toxic effects, cells employ various protective and repair mechanisms, such as mitochondrial proteolytic mechanisms and mitophagy.¹⁵⁵

The caseinolytic mitochondrial matrix peptidase proteolytic subunit (ClpP) plays a critical role in mitochondrial protein quality control through the selective degradation of proteins involved in various metabolic processes.^{212,213} ClpP is assembled into a double-ringed tetradecameric structure with a hollow chamber containing proteolytic active sites after import into the mitochondria. An AAA ATPase chaperone, ClpX, capped the tetradecameric structure at each end.²¹⁴ In cancers, a high level of ClpP was reported to eliminate ROS-damaged proteins and favour oncogenesis.^{214,215} Moreover, ClpP modulated mitochondrial functions through mitophagy and ferroptosis²¹⁶ and the mitochondrial unfolded protein response, which is crucial for ATP production, oxidative stress control, and apoptotic regulation.^{217,218} Manipulation of mitochondrial ClpP expression has been reported to significantly suppress leukaemia and lung, breast, and pancreatic cancers.^{68,71,219,220}

Mitophagy, an essential process for degrading dysfunctional or damaged mitochondria, is critical for maintaining ATP production and inhibiting oxidative stress. The BNIP3 (BCL2 interacting protein 3)/LC3A/B (Microtubule-Associated Protein 1 Light 3 A/B) system is at the centre of mitophagy and is involved in increased ROS production.^{155,162} In cancer cells, ineffective mitophagy leads to an accumulation of defective mitochondria, impacting cellular metabolism and potentially hindering progression.²²¹⁻²²³ An intriguing question is whether the newly identified mitochondrial chaperone ClpP can regulate mitophagy (**Figure 1-9, First step**). Furthermore, the mitochondrion, as the primary site of ROS production and iron metabolism,

likely plays a crucial role in the regulation of ferroptosis. Ferroptosis, a unique form of cell death mediated by iron-dependent lipid peroxidation (LPO) and ROS accumulation, represents a novel pathway distinct from apoptosis, necrosis, and autophagy.¹⁸³ Glutathione peroxidase 4 (GPX4) is one of the major defence systems against ferroptosis,²²⁴⁻²²⁷ but the role of ClpP activity on GPX4 and cancer cell ferroptosis is unknown **(Figure 1-9, Second step)**. -Thus, the current study investigated the associations between ClpP, mitophagy, and ferroptosis in RCC. I will deploy various assays at molecular, cellular, and *in vivo* levels. Lastly, I will further investigate clinical outcome data from patients with RCC.

Objectives

- 1) To perform bioinformatics analysis and exploration of Clinical Data Related to ClpP Expression
- 2) To *explore* mechanistic mitophagy in *in vitro*
- 3) To investigate mechanistic ferroptosis in *in vitro*
- 4) To further investigate of cancer biology and behaviour, both *in vitro* and *in Vivo*
- 5) To determine the association of ClpP expression in kidney cancer and clinical outcomes

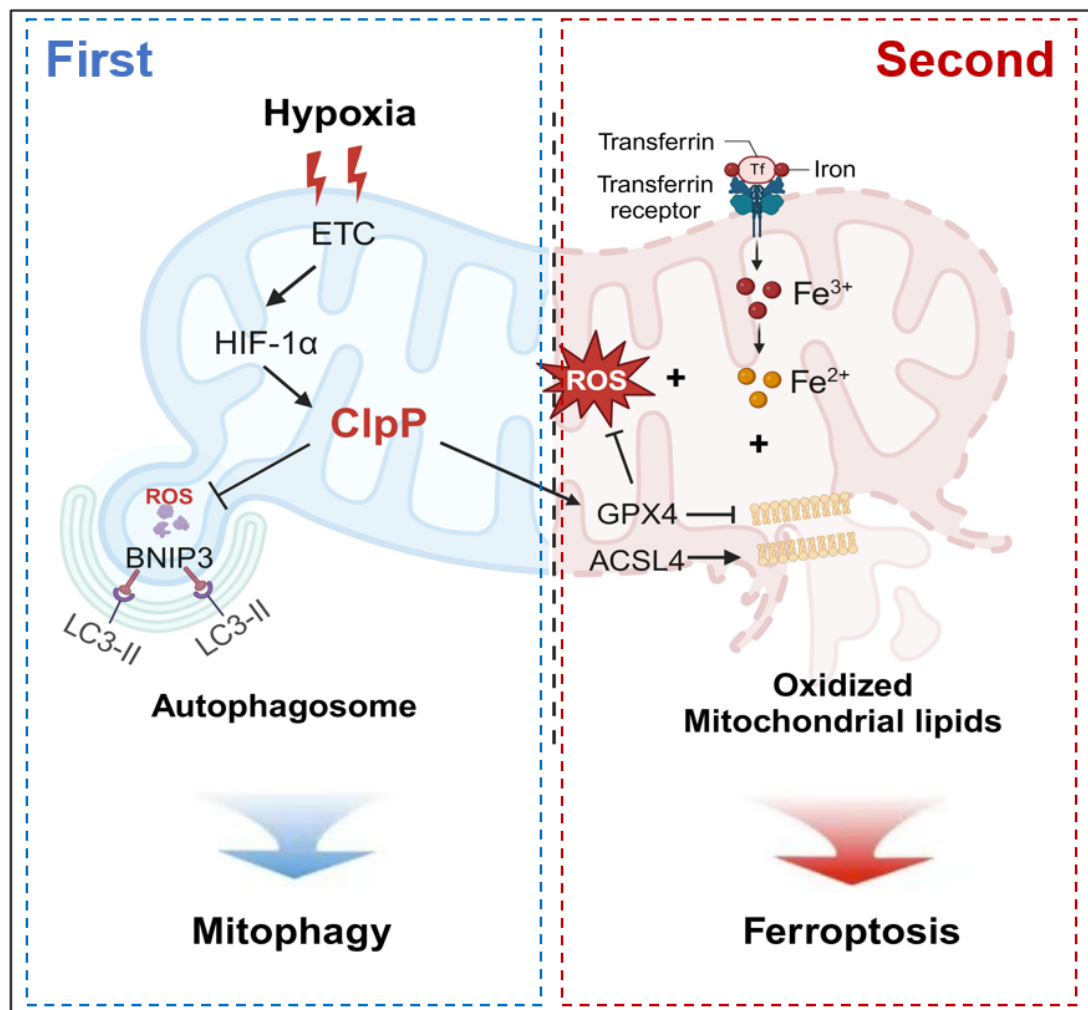
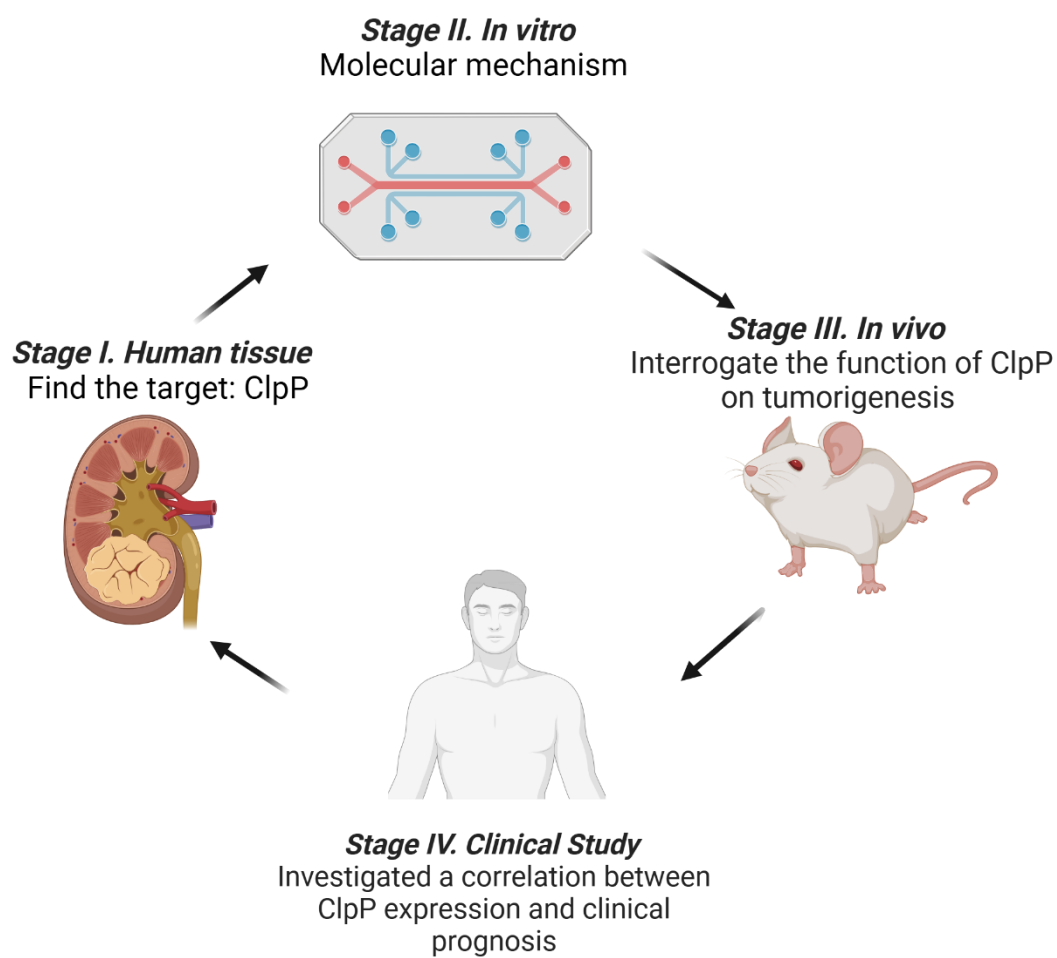


Figure 1-9. Hypothesis: ClpP(-) -- Mitophagy(-) -- Mitochondrial function (-) -- ROS(+) -- Ferroptosis(+).

First, ClpP engages with the hypoxia-induced mitophagy process through the BNIP3-related signalling pathway.

Second, ClpP regulates GPX4-mediated ferroptosis through ROS-related pathways.

CHAPTER TWO**Materials and methods****Graphical Method**

2.1 Stage I. Human samples study (Bioinformatics Analysis and Exploration of Clinical Data)

2.1.1 Ethics statement

The human part study was approved by the Ethics Committee of People's Hospital of Zhengzhou University (2019-44), Henan, China, and registered with the Chinese Clinical Trial Registry (www.chictr.org.cn; ChiCTR1900024468). Human renal cancer wax sections and fresh renal tissue were collected accordingly and used only for the current study. Three patients who underwent radical nephrectomy in Zhengzhou University People's Hospital from March 2020 to April 2020 were enrolled, and their tumour tissues and adjacent non-tumourous tissues were collected. Samples were prepared in pairs, including cancer and adjacent normal tissue. The samples were sequenced on an Illumina HiSeq X Ten platform, and 150 bp paired-end reads were generated. All analyses were performed by a Triple TOF 5600 mass spectrometer (SCIEX, USA) equipped with a Nanospray III source (SCIEX, USA).

2.1.2 Genomics, proteomics and bioinformatic analysis (human samples for bioinformatic analysis)

Transcriptome sequencing and analysis were performed by OE Biotech Co., Ltd. (Shanghai, China). Trimmomatic1 was used to process the raw data (raw readings). The data which includes poly-N sequences and low-quality reads were eliminated in order to get the clean reads. The clean reads were subsequently aligned to the reference genome with HISAT2^{228,229}. With the extensive application of gene chip technology and high-throughput sequencing techniques, the availability of cancer biological information database resources has significantly increased. Notably, the Cancer Genome Atlas (TCGA)^{230,231} and Oncomine databases are pivotal in cancer research. The TCGA database encompasses comprehensive data on the human cancer genome, including gene expression profiles and patient clinical information, originating from the Cancer and Tumour Gene Map Project. This database is a collaborative project between the National Cancer Institute and the National Human Genome Research Institute. The Oncomine database represents the most extensive oncogene-on-chip repository globally, functioning as a comprehensive data integration and mining platform. It amalgamates the gene-on-chip database (GEO), The Cancer Genome Atlas (TCGA), as well as RNA and DNA sequencing data reported in scientific literature.²³² This database encompasses cancer mutational profiles, gene expression data, and clinical information pertaining to the respective patients. Researchers are afforded the capability to reorganize and analyse the data within this repository from various perspectives, thereby facilitating the discovery of

underlying mechanisms associated with cancer development. This study employed bioinformatics analysis to investigate gene expression profile data of renal clear cell carcinoma from the TCGA and Oncomine databases. The objective was to identify key genes and elucidate their roles in the pathogenesis and progression of renal clear cell carcinoma, aiming to discover novel biomarkers and therapeutic targets.

Genomics (RNA-seq)

The mirVana miRNA Isolation Kit (Ambion) was employed to extract total RNA, and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was utilised to assess RNA integrity. Samples exhibiting an RNA Integrity Number (RIN) of 7 or above were utilised for the subsequent study. The TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) was employed for library construction, and sequencing was conducted on an Illumina platform (HiSeqTM 2500 or Illumina HiSeq X 10). **(Figure 2-1).**^{233,234}

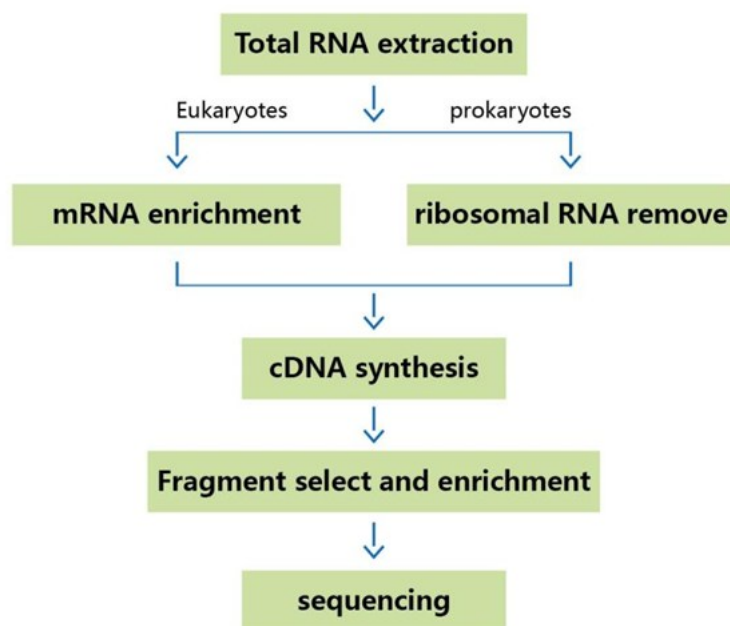


Figure 2-1. RNA-Seq experiment process

Bioinformation analysis process (**Figure 2-2**), Kits: mirVana™ miRNA ISolation Kit (Ambion-1561). Instrument, Refrigerated centrifuge: ST16R, Thermo. Gel imaging system: Tanon 2500, Biotanon Co., Ltd. UV spectrophotometer: NanoDrop 2000, Thermo.

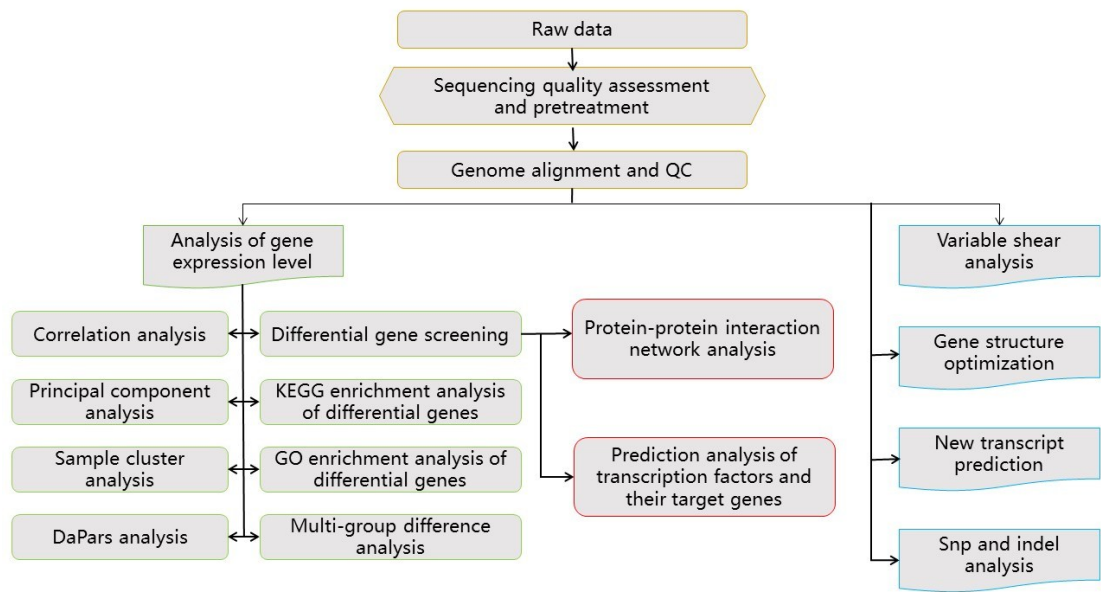


Figure 2-2. Biological information analysis process

Proteomics

Samples, comprising paired cancer and adjacent normal tissues, were preserved at -80°C. Sequencing was performed on the Illumina HiSeq X Ten platform, generating 150 bp paired-end reads. Proteomic analyses were carried out using a Triple TOF 5600 mass spectrometer (SCIEX, USA) equipped with a Nanospray III source (SCIEX, USA).^{235,236}

Experimental process

The basic process of labelled quantitative proteomics experiment is as follows: the total protein in the sample is extracted, a portion of the sample is extracted for protein concentration assessment and SDS-PAGE analysis, while another portion undergoes trypsin digestion and labelling. Subsequently, equal quantities of each labelled sample are combined for chromatographic separation, followed by analysis via LC-MS/MS and data interpretation. (Figure 2-3).

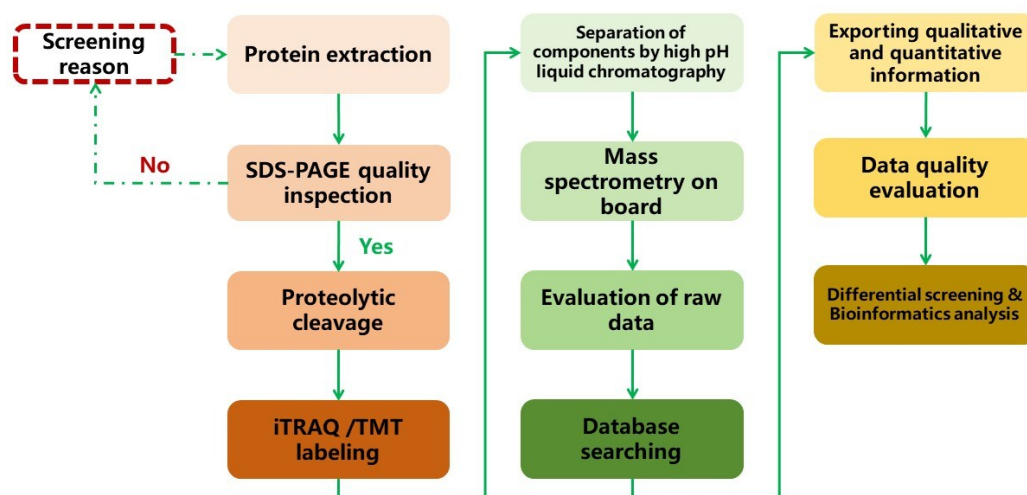
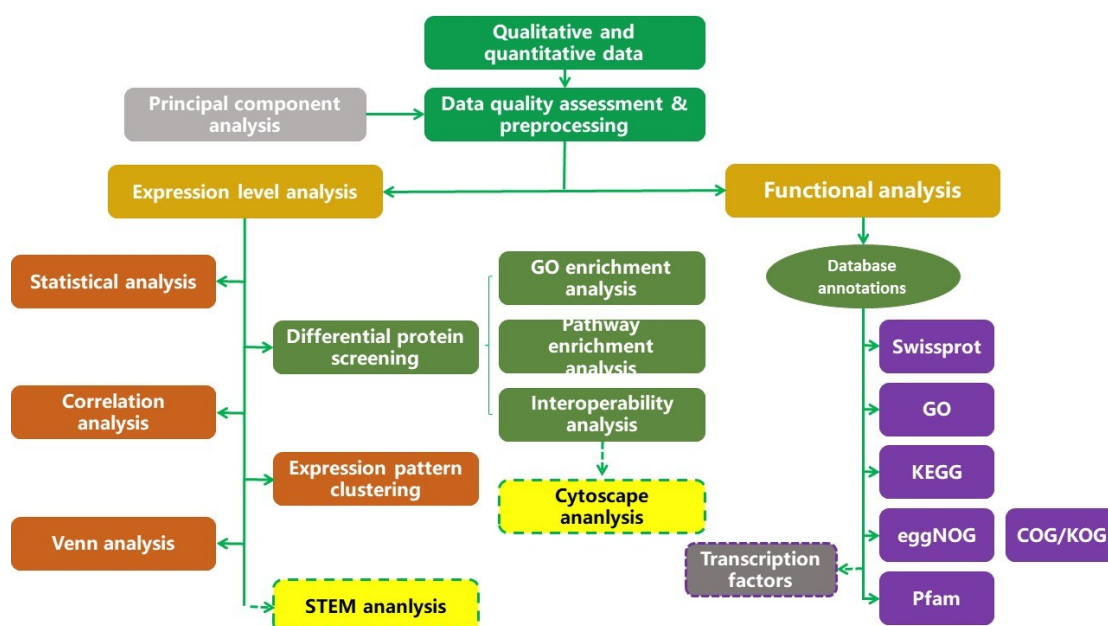


Figure2-3. Proteomics experiment process

Bioinformation analysis process

Conducting a search for qualitative and quantitative data inside the database, followed by expression level analysis and functional analysis, subsequent to quality assessment and preprocessing. The functional annotation analysis of the discovered proteins was conducted utilising various prevalent databases. Gene Ontology, pathway, and interaction analyses were conducted for the identified differential proteins. Simultaneously, correlation analysis, expression pattern clustering heat map, and Venn analysis were conducted on the data from the various comparison groups. Furthermore, based on the data, the correlation or area of interest was analysed, followed by the selection of essential proteins and their respective functions or pathways (Figure2-4).



Bioinformatic analysis

Figure 2-4. Biological information analysis process

Expression patterns for ClpP, GPX4, MAP1LC3B, BNIP3, HIF-1A, and ACSL4 were compared using cancer databases from TCGA (<http://tcga-data.nci.nih.gov/tcga/>). Kaplan–Meier analysis was based on the TCGA PRAD database, and a log-rank (Mantel-Cox) test was performed to evaluate statistical significance.

HDOCK was used to investigate protein-docking between ClpP and HIF1a, ClpP and BNIP3, ClpP and GPX4. The server information should be listed in the Method section (<http://hdock.phys.hust.edu.cn/>). The crystal structures of ClpP, BNIP3, GPX4, and HIF-1α were downloaded from the protein databank and prepared following the standard methods of the Protein Preparation Wizard²³⁰ (Schrödinger Suite). After removing all water molecules, hydrogen atoms were adjusted to a pH of 7. ClpP was used for docking following the default parameters of the LigPrep program. Then, ClpP was docked to BNIP3, GPX4, and HIF-1α using the extra precision mode of Glide.

2.2 Stage II *In vitro* study

2.2.1 Cell culture

Human clear cell renal carcinoma cells (RCC) BA85#21 (ECACC, Salisbury, UK) were grown in RPMI-1640 media, augmented with 10% foetal bovine serum, 2 mM L-Glutamine, and 100 U/mL penicillin-streptomycin (Life Technologies, Paisley, UK). Cells were cultured in a 5% CO₂ incubator at 37°C and propagated in Corning 150cm² cell culture flasks until reaching 60%-70% confluency prior to passaging. For passaging, adherent RCC cells were detached from the culture flask utilising 0.25% trypsin-EDTA, followed by centrifugation at 1500g for 5 minutes and re-suspension in RPMI 1640 media at the specified volume.

2.2.2 Hypoxia Challenge model

To induce hypoxia, RCC cells were incubated in a 1.5L custom-designed airtight, temperature-regulated chamber connected to calibrated flow meters for the delivery of a specific gas composition (Datex gas monitor, Helsinki, Finland) consisting of 1% oxygen and 5% CO₂, balanced with 94% nitrogen (BOC, Guildford, UK), and sealed for 24 hours at 37°C. The chamber was pre-flushed with the aforementioned gas combination to guarantee a steady gas composition.

2.2.3 Cell transfection and virus infection protocol

Small interfering RNA (siRNA)-mediated knockdown, along with short-hairpin RNA (shRNA), represents a widely used RNA interference (RNAi) approach targeting specific messenger RNA (mRNA) sequences to degrade mRNA, thereby silencing specific genes at the post-transcriptional level and inhibiting protein expression.²³⁷ Typically, siRNA molecules are delivered into the cytoplasm as double-stranded RNA (dsRNA) duplexes, where they accumulate and are processed by the dsRNA-specific endonuclease RNase III enzyme, Dicer, into siRNA molecules of 20-30 nucleotides in length.²³⁸ These cleaved siRNA strands are then loaded onto the RNA-induced silencing complex (RISC), a ribonucleoprotein complex capable of various compositions, enabling it to suppress gene expression through multiple mechanisms. Within RISC, the siRNA duplex separates, incorporating only one strand as an anti-sense guide to pair with the targeted sense mRNA sequence. At the core of RISC is the conserved Argonaute protein family member (such as Ago-2), which exhibits RNase-H like activity and associates closely with the anti-sense guide siRNA and its bound target mRNA,

catalyzing the cleavage of the mRNA sequence.

RCC cells were treated with siRNAs specifically for mouse ClpP, HIF-1 α , GPX4 or Bnip3, using the transfection reagent Lipofectamine RNAiMAX (#13778030, Thermo Fisher Scientific, USA) following the manufacturer's protocol. As a control, an additional group of RCC cells was incubated with AllStars Negative Control siRNAs (SI03650318; Qiagen), which are non-specific towards any known mammalian genes. The RCC cells were plated in 35mm Nunclon™ Δ -coated cell culture plates (#153066, VWR International, USA) and allowed to reach 30-50% confluency. Both siRNA and Lipofectamine RNAiMAX were separately pre-diluted in serum-free, antibiotic-free Eagle's minimum essential medium (MEM, #11090081, Gibco™, USA) and incubated at room temperature for 10 minutes to form a stable siRNA-lipid transfection complex. For each transfection in a 35mm plate, 10nM siRNA (from a 10 μ M stock) and 5 μ L Lipofectamine RNAiMAX were added to the RCC cells in a total volume of 300 μ L of MEM. The cells were incubated with this transfection mixture for 24 hours at 37°C with 5% CO₂ and then recovered in complete DMEM for 48 hours before undergoing OGD/Rep, as described previously.

Lentiviruses expressing a short hairpin RNA targeting ClpP and lentiviruses selectively overexpressing ClpP, along with their respective negative controls, were obtained from GENECHM (Shanghai, China). RCC cells were infected with the lentiviruses following the manufacturer's protocol. Infected cells were subjected to selection with 2 μ g/mL puromycin (Invitrogen, San Diego, CA, USA) for two weeks. The efficiency of ClpP knockdown and overexpression was assessed using quantitative PCR (qPCR) and Western blot (WB) analysis.

2.2.4 Immunofluorescence Staining

Immunofluorescence Staining Overview

Immunofluorescence (IF) is a powerful molecular technique used for visualizing and semi-quantifying different antigens within cellular or tissue samples. This method relies on specific interactions between target antigens and antibodies, where antibodies are labeled with a fluorophore that emits fluorescence upon excitation by a specific wavelength of light.

Proper fixation of cell or tissue samples is crucial for effective immunofluorescence staining. Fixation not only prevents the natural digestion of the tissue or autolysis, bacterial putrefaction, and protein degradation, but also preserves the needed cell structure and

antigen immunoreactivity. The most commonly used cross-linking agents include glutaraldehyde and formalin. Glutaraldehyde cross-links nucleophilic groups such as the amines in cellular protein by without any external reagent, while formalin forms radicals that are freely available to react with nearby amines, thus cross-linking the proteins. This process excludes proteins from proteins. The fixed tissues can subsequently be embedded in optimal cutting temperature (OCT) compound for cryo-sectioning or in paraffin for microtome sectioning. Xylene departs from the paraffin in the tissues when deparaffinization is conducted with xylene and subsequently with a series of ethanol solutions for gradually wetting the sections.

When tissues are fixed with cross-linking agents and embedded in paraffin, antigen retrieval is usually required to restore antigenicity and enhance antibody binding. Formalin fixation helps epitopes' sites by creating links among amino acids; however, it could be a step away from the correct peptide. Antigen retrieval methods, namely, heat-induced epitope retrieval (HIER) or protease-induced epitope retrieval (PIER), actually reduce these disulfide bonds thus facilitating antibody binding.

Before antibody incubation, blocking is performed to minimize nonspecific antibody binding. Common blocking agents in IF include bovine serum albumin (BSA) and normal serum. BSA's high protein concentration binds to non-target sites, reducing nonspecific binding of the primary antibody. Normal serum, derived from the same species as the secondary antibody, provides abundant antibodies that bind non-target reactive sites, further preventing nonspecific binding. When tissue samples express high levels of endogenous immunoglobulins, unconjugated Fab fragments can be used to block these immunoglobulins, preventing high background fluorescence. This step ensures that the secondary antibody selectively binds the primary antibody. The step-by-step workflow of immunofluorescence staining is showing in **Figure 2-5**.

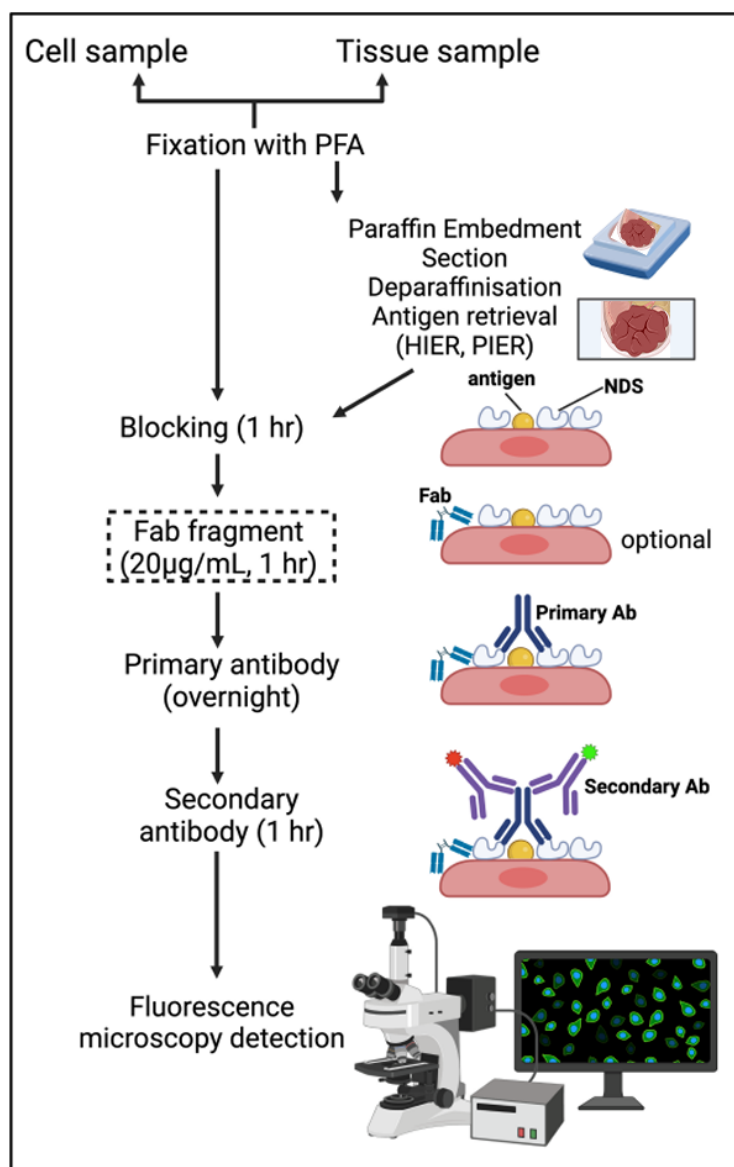


Figure 2-5. Workflow of immunofluorescence staining of cell and tissue samples.

Immunofluorescence Staining for Cell Samples

Primary RCC cells were fixed in ice-cold 4% paraformaldehyde (PFA) with two 10-minute cycles. After fixation, cells were permeabilized with 5% normal donkey serum (NDS) in PBS with 0.3% Tween 20, blocking non-specific binding from fluorescent donkey secondary antibodies. The cells were then incubated overnight at 4°C with primary antibodies specific to ferroptosis or mitophagy markers, diluted in PBS-Tween 20 containing 2% NDS. The following primary antibodies were used: Bnip3, anti-LC3A/B, ClpP, GPX4, HIF-1α and mouse anti-β tubulin III.

On the second day, cells were washed with PBS-Tween 20 to remove unbound antibodies and incubated with secondary antibodies, such as donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 568, for one hour at room temperature. Detailed antibody information is available in **KEY RESOURCES TABLE** (see page 91). Cells were subsequently washed, mounted with DAPI anti-fade medium, and observed under a Zeiss Axio Observer widefield microscope.

Immunofluorescence Staining for Tissue Samples

On the day of sample collection, mice were anesthetized with pentobarbital (50mg/kg, i.p.) and transcardially perfused with ice-cold 4% PFA in PBS (pH 7.4). Tissues were harvested, post-fixed in 4% PFA overnight at 4°C, and then sequentially dehydrated in increasing sucrose concentrations. The dehydrated tissues were further processed through a series of ethanol and xylene immersions before paraffin embedding. Coronal sections (15-20 µm thick) were obtained using a microtome, between -1.70mm and -2.46mm. Deparaffinization involved three 5-minute xylene washes, followed by rehydration in decreasing ethanol concentrations and a final rinse in deionized water. Heat-induced antigen retrieval was performed by heating tissue sections in 10mM sodium citrate buffer (pH 6.0) for 15 minutes.

Recovered antigens were then blocked with 5% NDS in PBS with 0.3% Triton X-100, a more effective surfactant than Tween 20, allowing better antibody penetration into the tissue. For mouse primary antibody reactions, sections were additionally incubated with an anti-mouse Fab fragment to prevent secondary antibody binding to endogenous immunoglobulins. Tissue sections were incubated overnight at 4°C with primary antibodies specific to necroptosis and mitophagy markers, diluted in 2% NDS in PBST. Primary antibodies used included Bnip3, anti-LC3A/B, ClpP, GPX4, HIF-1α and mouse anti-COX IV.

On day two, tissue sections were incubated with fluorescent secondary antibodies, including donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 568, at room temperature for one hour. Detailed information on primary and secondary antibodies is available in **KEY RESOURCES TABLE** (see page 91). The stained sections were mounted with DAPI anti-fade medium and examined using a Zeiss Axio Observer widefield microscope.

2.2.5 Live cell imaging of lipid peroxide

RCC cells were placed in 35-mm glass-bottom tissue culture dishes (MatTek Corp.) to observe internal lipid peroxidation. The cells received treatment with ClpP siRNA (50 nM), Fer-1 (10

μM), or Erastin ($10\ \mu\text{M}$) following a 48-hour period. The subjects were initially labelled with Liper fluo ($20\ \mu\text{M}$) for 30 minutes, thereafter washed, and ultimately treated with siRNA. The specimen was placed in a sealed, 37°C stage top incubator (Tokai Hit Co.) situated on the motorised stage of an inverted Nikon TiE fluorescence microscope equipped with an oil immersion lens (Nikon, CFI Plan Fluor, NA 1.43) and NIS Elements Software. We utilised Liperfluor with a diode-pumped light engine (SPECTRA X, Lumencor) and captured images using an ORCA-Flash 4.0 sCMOS camera (Hamamatsu) along with Chroma's excitation and emission filters. Data were collected every ten minutes for three hours, encompassing around 10 to 20 cells at each stage location. Each of the four distinct trials had 10 to 15 stage positions for each condition. NIS Elements (Nikon) was utilised to analyse the data. The region of the image devoid of cells was utilised to create a glowing effect for background removal.

2.2.6 Western blot

Western blotting is a prevalent molecular technique for detecting specific proteins within a mixture extracted from cell cultures or tissue samples. It often facilitates subsequent quantification of protein expression levels. With specific adaptations in sample preparation and buffer systems, western blotting can also isolate multiprotein complexes, enabling the analysis of protein-protein interactions, as discussed further below. Although “western blot” technically refers to the transfer of proteins from a gel onto a blotting membrane, the term broadly describes the entire protein detection process, encompassing sample preparation, gel electrophoresis, protein transfer, antibody incubation, and detection (**Figure 2-6**).

Before loading samples for gel electrophoresis, it’s necessary to determine the concentration of the protein lysate to ensure equal loading, allowing for accurate comparison of protein levels across samples. The Bradford assay, a straightforward colorimetric method, is frequently used for protein quantification. This assay employs Coomassie Blue dye, which changes from red (cationic, protonated form) to blue (anionic, unprotonated form) upon stable binding to proteins.

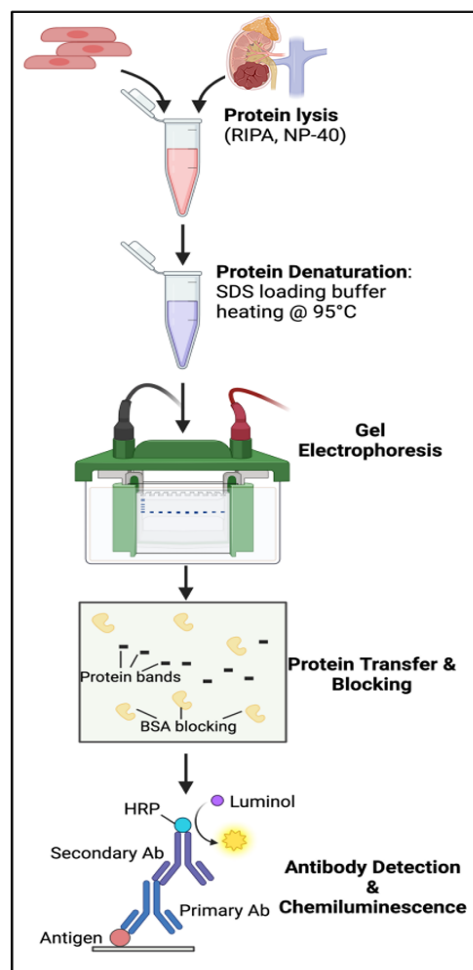


Figure 2-6. Overview of the key steps in western blot with cell or tissue samples.

During electrophoresis, proteins migrate toward the electrode with the opposite charge. Their movement speed and direction are influenced by their size, shape, and overall charge. Polyacrylamide gel serves as the matrix for protein separation, and the gel’s percentage, or polyacrylamide monomer concentration, dictates pore size. Adjusting gel composition allows for optimized separation based on protein size, with smaller proteins migrating faster than larger ones. Additionally, various buffer compositions, pH adjustments, or detergents can alter protein conformation and charge, facilitating distinct separation through the gel.

The most common type of gel electrophoresis involves the detergent sodium dodecyl sulfate (SDS) in the buffer system (e.g., lysis, sample, and running buffers). SDS acts as a denaturing agent, breaking intramolecular disulfide bonds and linearizing proteins. It also imparts a uniform negative charge to proteins, enabling consistent migration from the gel's negative end (cathode) toward the positive end (anode). Further reduction of protein structure can be achieved with β -mercaptoethanol (β -ME) or dithiothreitol (DTT) in the sample or lysis buffer.

After separating proteins on the polyacrylamide gel, they are transferred onto a hydrophobic blotting membrane (such as nitrocellulose or polyvinylidene fluoride) for antibody-based detection. Following transfer, the membrane is blocked with a protein-rich solution to prevent non-specific binding of the primary antibody to unintended targets. Skim milk (5% w/v) and bovine serum albumin (BSA, 1% w/v) are commonly used blocking agents. The blocked membrane is then incubated with a primary antibody overnight, followed by a conjugated secondary antibody for specific detection of the protein of interest.

For visualization, fluorophore-conjugated secondary antibodies can provide direct observation of the protein bands, although chemiluminescence is often preferred. In chemiluminescent detection, secondary antibodies are conjugated to reporter enzymes like horseradish peroxidase (HRP) or alkaline phosphatase (AP). Upon binding, these enzymes catalyze a reaction that generates a transient light signal proportional to the protein expression level. HRP is particularly sensitive, as it catalyzes the oxidation of luminol in the presence of hydrogen peroxide, producing a 425nm wavelength light at the protein band's location. This light signal can be captured with a luminescent CCD camera, and the signal's intensity is then quantified to determine protein expression levels.

2.2.7 Whole-cell Protein Lysis

Adherent RCC cells were rinsed with ice-cold phosphate buffered-saline (PBS) and were lysed in RIPA buffer (R0278, Sigma-Aldrich) supplemented with protease inhibitor (#4693159001, Roche, Switzerland) and phosphatase inhibitor (#4906845001, Roche). For tissue samples, the tissues were snap-frozen liquid nitrogen (N₂) and were homogenised in protease inhibitor and phosphatase inhibitor-supplemented cell lysis buffer (2mL RIPA buffer per 100mg tissue in weight) on ice by a tissue sonicator. Cell and tissue samples were incubated on ice for 30 minutes with gentle shaking, before centrifugation at 12,000g for 15 minutes to collect the

protein-rich supernatant.

2.2.8 Mitochondrial Isolation

Overview of Mitochondrial Isolation

The process of isolating mitochondria from cultured cells or tissue samples relies on a two-step differential centrifugation technique. This begins with a low-speed centrifugation step, which removes intact cells, cellular debris, and nuclei from the cell suspension.²³⁹ Next, a high-speed centrifugation is performed to enrich the mitochondrial fraction, effectively separating mitochondria from other organelles. Variations in the dissociation or homogenization method, buffer composition, and centrifugation speeds are sometimes applied, depending on the specific requirements of subsequent analyses, such as western blot, cytochrome c release, ROS synthesis, or ATP measurement.^{240,241}

For mitochondrial protein extraction, a mitochondria isolation kit (ab110170, Abcam, UK) was utilized according to the manufacturer's instructions, with all steps carried out on ice or at 4°C to minimize protein degradation. Adherent RCC cells were washed with ice-cold PBS (pH 7.4), detached with a cell scraper, and centrifuged at 1,000g at 4°C for 5 minutes to pellet the cells. The cell pellet was then resuspended in Reagent A from the kit and homogenized using a Dounce homogenizer with a B pestle, with approximately 40-50 strokes per sample to ensure complete dissociation (**Figure 2-7**).

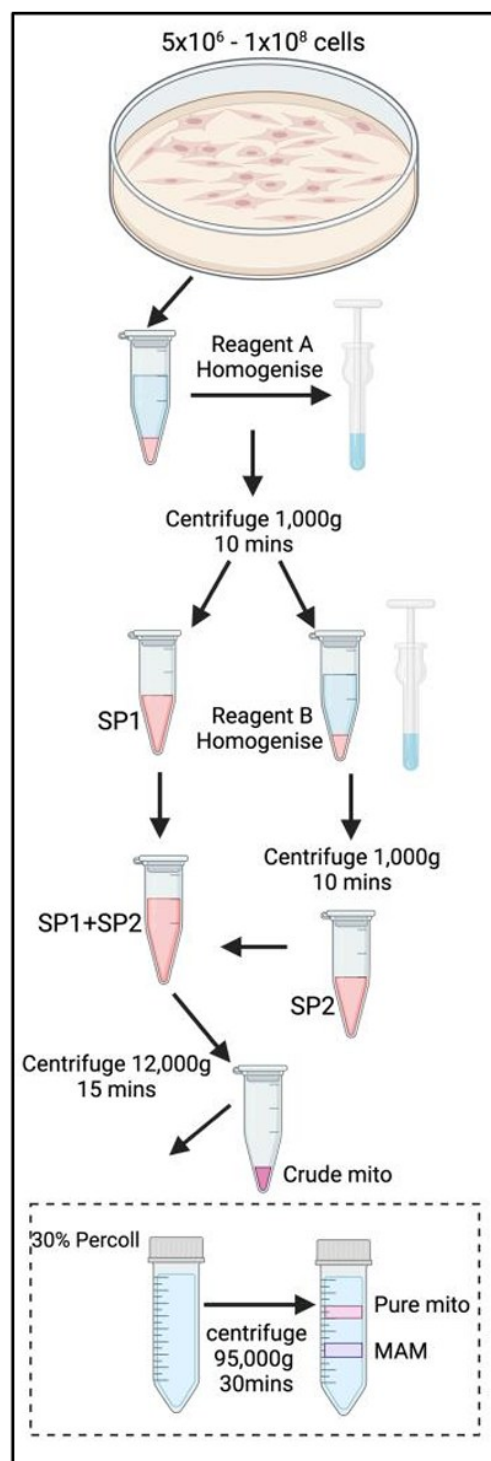


Figure 2-7. Overview of mitochondrial fraction extraction from cell samples.

Following homogenization, the cell mixture was centrifuged at 1,000g for 10 minutes at 4°C, and the supernatant (SP1) was saved. The pellet was resuspended in Reagent B, homogenized, centrifuged, and the supernatant (SP2) was collected and combined with SP1. This low-speed centrifugation step effectively removes cellular debris and larger organelles, such as nuclei. The combined supernatants were then subjected to high-speed centrifugation at 12,000g for 15 minutes, isolating the mitochondrial fraction from other organelles. The resulting crude mitochondrial pellet was resuspended in Reagent C, supplemented with protease inhibitor (#4693159001, Roche) and phosphatase inhibitor (#4906845001, Roche). Buffer volumes were adjusted based on the initial cell count, following the manufacturer's protocol.^{242,243}

After 24 hours of normoxic or hypoxic exposure, RCC cells underwent mitochondrial extraction utilising mitochondrial isolation kit #ab110170 (Abcam, Cambridge, UK). The mitochondrial preparation, as outlined in the manufacturer's instructions, involved cell lysis, centrifugation to eliminate big particles, and subsequent centrifugation to separate mitochondria. In summary, adherent cells were meticulously scraped and collected prior to being frozen at -20°C for 10 minutes to compromise the cell membrane. Samples were suspended in Reagent A, incubated on ice for 10 minutes, and subsequently homogenised with a Dounce Homogeniser. The homogenates underwent centrifugation at 1000g for a duration of 10 minutes. The supernatant was obtained (SPN1). The pellet was re-suspended in Reagent B. The stages of homogenisation and centrifugation were reiterated after the incorporation of Reagent B. The second supernatant was obtained (SPN2). SPN1 and SPN2 were amalgamated and underwent final centrifugation at 12,000g for 15 minutes at 4°C. The pellet was re-suspended in Reagent C, which contained a protease inhibitor cocktail (Roche), prior to protein measurement or freezing at -80°C.

2.2.9 Co-immunoprecipitation

Co-immunoprecipitation (co-IP) is a specific application of western blot and is regarded as the gold standard method for studying interactions between proteins and functions of protein complexes. The principle of co IP is that a primary antibody X recognising a target protein X as a constituent of a larger protein complex will precipitate protein X along with the entire protein multimer from the cell lysate, when the antibody X is conjugated to a solid bead support. The immuno-precipitated protein complex is eluted from the bead, separated by gel electrophoresis and blotted onto transfer membrane to be probed with antibodies against other constituents (Y, Z, etc.) of the protein complex, in order to reveal physical interactions between proteins X, Y and Z (**Figure 2-8**). It should be emphasised that since the different components of the (potential) protein complex is held together by weak bonds/associations, cautions must be taken during protein lysis, beads incubation and elution steps, with extensive optimisations to preserve the delicate protein-protein interactions until the detection step. For example, denaturing agents (e.g. SDS) should be avoided during protein extraction and replaced with mild detergents/surfactants, as denaturing agent will disrupt inter protein bonds/associations.

In order to establish whether ClpP exhibit physical interaction with GPX4, BNIP3 or HIF-1 α during OGD/Rep, co-immunoprecipitation with ClpP specific antibody was performed to pull-down proteins, using the PierceTM direct magnetic IP/Co-IP kit (88828; Thermo Scientific) as per manufacturer's instructions. In brief, the magnetic beads were incubated with rabbit anti-mouse ClpP antibody (3 μ g per reaction; 37705, Cell Signalling Technology) for 30 minutes at room temperature on a rotating platform. The antibody-coated magnetic beads were then incubated with RCC whole cell lysates for 2 hrs at room temperature on a shaking platform. Magnetic beads bound by antibody and target antigens were collected using a magnetic stand, before elution of the bound antigens for analysis by western blot. A non-specific rabbit IgG

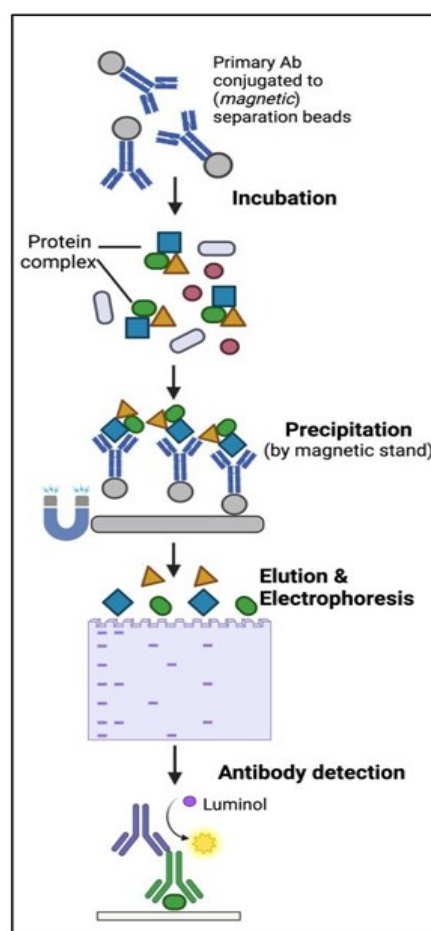


Figure 2-8. Principle and workflow of co immunoprecipitation using magnetic separation beads.

antibody was used as isotype control (3 μ g per reaction; #02-6102, Invitrogen) and was incubated with magnetic beads and cell lysate and eluted as above. Eluted samples were prepared for gel electrophoresis and protein transfer, as described above.

RCC cell lysates were precleared and antibodies for ClpP/Bnip3, ClpP/HIF-1 α , and ClpP/GPX4 were incubated overnight at 4°C for immunoprecipitation. Immediately following antibody binding, 40 μ L of Protein A/G Sepharose® beads slurry was added to each tube and incubated for 1 hour at 4°C. After centrifugation at a low speed at 4°C (2000 x g, 2 minutes), the Protein A/G Sepharose® beads were collected and washed three times. Immune complexes were eluted using an SDS-sample buffer and boiled at 95°C for 7 mins. Western-blot analysis was used for detection. For western blot examinations, cells were lysed using Laemmli buffer with 2.5% β -mercaptoethanol, subjected to boiling for 5 minutes, separated on SDS-PAGE denaturing gels (Bio-Rad), and subsequently transferred to PVDF membranes (Bio-Rad). Membranes were obstructed using 5% dry milk powder, and signals were detected employing a Super Signal West Pico or Femto Chemiluminescent Substrate kit (Pierce). Loading was standardised to action by densitometry and ImageJ. Subcellular fractionation tests were conducted by fractionating cells with the Proteome mitochondria isolation kit (QIAGEN) in accordance with the manufacturer's guidelines.

2.2.10 Electron Microscopy

We employed an electron microscope to examine the tagging of GFP-ClpP at the ultrastructural level, GFP-ClpP was purchased. We employed 4% paraformaldehyde (Electron Microscopy Sciences) and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) to preserve RCC cells at ambient temperature for 15 minutes. Cells were permeabilized with 0.25% saponin in PBS for 30 minutes. The cells were subsequently incubated for one hour in 5% goat serum containing 0.01% saponin in PBS. Subsequently, antibodies targeting ClpP, Bnip3, GPX4, and ACL4 were administered to the cells. Subsequently, they were maintained at ambient temperature for a duration of one night. The samples underwent five washes with PBS and were thereafter placed in a container containing 1.4 nm (12 nm) gold-conjugated Fab goat antibodies to mouse IgG and donkey antibodies to rabbit (Nanoprobes, A24921, 1:100/Jackson ImmunoResearch, 711-205-152, 1:100) for a duration of two hours at 4°C. Cells were cleaned with PBS five times, followed by the addition of 1% glutaraldehyde. PBS was subsequently employed three further times for cleaning. The immunogold-labeled samples were rinsed with water and subsequently subjected to the HQ silver enhancement kit (Nanoprobes) for 2.5

minutes to intensify the silver deposition. Subsequently, 0.2% OsO₄ was incorporated into the samples and incubated for 30 minutes at 4°C. The samples were subsequently purified with water and dyed with 1% uranyl acetate. Samples were desiccated in varying concentrations of ethanol and subsequently embedded with Epon. Uranyl acetate and lead citrate were employed to stain thin specimens prior to imaging with a transmission electron microscope.

2.2.11 Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell lines and tissues using TRIzol reagent (Thermo Fisher Scientific, Shanghai, China). Subsequently, Total RNA (1000 ng) was reverse transcribed into cDNA with a HiScript III 1st Strand cDNA Synthesis Kit with cDNA wiper (Vazyme Biotech, Nanjing, China). RT-PCR was conducted using SYBR Green Master Mix (Vazyme Biotech, Nanjing, China) to quantify the expression of each mRNA. The 2^{-ΔΔCt} method was used to calculate the relative mRNA expression, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene. All primers used were provided by Tsingke Biotech (Beijing, China), and the qRT-PCR primer sequences are listed in the **KEY RESOURCES TABLE** (page 91).

2.2.12 Flow Cytometry

The Principle of Flow Cytometry

A flow cytometer is an advanced instrument capable of simultaneously and quantitatively analyzing multiple physical and biochemical properties (such as cell size, granularity, and fluorescence intensity) across large cell populations in a single suspension as they move through the device.^{244,245}

Flow cytometry operates on the principles of light scattering and deflection from individual cells. As cells pass through the instrument, the scattered or deflected light is captured, converted into an electrical signal, and subsequently digitized for analysis. The process is supported by three main systems: fluidics, optics, and electronics, as illustrated in **Figure 2-9**.^{246,247}

In the fluidics system, an "ambient" sheath fluid surrounds a core stream of suspended cells, creating a pressure difference that arranges cells into a single file as they pass through the laser beam, a process called hydrodynamic focusing. The optical system comprises a laser light source and fixed excitation and collection optics. The excitation optics shape and focus the laser beam on the cells, while the collection optics, including lenses, mirrors, and filters, direct

specific wavelengths of emitted light to the appropriate detectors. When the laser encounters a cell or particle, it generates forward-scattered light (FSC), aligned with the laser beam, and side-scattered light (SSC), perpendicular to the laser. FSC, detected by a photodiode (PD), reflects cell size or surface area, while SSC, captured by a photomultiplier tube (PMT), provides insights into granularity and internal complexity.²⁴⁷

To detect fluorescence from fluorochrome-labeled cells or particles (such as those stained with fluorescent antibodies or dyes), various lasers can be used, like the argon-ion laser (488 nm), ultraviolet laser (300-400 nm), or red diode laser (630 nm). Upon laser illumination, emitted fluorescence is collected by PMTs, each equipped with an optical filter that allows only specific wavelengths to pass. For instance, a bandpass filter for FITC transmits light in the 515-545 nm range, matching FITC's emission spectrum.

Photodiodes (PDs) and photomultiplier tubes (PMTs) serve as photodetectors, transforming light and fluorescence signals into electrons, which are then multiplied to produce an electrical current. This current is amplified into a voltage pulse, proportional to the light or fluorescence intensity. The pulse size can be increased by adjusting the PMT voltage (pre-amplifier gain) or amplification gain, using either Log or Lin amplification. Finally, an Analog-to-Digital Converter (ADC) converts the 0-10V voltage pulse into a digital number. Each analog pulse is assigned a digital value between 0 and 1000 channels, which is relayed to a computer, where the light signal is plotted at the correct position on the data display.

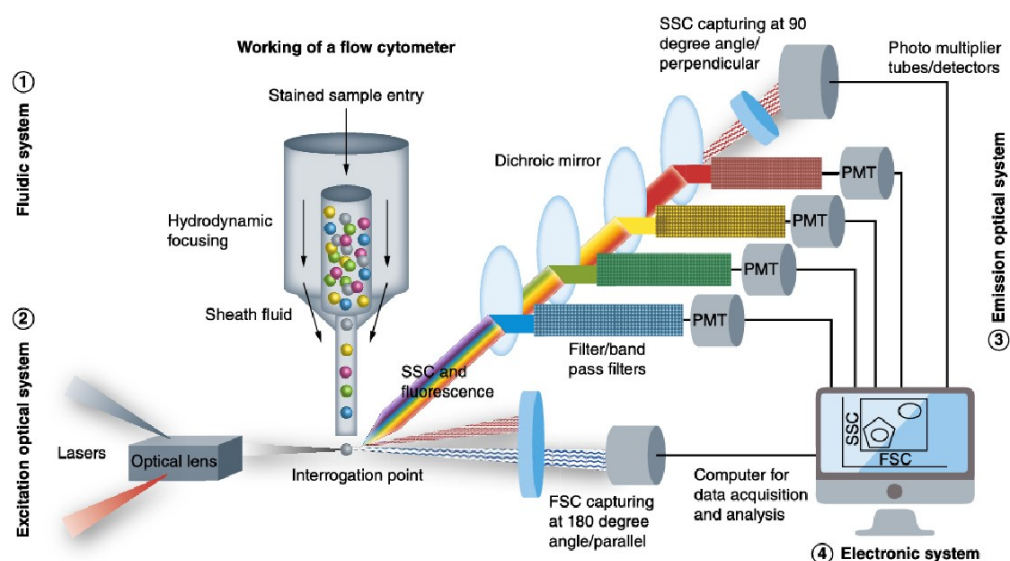


Figure 2-9. The principle of flow cytometry involves using hydrodynamic focusing to align cells in suspension into a single-file stream, guiding them through one or more laser beams for uniform interaction. Light scattered by each cell, along with any fluorescence emitted, is captured by lenses and routed through a network of mirrors and optical filters toward the detectors. The resulting signals are first converted into electrical currents and subsequently digitized through an analog-to-digital converter, allowing the data to be visualized using dedicated software tools. Key components include FSC (forward scatter), PMT (photomultiplier tube), and SSC (side scatter).

Following the 24-hour normoxic or hypoxic challenge, RCC cells cultivated on 24-well culture plates were treated with pre-warmed, enzyme-free cell dissociation buffer (Gibco) at 37°C for 5 minutes. Adherent cells were detached through repeated pipetting, rinsed with FACS running buffer, then centrifuged at 1000g for 5 minutes. RCC (business name, city name, country name) were re-suspended in FACS running buffer and treated with the ROS dye (Invitrogen) dihydroergotamine-123 (DHE-123; concentration 1:200) for 5 minutes in darkness. Excess DHE-123 stain was removed using FACS running buffer through centrifugation at 1500g for 5 minutes. The cell pellet was re-suspended in FACS running buffer and subjected to flow cytometric analysis.

2.2.13 JC-10 Detection of Mitochondrial Membrane Potential

JC-10, a type of mitochondrial probe from the carbocyanine class, is an improved version of the well-known 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) with enhanced water solubility. In functional, polarized mitochondria, JC-10 accumulates and forms J-aggregates, emitting an orange-red fluorescence at a wavelength of 590 nm. In contrast, in damaged or depolarized mitochondria, JC-10 remains in monomer form, emitting green fluorescence at 525 nm upon excitation at 488 nm (**Fig. 2-10**). These fluorescence shifts, reflecting changes in mitochondrial membrane potential, can be detected by flow cytometry with high sensitivity.

After the specified period of OGD/Rep treatment, CLpP knockdown, RCC cells were detached from the culture plate using Accutase solution (00-4555-56; ThermoFisher Scientific). Cells were then prepared and stained with the JC-10 mitochondrial membrane potential assay kit (Ab112133; Abcam) as per the manufacturer's protocol. Cells pre-treated with 2 μ M mitochondrial uncoupler FCCP (Ab120081; Abcam) for 30 minutes served as a positive control. For each sample, approximately 5×10^5 cells were resuspended in 500 μ L of dye-loading solution containing 2.5 μ L JC-10 dye and incubated in the dark at 37°C for 30 minutes.

JC-10-stained cells were analyzed using FITC and PE channels on a flow cytometer (BD LSR-II; BD Biosciences), with PMT voltages set to 300 for FITC and 400 for PE. Naïve control (NC) cells and FCCP-treated positive control cells established the compensation threshold due to emission spectral overlap between FITC and PE channels. Color compensation involved adjusting the FITC signal in the PE channel and vice versa. In this study, compensation was set at 27% for FITC – PE and 40% for PE – FITC to achieve clear separation between green JC-10 monomers and red-orange JC-10 aggregates. For each sample, the percentages of FITC-positive and PE-positive cells were recorded, with the mitochondrial membrane potential expressed as the PE/FITC ratio.^{248,249}

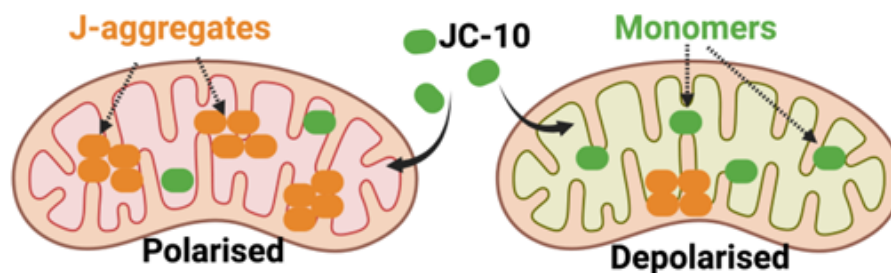


Figure 2-10. Principle of JC-10 detection of mitochondrial membrane potential.

JC-10 staining was used to determine mitochondrial membrane potential ($\Delta\psi_m$). RCC cells were transfected with ClpP siRNA or scramble siRNA for 48 h at 5×10^5 cells/mL. RCC cells treated with 10 μ L FCCP for 10 mins were used as a positive control. ClpP siRNA or scramble siRNA transfected RCC cells and FCCP-treated RCC cells were resuspended in 500 μ L of 1x JC 10 (NO. ab112133; Abcam) dye-loading solution for 30 mins at room temperature. The fluorescence intensities of JC-10 monomers and aggregation forms were measured on a flow cytometer.²⁵⁰

2.2.14 MDA

The procedure followed the instructions provided in the MDA Assay kit (Sigma-Aldrich, MAK085). Lipid peroxidation denotes the deterioration of lipids resulting from oxidative damage, functioning as a marker of oxidative stress. Polyunsaturated lipids are especially susceptible to oxidative damage, usually triggered by reactive oxygen species, resulting in a cascade that generates byproducts such as malondialdehyde (MDA). This assay quantifies lipid peroxidation by assessing the reaction between malondialdehyde (MDA) and Thio barbituric acid (TBA), resulting in a colorimetric product at 532 nm or a fluorometric product with excitation at 532 nm and emission at 553 nm, which correlates with the concentration of MDA.²⁵¹

Procedure

All standards and samples should be prepared in duplicate, using ultrapure water for consistency.

MDA Standards for Colorimetric Detection

To prepare a 0.1 M MDA Standard Solution, dilute 10 μ L of the 4.17 M MDA Standard with 407

μL of water. Then, dilute 20 μL of this solution with 980 μL of water to achieve a 2 mM MDA Standard. Prepare standards of 0, 4, 8, 12, 16, and 20 nM by adding 0, 2, 4, 6, 8, and 10 μL of the 2 mM solution into separate tubes, adjusting each to a final volume of 200 μL with water.

MDA Standards for Fluorometric Detection

Prepare the 2 mM solution as described above. Dilute 100 μL of this solution with 900 μL of water to make a 0.2 mM MDA standard. To create standards of 0, 0.4, 0.8, 1.2, 1.6, and 2.0 nM, add 0, 2, 4, 6, 8, and 10 μL of the 0.2 mM solution into separate tubes, bringing each to 200 μL with water.

Sample Preparation

For serum or plasma samples, mix 20 μL with 500 μL of 42 mM sulfuric acid in a microcentrifuge tube. Add 125 μL of Phosphotungstic Acid Solution, vortex, incubate for 5 minutes at room temperature, then centrifuge at $13,000 \times g$ for 3 minutes. Prepare a solution with 2 μL of BHT (100 $\times$) in 100 μL of water, and resuspend the pellet in this solution on ice, adjusting to 200 μL with water.

For tissue (10 mg) or cells (1×10^6), homogenize on ice in 300 μL of MDA Lysis Buffer with 3 μL of BHT (100 $\times$). Centrifuge at $13,000 \times g$ for 10 minutes to remove insoluble material. Alternatively, homogenize 10 mg of sample in 150 μL of water with 3 μL of BHT (100 $\times$), add 2 N perchloric acid to precipitate protein, vortex, centrifuge, and transfer 200 μL of supernatant to a new tube.

Calculations

To account for background noise, subtract the value of the 0 (blank) MDA standard from all readings. Background values can significantly affect results and must be removed from each reading. Construct a standard curve using values from the MDA standards, then determine the amount of MDA in samples based on this curve.

2.2.15 LDH

The procedure followed the instructions provided in the LDH-Cytotoxicity assay kit II (Abcam, ab65393).

The Cytotoxicity LDH Assay measures cell damage by detecting lactate dehydrogenase (LDH) activity released from compromised cells. LDH is a stable cytoplasmic enzyme found in all cell

types, and it enters the cell culture medium when the plasma membrane is damaged. The Cytotoxicity LDH Assay Kit-WST measures released LDH activity through the following reaction: LDH catalyzes the conversion of lactate to pyruvate, reducing NAD to NADH in the process. NADH, in turn, reduces a water-soluble tetrazolium salt (WST) with the help of an electron mediator, producing an orange formazan dye. The quantity of this dye is directly proportional to the amount of LDH released, serving as an indicator of cytotoxicity.

This assay can measure cytotoxicity in living cells without affecting their viability (homogeneous assay), as the Cytotoxicity LDH Assay Kit-WST does not interfere with live cell function. Additionally, a non-homogeneous assay can be performed using cell culture supernatant alone. Unlike similar products, the reconstituted Working Solution in this kit remains stable under refrigeration and can be stored for extended periods as a ready-to-use solution. (Figure 2-11)

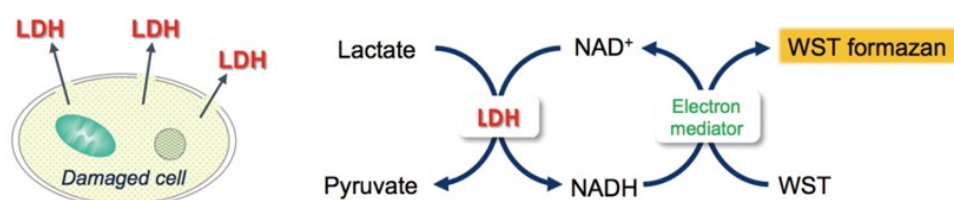


Figure2-11 Principle of cytotoxicity measurement

2.2.16 Fe²⁺ assay

The procedure followed the guidelines in the ion assay kit (Sigma-Aldrich, MAK025). Iron is an essential mineral involved in numerous biological processes, including transport and redox reactions. As a transition element, iron can exist in various oxidation states, with iron II (Fe²⁺ or ferrous iron) and iron III (Fe³⁺ or ferric iron) being the most common. Iron-containing proteins facilitate many reactions, often relying on temporary shifts in iron's oxidation state to drive chemical processes. In this assay, an acidic buffer is used to release iron from samples. Fe²⁺ can be measured directly, or samples can be reduced to determine total iron (Fe²⁺ and Fe³⁺). The released iron reacts with a chromogen, producing a colorimetric (593 nm) signal proportional to the iron concentration, offering a straightforward method for quantifying iron in biological samples.²⁵²

Procedure

Iron Standards for Colorimetric Detection. To prepare a 1 mM iron standard solution, dilute 10 μL of the 100 mM Iron Standard with 990 μL of water. For standards of 0, 2, 4, 6, 8, and 10 nmol per well, add 0, 2, 4, 6, 8, and 10 μL of this 1 mM solution to separate wells in a 96-well plate. Bring each well to 100 μL by adding Iron Assay Buffer, and then add 5 μL of the Iron Reducer to each standard.

Sample Preparation

For tissue samples (10 mg) or cells (2×10^6), homogenize rapidly in 4–10 volumes of Iron Assay Buffer. Centrifuge at $16,000 \times g$ for 10 minutes at 4 °C to remove insoluble debris. Serum and other liquid samples can be added directly to the wells; however, this kit is unsuitable for plasma samples. Adjust each sample to a final volume of 100 μL using Iron Assay Buffer. For unknown samples, test various dilutions to confirm that readings fall within the standard curve's linear range.

2.2.17 Transwell assays

Cell invasion was tested using Transwell plates (24-well; Corning, NY, USA) coated with Matrigel (Corning, NY, USA). Cells were seeded in the upper chamber with serum-free medium, and DMEM containing 10% FBS was loaded into the lower chamber. After 24 h incubation, cells invading the bottom of the filter membrane were stained using crystal violet and observed under a microscope. Five fields were randomly selected to calculate the number of invasive cells.

2.2.18 Wound-Healing Assay

Cells were seeded into 6-well plates, and when they reached 95% confluence, a sterile 200- μL pipette tip was used to scratch the cell layers. The plates were washed twice with PBS to remove the suspended cells, and then fresh serum-free medium was added. Cell migration images were recorded in three defined fields at 0 h and 24 h after the scratch. Wound-healing rate was calculated with the formula: wound healing (%) = (scratch width at 0 h – width at 24 h)/width at 0 h.

2.2.19 Cell Viability Assay

Cell Viability by Cell Counting Kit-8 (CCK8)

The Cell Counting Kit-8 (CCK-8) is a reliable and sensitive method for assessing cell viability, frequently applied in cytotoxicity screenings. This kit contains WST-8, a water-soluble

tetrazolium salt, specifically [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], which undergoes reduction by cellular dehydrogenases in the electron transport chain to form an orange formazan dye.²⁵³ The amount of dye produced correlates with the dehydrogenase activity and cell viability, as it is proportional to the number of viable, actively respiring cells.²⁵⁴

In the experiment, RCC cells were seeded in a 96-well plate at a density of 5×10^4 /mL (approximately 5000 cells per well) and allowed to adhere. Each well then received 10 μ L of CCK-8 reagent, reaching a final volume of 110 μ L (10 μ L CCK-8 + 100 μ L DMEM). The cells were incubated in the dark at 37°C for 2 hours, coinciding with the reperfusion period. Blank controls (BC) without cells were included, with 10 μ L of CCK-8 added to 100 μ L of DMEM and incubated similarly. The color intensity was measured at 450 nm using a microplate reader, with blank control values averaged and subtracted from all other readings. The naïve control (NC) group was set to represent 100% viability, and results from OGD/Rep and treated groups were calculated as percentages relative to NC.

$$\% \text{ viability} = \frac{(\text{treatment} - \text{blank control})}{(\text{NC} - \text{blank control})}$$

2.3 Stage III

2.3.1 Mouse Xenograft In Vivo Study

The experimental procedures in this study were approved by the Animal User Committee of the People's Hospital of Zhengzhou University in China, (Approval Number 2019-44). All of the experiments have been conducted according to ARRIVE guidelines which were aimed at preventing unethical and inhumane treatment of animal subjects. The study involved six-week-old, pathogen-free, female BALB/c nude mice acquired from Weitong Lihua Laboratory Animal Co., LTD, Beijing (Certification No.110011211113230175). The mice were kept in a well-regulated environment, which made sure that temperature and humidity were stable, light/dark cycles lasted for 12 hours, and they were offered maximum access to water and food. This homogeneous training system was imperative for minimizing environmental variability and securing the robustness of the experimental results.

In the experiment, there were five groups, each with nine nude mice, which enabled us to precisely measure different variables by sufficient statistical power. Mice in each group got flank injections of 5 times 10^6 RCC cells (output of 150 μ L of PBS), carrying specific constructs:

NC (phosphate-buffered saline as negative control), sh-ClpP, sh-NC, oe-ClpP, and oe-NC. The transfected RCC cells were applied to estimate the impacts of both ClpP genes knockdown and overexpression (sh-ClpP oe-ClpP) during tumour development and the specific control groups for each site-specific ClpP gene manipulation, respectively.

Moreover, every day in the period of the experiment, mice were examined for signs of distress or diseases to make sure that animal welfare and ethical standards are complied with. Tumour growth has been taken in two dimensions namely the shortest things (a) and the longest things (b) by digital calipers. Tumour volume was obtained using the equation ($V = (a^2 \cdot b) / 2$), where the shortest diameter ('a') and the longest diameter ('b') are the input. Estimations were done at constant time intervals along the duration of the tumour development so that it was possible to compare the effects of each transfection type.

After 60 days, all mice euthanized per institutional guidelines, tumours were excised and weighed after which the average weight of each group was recorded to facilitate comparison tumour growth. The tumours were fixed in paraformaldehyde, embedded in paraffin, and sectioned after they were collected. The sections were stained with immunofluorescence to visualize protein expression and explore the molecular mechanisms affected by ClpP modulation.

This approach ensured a comprehensive assessment of tumour growth dynamics and cellular changes due to ClpP knockdown and overexpression, contributing valuable data to the understanding of ClpP's role in renal cell carcinoma progression.

2.4 Stage IV

2.4.1 Clinical Study (Human Samples)

Participants

The research included a group of 437 patients with renal cell carcinoma (RCC), all of whom had either a partial or radical nephrectomy performed at the People's Hospital of Zhengzhou University from June 2019 to May 2024. The inclusion criteria were determined with great precision to guarantee the study's main focus on primary RCC cases with no prior treatments that may have changed baseline tumour characteristics. The conditions were as follows: (1) Surgical specimens were confirmed as primary RCC through postoperative pathological

diagnosis, and (2) There was no history of preoperative interventions, like radiotherapy, chemotherapy, or immunotherapy, thus, providing a clear evaluation of tumour biology. Patients were disqualified if they had (1) severe comorbidities (heart, lung, liver, or others) that could be related to survival independent of RCC, (2) another tumour in carbon which existed or (3) lack of complete clinical data or few follow-ups that may have brought inaccuracy to the drawn results.

The researchers took primary RCC tissue and nearby normal tissues from each patient during the surgery. Namely, in order to separate the two, para-cancerous tissues, at least 2 centimeters away from the tumour margin, were taken and confirmed as normal by a pathologist. The demarcation between cancerous and non-cancerous tissues permitted a side-by-side examination of the ClpP protein expression levels in the two tissue types, therefore, exploring ClpP's significance in RCC pathology.

Immunohistochemical Detection of ClpP Protein Expression

In the course of the study, a SP method was performed to detect ClpP protein expression in RCC tumour tissues and adjacent normal tissues. Fresh tissue samples were immediately fixed in a 10% formalin solution after resection, making sure that the tissue was preserved in its optimal condition. To prepare 3 to 5 consecutive sections from each sample, normal paraffin embedding was used: each section 4 μm in thickness.

Sections were then processed through a series of preparative steps: they were dewaxed in xylene and dehydrated through a graded ethanol series. Endogenous peroxidase activity was inhibited using a 3% hydrogen peroxide solution to prevent nonspecific staining. Antigen retrieval was achieved via microwave treatment, which helps to unmask epitopes and improve antibody binding. A mouse monoclonal antibody against ClpP, diluted at 1:100, was applied and incubated overnight at 4°C to ensure precise targeting of the ClpP protein. The following day, an HRP-conjugated secondary antibody was added to facilitate visualization. The color development was achieved using a diaminobenzidine (DAB) substrate solution, resulting in a brown stain in areas of ClpP expression. The sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and sealed with neutral resin for preservation. Micrographs were taken under a microscope to capture images of stained tissue sections, providing visual data for analysis.

Assessment of Wax Sections

The immunohistochemical images were evaluated using a two-tiered scoring method, calculating a total immunohistochemical score based on the percentage of ClpP-positive cells and staining intensity. To ensure accuracy, five high-magnification fields ($\times 400$) were randomly selected per section to assess positive cells. ClpP protein expression was considered positive if brown or brownish-yellow particles were observed in the cell membrane and/or cytoplasm. The percentage of positive cells was assigned scores as follows: $\leq 5\%$ = 0 points, 6%–25% = 1 point, 26%–50% = 2 points, and $> 50\%$ = 3 points. Staining intensity was scored separately: no staining = 0 points, light yellow = 1 point, brown = 2 points, and dark brown = 3 points. The overall score, calculated by multiplying these two scores, was categorized as follows: 0 as negative (-), 1-2 as weakly positive (+), 3-4 as moderately positive (++), and 6-9 as strongly positive (+++). Low expression was defined as a total score of 0-2, while high expression ranged from 3-9. All scoring was independently performed by two senior pathologists to ensure objectivity and reliability.

Follow-Up Visits

Postoperative follow-up data were collected annually through outpatient reviews or telephone interviews, with the final follow-up recorded on May 31, 2024. The primary outcome measure was overall survival (OS), defined as the interval from the date of surgical treatment to death from any cause. This longitudinal data collection aimed to correlate ClpP expression levels with patient prognosis, providing insights into its potential role as a prognostic biomarker in RCC.

2.5 Quantification and statistical analysis

For all experimental data, data from each experimental group was tested for normality and deviation from the Gaussian distribution using the D'Agostino-Pearson normality test, and a QQ-plot is generated for each group of data. The null hypothesis is that all data sampled from a population follows a Gaussian distribution. When $P > 0.05$, the null hypothesis is accepted, and the data is considered to be normally distributed, when $P < 0.05$ was considered to be statistically significant. All analysis was performed with Graphpad Prism 9.0 (La Jolla, USA).

For *in vitro* study, each *n* represents an independent cell experiment and at least 3 independent experiments ($n=3$) were performed for each set of data. For semi-quantitative analysis of *in vitro* immunofluorescence staining, each *n* corresponds to 3 replicates of the

same experiment and represents the averaged value from 3 replicates. For *in vivo* study, each n represents an individual animal, and each experiment included at least 9 animals (n=9) for statistical analysis.

Patient characteristics were analyzed using Fisher's exact test (categorical variables), independent t-test, or Mann-Whitney U test (continuous variables) as appropriate for comparison of two groups. Hodges-Lehmann calculated the difference between the two medians with 95% CI. Kaplan-Meier curve was used to estimate the time-to-event results, and the log-rank test was used to analyze the differences between groups. Significance was assumed at p-value less than 0.05. To eliminate confounding effects, we also employed multivariate Cox regression analysis. All statistical analyses were conducted with Graphpad Prism version 8.3 (Graphpad), SPSS version 14.0 (SPSS, Chicago, IL) or SAS version 9.2 software (SAS Institute, Cary, NC).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-ClpP	Santa Cruz	Sc-271284
Rabbit Polyclonal anti-HIF-1 α	Novus biologicals	NB100-479
Rabbit monoclonal anti-LC3A/B	Cell Signalling Technology	12741
Rabbit monoclonal anti-BNIP3	Cell Signalling Technology	12396
Rabbit monoclonal anti-GPX4	Abcam	ab125066
Rabbit Polyclonal anti-ACSL4	Invitrogen	PA5-27137
Mouse monoclonal anti-GAPDH	Merck Millipore	MAB374
Mouse monoclonal anti-VDAC1	Santa Cruz	sc-390996
HRP-conjugated donkey anti-mouse	Cell Signalling Technology	7076
HRP-conjugated donkey anti-rabbit	Cell Signalling Technology	7074
Donkey Anti-rabbit Alexa Fluor 488	Invitrogen	A21206
Donkey Anti-rabbit Alexa Fluor 568	Invitrogen	A10042
Donkey Anti-mouse Alexa Fluor 488	Invitrogen	A21202
Donkey Anti-mouse Alexa Fluor 568	Invitrogen	A10037
1.4 nm gold-conjugated fragment antigen-binding (Fab) goat antibody to mouse IgG	Nanoprobe	A24921
12 nm gold-conjugated fragment antigen-binding (Fab) Donkey antibody to Rabbit IgG	Jackson ImmunoResearch	711-205-152
Anti- monomeric Keima-Red	Medical and Biological Laboratories	2F7
Biological samples		
Fresh human renal cancer tissues	People's Hospital of Zhengzhou University, Henan, China	N/A
Human renal cancer wax-imbedded sections	People's Hospital of Zhengzhou University, Henan, China	N/A
Chemicals, peptides, and kit		
MitoTracker Red (CMXRos)	Invitrogen	M7512
HIF-1A siRNA	Qiagen	NM_001530

BNIP3 siRNA	Qiagen	NM_004052
ClpP siRNA	Qiagen	NM_006012
GPX4 siRNA	Qiagen	NM_002085
RNAi MAX	Qiagen	13778150
Mitochondrial isolation kit	Abcam	ab110170
Lipofectamine 2000 kit	Invitrogen	11668019
Co Immunoprecipitation Kit	Abcam	ab206996
Cell counting kit-8	Sigma-Aldrich	96992
Dihydrorhodamine 123	Invitrogen	D23806
JC 10	Abcam	ab112133
ATP Assay Kit	Abcam	ab83355
Ferrostatin-1 (Fe-1)	Sigma-Aldrich	SML0583
Liperfluo	Dojindo	1448846-35-2
Iron Stain kit	Abcam	ab150674
Iron Assay Kit	Sigma-Aldrich	MAK025
Lipid Peroxidation (MDA) Assay Kit	Sigma-Aldrich	MAK085
LDH-Cytotoxicity Assay Kit II	Abcam	ab65393
Triton X-100	Solarbio Life Science	T8200
Erastin	Sigma-Aldrich	E7781
Lentiviral-CLPP-RNAi	GENECHEM	N/A
luminol reagent	Santa Cruz	sc-2048
Sequence-Based Reagents		
Primer: HIF-1A 5'-ATCCATGTGACCATGAGGAAATG-3' 3'-ATCCATGTGACCATGAGGAAATG-5'	Tsingke Biotech	N/A
Primer: ClpP 5'- TTGCCAGCCTTGTTATCGCA -3' 3'- GGTGAGGATGTACTGCATCG -5'	Tsingke Biotech	N/A
Primer: LC3A/B 5'- AAGGCGCTTACAGCTCAATG -3' 3'- CTGGGAGGCATAGACCATGT -5'	Tsingke Biotech	N/A
Primer: BNIP3 5'- CAGGGCTCCTGGGTAGAACT -3' 3'- CTACTCCGTCCAGACTCATGC -5'	Tsingke Biotech	N/A

Primer: GPX4 5'- GAGGCAAGACCGAAGTAACTAC -3' 3'- CCGAACTGGTTACACGGGAA -5'	Tsingke Biotech	N/A
Primer: ACSL4 5'- GCTGTGCCTGTCATGGTGTT -3' 3'- CACTGGATCTTGGTGCCATTT -5'	Tsingke Biotech	N/A
Primer: GAPDH 5'- GGAGCGAGATCCCTCCAAAAT -3' 3'- GGCTGTTGTCATACTTCTCATGG -5'	Tsingke Biotech	N/A
Deposited data		
Raw and analyzed data	This paper	NCBI SRA: PRJNA1119576
Software and algorithms		
ImageJ	Imagej	https://wsr.imagej.net/distros/win/ij-153-win-java8.zip
Graphpad Prism	Graphpad	https://www.graphpad.com/scientific-software/prism/
CaseViewer	3DHISTECH	https://www.3dhistech.com/solutions/caseviewer/
Other		
Q Exactive™ GC Orbitrap™ GC-MS/MS	ThermoFisher	0725500
Q Exactive™ Plus	ThermoFisher	IQLAAEGAAPFALG MBDK
HiSeq X™ Ten Sequencing System	Illumina	N/A
Hypoxia Incubator Chamber	Stemcell	27310

CHAPTER THREE:

INTEGRATED RNA-SEQ, PROTEOMIC AND BIOINFORMATIC ANALYSES

3.1 INTRODUCTION

Renal clear cell carcinoma (RCC) is a highly prevalent and aggressive form of kidney cancer, representing over 90% of kidney malignancies. RCC affects hundreds of thousands globally, with an estimated 430,000 new cases diagnosed each year.²⁵⁵ The disease's multifaceted resistance to standard treatments - including chemotherapy, radiotherapy, and even some targeted therapies - poses significant clinical challenges, leading to persistently poor outcomes, especially in advanced stages.⁴³ The substantial cellular heterogeneity within RCC tumours is an essential contributor to this resistance, as it facilitates the ability of cancer cells to evade treatment and establish metastases.³⁸ RCC is typically a disease that is characterized by the cellular responses to changes in the tumour microenvironment, a space that is mainly known for being poor in nutrients, under oxidative stress, and hypoxia.⁴ These conditions induce physiological stresses that cancer cells must live through thus selecting the ones that can flicker between aerobic and anaerobic metabolism and have a high level of cell death resistance which highlights the need to throw light on mechanisms that support these cellular changes.⁵

RCC is frequently associated with genetic mutations, particularly in the von Hippel-Lindau (VHL) tumour suppressor gene, which plays a foundational role in RCC pathogenesis. VHL loss of function results in the stabilization of hypoxia-inducible factors (HIF-1 α and HIF-2 α), known to upregulate angiogenesis-promoting genes and reprogram metabolism, thereby enhancing RCC cells' survival and proliferation under hypoxic conditions.²⁵⁶ However, mutations and genetic alterations alone cannot fully explain RCC's complex biochemical adaptations, particularly its ability to maintain cellular homeostasis despite severe stressors in the tumour microenvironment. Proteomic and transcriptomic adaptations are increasingly recognized as critical for RCC cell survival, driving interest in multi-omic approaches that can provide deeper insights into RCC's regulatory networks.²⁵⁷ Specifically, recent studies suggest that metabolic pathways involving mitochondrial function, autophagy, ferroptosis resistance, and oxidative stress regulation play fundamental roles in supporting RCC's survival and progression.^{187,258}

In recent years, multi-omic methodologies integrating transcriptomics and proteomics have

advanced the field's ability to comprehensively analyze RCC's molecular profile. Transcriptomic profiling, mainly through RNA sequencing (RNA-seq), has proven invaluable in identifying gene expression changes that differentiate RCC cells from normal cells.²⁵⁹ Proteomics, in contrast, provides insights into post-transcriptional modifications, protein interactions, and functional dynamics that reveal how RCC cells achieve metabolic resilience.²⁵⁹ Together, these approaches offer a powerful toolset to uncover RCC's adaptive strategies at the molecular level. While previous research has shed light on pathways involved in autophagy and oxidative stress in RCC, the precise regulatory networks allowing RCC cells to circumvent ferroptosis and sustain mitochondrial function under stress remain poorly understood.²⁶⁰

This part of my PhD project uses a comprehensive, multi-layered analytical approach that combines high-throughput RNA-seq and quantitative proteomic profiling to delineate RCC's molecular landscape. By comparing malignant RCC tissue samples with adjacent non-tumorous tissue, we gain an in-depth view of differential gene and protein expression patterns associated with RCC progression. This integrative approach allows us to focus on critical metabolic pathways, including those regulating mitochondrial function, autophagy, and ferroptosis resistance - pathways essential to RCC's metabolic resilience and adaptability to stress. Our analyses spotlight the role of ClpP, a mitochondrial protease that plays a significant role in mitochondrial protein maintenance, as an emerging regulator of RCC survival. Traditionally, ClpP has been recognized for its role in ensuring mitochondrial protein quality control; however, recent evidence suggests that ClpP may also facilitate cellular stress responses, particularly under conditions of oxidative stress and hypoxia.³⁸ This study examines the interactions between ClpP and other vital proteins implicated in RCC stress responses, such as GPX4, BNIP3, and HIF-1 α , to better understand the regulatory role of ClpP within RCC's adaptive framework.

The findings presented here demonstrate that ClpP and associated proteins involved in autophagic and antioxidative pathways are pivotal in RCC's ability to resist ferroptosis - a regulated cell death process triggered by lipid peroxidation.^{220,261} This process is critical for RCC cell survival, given the high oxidative stress within the tumour microenvironment⁶⁸. Furthermore, pathway enrichment analyses reveal that ClpP contributes to RCC's capacity to adapt to hypoxic environments, an adaptation that is critical to RCC's progression and metastatic potential.¹⁵⁹ The results underscore ClpP's potential as a therapeutic target in RCC. Given that ClpP expression is consistently upregulated in RCC tissues, therapies designed to

inhibit ClpP may disrupt RCC's adaptive mechanisms, potentially sensitizing tumour cells to ferroptosis and other forms of cell death. Such therapeutic strategies may provide a novel approach to improve outcomes for RCC patients by targeting the molecular drivers of RCC resilience.^{18,175}

3.1.1 Aims, Hypothesis, and Objectives

This study seeks to deepen the understanding of RCC by investigating the molecular profile of RCC tissues relative to adjacent non-tumourous tissues. By integrating RNA-seq, proteomic analyses, and bioinformatics, we aim to delineate RCC's critical regulatory networks and identify ClpP as a potential therapeutic target. Our findings may inform the development of targeted treatments designed to disrupt RCC's adaptive capacity by interfering with pathways that underpin its metabolic flexibility and resistance to cell death.

3.1.2 Specific objectives include:

Objective 1: To analyze differential gene expression patterns in RCC tissues compared to adjacent non-tumourous tissues through RNA sequencing (RNA-seq) and proteomic profiling to identify critical genes and proteins associated with RCC pathogenesis and progression.

Objective 2: To investigate mitochondrial metabolism, ferroptosis, and hypoxia-responsive pathways in RCC through pathway enrichment analyses, elucidating the critical molecular mechanisms that allow RCC cells to survive oxidative and hypoxic stress within the tumour microenvironment.

Objective 3: To explore the regulatory role of ClpP, a mitochondrial protease, in RCC adaptation and survival, particularly in autophagy, oxidative stress, and ferroptosis resistance, with a focus on ClpP's interactions with essential proteins such as GPX4, BNIP3, and HIF-1 α .

Objective 4: To assess the expression levels and interactions among ClpP, GPX4, BNIP3, ACSL4, and HIF-1 α in human RCC and adjacent tissues, identifying potential correlations that underscore ClpP's functional importance in RCC's metabolic and stress response networks.

These objectives aim to clarify RCC's molecular framework, highlighting pathways that may inform novel therapeutic strategies for improving RCC treatment outcomes.

3.2 RESULTS

This large-scale study covering many levels of investigation adopted an approach of high-throughput RNA sequencing (RNA-seq) and proteomic profiling for the dissection of the molecular complexity of RCC. In RCC, the differences between the three cancerous and non-cancerous tissue samples of two patients were not just on the tissue level but were elaborated in great detail at the genetic and protein levels. This comprehensive methodology has as its driving force the identification of the molecular basis in the RCC pathogenesis for the whole situation thus the creation of a new understanding of the disease.

3.2.1 RNA-seq Analysis

The RNA-seq analysis meticulously examined RNA expression profiles from RCC tissues and adjacent non-tumourous samples. Principal Component Analysis (PCA) demonstrated distinct clustering patterns for each group, underscoring the experimental design's robustness and reproducibility while verifying substantial inter-group divergence (**Figure 3-1**). These clustering patterns highlighted genetic coherence within the groups, marking a clear molecular division between malignant and benign cellular environments.

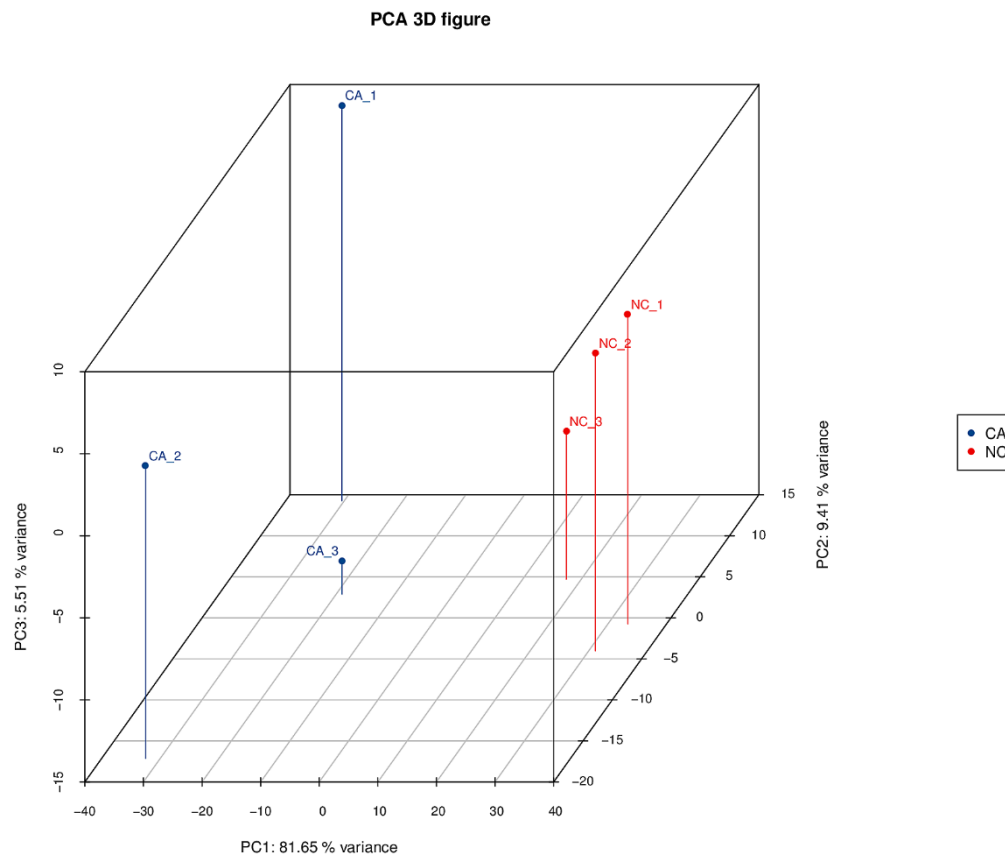


Figure 3-1. The RNA-seq of tumor tissues and adjacent tissues on RCC. Principal component analysis 3D scores plot of tumor and adjacent tissues. All samples were clustered into two groups (n = 3 per group). Red represents the non-cancer group , and green indicates the cancer patient group.

Volcano plots were generated to represent the distribution of differentially expressed genes, providing a visual encapsulation of gene expression alterations' magnitude and statistical significance across sample groups. Further refinement was achieved through hierarchical clustering of gene expression profiles, revealing unique expression patterns specific to each group, thereby deepening our understanding of the molecular heterogeneity between tumourigenic and non-tumourigenic tissues (**Figure 3-2**).

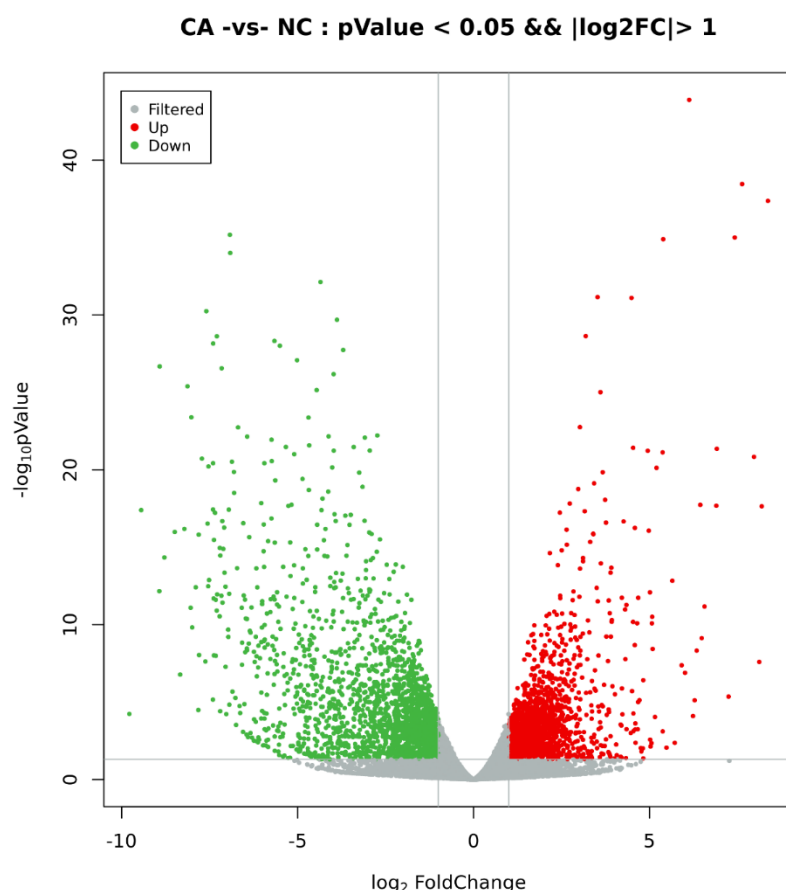
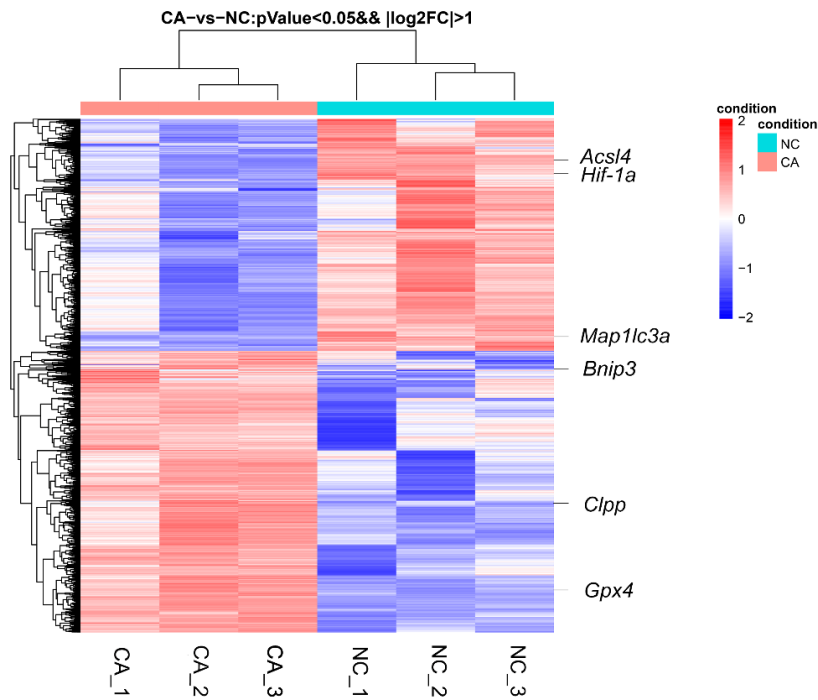


Figure 3-2. Differentially expressed volcano map

The differences generated by the comparison are reflected in the volcano map. Gray is the gene with a non-significant difference; red is the high expression of significantly different genes, and green is the low expression of significantly different genes.

The hierarchical clustering of RNA expression profiles across samples enabled us to visualize co-expression patterns, revealing heterogeneity within each group's proteomic landscape and resulting in a heat map (**Figure 3-3A**). To examine pathway-level alterations underpinning these genetic changes, we conducted the results of pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We identified the top 20 significantly enriched pathways using a hypergeometric distribution test, providing a structured view of RCC-associated pathways (**Figure 3-3B**). This analysis illuminated critical pathway perturbations essential to RCC progression.

A



B

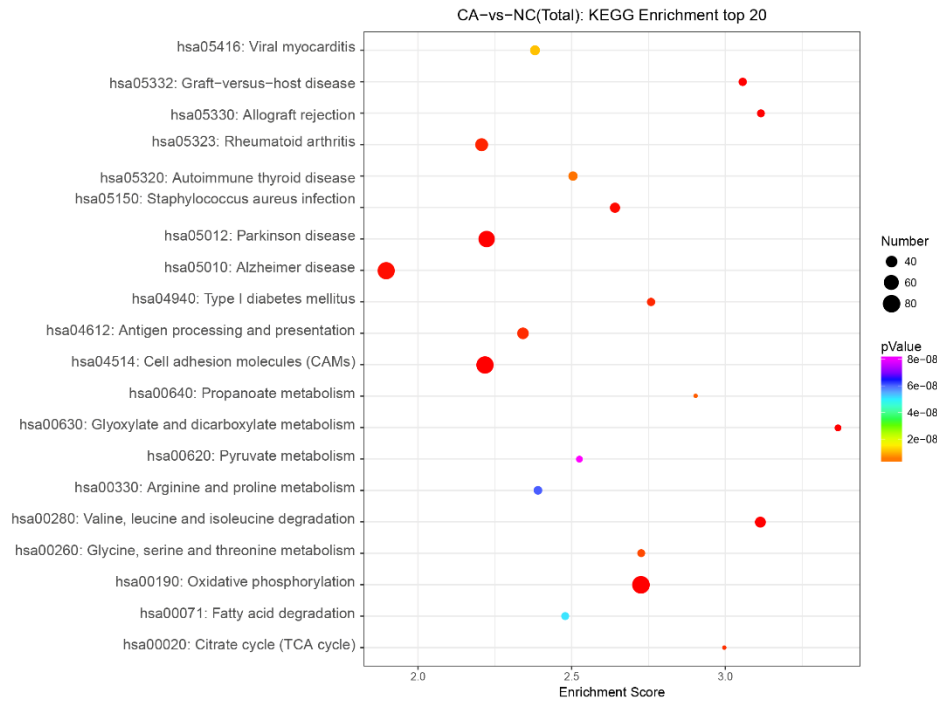


Figure 3-3. The gene heatmap and enriched signalling pathway**A) Differential gene grouping cluster map.**

Comprehensive RNA-seq analyses were conducted on RCC and adjacent non-cancerous tissues from three distinct patients. Instances of upregulation are marked in red, while downregulation is indicated in blue. Green represents the non-cancer group, and red indicates the cancer patient group.

B) KEGG enriched top20 bubble diagram.

A graphic representation of the top 20 signalling pathways from KEGG enrichment distribution points reveals differentially expressed genes between cancer and non-cancerous tissues (NC) groups. Number on the circle chart indicates the number of genes enriched under the term.

3.2.2 Proteomics Analysis

For proteomic analysis, data processing was executed with rigorous standards to retain only those proteins with consistent detection in a minimum of 50% of samples per group, confirmed through unique peptide identification. To maintain data integrity, missing values were substituted with the group mean, followed by median normalization and log2 transformation, ensuring the reliability of the dataset across samples.

Principal Component Analysis

The PCA applied to the protein expression data revealed distinct spatial separation between sample groups, highlighting intrinsic biological differences that validated our proteomic findings (**Figure 3-4**). This separation illustrates molecular discrepancies between tumourigenic and non-tumourigenic samples, further substantiating the distinct proteomic landscape associated with RCC.

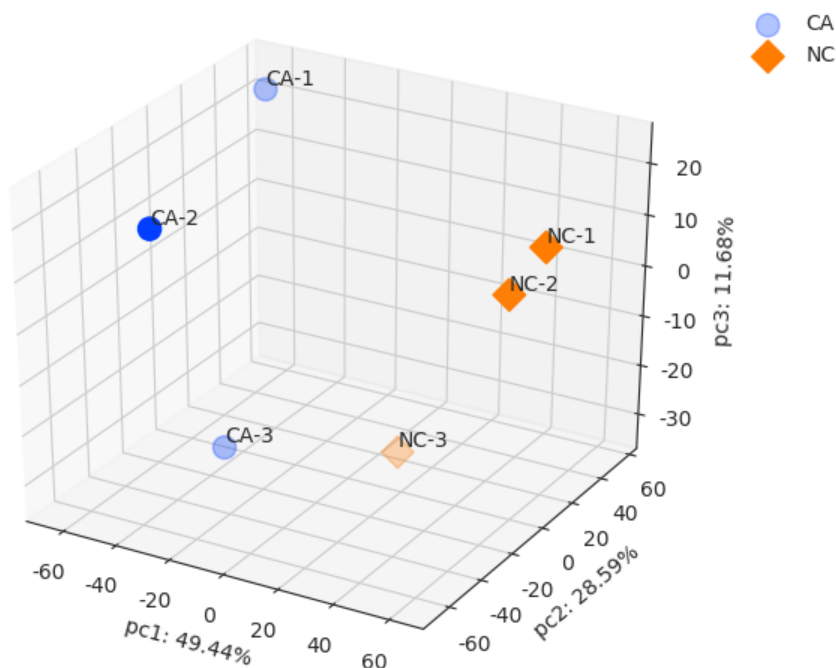


Figure 3-4. The proteomics of tumor tissues and adjacent tissues on RCC. Principal component analysis 3D scores plot of tumor and adjacent tissues. All samples were clustered into two groups ($n = 3$ per group). The orange diamond ($\diamond$) represents the non-cancer group, and the blue circle ($\circ$) indicates the cancer patient group.

Differential Protein Expression Analysis

A rigorous approach was employed to identify differentially expressed proteins, applying criteria including a fold-change threshold of 1.5 and a p-value of <0.05 . The differential expression patterns observed were visually depicted in a volcano plot (**Figure 3-5**), spotlighting proteins with substantial deviations in expression, thus emphasizing proteins critical to RCC pathophysiology.

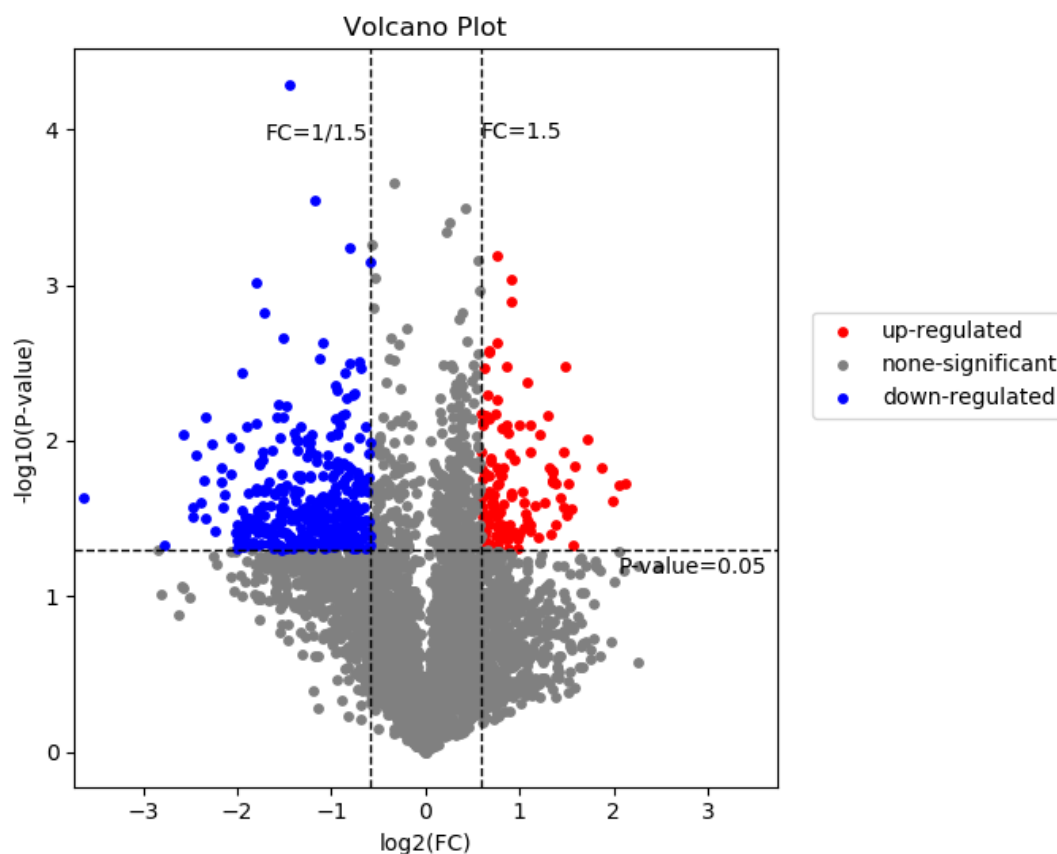
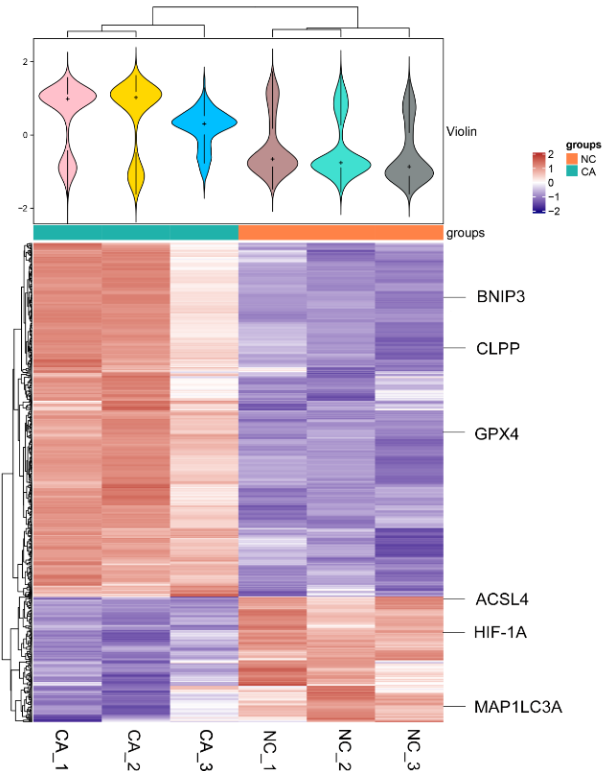


Figure 3-5. The horizontal coordinate of the volcano map is $\log_2(\text{FC})$, and the farther the value is from 0, the more significant the difference; the right side is up, and the left side is down. The ordinate is $-\log_{10}(\text{P-value})$, and the further the ordinate value is from 0, the more significant the difference. The blue dots represent down-regulated differentially expressed proteins, the red dots represent up-regulated differentially expressed proteins, and the gray dots represent non-significant differentially expressed proteins.

Cluster Analysis of Protein Expression

Hierarchical clustering of protein expression profiles across samples enabled us to visualize co-expression patterns, revealing heterogeneity within the proteomic landscape of each group (**Figure 3-6A**). The resulting heatmap illustrated sample clustering based on protein expression profiles. Pearson correlation coefficients were calculated to assess expression similarity, with high coefficients indicating a strong correlation among the top 50 significantly different proteins, as visualized in the correlation heatmap (**Figure 3-6B**).

A



B

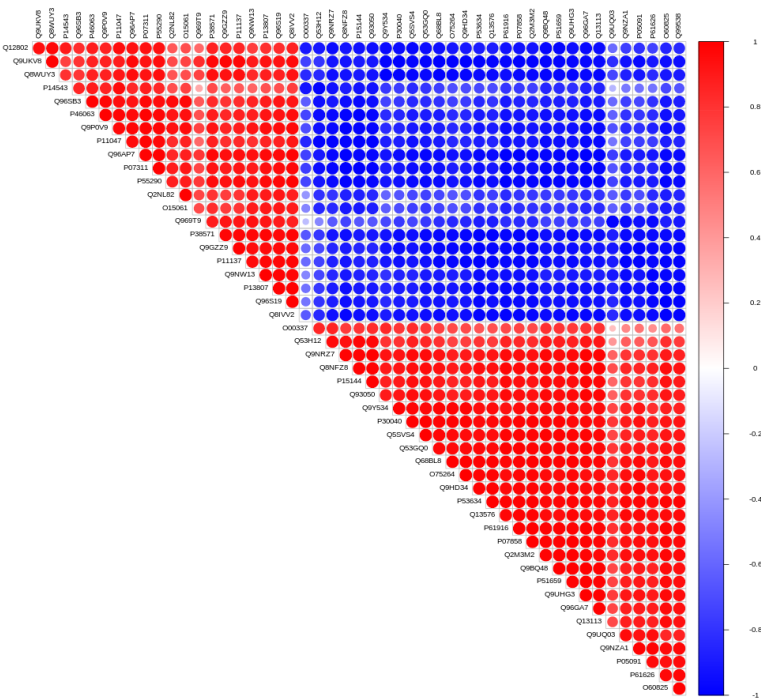


Figure 3-6. The gene heatmap and correlation analysis.

A) The violin diagram at the top combines a box plot and a density map. The flatter the violin case, the more concentrated the data, the probability distribution of the violin case contour reflects the expression value, different color fillings represent different samples, the "+" in the middle of the violin represents the median of the data, and the vertical axis represents the protein coding gene expression. Below the violin is the column annotation of the heat map, and the samples in the same group correspond to the same color block annotation. The clustering heat map below is clustered according to protein expression. Red represents a high-expression protein, blue represents a low-expression protein, and each line represents the expression level of each protein in different groups.

B) Expression correlation analysis. The red is a positive correlation, the blue is a negative correlation between genes, the circle's size represents the significance of the correlation test, and the color's depth represents the correlation coefficient's size.

KEGG Pathway Enrichment Analysis of Differential Proteins

KEGG pathway enrichment was performed on differentially expressed proteins to analyze RCC-related pathway disruptions further. Pathways were evaluated based on $-\log_{10}P$ values, with the top 20 pathways represented in a bubble plot (**Figure 3-7**). These pathways represent essential cellular functions, encompassing metabolic and regulatory processes foundational to RCC's biology and progression.

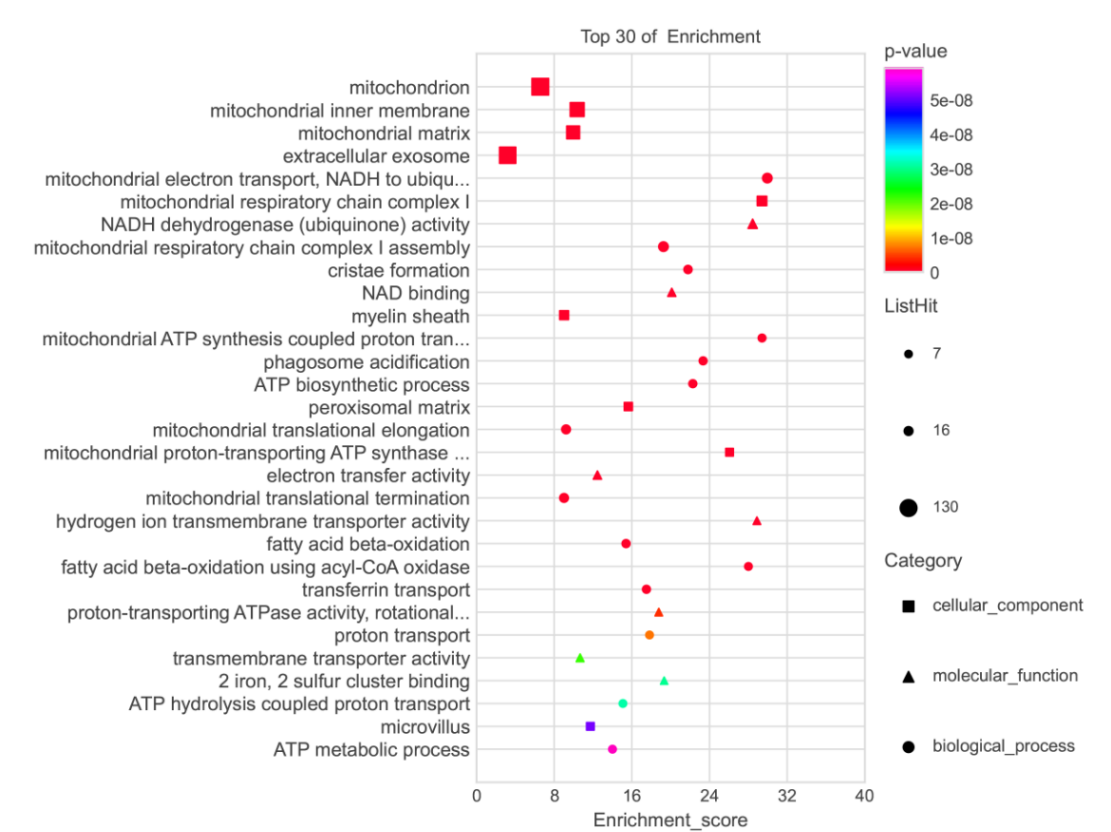


Figure 3-7. KEGG enriched top20 bubble diagram. The X-axis Enrichment Score is the enrichment score, and the Y-axis is the pathway information of the top 20. Items with more giant bubbles contain more different proteins, and the color of bubbles varies from red to green to blue to purple. The smaller the enrichment p-value, the greater the significance.

3.2.3 The Venn diagram analysis

Among the many differentially expressed proteins, a notable subset of 334 proteins displayed consistent upregulation in both RNA-seq and proteomic datasets specific to malignant tissues. Mitochondrial proteins such as ClpP (caseinolytic mitochondrial matrix peptidase proteolytic subunit) and ClpX (caseinolytic mitochondrial matrix peptidase chaperone subunit) emerged as prominent factors within mitochondrial catabolic processes, suggesting a shift in mitochondrial dynamics critical to RCC pathogenesis (Figure 3-8).

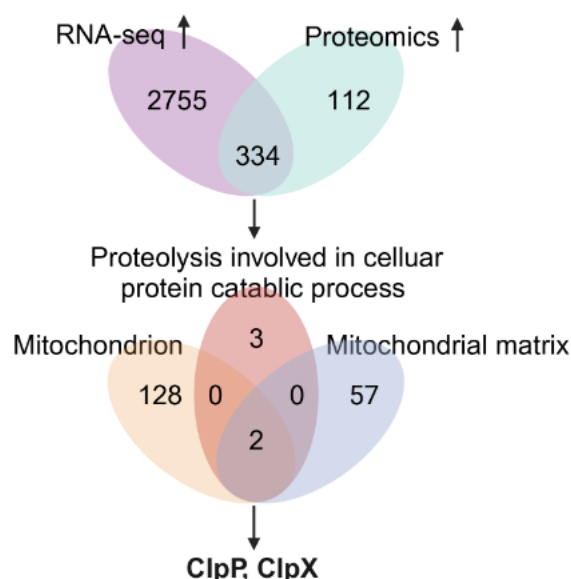
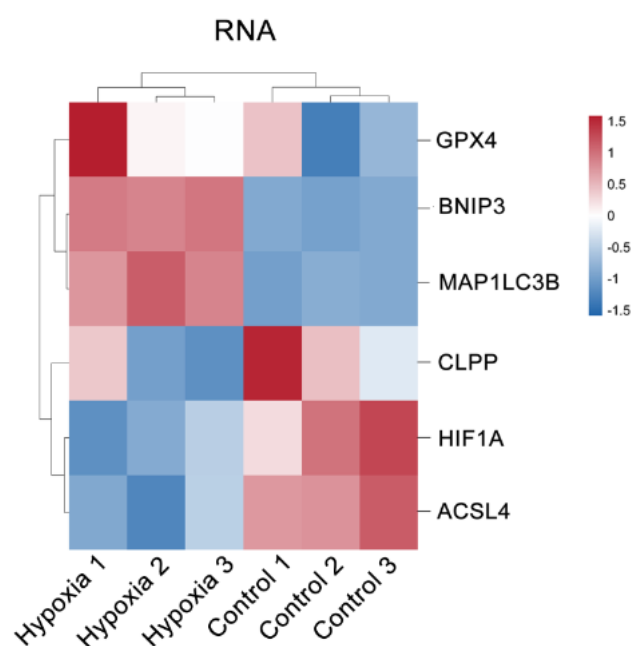


Figure 3-8. The Venn diagram displays overlapping signalling pathways identified through genomic and proteomic analyses of RCC and adjacent non-cancerous tissues from three patients.

3.2.4 Integration of Ferroptosis and Mitochondrial Metabolism

Further pathway enrichment analysis revealed significant associations with ferroptosis-related proteins, which play pivotal roles in iron-dependent programmed cell death. Key regulators such as GPX4 (glutathione peroxidase 4) and ACSL4 (Acyl-CoA Synthetase Long-Chain Family Member 4) emerged as central molecular players. Disruptions in GPX4 and ACSL4, known to affect cellular redox balance and lipid peroxidation, may initiate ferroptosis mechanisms within the RCC microenvironment. Concurrently, mitochondrial proteins, including ClpP, BNIP3 (BCL2 interacting protein 3), MAP1LC3A (LC3A), and HIF-1 α (hypoxia-inducible factor-1 alpha) demonstrated alterations indicative of an interplay between ferroptosis and mitochondrial pathways, suggesting a combined adaptive response to oncogenic stressors within RCC (**Figure 3-9 A and B**).

A



B

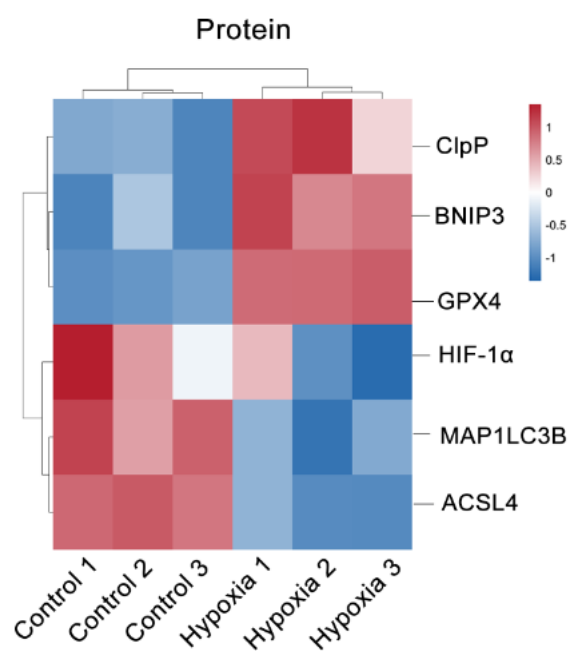


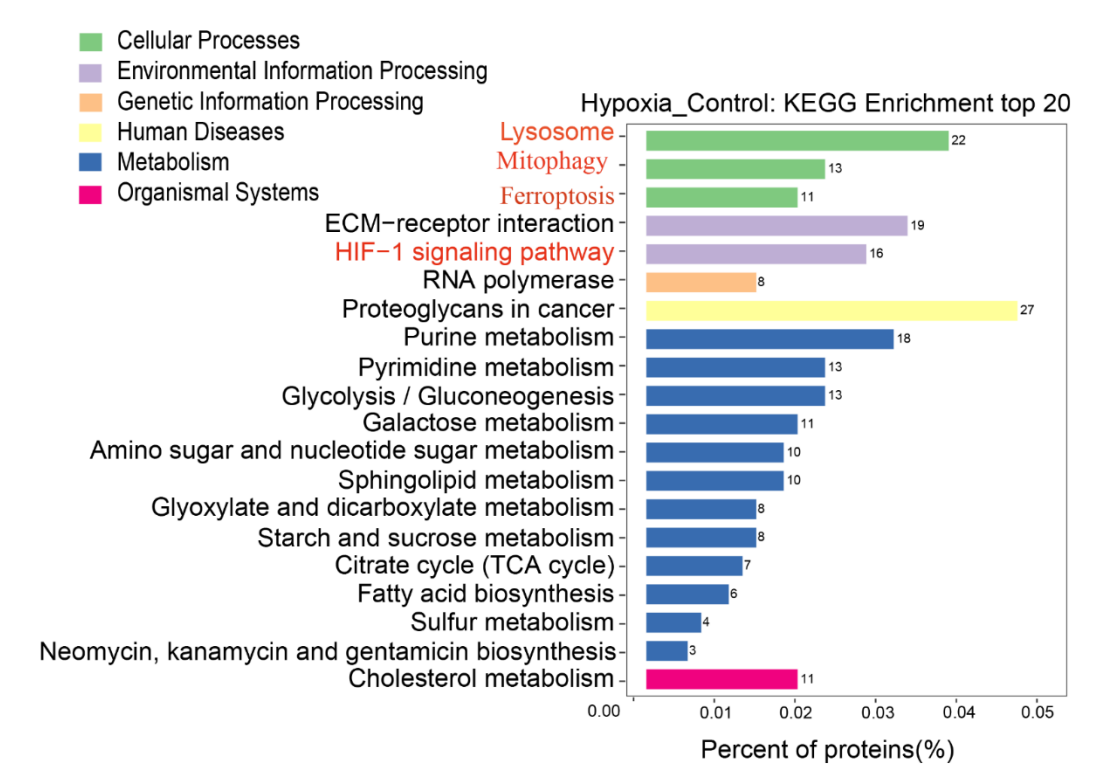
Figure 3-9. The gene and protein heatmap of three patients samples.

A) The heatmap of RNA between hypoxia-treated RCC4 cells and normoxia-treated RCC4 cells.

B) The heatmap of protein between hypoxia-treated RCC4 cells and normoxia-treated RCC4 cells.

3.2.5 Comparative Analysis in Hypoxic Conditions

To investigate the molecular adaptations of RCC under hypoxic conditions, we conducted RNA-seq analyses on RCC cells cultured under hypoxic versus normoxic environments. The KEGG pathway analysis identified significant activation within lysosomal, mitophagy, HIF-1 α , and ferroptosis pathways, underscoring the cellular responses to hypoxia-induced metabolic stress. ClpP’s role across these pathways (**Figures 3-10**) highlights its potential centrality in RCC’s survival and hypoxic adaptation mechanisms.



Figures 3-10. The top 20 signaling pathways in KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis highlights differentially expressed pathways in NC (standard control) and hypoxia groups in RCC4 cells, as determined by RNA-seq.

3.2.6 Differential Protein Interaction Network Analysis

Using the STRING database, we constructed a protein-protein interaction (PPI) network comprising the top 25 proteins ranked by connectivity (**Figure 3-11**). The network visualization, achieved via the Python network package, presented nodes color-coded by expression status (red for upregulated, green for downregulated), with node size indicating connectivity level. This PPI network reinforces ClpP’s involvement across multiple pathways, underscoring its regulatory potential within RCC.

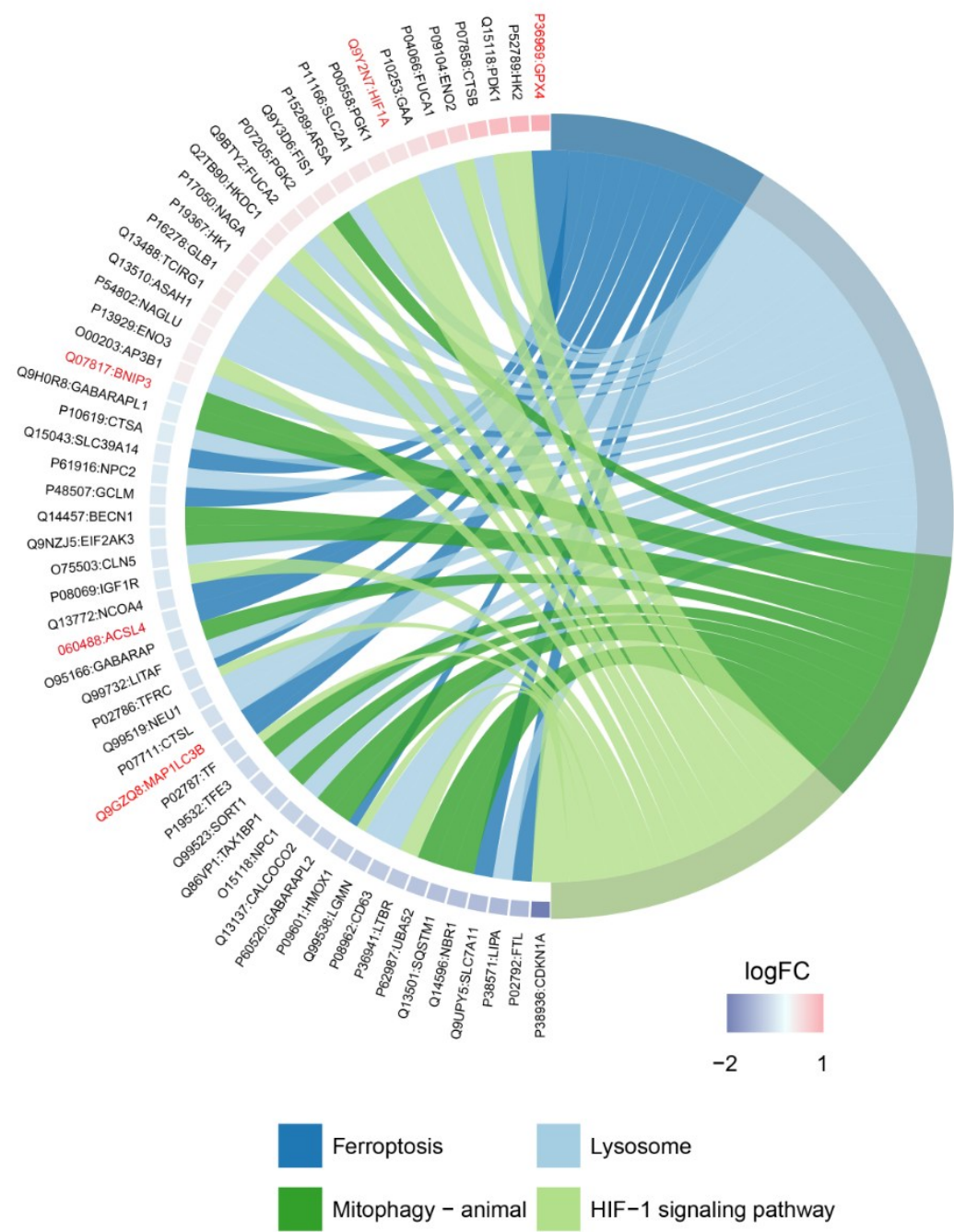


Figure 3-11. The RNA-Seq and proteomic data corroborate the hypothesis of a connection between ClpP, GPX4, HIF-1A, BNIP3, ACSL4, and MAPLC3B in hypoxia-treated RCC cells.

The complex interplay among mitochondrial metabolism, ferroptosis regulation, and hypoxia-induced signalling in RCC reveals a sophisticated molecular landscape. ClpP's significant role within these networks supports its potential as a therapeutic target, as disrupting ClpP-mediated pathways could destabilize the metabolic flexibility critical for RCC cell survival.

3.2.7 Bioinformatic Analyses

Through an analysis of 714 RCC patient samples within TCGA and ICGC datasets, we observed elevated expressions of ClpP, BNIP3, and GPX4, along with a notable downregulation of ACSL4 and HIF-1A in malignant tissues compared to non-malignant samples. This expression pattern suggests that ClpP plays a regulatory role in modulating ferroptosis and mitophagy, thereby influencing RCC's response to cellular stress and metabolic reprogramming (**Figure 3-12**).

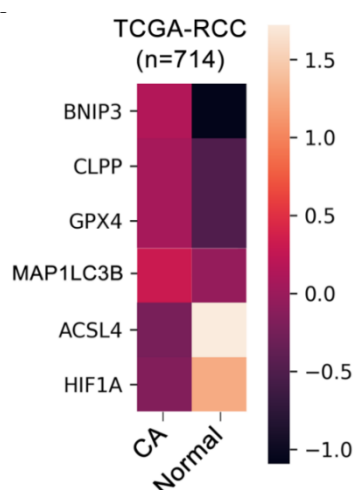


Figure 3-12: Heatmap summarizes mRNA expression profiles from the TCGA database (n=714) for ClpP, HIF-1 α , LC3A/B, BNIP3, ACSL4, and GPX4 in RCC, compared to paired normal tissues, highlighting statistically significant differences ($p < 0.05$). (cancer vs. normal, light: upregulation; dark: downregulation).

Correlation analyses further revealed significant positive associations between ClpP and proteins such as GPX4, BNIP3, and MAP1LC3A, while inverse correlations were noted with ACSL4 and HIF-1A (**Figure 3-13**). These relationships imply ClpP's involvement in oxidative stress regulation, enhancing cellular resilience under hypoxic, nutrient-limited conditions.

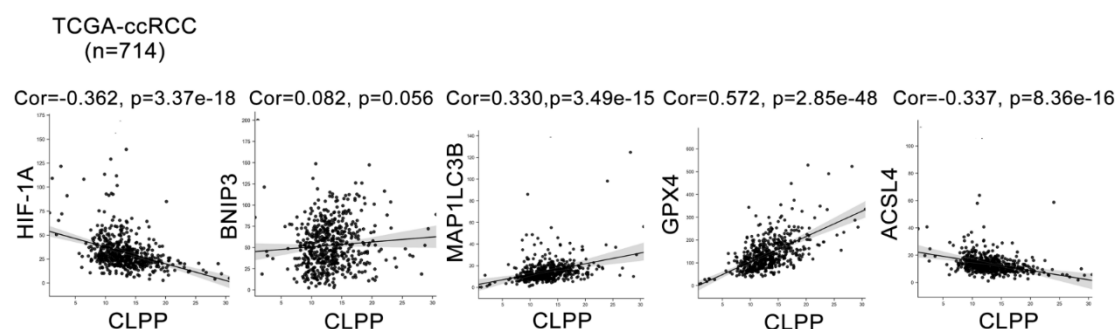


Figure 3-13. Co-expression analysis of ClpP with HIF-1 α , LC3A/B, BNIP3, ACSL4, and GPX4 mRNA levels was performed using RNA-Seq FPKM data from the TCGA database (n=714). (Cor, Pearson correlation coefficients R-value; p, p-values)

Protein-Protein Interaction Insights

STRING database analysis illustrated ClpP's interactions with proteins, including GPX4, MAP1LC3A, and BNIP3 (**Figure 3-14**), suggesting that ClpP contributes to a protective mechanism against ferroptosis cell death, primarily by detoxifying lipid peroxides—a key element in ferroptosis regulation. ClpP's interactions with MAP1LC3A and BNIP3 indicate its role in autophagy and mitochondrial integrity, which are crucial for supporting the high metabolic demands of RCC cells.

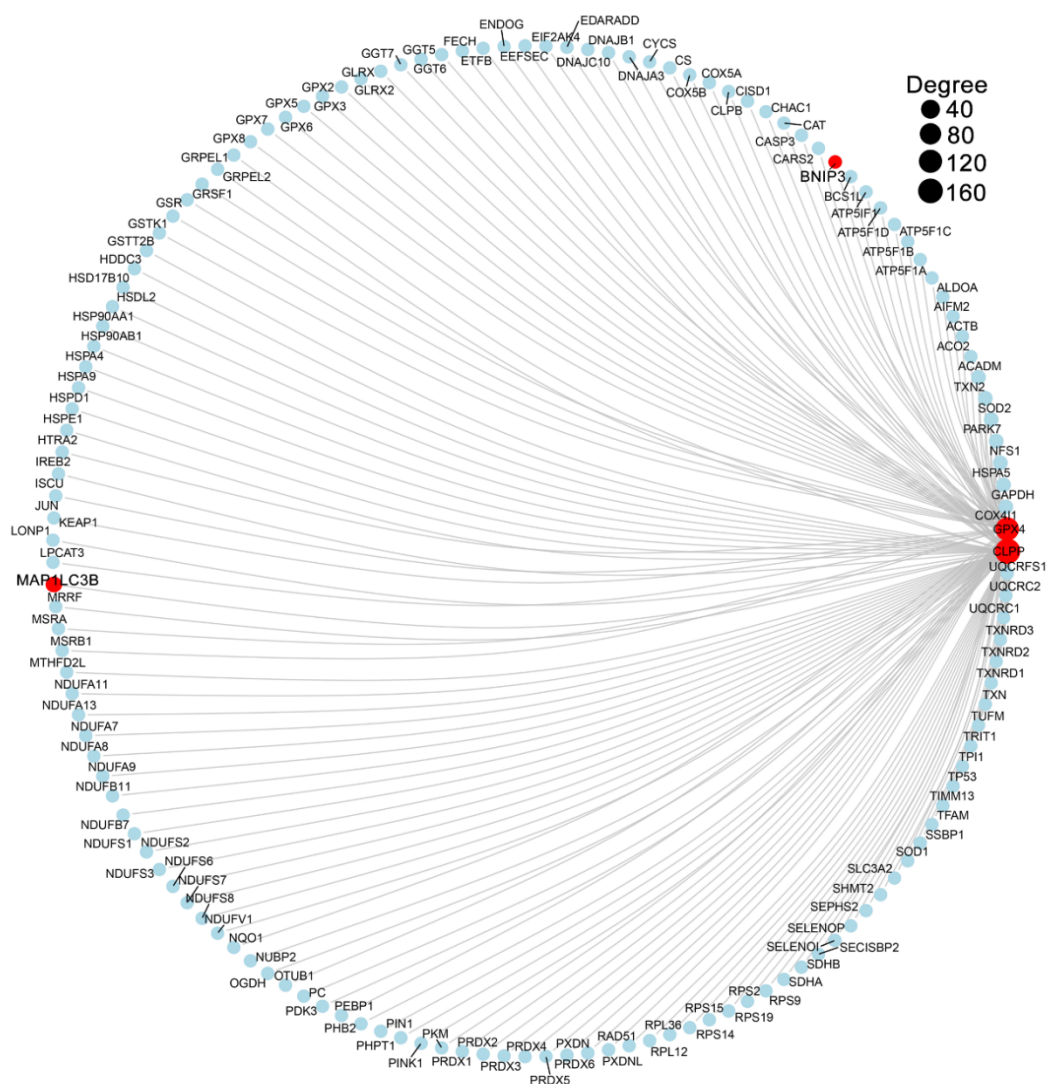


Figure 3-14. Protein-protein interaction (PPI) networks sourced from the STRING database demonstrate interactions between ClpP and the GPX4, MAPLC3A, and BNIP3 proteins.

Conclusion and Future Directions

The description of this protein as a central connection in the molecular network of RCC implies that it is a potential option of treatment. It is a part of the mechanism that the tumour uses to adapt to the new situation and is involved in the processes of autophagy, modification due to hypoxia, and resistance to ferroptosis, which is RCC's intricate molecular communication responsible for sustaining its metabolic resilience. Further research should be focused on thorough mechanistic studies to additionally demonstrate ClpP's effects on redox

balance, hypoxia adaptation, and metabolic flexibility. It is likely that by modulating ClpP's activity, it will be easier to impair RCC's adaptive mechanism, and this can potentially be a breakthrough in improving the clinical outcomes of these patients.

Ultimately, ClpP functions as the core mediator within RCC's adaptable framework, which outlines the cellular tactics that RCC uses and the types of which RCC employs to sustain its resilience. The therapy that aims to influence ClpP's regulatory roles can be the primary means of RCC pathway disruption, hence, the way to more remissive cancer synthesis materialized.

3.2.8 Analysis of RCC Patient Tissues

To critically confirm the spatial closeness of ClpP with major molecular markers such as BNIP3, LC3A/B, HIF-1 α , GPX4, and ACSL4 within human renal cell carcinoma (RCC) tissues, we enacted double immunostaining methodologies. This technique of imaging enabled the visualization and confirmation of ClpP's coherent location in addition to the primary proteins for a detailed view of the regions across the cells (Figure 3-15, upper panel). The study revealed a distinct and statistically significant positive correlation between ClpP and BNIP3, LC3A/B, HIF-1 α , and GPX4 fluorescence intensities, as evidenced by Pearson's correlation coefficients of more than 0.5. The fact that almost all of the fluorescence could be seen along with the fibers shows that ClpP is highly co-localized and intertwined with the endoskeleton, ~~indicating that there may be a potential partnership between ClpP and the proteins in the moments of mitophagy and ferroptosis in RCC cells.~~ The co-localization analysis of the incubation process, in contrast to the positive visualization protocol (used, Figure 3-15, lower panel), unravel the negative correlations of ClpP with ACSL4 ($R < 0$), which is a lipid metabolism enzyme usually linked to the ferroptosis, thus the interaction overall points that RCC metabolic regulation could be counteracted by the potential antagonistic roles.

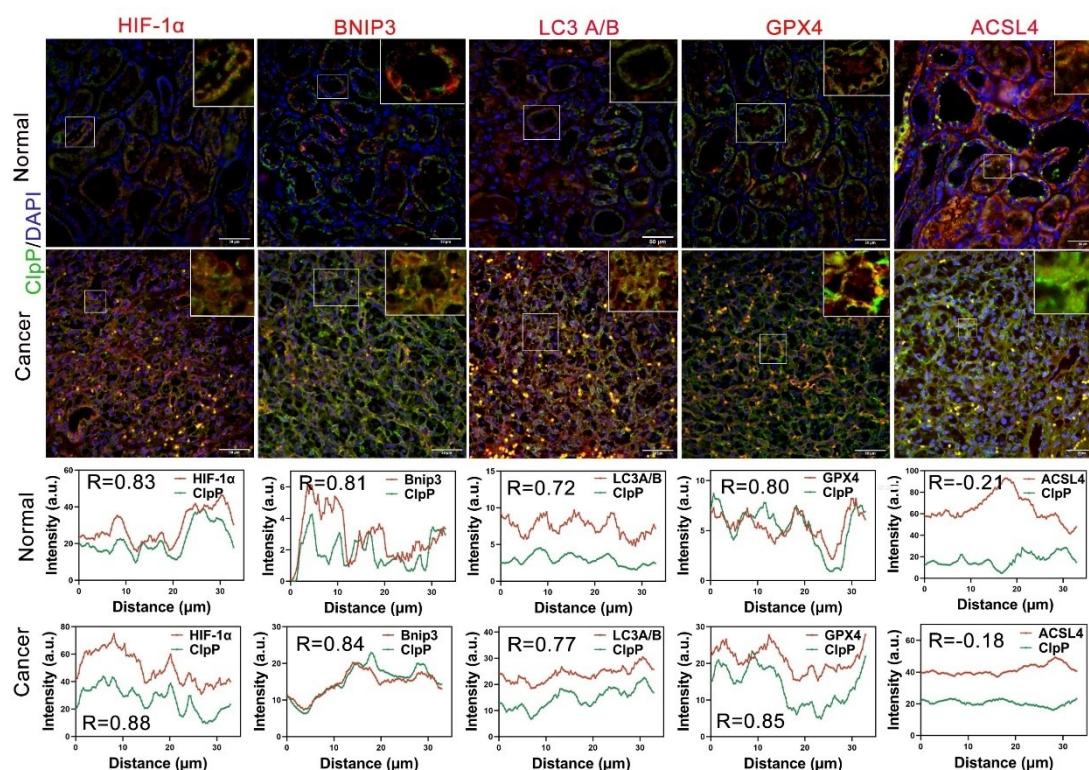


Figure 3-15. Upper part: Dual immunostaining was performed on RCC and adjacent non-tumor tissues to detect the expression of ClpP along with BNIP3, LC3A/B, HIF-1 α , GPX4, and ACSL4. Scar bar: 50 μ m. Lower part: Colocalization analysis for ClpP along with BNIP3, LC3A/B, HIF-1 α , GPX4, and ACSL4. Pearson correlation coefficient (R) is a number between -1 and 1 that measures the strength and direction of the relationship between two variables. 0- 1: positive correlation, 0: no correlation, -1- 0: negative correlation.

Further validating the differential expression patterns of ClpP and its associated proteins, we conducted a comprehensive suite of molecular assays encompassing Western blotting, quantitative reverse transcription PCR (qRT-PCR), and immunohistochemical staining. These assays revealed a consistent upregulation of ClpP alongside BNIP3, LC3A/B, HIF-1 α , and GPX4 within RCC tumour tissues relative to adjacent noncancerous (NC) tissues. Western blot analyses provided quantitative confirmation of increased protein expression levels (**Figure 3-16A and B**), while qRT-PCR corroborated these findings at the mRNA level, reinforcing the transcriptional upregulation of ClpP and its co-regulated partners in RCC (**Figure 3-16C**).

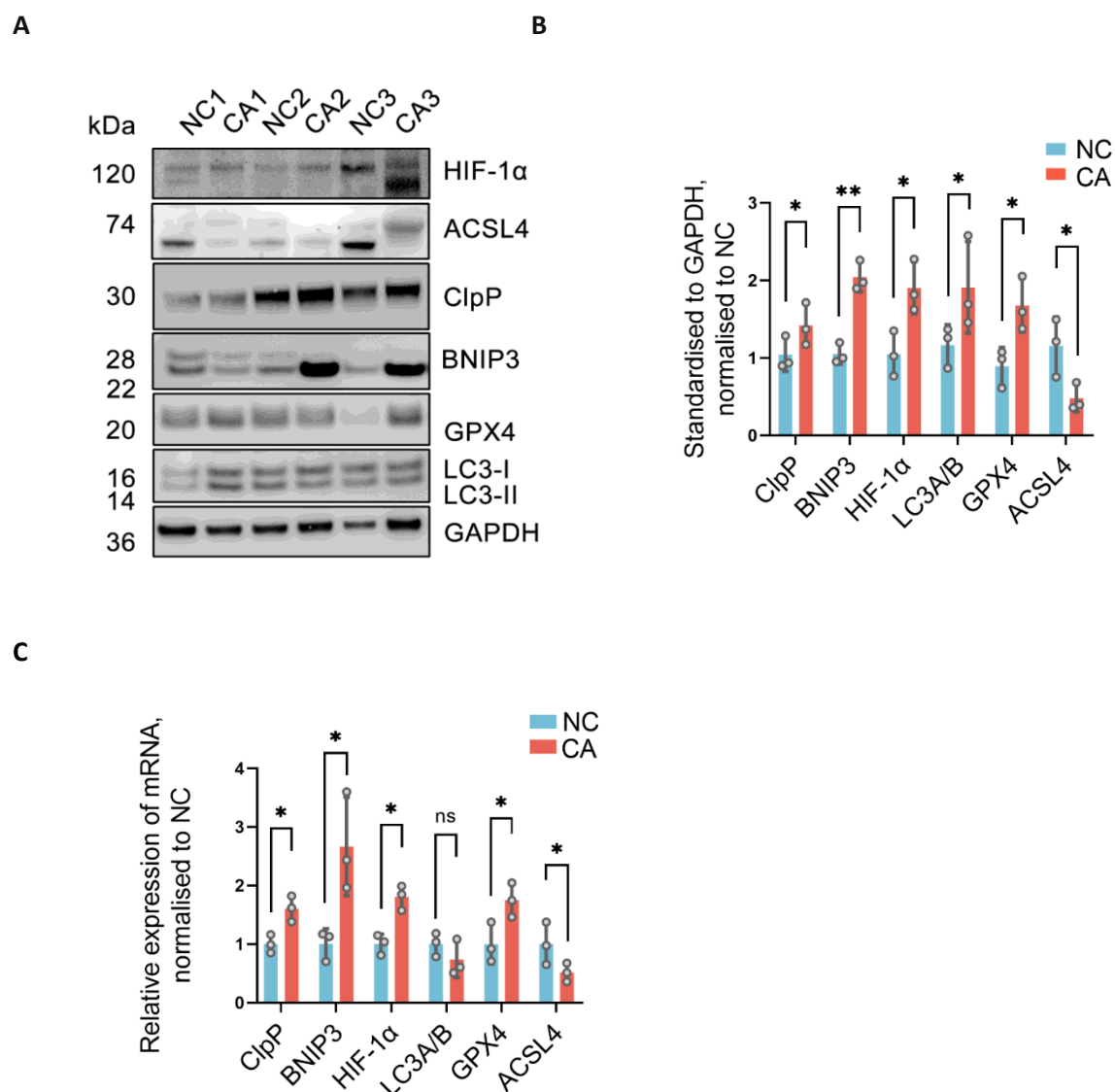


Figure 3-16. The RNA and protein expression levels.

A) and B). Western blot analysis of ClpP, BNIP3, LC3A/B, HIF-1 α , GPX4, and ACSL4 protein expression in tumor tissues compared to adjacent non-tumor tissues. * $p < 0.05$ and ** $p < 0.01$.

C). Comparative analysis of RNA expression of ClpP, BNIP3, LC3A/B, HIF-1 α , GPX4, and ACSL4 in tumor versus adjacent non-tumor tissues in human samples. * $p < 0.05$ and ** $p < 0.01$.

Meanwhile, Immunohistochemical staining afforded spatial insight, showcasing pronounced ClpP staining intensity in tumour regions, affirming its enhanced presence within malignancies and underscoring its potential role as a biomarker of oncogenic processes. **(Figure 3-17)**

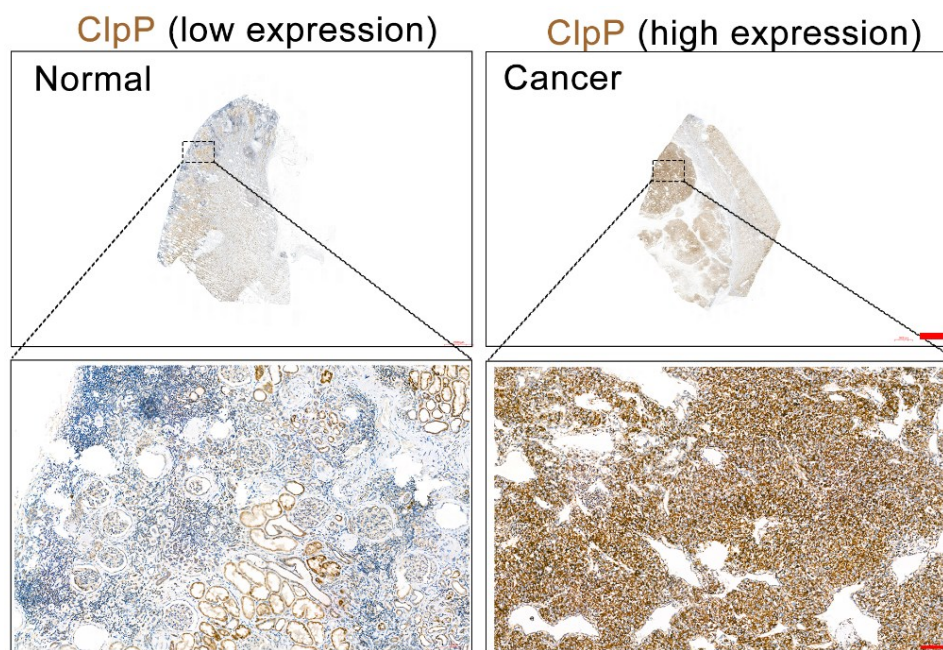


Figure 3-17. Immunohistochemical assessment of ClpP expression in tumor tissues relative to adjacent non-tumor tissues. Left: Shows that the pathological immunohistochemical results are low expression for ClpP; Right: Shows that the pathological immunohistochemical results are high expression for ClpP.

ClpP's Multifaceted Role in RCC: Co-Localization with Autophagic, Hypoxic, and Antioxidative Proteins

Our findings reveal that ClpP expression is notably upregulated and co-localizes with several critical proteins involved in autophagy, hypoxic response, and antioxidative defense mechanisms within RCC cells. This co-localization pattern suggests that ClpP actively contributes to RCC cell survival, aligning with its proposed role in regulating both ferroptosis and mitophagy. The strong positive correlations observed between ClpP and BNIP3 and LC3A/B point to ClpP's involvement in autophagic and mitophagy processes. ClpP is associated with these proteins and appears to support cellular turnover/metabolic flexibility in the stressor hypoxia that promotes RCC cells. Similarly, the evident relation to the transcription factor HIF-1 α , a critical player of hypoxia adaptation, further emphasizes ClpP as a catalyst of

cellular responses to hypoxia, consequently ensuring longer survival of RCC cells in highly oxygen-deficient microenvironments.

Concurrent with its intersecting with the autophagic machinery, ClpP's positive correlation with GPX4 is a testimony to ClpP's defense-based activities. GPX4, which is a perpendicular enzyme and has indispensable functioning in lipid peroxidation alleviation, is shown to be with ClpP, a fact hinting that ClpP is closer to cells' protection from ferroptosis, which is a programmed form of death induced by the regular lipid peroxidation, as the mechanism of interaction has been determined. The connection indicates that ClpP may act as an umbrella, giving oxidative protection against oxidative damage, which ensures RCC cell viability through this limiting-specific condition where the attack of lipid peroxidation is maintained at bay. On the other hand, ClpP's negative correlation with ACSL4, a pro-ferroptosis enzyme that boosts lipid peroxidation, points to ClpP linking to a protective anti-ferroptosis tool's functioning. The GPX4 and ACSL4 coordination of regulation processes parallels the metabolic requirements of RCC cells, which are highly dependent on controlled lipid metabolism for massive growth and survival of the cells.

Implications of ClpP as a Central Mediator in RCC Cellular Adaptation and Ferroptosis Resistance

All in all, these results of the research show that ClpP is the main player in autophagy, hypoxic adaptation, and ferroptosis resistance networks which are essential for the maintenance of the complex metabolic resilience unique to RCC cells. In the interaction with proteins that are involved in cell quality control and adaptation to stress, ClpP regulates RCC cells' capabilities of enduring the harsh tumour microenvironment characterized by nutrient deprivation and oxidative stress. Overriding all of ClpP's links to microenvironmental conditions orientation one can view the molecule as the chief component arranging multiple courses to RCC cell broadening, and yet, such cells can elude cell-death mechanisms and flexibly adjust metabolic pathways.

The indispensableness of ClpP in these basic processes necessitates further mechanistic studies to determine the particular molecular paths through which ClpP regulates these reactions. Investigating the comprehensive mechanisms by which ClpP affects RCC progression will be crucial for grasping RCC's adaptive and survival strategies, specifically regarding the way ClpP alters lipid metabolism and antioxidant defenses to cause the inhibition of ferroptosis.

Potential for ClpP as a Therapeutic Target in RCC

Considering the ClpP's pivotal role in RCC cell survival via autophagy, hypoxic adaptation, and ferroptosis regulation, the protein seems like a viral candidate for selective therapy treatment. ClpP targeting is a promising technology to damage RCC cells' adaptability, make them more deficient in resistance, and encourage their apoptosis. Therefore, future study's aim should be the creation of ClpP inhibitors or modulators which bring new therapy approaches, focused on RCC's defense mechanisms turning off. On the other hand, through rendering RCC cells more sensitive to ferroptosis, ClpP-targeted therapies overcome the limitations of present treatments and enable better therapeutic outcomes for RCC patients.

3.3 DISCUSSION

This paper also reports the most detailed molecular study of renal clear cell carcinoma (RCC) using a combined multi-omic technique. Therefore, ClpP remains an influential host in RCC's resilience and adaptation pathways. The application of RNA sequencing, proteomic profiling, and bioinformatics lead to the delineation of RCC's molecular landscape, and the pathways contributing to the disease's survival and therapy-resistance are highlighted. The findings demonstrate how renal cell carcinoma (RCC) networks depend on the interplay of these processes to survive within the challenging tumour microenvironment (TME).

3.3.1 Molecular Insights into RCC Pathogenesis

We have shown that our RNA-seq and proteomic analyses provided proof of enormous gene and protein expression differences between malignant RCC and non-cancerous tissues through the acquisition of unique molecular signatures related to RCC pathogenesis. The principal component analysis and hierarchical clustering methods both confirmed the considerable divergence between RCC and the surrounding non-tumorous tissues, which guarantees that our approach sustains the characterization of RCC on the molecular level. The analyses of differences in gene and protein expression presented in the volcano and heatmap plots were sufficient to profile through specific genes and proteins essential to RCC progression. Notably, proteins involved in mitochondrial function, oxidative stress regulation, and autophagy showed differential expression, reinforcing the significance of metabolic and stress response adaptations in RCC survival.

Our data revealed several pathways perturbed in RCC, including ferroptosis, mitophagy, and HIF-1 α signalling. These pathways, implicated in cellular adaptation to oxidative stress and hypoxia, appear to work in concert to enhance RCC's resilience. Notably, the KEGG pathway enrichment analysis identified clusters of differentially expressed genes and proteins associated with these pathways, further validating their roles in RCC's adaptive mechanisms. These pathway disruptions align with prior research highlighting the centrality of oxidative and metabolic stress responses in RCC's pathophysiology.^{262,263}

3.3.2 ClpP as a Central Mediator in RCC Survival Mechanisms

ClpP was identified as a key regulator in RCC's molecular network, with consistent upregulation observed across multiple datasets. ClpP's association with proteins involved in

autophagy (BNIP3, LC3A/B),¹⁶² hypoxic adaptation (HIF-1 α), and antioxidative defense (GPX4) points to its integral role in RCC's cellular maintenance under stress. Our results suggest that ClpP may be a crucial factor in RCC's ability to resist ferroptosis, a form of programmed cell death driven by lipid peroxidation. Specifically, ClpP's positive correlation with GPX4 suggests its involvement in protecting RCC cells from oxidative damage, which is critical in the high-stress environment of the RCC TME.^{264,265}

The ClpP and GPX4 connection and the regulation of lipid peroxidation are clues to the involvement of ClpP in lipid peroxidation, therefore, in ferroptosis resistance. On the other hand, through the negative correlation between ClpP and ACSL4, a pro-ferroptosis enzyme, the mechanism involved might be a defense mechanism against lipid peroxidation-induced cell death in RCC. This balancing act between oxidative stress and lipid metabolism by ClpP points out another highlight, which is that the survival and growth of RCC depend on the latter's ability to cope with the nutrient-scarcity and hypoxic conditions. ClpP's feedback control in RCC cells would have provided RCC cells with the ability to keep the mitochondria strong and metabolic flexibility even with ferroptosis, which is the result of a perfect transferred system between RCC and apoptosis.^{187,206}

3.3.3 Hypoxic and Autophagic Adaptations in RCC

The treatment of RCC cells in hypoxia strengthened the central hypothesis and indicated the further participation of ClpP in stress adaptive responses through the effects of environmental stressors. ClpP's crucial role in RCC survival was evidenced by a plethora of pathways involved in the mechanisms of lysosomal degradation, mitophagy, HIF-1 α signalling, and ferroptosis, which were revealed by the RNA-seq and KEGG pathway analyses. ~~Thus, the results demonstrate that ClpP plays a crucial role in RCC year-round sustenance by abating those pathways that are proper for cells' homeostasis during hypoxia.~~ By maintaining mitochondrial quality control and regulating oxidative stress responses, ClpP enables RCC cells to survive and proliferate in low-oxygen environments, a characteristic feature of the RCC TME. This aligns with prior findings suggesting that hypoxia-driven metabolic reprogramming is critical in RCC's aggressive behaviour and metastatic potential.^{165,177}

3.3.4 Therapeutic Potential of Targeting ClpP in RCC

Since ClpP is at the center of RCC's adaptive networks, it creates a viable target for therapeutic intervention. Regulating ClpP can lead to the impairment of RCC's flows of metabolism,

thereby inducing tumour cells into ferroptosis and other forms of cell death. ClpP-focused treatments will allow the RCC's defense mechanisms to be compromised, thereby empowering conventional and targeted therapies. Besides, our results also show that ClpP is a good biomarker for RCC progression due to its stable overexpression in malignant tissues. Future studies should put a spotlight on ClpP inhibitors or modulators development, and that may in turn open up new treatment doors to RCC's survival pathways by targeting the concepts that would lead to cell death.^{220,266}

The results of the study reveal the role of ClpP, together with the proteins of the autophagy and antioxidative pathways, in RCCs ability to prevent cell death through ferroptosis. Given the highly hypoxia-inducing conditions in the RCC tumour microenvironment, this is likely a predominant phenomenon for cancer cell survival.¹⁸⁷ Apart from this, pathway enrichment analysis indicates that ClpP promotes RCC's adaptability in response to low-oxygen conditions, which is essential for the tumour's progression and metastasis.¹⁷ On the basis of the data, the location of ClpP in RCC is that it might be an attractive target for therapeutics. ClpP upregulation is seen constantly in RCC tissues. Therapy approaches that are designed specifically to target ClpP could disrupt RCC's adaptive responses to the environment and thereby sensitize tumour cells to ferroptosis and other cell death processes. Targeting ClpP could, therefore, provide a novel avenue to enhance treatment outcomes for RCC patients by undermining RCC's molecular foundations of resilience.^{163,214}

3.3.5 Limitations and Future Directions

Despite the promising insights gained from this study, certain limitations warrant further investigation. Our sample size was limited to three RCC patients, and additional studies with larger cohorts are essential to validate these findings across diverse patient populations. Moreover, while our research suggests a protective role for ClpP in RCC, in-depth mechanistic studies are needed to elucidate the precise molecular pathways through which ClpP modulates ferroptosis resistance and hypoxia adaptation. Investigating ClpP's interactions with other RCC stress response network components will be crucial for understanding its full therapeutic potential.

The development and evaluation of ClpP-targeted drugs should be prioritized in future research, encompassing both in vitro studies and subsequent clinical trials, to assess their efficacy. RCC exhibits inherent resistance to numerous existing treatments; therefore,

inhibiting ClpP may facilitate the creation of innovative therapeutic strategies, ultimately improving patient outcomes. Besides, another link is ClpP's role of autophagy and the relationship thereto the ferroptosis process. This has a huge opportunity for new tests that investigate cancers with this kind of metabolism.

3.3.6 Conclusion

Our research highlights the role of ClpP in RCC's network of molecules, particularly in the activation of autophagy, hypoxia, and antioxidative pathways that make RCC cells be able to gain a foothold in host tissues even under stressful conditions. The survival of RCC ClpP is a probable central mediator of hypoxia and overproduction of oxidative species in RCC. Using ClpP's regulatory functions as a weapon may lessen RCC's adaptive capacity, making the tumour more susceptible to ferroptosis and subsequent treatments. Exploring the molecular aspects of RCC resistance to treatment, this study provides a platform for the development of ClpP-targeted therapies, thus adding to a new era of personalized treatments and even still unexplored options conceivable only for RCC patients.

CHAPTER FOUR: TO EXPLORE THE MOLECULAR MECHANISM OF MITOPHAGY AND FERROPTOSIS *IN VITRO*

4.1 INTRODUCTION

The RCC4 cell line, which originates from clear cell renal cell carcinoma (ccRCC), represents a widely adopted *in vitro* model system that offers valuable insights into the molecular and metabolic characteristics underlying renal cancer.²⁶⁷ Clear cell renal cell carcinoma is the most prevalent subtype of kidney cancer and is frequently associated with inactivation of the von Hippel-Lindau (VHL) tumour suppressor gene, a genetic alteration critical to ccRCC pathogenesis.⁴⁸ The VHL gene functions as a primary regulator of hypoxia-inducible factors (HIFs),²⁶⁸ transcription factors that mediate cellular adaptation to oxygen fluctuations.²⁶⁸ In RCC4 cells, the loss of VHL functionality results in the persistent stabilization of HIFs even under normoxic conditions, which effectively mimics the hypoxic environment often observed within ccRCC tumours. This distinctive genetic profile renders RCC4 cells an essential model for exploring hypoxia's role in cancer progression and provides an experimental platform for testing therapeutic strategies targeting the VHL-HIF signalling axis.¹³⁸

Moreover, the RCC4 cell line is instrumental in advancing our understanding of metabolic reprogramming in renal cancer. With VHL inactivation, RCC4 cells shift their energy production from oxidative phosphorylation to glycolysis, a hallmark metabolic alteration in cancer known as the "Warburg effect." This metabolic switch supports rapid cellular proliferation and tumour growth, while simultaneously contributing to a high rate of reactive oxygen species (ROS) production and an altered oxidative stress profile characteristic of aggressive ccRCC. Consequently, RCC4 cells are commonly employed in studies aimed at elucidating mechanisms of oxidative stress, mitochondrial function, and metabolic vulnerability in VHL-deficient cells, offering a valuable resource for identifying therapeutic targets that exploit these cancer-specific metabolic adaptations.²⁶⁹

Other than metabolic studies, RCC4 cells are a useful tool for examining the action of various drugs, such as inhibitors, which target HIF signalling and angiogenesis, the crucial processes in ccRCC pathophysiology. RCC4 cells' VHL genome is an object that scientists use to study the consequences of both VHL repair and further inhibition of the gene showing how this may reflect in drug efficacy and resistance mechanisms. Furthermore, since the VHL-HIF pathway

is central to immune modulation in the tumour microenvironment, RCC4 cells furnish a model for examining immune evasion, thereby accelerating the development of efficient immunotherapy approaches for ccRCC, where immune invasion is the key.

Thus, the use of RCC4 cells as a prototype proved to be a highly successful model to study the difficult biology of ccRCC. Despite the fact that the studies of hypoxic signalling, metabolic reprogramming, and therapeutic responses can be applied to only limited number of RCCs, they play a key role in renal cancer research and provide more successful treatment options for ccRCC.

Mitophagy, a selective autophagic process targeting dysfunctional mitochondria, is increasingly recognized as a significant factor in cancer progression and survival.²⁷⁰ This process is regulated by several key proteins, including BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3),²⁷¹ microtubule-associated proteins 1A/1B light chain 3B (LC3), and hypoxia-inducible factor 1- α (HIF-1 α), which act in concert, particularly under hypoxic conditions. HIF-1 α , as a major mediator of cellular response to hypoxia, is stabilized in low-oxygen tumour environments, where it activates BNIP3 expression. BNIP3 disrupts interactions with anti-apoptotic proteins and recruits LC3 to damaged mitochondria, thereby marking them for degradation.²⁷² This selective removal of damaged mitochondria enables cancer cells to maintain mitochondrial integrity, energy production, and avoid oxidative stress that could otherwise initiate cell death. Consequently, mitophagy facilitates metabolic adaptation in tumours, helping cells survive in low-nutrient, low-oxygen environments. The potential of targeting mitophagy-related pathways is an emerging area in cancer therapy, as inhibiting mitophagy may impair mitochondrial health in cancer cells, reducing their survival and proliferation.²⁷³

The fact that ferroptosis, a type of programmed cell death that is dependent on the iron-controlled lipid peroxidation, is now recognized as a new cancer target is very significant. It restricts the tumour growth and is also a possible therapeutical option¹. Two primary regulators of ferroptosis are GPX4 which is glutathione peroxidase 4 and acyl-CoA synthetase long-chain family member 4 which is ACSL4. Thus, GPX4 prevents fatal oxidative damage that leads to ferroptotic cell death by the removal of lipid peroxides, the oxidation products of unsaturated fatty compounds¹⁰. Cancer cells that express high levels of GPX4 are able to survive in spite of the fact that they are killed by ferroptosis, which is a major advantage, and

are able to divide and grow further. However, ACSL4 enhances the accumulation of polyunsaturated fatty acids (PUFAs) into the membranes of cells, which subsequently enables lipid peroxidation of cells¹¹. The elevated expression of ACSL4 in cancer cells can enhance the cellular susceptibility to ferroptosis by promoting PUFA peroxidation in the case of oxidative stress. GPX4 and ACSL4 are the newest vulnerabilities of ferroptosis in cancer, which rely on selective ferroptosis-to-death strategies through GPX4 inhibition and ACSL4 upregulation in tumour cells, thus allowing cancer treatment to evolve.

ClpP a Mitochondrial Protein Quality Control Protease Dually Acts to Cause the Decline of Cancer Cells.^{97,274} ClpP can perform the task of degrading deteriorated or improperly folded mitochondrial proteins, through which mitochondrial function realization in cancer cells that are capable of growing and surviving rapidly via mitochondria activity may aid the process²⁶⁴. Such a situation along with mutated or hyperactive ClpP states has been detected in some of the cancers, and it is responsible for the tightened mitochondrial proteostasis, which in turn is a probable facilitating factor of tumour progression.¹¹⁸ Its distinguished function in regulating mitochondrial protein turnover makes ClpP a great therapeutic target in the case of the cancers that are mainly based on mitochondrial bioenergetics for their survival.²⁷⁵ Targeting ClpP with specific inhibitors or activators can disrupt mitochondrial proteostasis, inducing metabolic stress and cell death in cancer types reliant on mitochondrial function for growth and chemotherapy resistance.²⁷⁶

The hypoxia-inducible factor 1- α (HIF-1 α) transcription factor is a pivotal player in cellular adaptation to hypoxia and is notably implicated in the development and progression of kidney cancers, including ccRCC.²⁷⁷ Under normal oxygen conditions, HIF-1 α is rapidly degraded via the VHL pathway, preventing its accumulation.²⁷⁸ However, in ccRCC, where VHL mutations frequently disrupt this degradation pathway, HIF-1 α accumulates even under normoxic conditions, triggering the transcription of genes involved in angiogenesis, glucose metabolism, and cell proliferation. These gene products collectively enhance tumour growth and survival, with vascular endothelial growth factor (VEGF) as a prominent target of HIF-1 α , promoting angiogenesis to ensure an adequate blood supply to the tumour.²⁷⁹ Given its fundamental role in ccRCC pathogenesis, HIF-1 α is a central focus in therapeutic research, with significant efforts directed toward inhibiting this pathway to improve treatment outcomes in ccRCC.¹²³

This research uncovers ClpP's multifaceted role in RCC, suggesting that ClpP supports

mitochondrial integrity and interacts with proteins critical to autophagy, oxidative stress response, and ferroptosis inhibition. These findings position ClpP as a promising therapeutic target, as modulating its function may expose RCC's vulnerabilities, particularly its reliance on mitochondrial proteostasis for survival. Future therapeutic strategies aimed at ClpP could weaken RCC's adaptive responses, enhancing sensitivity to ferroptosis and improving patient outcomes in ccRCC. This study lays the groundwork for further investigations into ClpP-centered therapies, offering a novel pathway for disrupting RCC's metabolic and stress adaptation mechanisms and expanding treatment possibilities for this challenging malignancy.

4.1.1 Research Focus and Objectives

The regulatory functions of HIF-1 α in RCC encompass interactions with numerous proteins, including BNIP3, LC3, GPX4, and ACSL4, which together orchestrate processes like autophagy, oxidative stress management, and ferroptosis inhibition—adaptations essential for RCC cells to survive in the hostile tumour microenvironment. Examining the cooperation between mitophagy and ferroptosis could be a strategy to overthrow mitochondrial homeostasis by damaging and making cancer cells outmatch ferroptosis-induced cell death. The main advantage of this strategy over drug resistance is that it may be used to target cancer-specific metabolic dependencies and lead to the selective destruction of cancer cells.

The object of the present study is ClpP, a component of the mitochondria that is observing increasing connections with renal cell carcinoma's adaptation mechanisms, including at oxygen-deficient conditions. Our goal is to highlight the dynamics that existed between ClpP, HIF-1 α , and other RCC-related proteins in hypoxic conditions. The analysis of RCC adaptability mainly based on the gene expression and protein production patterns as well as the bioinformatics analysis is aimed at mapping the molecular network that supports RCC adaptability. Experiments such as western blotting, immunofluorescence, electron microscopy, and protein docking will give the data on the expression, localization, and functional interactions of ClpP under hypoxic conditions.

4.1.2 Specific objectives include

Objective 1: To analyze the interactions among HIF-1 α , BNIP3, LC3A/B, GPX4, ACSL4 and ClpP in RCC cells under hypoxic stress.

Objective 2: To investigate the impact of ClpP depletion on mitochondrial morphology and

function in RCC cells.

Objective 3: To explore the role of ClpP depletion in modulating mitochondrial Fe^{2+} dynamics and ferroptosis in renal cell carcinoma cells.

4.2 RESULTS

4.2.1 Interactions Between HIF-1 α , BNIP3, LC3A/B, GPX4, ACSL4, and ClpP in Renal Cell Carcinoma Cells Under Hypoxic Stress

Hypoxia is a characteristic of solid tumours and is especially verified in renal cell carcinoma (RCC) where the availability of oxygen within the core of a tumour can fall below 1%, as a result of limited vascular supply and highly proliferative cancer cells.²⁸⁰⁻²⁸² Under these anoxic conditions, the RCC cells change significantly at transcriptional and translational levels, so they gain the ability to undergo adaptation and resilience. In this study, we examined the adaptive molecular responses triggered by hypoxia in RCC, focusing on the regulatory interactions involving HIF-1 α , BNIP3, LC3A/B, GPX4, ACSL4, and ClpP. During hypoxic exposure, we observed marked upregulation of HIF-1 α , BNIP3, LC3A/B, GPX4, and ClpP, with a distinct nuclear translocation of HIF-1 α , emphasizing its critical role as a hypoxia-responsive transcriptional regulator essential for RCC cell adaptation (**Figure 4-1**).

Consistent with expression profiles from normoxic RCC tissues, ClpP exhibited robust positive correlations with BNIP3, LC3A/B, and GPX4 under hypoxia (Pearson's correlation coefficient $R > 0.5$), while showing inverse correlations with ACSL4 and HIF-1 α ($R < 0$). The observed co-localization of BNIP3, LC3A/B, and GPX4 with the mitochondrial marker MitoTracker implicates these proteins in mitochondria-specific autophagy, or mitophagy, as an adaptive response to hypoxic stress (**Figure 4-1**). This mitochondrial localization suggests that ClpP, in conjunction with BNIP3 and LC3A/B, plays an active role in mitochondrial quality control, potentially mitigating cellular damage and promoting survival under challenging conditions.

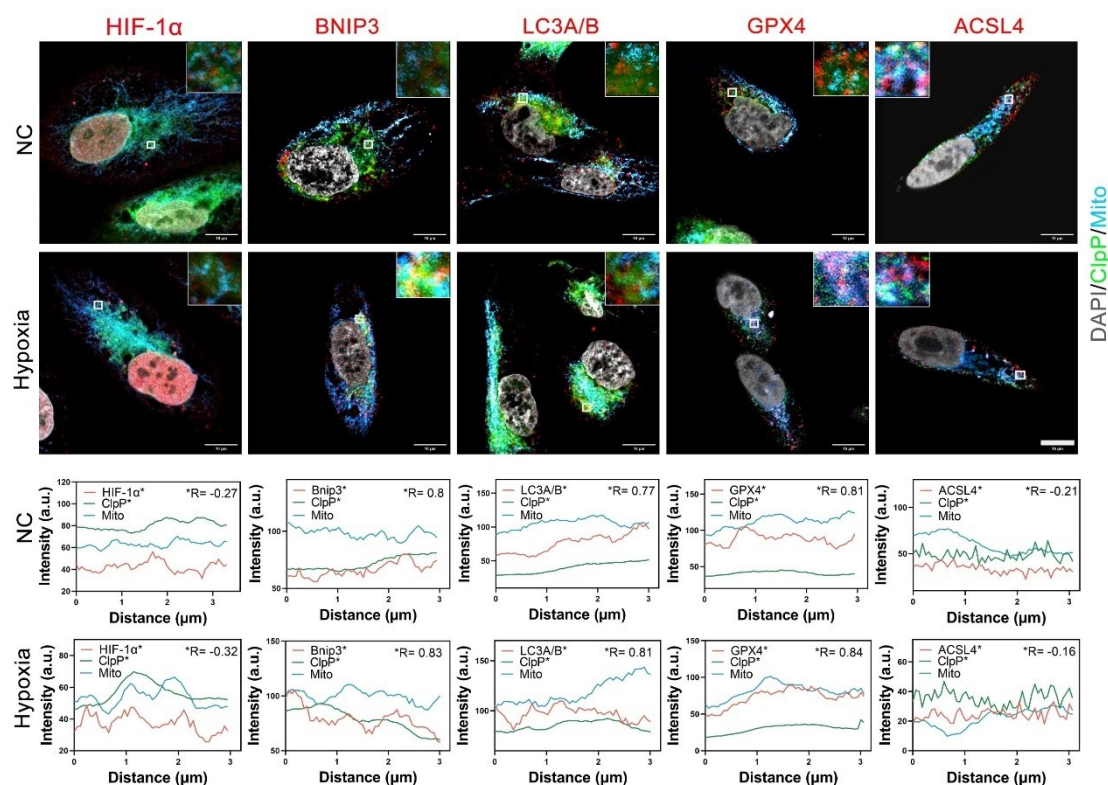


Figure 4-1. Presents immunofluorescence staining results. Upper part: Multiplex immunostaining was conducted on RCC cells under normoxic and hypoxic conditions to detect the expression of ClpP along with BNIP3, LC3A/B, HIF-1α, GPX4, and ACSL4. Scale bar: 10μm. Lower part: Colocalization analysis of ClpP along with BNIP3, LC3A/B, HIF-1α, GPX4, and ACSL4. (Pearson correlation coefficient, R)

Western blot analysis of mitochondrial fractions isolated from hypoxia-exposed RCC cells revealed elevated expression levels of ClpP, BNIP3, LC3A/B, and GPX4, alongside a reduction in ACSL4 and HIF-1α expression, supporting the hypothesis that hypoxia selectively modulates a suite of proteins that regulate mitophagy and inhibit ferroptosis (**Figure 4-2 A-B**). These expression patterns were further validated at the mRNA level via qRT-PCR, with the exception of HIF-1α, which was predominantly localized in the nucleus under hypoxia, reinforcing its role as a hypoxia-induced transcriptional regulator (**Figure 4-2 C**). Collectively, this molecular profiling indicates that hypoxia promotes the expression of mitophagy-related proteins (ClpP, BNIP3, and LC3A/B) and the ferroptosis inhibitor GPX4, while suppressing ACSL4, a known mediator of ferroptosis pathways.

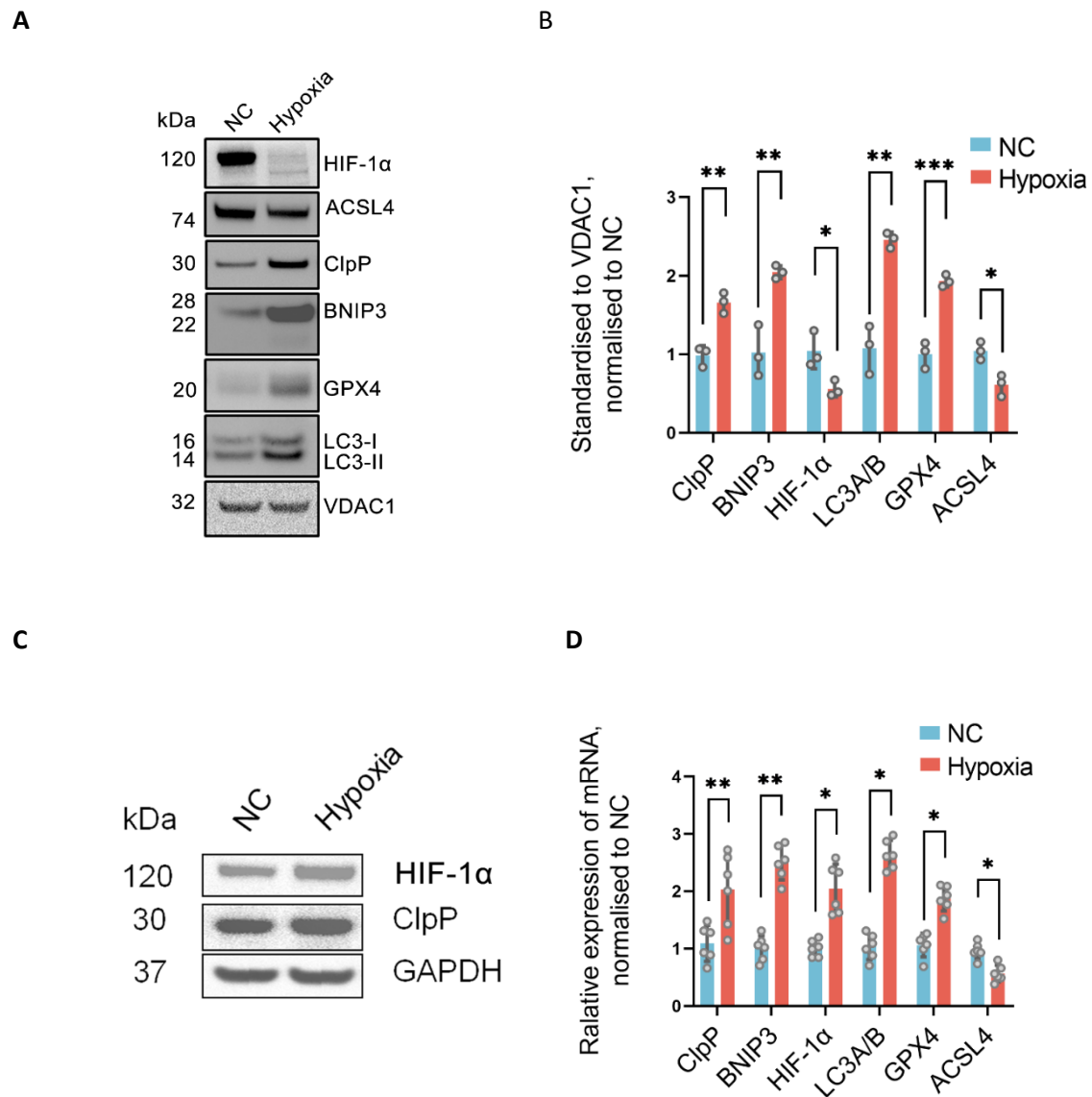


Figure 4-2. The RNA and protein expression levels.

A) and B). Post-hypoxia evaluation of protein levels in the mitochondria of RCC cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

C) Western blot analysis assessing HIF-1 α and ClpP protein expression from whole cells.

D) Assessment of mRNA levels of RCC cells after hypoxia treatment, * $p < 0.05$ and ** $p < 0.01$.

To elucidate ClpP’s molecular interactions in RCC under hypoxic conditions, we employed HDOCK, a high-precision protein-protein docking tool, to eanalyze the binding affinity and structural compatibility of ClpP with HIF-1 α . The docking analysis identified a high-confidence interaction between ClpP and HIF-1 α , with a top docking score of -282.46 kcal/mol and a confidence score of 0.934 (**Table 4-1**). Detailed assessment of the binding interface revealed eight hydrogen bonds stabilizing this interaction, suggesting a strong and likely functional association between ClpP and HIF-1 α (**Figure 4-3, Table 4-2**).

Table 4-1. Statistics of HIF-1 α -ClpP interconnection Docking scores

Rank	Docking Score	Confidence Score
1	-282.46	0.934
2	-271.32	0.9188
3	-270.2	0.9171
4	-264.36	0.9078
5	-262.57	0.9048
6	-257.83	0.8963
7	-253.1	0.8872
8	-252.73	0.8864
9	-248.98	0.8786
10	-244.79	0.8694

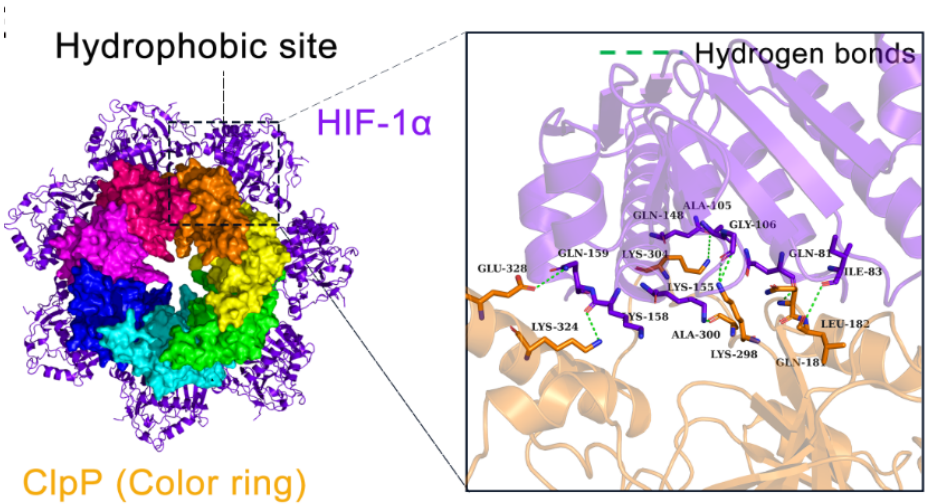


Figure 4-3. Molecular docking and affinity analysis between ClpP (polymer structure) and HIF-1 α . (Hydrogen bonds, green lines; purple, BNIP3; Color ring, ClpP). Docking scores are computational metrics used to evaluate and rank the predicted binding poses of two interacting molecules.

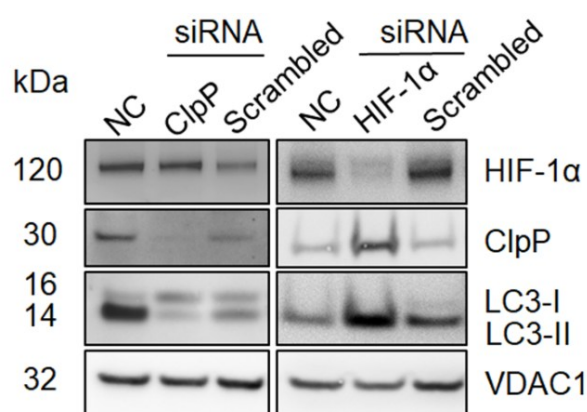
Table 4-2. Statistical table of HIF-1 α -ClpP Specific interaction

ClpP	HIF-1 α	Distance (Å)	Specific Interactions
C:Lys 155	H:Ala 300	2.6	1x hb to H:Ala 300
C:Gln 159	H:Glu 328	3	1x hb to H:Glu 328
C:Ile 83	H:Leu 182	3.1	1x hb to H:Leu 182
C:Gly 106	H:Lys 298	2.5	1x hb to H:Lys 298
C:Ala 105	H:Lys 298	3	1x hb to H:Lys 298
C:Gln 148	H:Lys 304	1.9	1x hb to H:Lys 304
C:Gln 81	H:Gln 181	1.7	1x hb to H:Gln 181
C:Lys 158	H:Lys 324	1.5	1x hb to H:Lys 324

hb: Hydrogen bond

Functional assays revealed that ClpP knockdown, unlike HIF-1 α silencing, led to enhanced mitochondrial ClpP expression and increased conversion of LC3-I to LC3-II during hypoxia (**Figure 4-4A**). Co-immunoprecipitation assays confirmed a direct interaction between ClpP and HIF-1 α (**Figure 4-4B**), emphasizing ClpP's regulatory role in hypoxia-induced autophagic responses.

A



B

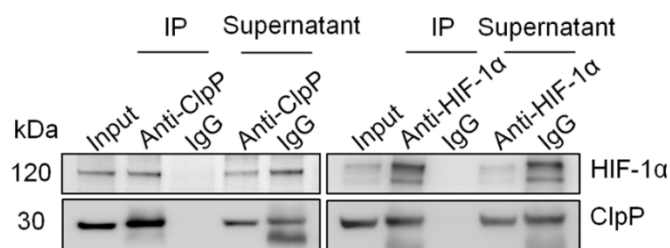


Figure 4-4. Western blot analysis assessing HIF-1 α and ClpP protein expression

A) Impact of ClpP and HIF-1 α silencing on the conversion of LC3-I to LC3-II under hypoxic conditions

B) Co-immunoprecipitation (CO-IP) experiments examining interactions between ClpP and HIF-1 α .

Moreover, HDock analysis of ClpP's interaction with BNIP3 yielded a docking score of -244.92 kcal/mol and a confidence score of 0.908 (**Table 4-3**). This interaction, supported by four stabilizing hydrogen bonds (**Figure 4-5, Table 4-4**), highlights a functional association between ClpP and BNIP3 in RCC cells under hypoxic conditions.

Table 4-3. Statistics on BNIP3-ClpP interconnection Docking scores

Rank	Docking Score	Confidence Score
1	-244.92	0.9081
2	-232.16	0.9074
3	-230.04	0.9073
4	-229.985	0.9073
5	-225.895	0.9069
6	-225.115	0.9068
7	-222.5	0.9066
8	-220.285	0.9064
9	-220.1	0.9064
10	-219.29	0.9063

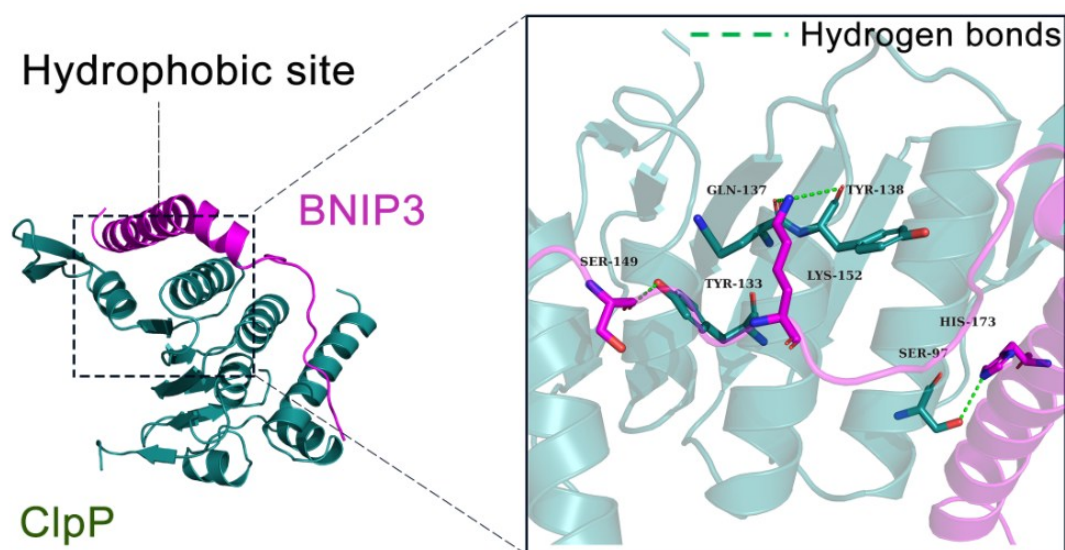


Figure 4-5. Molecular docking and affinity analysis between ClpP (Monomer structure) and BNIP3. (Hydrogen bonds, green lines; pink, BNIP3; cyan, ClpP)

Table 4-4. Statistical table of BNIP3-ClpP Specific interaction

BNIP3	ClpP	Distance (Å)	Specific Interactions
B:Lys 152	C:Tyr 138	2.3	1x hb to C:Tyr 138
B:Ser 149	C:Tyr 133	2.9	1x hb to C:Tyr 133
B:His 173	C:Ser 97	2.6	1x hb to C:Ser 97
B:Lys 152	C:Gln 137	3	1x hb to C:Gln 137

hb: Hydrogen bond

Notably, knockdown of ClpP significantly reduced BNIP3 expression, indicating that ClpP may act as an upstream regulator of BNIP3. Conversely, BNIP3 silencing did not alter ClpP levels (**Figure 4-6A**), suggesting a unidirectional regulatory influence of ClpP on BNIP3. Co-immunoprecipitation further validated the ClpP-BNIP3 interaction (**Figure 4-6B**), underscoring a hierarchical organization within the mitophagy signalling pathway in hypoxic environments.

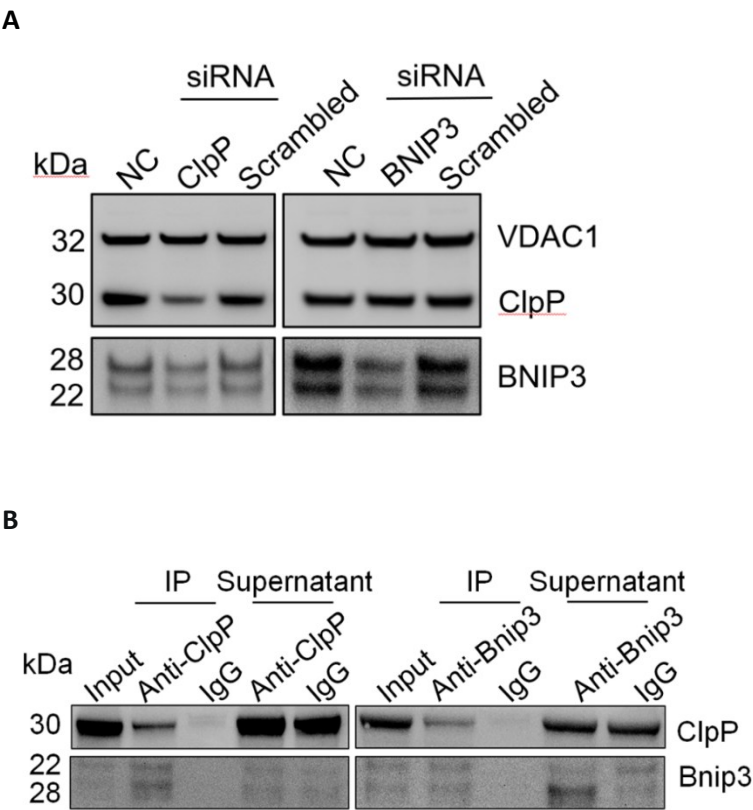


Figure 4-6. Western blot analysis assessing Bnip3 and ClpP protein expression

A) Effects of ClpP and BNIP3 silencing on their mutual expression under hypoxia.

B) Co-immunoprecipitation (CO-IP) experiments investigating interactions between ClpP and BNIP3.

These findings collectively suggest that ClpP orchestrates a complex regulatory network in RCC, facilitating cellular adaptation to hypoxia through interactions with proteins integral to mitophagy, ferroptosis inhibition, and hypoxia response. ClpP appears to regulate HIF-1 α nuclear translocation, thereby activating hypoxia-inducible gene expression critical to RCC survival. The positive correlation between ClpP and GPX4 implies a collaborative role in

ferroptosis suppression, which may shield cells from oxidative stress. Meanwhile, the negative correlation with ACSL4, a pro-ferroptosis enzyme, aligns ClpP with pathways that inhibit ferroptosis, thereby enhancing cell viability under hypoxic and nutrient-limited conditions in the tumour microenvironment.

In summary, these insights into ClpP's regulatory functions under hypoxia underscore its pivotal role as a molecular hub in RCC. ClpP's influence on key survival pathways, especially mitophagy and ferroptosis resistance, suggests its potential as a therapeutic target. Further mechanistic studies are warranted to explore ClpP's potential in therapeutic strategies designed to weaken RCC's adaptive mechanisms and sensitize tumour cells to cell death. Mapping ClpP's interaction networks provides a foundation for developing targeted therapies to inhibit RCC progression, thereby enhancing patient outcomes.

4.2.2 Impact of ClpP Depletion on Mitochondrial Morphology and Function in RCC Cells

To assess ClpP's role in mitophagy, we employed a mitophagy-specific fluorescent reporter protein, Keima, which localizes within the mitochondrial matrix and serves as a marker of autophagic degradation. Analyses of mito-Keima fluorescence in normoxic and hypoxic conditions, using scrambled siRNA as a control, revealed a marked decrease in the mitophagy signal following ClpP knockdown (**Figure 4-7A vs. B**). This reduction underscores ClpP's essential role in sustaining mitophagy flux under hypoxic stress - a hallmark of the tumour microenvironment.

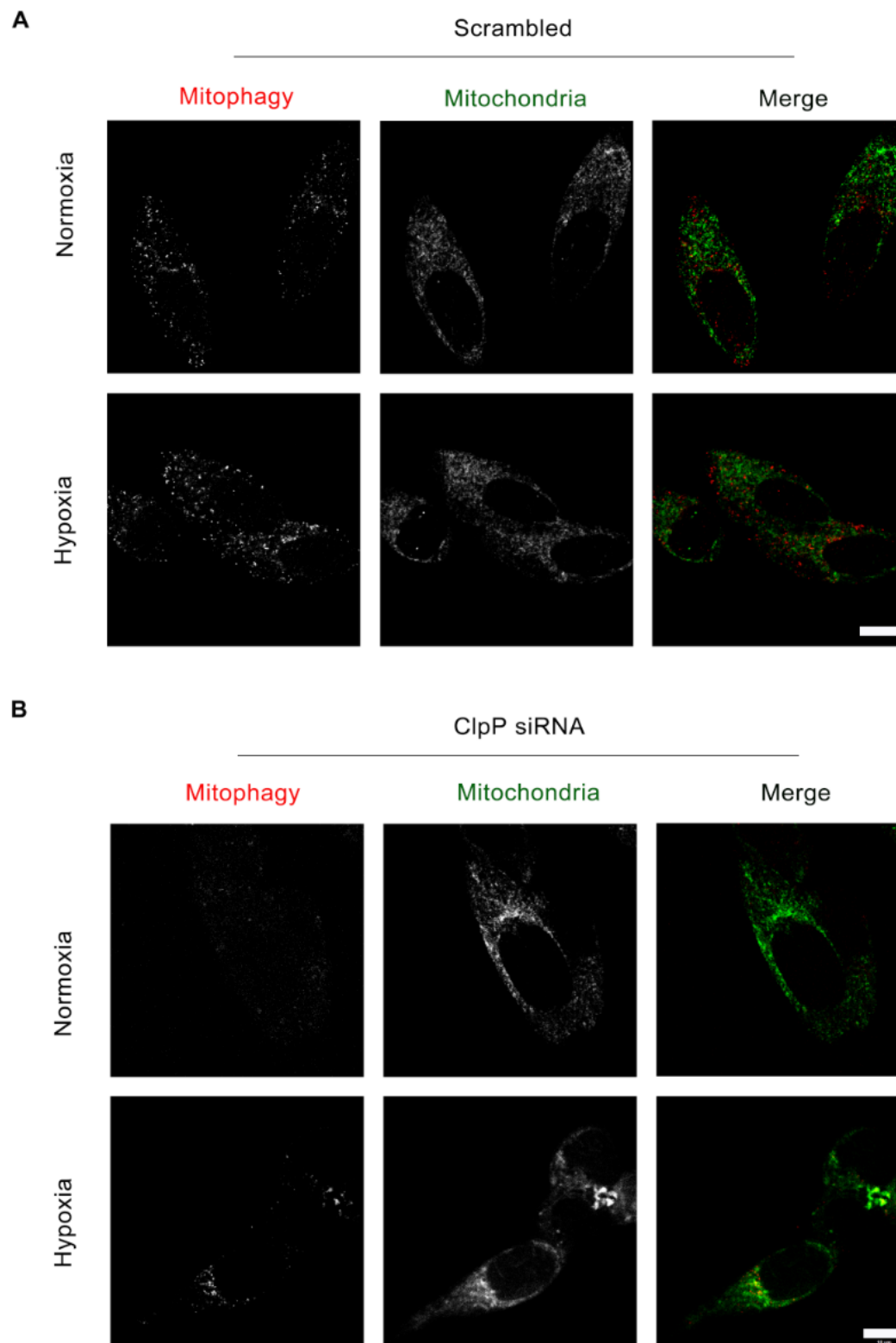


Figure 4-7. illustrates the correlation between mitophagy (mito-Keima) and ClpP expression levels

A) Depicts the localization and expression of mito-Keima about ClpP in RCC cells under both hypoxic and normoxic conditions. (Scale bars, 10 μ m)

B) Demonstrates the suppression of ClpP and mito-Keima following ClpP knockdown. (Scale bars, 10 μ m)

To further elucidate the impact of ClpP on mitochondrial architecture and function, we visualized RCC cell mitochondria with MitoTracker dye and measured mitochondrial membrane depolarization under normoxic and hypoxic conditions. Control cells displayed a well-structured reticular network and predominantly tubular morphology, indicative of healthy mitochondria (**Figure 4-8A**). In contrast, treatment with FCCP (10 μ M), a known disruptor of mitochondrial function, led to extensive fragmentation, which was similarly observed following ClpP knockdown (**Figure 4-8A**). Quantitative analysis indicated a significant increase in cells with fragmented mitochondria post-ClpP depletion, highlighting ClpP's critical role in preserving mitochondrial integrity (**Figure 4-8B**).

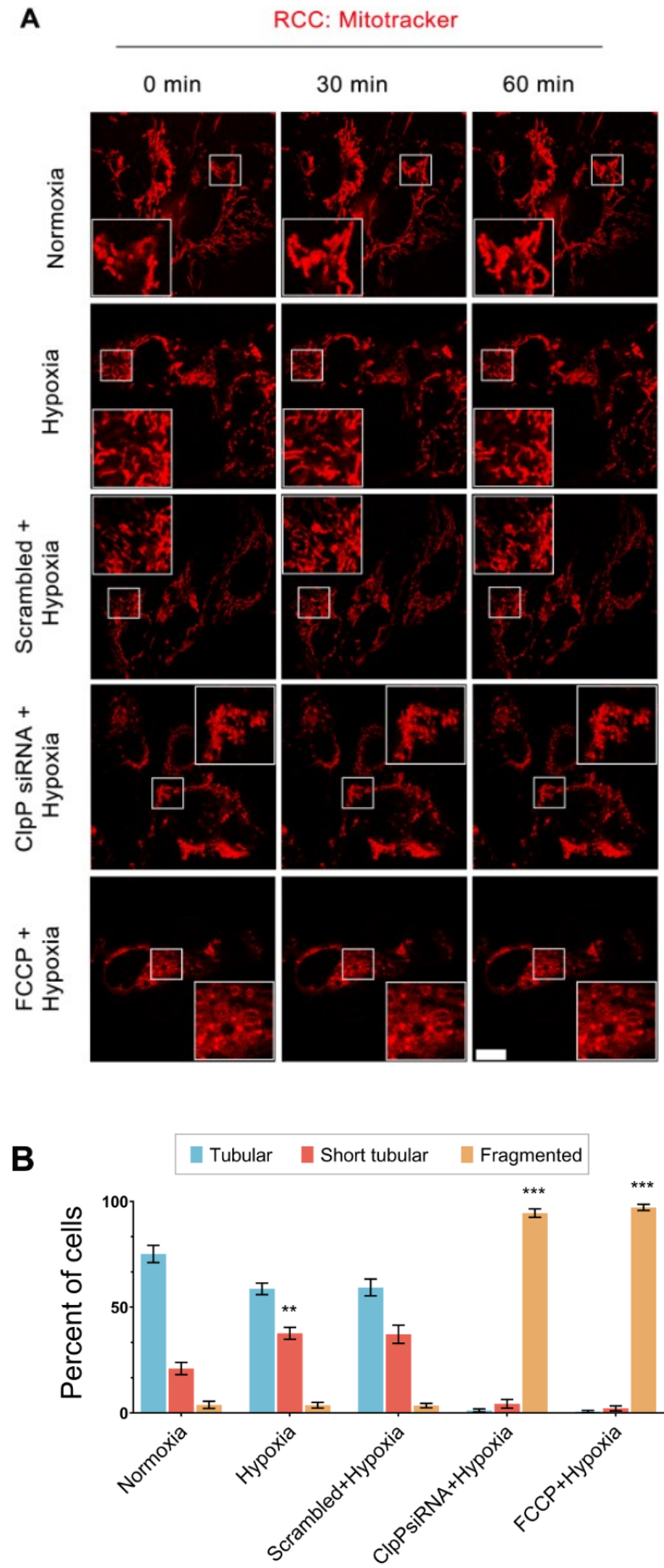
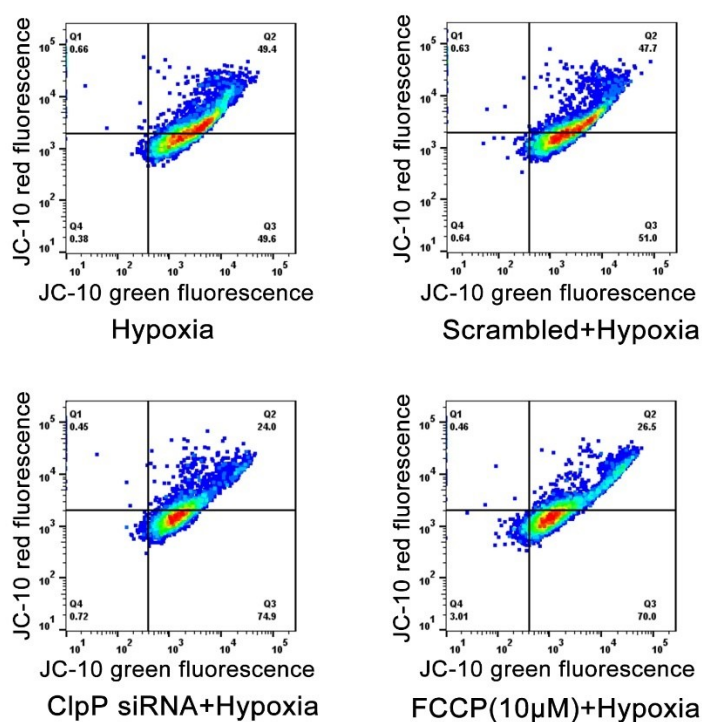
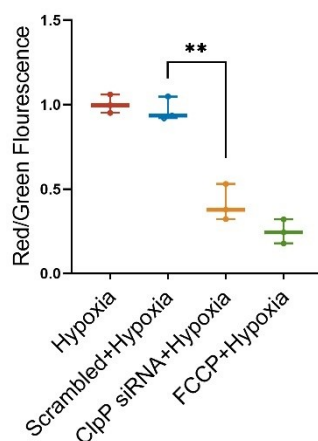
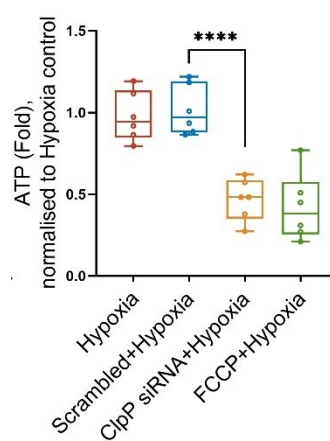


Figure 4-8. Mitochondrial morphology undergoes significant alterations under varying conditions.

A) 5 groups of RCC cells were stained with the stain for live mitochondria, mitotracker (red) and live-cell images were obtained by confocal microscopy. The images were obtained every 30 mins at x670 magnification. Magnified images of mitochondria alone are shown in the white boxes. Scale bars = 10 μ m.

B) Mitochondrial morphology described in (A) was counted according to the criteria detailed in methods. (**p < 0.01, ***p < 0.001).

To evaluate mitochondrial membrane potential ($\Delta\Psi_m$), we used JC-10 dye, which fluoresces red or green based on polarization state. RCC cells under hypoxia and scrambled siRNA treatment exhibited predominantly red fluorescence, indicating polarized, functional mitochondria ($45.7\% \pm 3.9\%$). In contrast, ClpP knockdown resulted in a shift towards green fluorescence, indicative of mitochondrial depolarization ($68.6\% \pm 4.6\%$, $p < 0.05$) (**Figure 4-9A**). FCCP treatment produced similar results, reinforcing the notion that ClpP deficiency disrupts mitochondrial membrane potential. A red-to-green fluorescence analysis further confirmed a reduction in $\Delta\Psi_m$ in ClpP-depleted cells (**Figure 4-9B**), while ATP levels in hypoxic RCC cells treated with ClpP siRNA were significantly lower than in the scrambled control group, indicating a substantial decline in mitochondrial bioenergetics (**Figure 4-9C**).

A**B****C****Figure 4-9. Assessment of mitochondrial function.**

A, B) Evaluation of mitochondrial membrane potential ($\Delta\Psi_m$) using JC-10 and flow cytometry. The red fluorescent is associated with healthy mitochondria while Green fluorescent signal is associated with increased mitochondrial membrane depolarization. The mean fluorescence intensity ratio of JC-10 in green and red was determined. (** $p < 0.01$).

C) Quantification of ATP levels in RCC cells post transfection with ClpP siRNA (**** $p < 0.0001$).

In contrast, ClpP-depleted RCC cells exposed to hypoxia exhibited extensive mitochondrial fragmentation, with abundant lysosomes and degraded mitochondrial structures, indicative of impaired mitophagy. GFP-BNIP3 signals were primarily localized in lysosomes, suggesting BNIP3-mediated mitophagy proceeded independently of ClpP. However, ClpP deficiency led to severe mitochondrial fragmentation and degradation (**Figure 4-10, panels g–l**), signifying ClpP's vital role in preserving mitochondrial integrity through BNIP3-LC3A/B-mediated mitophagy.

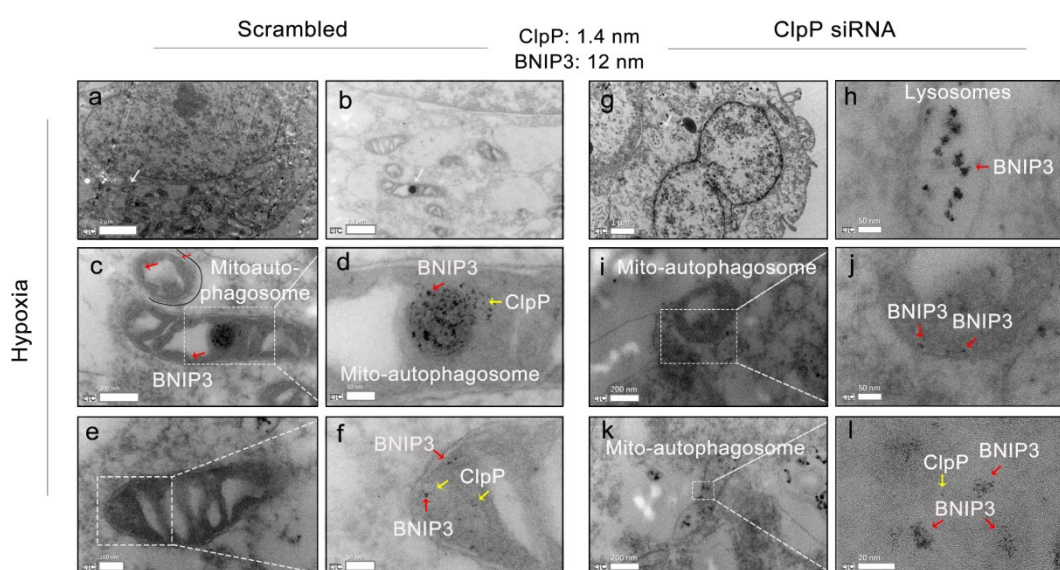


Figure 4-10. Mitochondrial ultrastructure in Scrambled and ClpP siRNA treated RCC cells after 24hr Hypoxia; data were analysed using transmission electron microscopy (TEM). The targeted use of antibodies in gold nanoparticles enables ultrasensitive detection of the relationship between ClpP and BNIP3. Red arrows indicate BNIP3 (12nm), yellow arrows indicate ClpP (1.4nm). Mito, mitochondrion; Scale bars, 20, 50, 100, 200nm; 0.5,

These findings highlight ClpP's essential role in mitochondrial quality control and mitophagy processes under hypoxic stress in RCC cells. By regulating the interplay between BNIP3, LC3A/B, and mitochondrial membrane dynamics, ClpP ensures that RCC cells can maintain functional mitochondria, a prerequisite for survival in the challenging tumour microenvironment. Understanding the precise molecular mechanisms through which ClpP controls mitochondrial homeostasis and autophagic responses may offer valuable insights for future therapeutic strategies targeting RCC progression.

4.2.3 The Role of ClpP Depletion in Modulating Mitochondrial Fe²⁺ Dynamics and Ferroptosis in Renal Cell Carcinoma Cells

This study sought to elucidate the intricate relationship between ClpP and ferroptosis pathways within renal cell carcinoma (RCC). To achieve this, a comprehensive RNA sequencing (RNA-seq) and proteomic analysis was conducted, with the goal of mapping ClpP's involvement in ferroptosis-related molecular mechanisms. Through molecular docking studies, the binding interactions between ClpP and GPX4, a key enzyme that mitigates lipid peroxidation in ferroptosis processes, were examined (**Table 4-5**). The docking analyses revealed robust interactions, stabilized by four hydrogen bonds and two salt bridges, between ClpP and GPX4, suggesting a stable protein complex (**Table 4-6, Figure 4-11**). These findings imply a regulatory role of ClpP over GPX4, positioning ClpP as a potential upstream effector within the ferroptosis pathway.

Table 4-5. Statistics on GPX4-ClpP interconnection Docking scores

Rank	Docking Score	Confidence Score
1	-253.506	0.91663
2	-242.737	0.88473
3	-226.919	0.83061
4	-226.6	0.8294
5	-219.857	0.80355
6	-217.613	0.79464
7	-215.996	0.78815
8	-209.891	0.76274
9	-209.836	0.76252
10	-209.319	0.76032

Table 4-6. Statistical table of GPX4-ClpP Specific interaction

Clpp	GPX4	Distance(A)	Specific Interactions
C:Tyr 118	G:Asn 132	2.3	1x hb to G:Asn 132
C:Lys 113	G:Asp 6	1.6	1x hb, 1x salt bridge to G:Asp 6
C:Glu 82	G:Arg 9	2.7	1x hb to G:Arg 9
C:Arg 81	G:Asp 111	1.7	1x salt bridge to G:Asp 111
C:Gln 65	G:Asn 109	3.1	1x hb to G:Asn 109
C:Val 63	G:Asn 109	2.6	1x hb to G:Asn 109

hb: Hydrogen bond

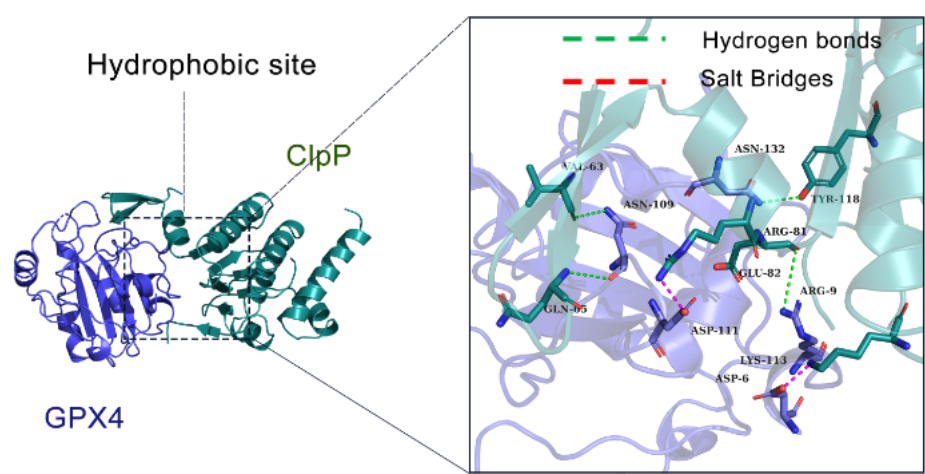


Figure 4-11. Molecular docking and affinity analysis between ClpP and GPX4. (Hydrogen bonds, green lines; salt Bridges, red lines; Blue, GPX4; cyan, ClpP)

Further functional studies substantiated these insights by showing a marked downregulation of GPX4 upon ClpP knockdown. Conversely, GPX4 silencing did not reciprocally affect ClpP expression, suggesting a unidirectional regulatory effect (**Figure 4-12A**). Co-immunoprecipitation assays confirmed the physical interaction between ClpP and GPX4, supporting the hypothesis that ClpP acts as a regulatory hub for GPX4-mediated ferroptosis suppression in RCC cells (**Figure 4-12B**).

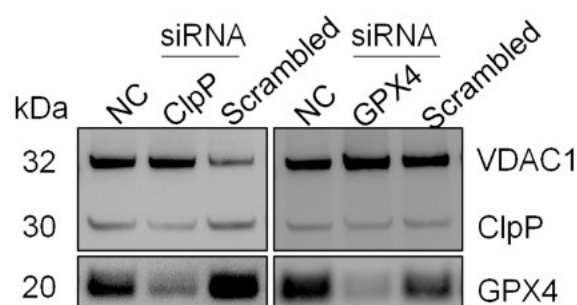
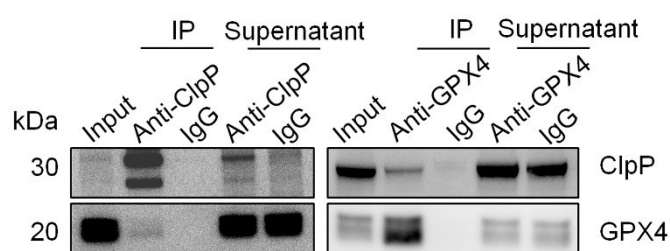
A**B**

Figure 4-12. Western blot analysis assessing GPX4 and ClpP protein expression

A) RCC cells transfected with control (Scrambled) and ClpP siRNA were lysed and analyzed by Western Blot.

B) Co-immunoprecipitation (CO-IP) experiments to study interactions between ClpP and GPX4.

Ferroptosis is characterized by intracellular iron accumulation, increased reactive oxygen species (ROS) production, and lipid peroxidation. To further elucidate ClpP's role in this process, lipid peroxidation levels were measured using the Liperfluo probe, which fluoresces upon reduction of lipid hydroperoxides. In RCC cells treated with ClpP-siRNA, a substantial, time-dependent increase in lipid peroxidation was observed, comparable to levels induced by the ferroptosis inducer Erastin (10 μ M) (**Figure 4-13A**). Quantitative analysis revealed that the fluorescence intensity of Liperfluo in ClpP-depleted cells was significantly higher than in control cells, underscoring an increased susceptibility to lipid peroxidation and ferroptosis ($P < 0.0001$, **Figure 4-13B**). Notably, pre-treatment with the ferroptosis inhibitor Ferrostatin-1 (Fer-1, 10 μ M) effectively reduced the Liperfluo signal in ClpP-knockdown cells to control levels, indicating that the observed lipid peroxidation was ferroptosis-dependent ($P > 0.05$, **Figure 4-13B**).

A

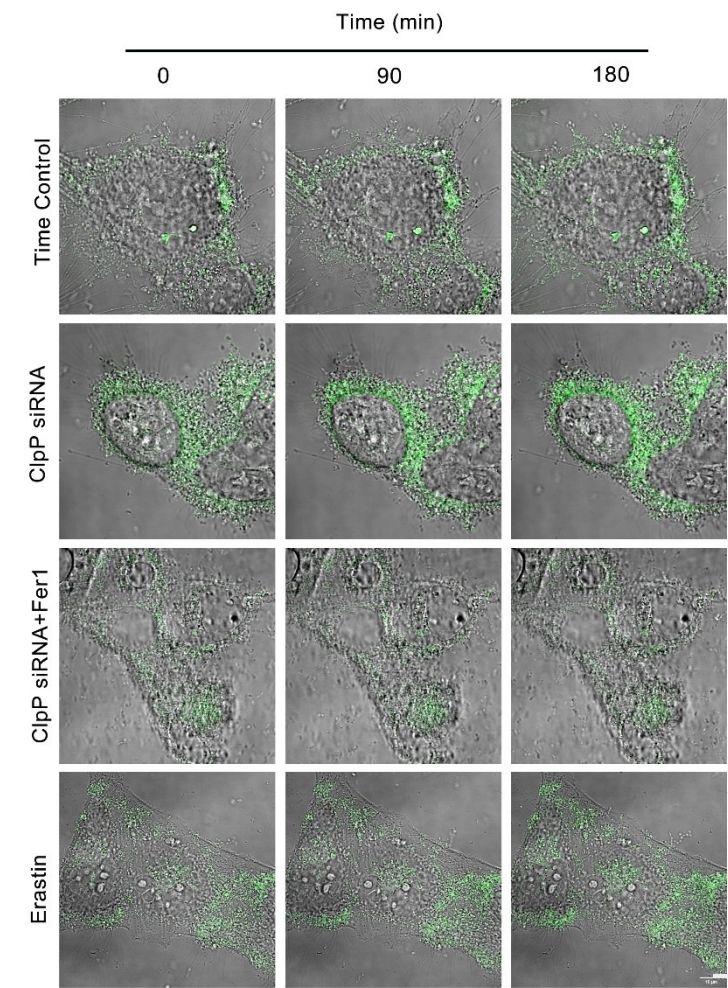
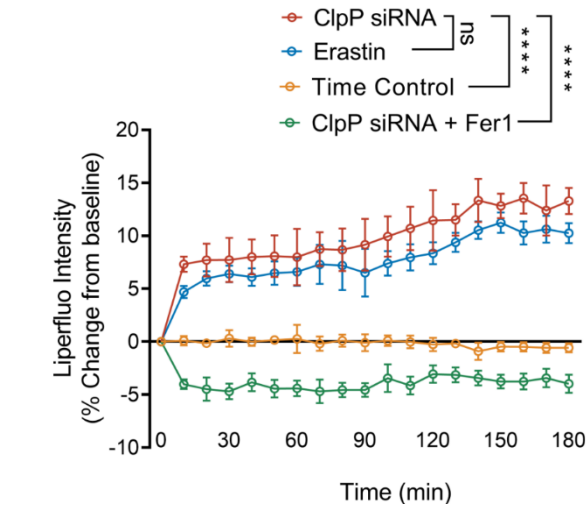


Figure 4-13. Detection of lipid hydroperoxides by live cell fluorescence imaging in RCC cells.

A) Detection of lipid hydroperoxides by live cell fluorescence imaging using Liperfluo (20μM) in RCC cells treated with Erastin (10μM), ClpP siRNA (50nM) or ClpP siRNA +Fer1 (10μM) for 3 hours. Time control is used as a negative control, Erastin as a positive control for ferroptosis, and Fer-1 as an inhibitor control.

B) The fluorescence intensity of the 4 groups at different time points, as depicted in Figure D. For statistical analysis, each stage position was counted as one data entry, n=6. (****p < 0.0001).

B



Immunofluorescence staining corroborated these results, revealing decreased GPX4 expression and increased ACSL4 expression in ClpP-depleted cells (**Figure 4-14A-C**).

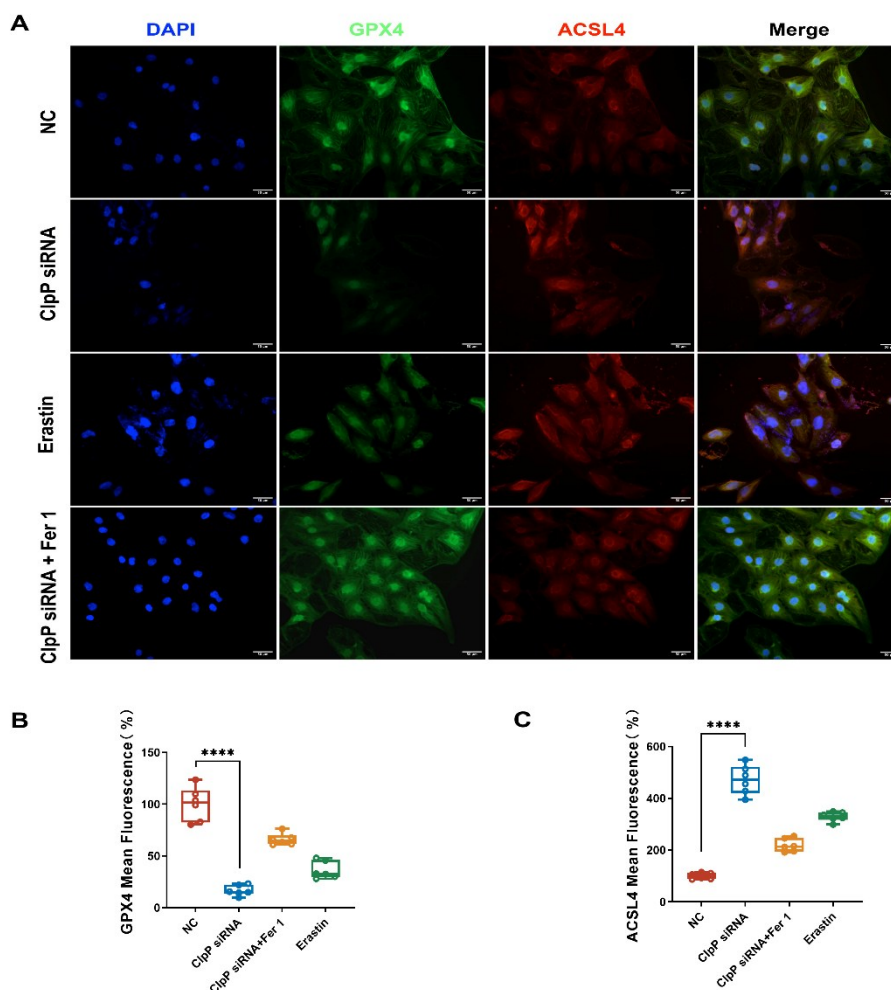


Figure 4-14. Presents immunofluorescence staining results, exploring the effects of ClpP knockdown on GPX4 and ACSL4

A) Showcases the immunofluorescence-stained images of GPX4 expression (green) and ACSL4 expression (red) under the treatments of ClpP-siRNA, erastin, and a combination of ClpP-siRNA and Fer 1. DAPI (blue) was used to indicate the location of nuclei. Representative images were acquired at 20x magnification, and the brightness and contrast were uniformly adjusted using Image J Fiji. (Scale bars, 50 μm)

B, C) Provide the fluorescent intensity analysis of ACSL4 and GPX4, respectively. Data are represented as box-Whisker plots (n=6). Statistical analysis was performed using the Student t-test, with ****p<0.0001, via GraphPad Prism.

Considering the central role of ROS in ferroptosis cell death, we assessed ROS levels using the Dihydrorhodamine 123 probe in ClpP-siRNA treated RCC cells under hypoxic conditions. ClpP knockdown led to a significant increase in ROS levels compared to hypoxic and scrambled siRNA-treated control cells ($P < 0.05$, **Figure 4-15A-C**).

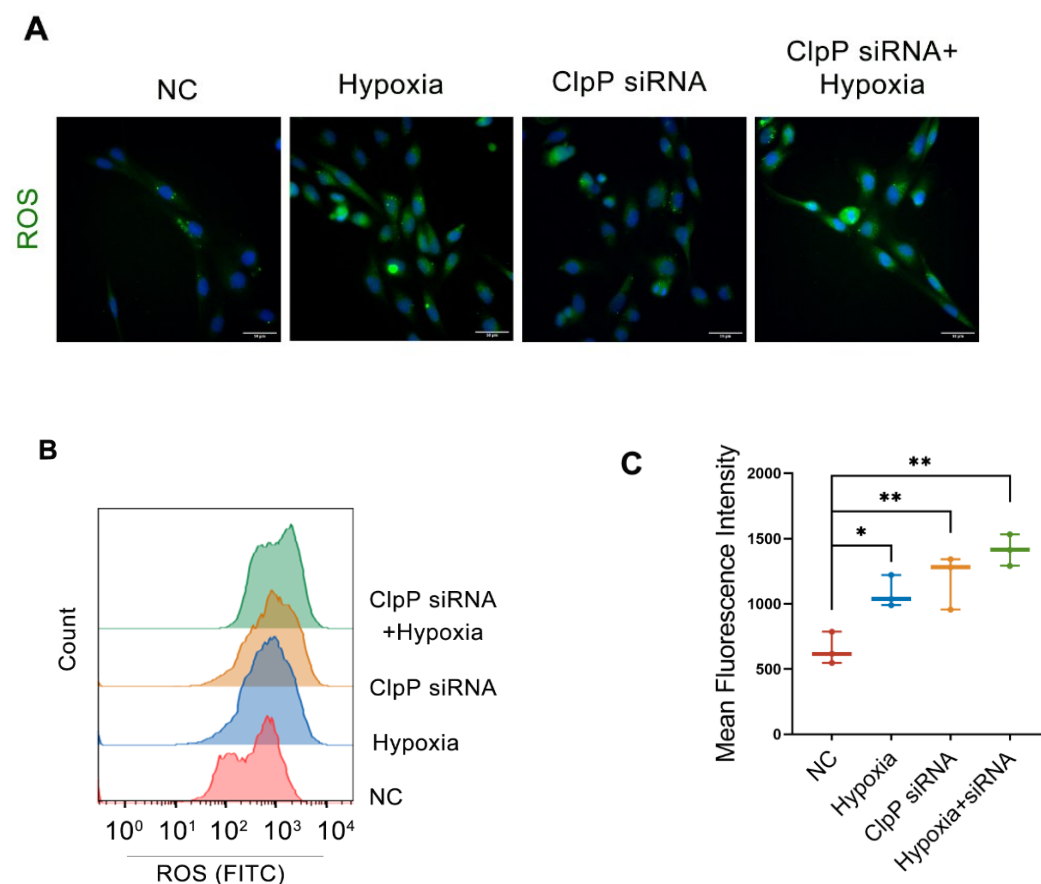


Figure 4-15. Reactive Oxygen Species (ROS) production by Renal Cell Carcinoma (RCC) cells after 24 hours of hypoxic culture and ClpP siRNA treatment was measured using immunofluorescence (**A**) and flow cytometry (**B, C**). (* $p < 0.05$ and ** $p < 0.01$).

Further validation of these findings was obtained by measuring intracellular Fe^{2+} concentrations and lipid peroxidation through a malondialdehyde (MDA) assay. Consistent with expectations, ClpP knockdown led to Fe^{2+} release and elevated MDA levels, mirroring the effects induced by Erastin treatment, indicative of enhanced ferroptosis activity (**Figure 4-16A and B**). Cell viability assays, including lactate dehydrogenase (LDH) release and cell counting kit-8 (CCK-8) assays, demonstrated that ClpP knockdown significantly increased LDH release and reduced cell viability to approximately 50% of baseline levels, comparable to the cytotoxic effects of Erastin treatment. Importantly, a two-hour pre-treatment with Fer-1 effectively

mitigated the cytotoxic effects of ClpP knockdown, indicating that ClpP deficiency-induced cell death is predominantly ferroptosis (**Figure 4-16C and D**).

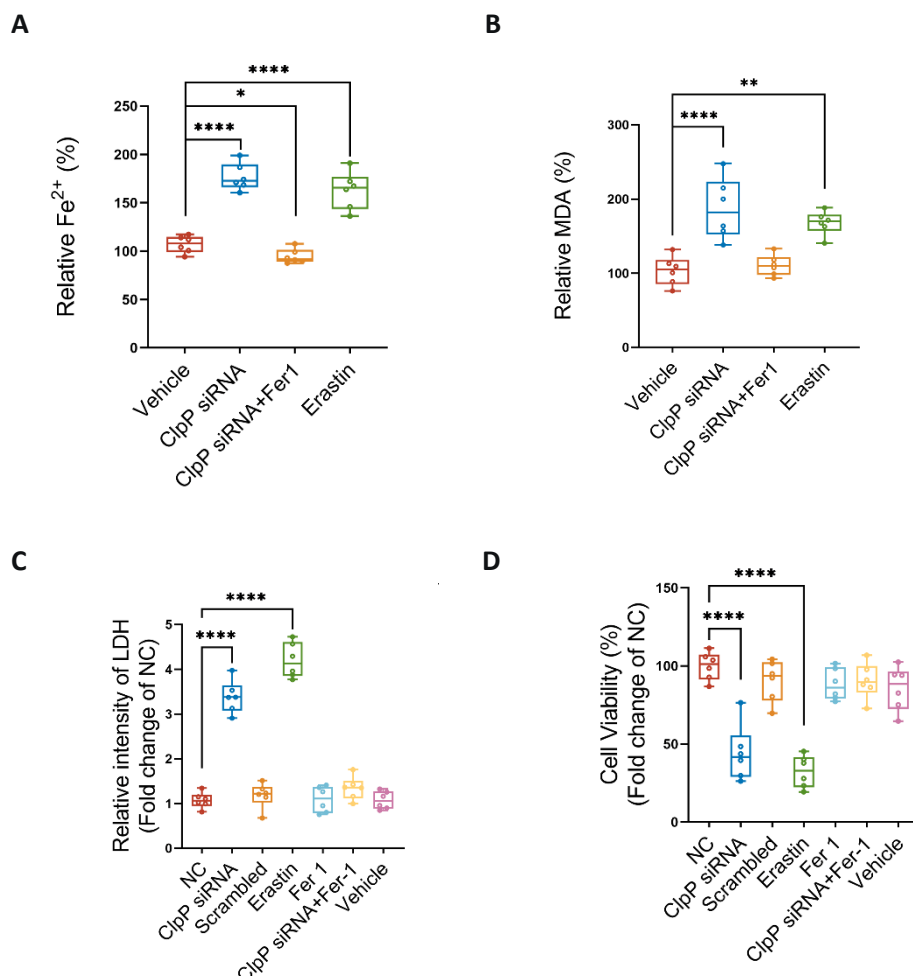


Figure 4-16. Renal cell carcinoma (RCC) cells were analyzed after 24 hours of hypoxic culture and treatment with ClpP siRNA.

A) Intracellular Fe^{2+} levels in ClpP siRNA treated RCC cells were measured using an iron assay kit and compared to the vehicle (DMSO) group. (* $p < 0.05$ and **** $p < 0.0001$).

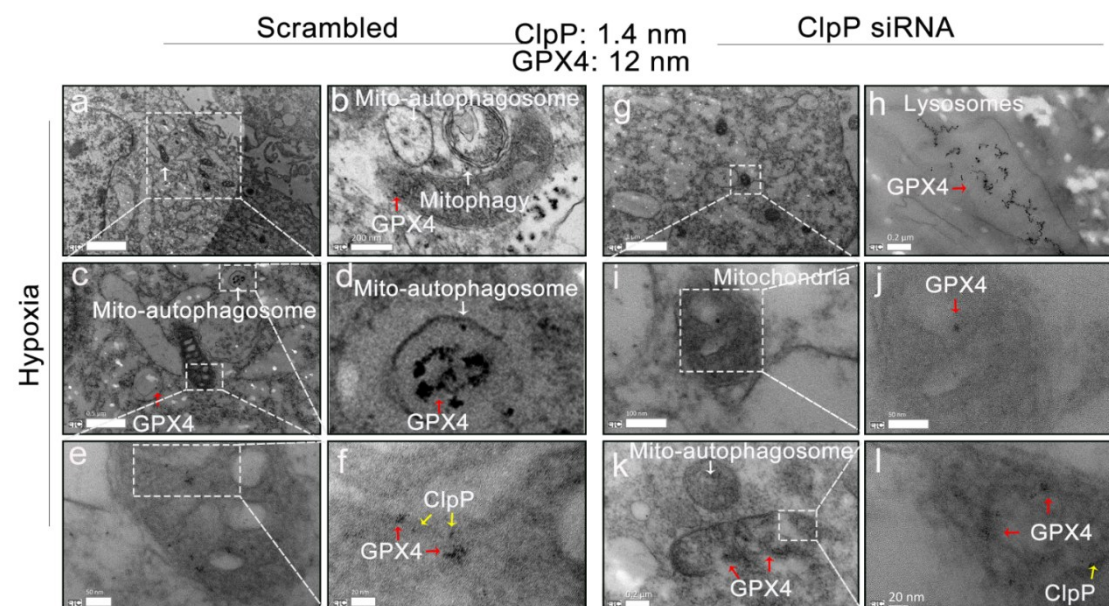
B) Malondialdehyde (MDA) analysis in ClpP siRNA treated RCC cells compared to the vehicle group. (** $p < 0.01$ and **** $p < 0.0001$).

C) The LDH (lactate dehydrogenase) cytotoxicity assay was conducted on RCC cells treated with NC (negative control), ClpP siRNA, scrambled (vector control), Erastin, Fer1, ClpP siRNA + Fer1, or vehicle group. (**** $p < 0.0001$).

D) Proliferative activity was measured using cell counting kit-8(CCK-8) in RCC cells transfected with NC, ClpP siRNA, scrambled, Erastin, Fer1, ClpP siRNA + Fer1, or vehicle group and cultured for 24 hours after hypoxic challenge. (**** $p < 0.0001$).

Electron microscopy provided additional insights into mitochondrial morphology in ClpP-knockdown cells. In control RCC cells under hypoxic conditions, mitochondria displayed structural integrity, as evidenced by elongated and reticular morphologies (**Figure 4-17A a,b**). In contrast, ClpP-depleted cells showed a marked shift towards spherical, fragmented mitochondria, indicative of mitochondrial damage under stress conditions (**Figure 4-17A i-l**). Immunogold labeling further confirmed the localization of GFP-tagged ClpP and GFP-GPX4 on the outer mitochondrial membrane of control cells, suggesting an intact mitophagy process (**Figure 4-17A c-f**). However, in ClpP-knockdown cells, GFP-GPX4 signals within mitochondria decreased and were predominantly redistributed within lysosomes, indicating disrupted mitophagy and increased lysosomal degradation (**Figure 4-17A g-l**). Additionally, GFP-ACSL4, a pro-ferroptosis marker, was localized at regions of ruptured mitochondrial membranes and lysosomal interfaces, reinforcing the hypothesis that ClpP depletion exacerbates ferroptosis by failing to inhibit ACSL4-mediated lipid peroxidation (**Figure 4-17B a-d**).

A



B

ClpP: 1.4 nm ClpP siRNA
ACSL4: 12 nm

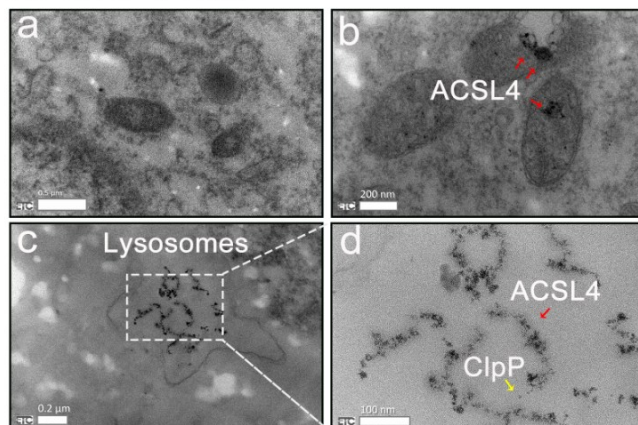


Figure 4-17. (A, B) Mitochondrial ultrastructure in Scrambled and ClpP siRNA treated RCC cells exposed to 24hr of Hypoxia was analyzed by transmission electron microscopy (TEM). The targeted use of antibodies in gold nanoparticles enables the ultrasensitive detection of the relationship between ClpP, GPX4 and ACSL4. Red arrows indicate GPX4 or ACSL4 (12nm), yellow arrows indicate ClpP (1.4nm). Mito, mitochondrion.

In conclusion, these findings reveal ClpP as a critical modulator at the intersection of ferroptosis and mitophagy, playing an essential role in RCC cells' response to hypoxic stress. ClpP's regulatory impact on GPX4 and ACSL4, alongside its function in maintaining mitochondrial Fe²⁺ homeostasis, ROS levels, and lipid peroxidation, underscores its role in modulating ferroptosis cell death. Through the preservation of mitochondrial integrity and the facilitation of mitophagy clearance of injured organelles, ClpP assists RCC cells' adaptive resistance to hypoxia-induced ferroptosis. These discoveries position ClpP as a candidate for therapeutic intervention, which could be targeted by means of strategies aimed at increasing ferroptosis sensitivity in RCC cells via the disintegration of ClpP's regulatory network. Further research is necessary to develop ClpP inhibitors that could destabilize RCC's metabolic adaptation, which would lead to the development of new treatments that take advantage of RCC's weaknesses in ferroptosis defence.

4.3 DISCUSSION

Our study gives significant knowledge of the important role of ClpP in cellular adaptation to hypoxia, the maintenance of the quality control of mitochondria, and the inhibition of ferroptosis in RCC cells.^{179,182} Hypoxia is a distinguishing feature of solid tumours, especially in RCC, because of the insufficient oxygen supply that results from the irregular vascularization and the high rate of proliferation of cells.^{25,271} In these hypoxic conditions, RCC cells need to switch on different adaptive pathways to survive and to proliferate.¹⁰⁸ Our results reveal the overexpression of ClpP, BNIP3, LC3A/B, and GPX4, and the nuclear translocation of HIF-1 α in reaction to hypoxic stress. In summary, these molecular events show that ClpP is the key player in the protection of mitochondria, the expression of hypoxia-induced genes, and the prevention of ferroptosis, which is a kind of iron-dependent oxidative cell death. These data reinforce the fact that ClpP is a crucial factor in the bioenergetic homeostasis of the cell and in the assistance of RCC cells to avoid the cell death mechanisms that are activated by oxidative stress in a low-oxygen microenvironment, making ClpP a promising therapeutic target for RCC treatment.

4.3.1 ClpP as a Regulator of Mitophagy and Mitochondrial Quality Control

Our findings suggest that ClpP functions as a vital regulator of mitophagy in RCC cells, an autophagic process selectively targeting damaged mitochondria for degradation to maintain cellular homeostasis under hypoxic conditions.¹⁶⁷ This is evidenced by positive interactions and correlations between ClpP, BNIP3, and LC3A/B under hypoxic conditions, implying ClpP's role in facilitating mitochondrial turnover through BNIP3- and LC3A/B-mediated autophagic pathways.^{265,283} The co-localization of ClpP, BNIP3, and LC3A/B with mitochondrial markers supports ClpP's active role in promoting mitochondrial degradation, especially in hypoxic environments where mitochondrial integrity is critical for cell survival. Functional assays support this hypothesis, showing that ClpP depletion disrupts mitophagy, leading to mitochondrial fragmentation and membrane depolarization, compromising mitochondrial function. ClpP's role in sustaining mitophagy flux suggests that targeting ClpP could impair RCC cells' ability to clear damaged mitochondria, thus disrupting cellular homeostasis and making RCC cells vulnerable to stress-induced apoptosis.

The critical role of ClpP in maintaining mitochondrial quality control extends beyond its impact on mitophagy.²⁸⁴⁻²⁸⁶ In our study, ClpP depletion resulted in pronounced mitochondrial

dysfunction, including reduced mitochondrial membrane potential, diminished ATP production, and enhanced mitochondrial fragmentation. These structural and functional mitochondrial disruptions underscore ClpP's role in preserving mitochondrial bioenergetics under hypoxic stress, crucial for sustaining RCC cell proliferation in the hostile tumour microenvironment.^{86,287,288} Previous studies have also highlighted the importance of mitochondrial stability in supporting RCC cell survival under hypoxic conditions, reinforcing the view that ClpP plays an essential role in protecting mitochondrial function.²⁸⁹⁻²⁹¹ The loss of mitochondrial integrity in ClpP-deficient cells implies that ClpP helps stabilize mitochondrial structure and maintains energy production essential for RCC cell survival.

4.3.2 ClpP's Role in Supporting Hypoxia-Responsive Gene Expression

Our research further suggests that ClpP directly interacts with HIF-1 α , which supports the nuclear translocation of HIF-1 α and the activation of hypoxia-inducible genes. Protein docking analyses and co-immunoprecipitation experiments validated a high-confidence interaction between ClpP and HIF-1 α , suggesting that ClpP may function as a molecular stabilizer of HIF-1 α . This regulatory mechanism potentially allows RCC cells to adapt to oxygen deprivation by modulating gene expression that promotes cell survival and growth.^{279,292} Given HIF-1 α 's established role in driving hypoxia-induced cellular adaptation in RCC^{123,126,292}, our data imply that ClpP plays a fundamental role in fine-tuning the hypoxia response pathway, supporting hypoxia-driven oncogenic signalling in RCC. This ClpP-mediated enhancement of HIF-1 α activity could represent a crucial adaptation that enables RCC cells to proliferate within the oxygen-limited core of the tumour microenvironment.⁴²

In addition to modulating gene expression, ClpP's interaction with HIF-1 α highlights its role in influencing metabolic reprogramming within RCC cells under hypoxic conditions. By stabilizing HIF-1 α , ClpP likely supports the shift to glycolytic metabolism, a common adaptation of cancer cells to hypoxia known as the Warburg effect.¹³⁰ As hypoxia drives metabolic alterations that favor glycolysis over oxidative phosphorylation,^{77,90} ClpP's regulatory effect on HIF-1 α implies a role in maintaining RCC cell metabolic flexibility. These observations suggest that therapeutic strategies targeting ClpP may disrupt RCC cells' hypoxia-driven metabolic adaptations, potentially limiting their survival in the tumour microenvironment.

4.3.3 ClpP's Role in Inhibiting Ferroptosis and Enhancing RCC Cell Viability

Our study also underscores ClpP's significant role in inhibiting ferroptosis, an iron-dependent

form of cell death characterized by excessive lipid peroxidation. The positive correlation between ClpP and GPX4, an enzyme essential in neutralizing lipid peroxides, alongside an inverse correlation with ACSL4, a pro-ferroptosis enzyme, suggests that ClpP actively shields RCC cells from ferroptosis under hypoxic conditions.^{187,206} GPX4 plays a vital role in preventing lipid peroxidation and, thus, ferroptosis, while ACSL4 promotes lipid peroxidation, sensitizing cells to ferroptosis^{184,216,293}. Our data demonstrate that ClpP knockdown leads to elevated levels of reactive oxygen species (ROS), increased lipid peroxidation, and intracellular iron accumulation, all of which are hallmarks of ferroptosis.²⁰⁷ This shift towards ferroptosis upon ClpP depletion was further validated using ferroptosis inhibitors, which effectively mitigated cell death in ClpP-deficient cells.

The role of ClpP in suppressing ferroptosis may serve as a protective mechanism, allowing RCC cells to withstand the oxidative stress and nutrient deprivation characteristic of the tumour microenvironment. By maintaining GPX4 activity and downregulating ACSL4, ClpP enables RCC cells to evade ferroptosis, thus promoting cellular viability. These results suggest that targeting ClpP in RCC may render tumour cells more susceptible to ferroptosis, providing a novel avenue for therapeutic intervention. Previous studies have indicated that disrupting ferroptosis resistance mechanisms in RCC cells can significantly reduce their survival,^{195,294,295} and our findings reinforce this potential therapeutic strategy.

4.3.4 Therapeutic Potential of ClpP Inhibition in RCC

Our study indicates that ClpP might be a therapeutic target for RCC due to its various regulatory roles in hypoxia adaptation, mitochondrial function maintenance, and ferroptosis suppression. The role of ClpP on mitophagy and mitochondrial integrity in RCC cells is a hint of a possible strategy for ClpP targeting that can result in the removal of RCC cells' ability to recycle damaged mitochondria, thereby disrupting the cellular energy production and leading to cell death. Since ClpP also has the function of stabilizing HIF-1 α and facilitating ferroptosis resistance, the inhibition of ClpP could be the trigger for the simultaneous suppression of multiple RCC survival pathways. The multifactorial method may be the superior one since it would result in RCC cells being more sensitive to oxidative stress, metabolic stress, and cell death mechanisms induced by the hypoxic tumour microenvironment.

4.3.5 Future Directions and Limitations

For the purpose of examining ClpP's therapeutic potential in RCC, the next research should

investigate the molecular mechanisms of ClpP's associations with mitophagy and ferroptosis-regulating proteins and its downstream signalling pathways. Also, the development of specific ClpP inhibitors would allow researchers to study the impact of ClpP inhibition on RCC cells in preclinical models. By characterizing ClpP's interactions with other proteins involved in autophagy and ferroptosis, researchers can comprehensively understand ClpP's role in RCC cell survival. Furthermore, assessing the effects of ClpP inhibition on RCC progression *in vivo* would offer valuable insights into its therapeutic potential and inform the development of targeted treatments for RCC.

A limiting factor of our study is the possibility of ClpP's regulatory functions being different across the RCC cell lines and tumour samples from the patient. Because RCC is characterized by a high degree of variability in its genetic and molecular features, it is necessary to conduct further research to study the function of ClpP in different RCC subtypes and to check the potential different responses to ClpP-targeted therapies. Another point is that our study was mainly focused on the role of ClpP in hypoxic conditions, but the tumour microenvironment is also influenced by factors such as nutrient deprivation and immune cell infiltration. Investigating how these factors interact with ClpP-mediated pathways could provide a more comprehensive understanding of ClpP's role in RCC and its potential as a therapeutic target.

4.3.7 Conclusion

In the end, this study emphasizes the complex role of ClpP in RCC, thus demonstrating its significance in the regulation of mitophagy, the promotion of hypoxia-induced gene expression, and the inhibition of ferroptosis. ClpP's function in ensuring mitochondrial quality control during hypoxia in RCC cells facilitates the process of getting rid of damaged mitochondria and thereby mitochondrial function can be maintained, which is crucial for survival in the tumour microenvironment. Along with that, ClpP through its interaction with HIF-1 α modulates the hypoxia-responsive gene expression which enhances RCC cells' ability to adapt to the low oxygen conditions. Furthermore, ClpP's ferroptosis inhibition represents an adaptive means by which RCC cells repel oxidative stress, thereby boosting their survival. These highlight that ClpP is a potentially interesting target for RCC cancer therapy, and this may involve breaking the very setup of the unknown RCC adaptive mechanisms, making tumour cells more prone to ferroptosis, and hence getting better diagnostic outcomes.

CHAPTER FIVE:

INVESTIGATION OF CANCER BIOLOGY AND BEHAVIOR IN RCC CELLS – *IN VITRO* AND *IN VIVO*

5.1 INTRODUCTION

Renal cell carcinoma (RCC) poses a significant global health challenge due to its high metastatic potential and poor response to standard treatments, accounting for a large proportion of kidney tumors.¹³⁸ RCC pathogenesis is driven by a complex interplay of metabolic, epigenetic, and genetic factors that promote tumor growth, survival, and metastasis.⁴⁸ A thorough understanding of the underlying molecular mechanisms is crucial for developing targeted therapies to address RCC's challenges, particularly its metabolic adaptability and resistance to cell death.²⁹⁶ Mitochondrial dynamics and related stress-response mechanisms are key regulators of cancer cell adaptability and malignancy in RCC progression.^{63,75}

Crucially important organelles for homeostasis and the generation of cellular energy are mitochondria. Essential processes linked to the pathogenesis of renal cell carcinoma (RCC) are mitophagy, control of oxidative stress, and apoptosis.²⁷² By creating an environment that favors continuous growth, evasion of death, and metastatic spread—that is, by means of which oncogenic transformation is facilitated—disruption in mitochondrial function can lead to tumour progression.^{140,144} Evaluating mitochondrial quality calls for the mitochondrial protease caseinolytic P (ClpP). Part of the mitochondrial proteostasis network, ClpP is essential for the selective destruction of aberrant or damaged proteins, hence maintaining mitochondrial integrity.^{101,112} In the demanding setting of renal cell carcinoma, defined by metabolic reprogramming and oxidative stress, this capacity is absolutely vital.^{297,298} Cellular homeostasis depends on ClpP, but, its exact function in RCC carcinogenesis and cancer cell dynamics is yet unknown.^{108,129}

Recent research emphasizes ClpP's several functions in cancer biology by showing its involvement in cellular motility, invasibility, and metabolic adaptation.^{71,74} Studies on several cancer types show that ClpP increases tumour cell survival under metabolic and oxidative stress mostly by controlling mitophagy, the selective destruction of damaged mitochondria by a separate autophagic pathway.^{71,299} This approach maintains mitochondrial activity and shields cancer cells against the negative effects of too much reactive oxygen species (ROS) buildup.^{152,300} ClpP is related to the control of ferroptosis, a regulated kind of cell death defined

by iron-dependent lipid peroxidation.²⁰⁷ Resistance to ferroptosis in renal cell carcinoma (RCC) is interesting since cellular modifications usually promote survival under unfavorable conditions, therefore stressing the crucial role of ClpP in carcinogenesis.²⁸⁸

Through *in vitro* and *in vivo* experimental models, this work investigated the function of ClpP in renal cell carcinoma (RCC) thereby addressing all sides of the research. Using RCC cell lines with genetically changed ClpP expression levels reflecting tumour aggressiveness, this study investigated the effects of ClpP activity on important cellular activities including proliferation, migration, and invasion. Our results show that whilst mitophagy is raised and susceptibility to ferroptosis is lowered, ClpP overexpression increases RCC cell motility and invading potential. Significant modifications in these processes resulted from the suppression of ClpP by short hairpin RNA (shRNA) technology, therefore underscoring the critical function of ClpP in preserving tumour cell resilience under metabolic and oxidative stress.

In cancer research, short hairpin RNA (shRNA) technology is a useful technique for precisely silencing genes, therefore enabling the study of their functional involvement in carcinogenesis, progression, and treatment resistance. To form stem-loop structures, shRNAs are delivered into cancer cells via viral vectors, such as lentiviruses, which are processed by the host cell's RNA interference machinery into active small interfering RNAs (siRNAs), hence generating stem-loop structures.³⁰¹ These siRNAs then control translation or cause mRNA transcripts to degrade, therefore modulating gene expression. Tumour models employ ShRNA-mediated knockdown to examine the roles of oncogenes or tumour suppressors in important processes like cell proliferation, apoptosis, invasion, and metastases. ShRNA libraries also enable high-throughput screening to find new cancer vulnerabilities, usually exposing hitherto unidentified genes vital for tumour cell survival in response to stressors including hypoxia or chemotherapy.²³⁷ While shRNA presents great potential for the identification of therapeutic targets, off-target effects and variability in knockdown efficacy demand careful experimental validation and correction. By use of *in vivo* tumour models, these tools enable researchers to investigate gene function within the tumour microenvironment and systemic connections, therefore enhancing the preclinical development of targeted medicines.³⁰¹

Further, *in vivo* studies employing xenograft models demonstrated that ClpP depletion results in attenuated tumour growth and reduced metastatic potential, providing strong evidence of its role as a driver of RCC progression. These findings were supported by analyses of signalling

pathways downstream of ClpP, which revealed its influence on key regulatory networks involved in mitochondrial dynamics and cellular stress responses. Collectively, our study positions ClpP as a central regulator of RCC tumourigenesis, with implications for its potential as a therapeutic target.

The xenotransplantation of tumour cells into athymic nude mice has emerged as a cornerstone methodology in oncological research, facilitating the *in vivo* recapitulation of tumourigenesis, progression, and therapeutic responsiveness under physiologically relevant conditions. Nude mice, characterized by a genetic mutation leading to a congenitally absent thymus and, consequently, a profound deficiency in T-cell-mediated immunity, serve as an indispensable host for xenografts, particularly of human origin, due to their inability to mount an immune rejection.³⁰² The preparation of tumour cell suspension involves meticulous optimization of cell concentration, viability, and the medium composition to ensure robust engraftment. These cells are subsequently introduced into the host via subcutaneous, intraperitoneal, or orthotopic injection routes, with each modality tailored to the experimental aims and the tumour's tissue of origin.

In the first therapeutic efficacy studies, subcutaneous injections are very useful because of their technical simplicity and simplicity of tracking tumour development kinetics. By means of the injection of tumour cells into the anatomical region corresponding to their tissue of origin, orthotopic transplantation offers a more biologically true model of tumour-stroma interactions, local invasion, and metastatic dissemination. In the study of complicated tumour behavior and pharmacodynamic assessments, this approach has become somewhat well-known. Because of its similarities to peritoneal carcinomatosis, the intraperitoneal method is sometimes used in studies of ovarian and gastrointestinal tumours.

Although this strategy is pragmatic, it requires strict respect to ethical guidelines including the application of humane endpoints and improved analgesic techniques as advised by the National Research Council (2011).³⁰³ Moreover, exact animal care and procedural consistency are essential for assuring experimental reproducibility and limiting variability resulting from elements like faults in cell preparation or discrepancies in injection sites. Real-time observation of tumour dynamics made possible by advances in imaging technology improves the translational value of this model in preclinical cancer research, therefore influencing its application continuously.

The data offer strong proof that ClpP is a necessary regulator of RCC growth. ClpP overexpression increases the migratory and invasive capacity of RCC cells, promotes mitophagy, and reduces sensitivity to ferroptosis, hence fostering tumour growth and progression. On the other hand, ClpP depletion compromises these adaptive systems, leading to lower cellular motility, less invasive potential, and a notable slowing down of *in vivo* tumour development. These results stress ClpP's possible therapeutic target as well as its functional relevance in renal cell cancer biology. We hypothesize that ClpP modulates critical oncogenic pathways in renal cell carcinoma (RCC), thereby advancing our understanding of RCC pathophysiology and uncovering novel therapeutic targets for intervention in this challenging malignancy.

5.1.1 Specific objectives include:

Objective 1: To Evaluate the Impact of ClpP Modulation on RCC Cell Behavior *In Vitro*:

- **Establish RCC Cell Models:** Generate and validate RCC cell models with altered ClpP expression using lentiviral transfection systems, including overexpression (oe-ClpP) and knockdown (sh-ClpP).
- **Evaluate Cell Behavior:** Assess the effects of ClpP modulation on cell motility, invasion, and related behaviors using wound healing and transwell migration assays, with a focus on underlying molecular mechanisms.

Objective 2: To Assess the Role of ClpP in Tumour Growth and Progression *In Vivo*:

- **Develop Xenograft Models:** Create a xenograft mouse model by injecting RCC cells with modified ClpP expression to investigate tumor formation and growth under overexpression or suppression of ClpP.
- **Analyze Tumor Progression:** Measure tumor volume and mass, correlating ClpP expression levels with tumor growth to determine its functional role *in vivo*.

Objective 3: To Elucidate the Relationship Between ClpP, Mitophagy, and Ferroptosis in RCC:

- **Examine Co-localization:** Use immunofluorescence to analyze ClpP expression alongside mitophagy markers (BNIP3, HIF-1A) and ferroptosis markers (GPX4, ACSL4) in RCC tissues.
- **Measure Biochemical Markers:** Assess ferroptosis-related biochemical markers

such as malondialdehyde (MDA) levels and Fe^{2+} concentrations to evaluate ClpP's regulatory effects on mitophagy and ferroptosis.

Objective 4: To Investigate the Mechanistic Interplay Between ClpP Expression and RCC Cell

Survival Pathways:

- Explore how ClpP modulates critical cellular mechanisms such as mitochondrial quality control and oxidative stress resistance, focusing on its dual role in enhancing mitophagy and suppressing ferroptosis.
- Provide mechanistic insights into how these pathways contribute to RCC cell adaptability, invasiveness, and tumour progression, laying the groundwork for potential therapeutic interventions targeting ClpP.

5.2 RESULTS

5.2.1 In Vitro Analysis: Impact of Modified ClpP Expression on RCC Cell Behavior

We investigated the role of ClpP in RCC cell biology and its influence on cancer cell behaviour by creating RCC cell lines with modified ClpP expression levels using lentiviral transfection. Two experimental cell models were created: one exhibiting ClpP overexpression (oe-ClpP) and another with reduced ClpP expression via short hairpin RNA (shRNA-ClpP). Western blot and quantitative reverse transcription PCR (qRT-PCR) assays validated the desired overexpression or knockdown of ClpP in the corresponding transfected cells (**Figure 5-1A and B**).

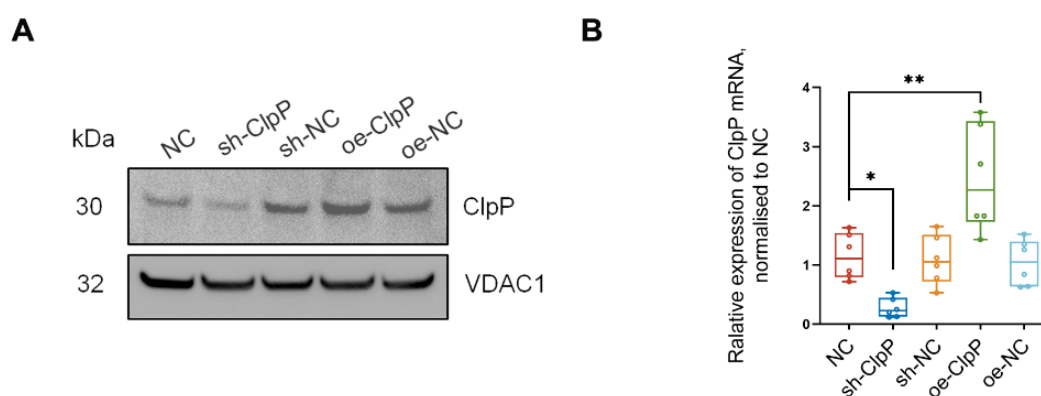


Figure 5-1. illustrates the lentiviral transfection of Renal Cell Carcinoma (RCC) cells

A) Western blotting was employed to investigate the construction of RCC cells transfected with sh-ClpP, sh-NC, oe-ClpP, and oe-NC.

B) Analyzed ClpP mRNA levels in RCC cells transfected with sh-ClpP, sh-NC, oe-ClpP, and oe-NC.

The findings from these modified RCC cell lines were consistent with results from prior siRNA knockdown experiments, wherein ClpP depletion through shRNA resulted in a substantial reduction in BNIP3 expression, a key marker of mitophagy. Conversely, ClpP overexpression significantly upregulated BNIP3 levels compared to control cells, suggesting a regulatory relationship between ClpP and BNIP3 (**Figure 5-2A and B**).

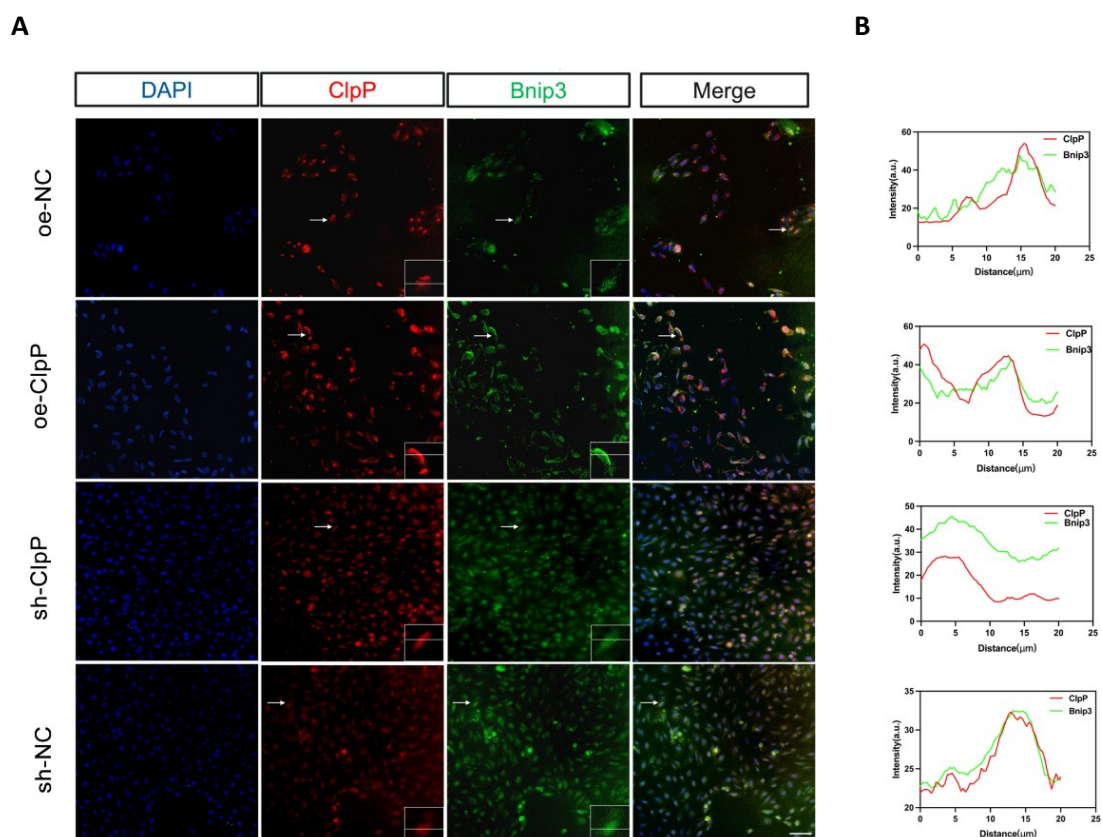


Figure 5-2 the subcellular co-localization of ClpP and Bnip3

A) RCC cells transfected with sh-ClpP, sh-NC, oe-ClpP, and oe-NC were subjected to double immunofluorescence staining with anti-ClpP antibodies (depicted in red) and anti-Bnip3 (in green) at 20x magnification. DAPI (blue) was used to counterstain the nuclei. White arrows indicate RCC cells where co-localization of ClpP and Bnip3 occurs. (Scale bars, 20μm)

B) Quantified the immunofluorescence intensity about the distance along the white solid lines drawn across the selected areas for Bnip3 (green) and ClpP (red) in lentivirus-transfected cells. This reflects the same cell's intrinsic ClpP (red line) and Bnip3 immunofluorescence values (green line). Statistical

Subsequently, we assessed the impact of altered ClpP expression on RCC cell migratory and invasive capabilities, employing wound healing and transwell migration assays to quantify these behavioral changes. The results demonstrated that ClpP knockdown severely impaired both migration and invasion, as cells exhibited reduced movement and invasiveness. In contrast, ClpP overexpression markedly enhanced these abilities, underscoring ClpP's critical role in modulating the motility and invasive potential of RCC cells (**Figure 5-3A-E**). Collectively,

these data highlight the importance of ClpP in RCC cell behavior regulation, wherein reduced ClpP expression dampens RCC cell movement, while elevated ClpP expression promotes aggressive cellular characteristics.

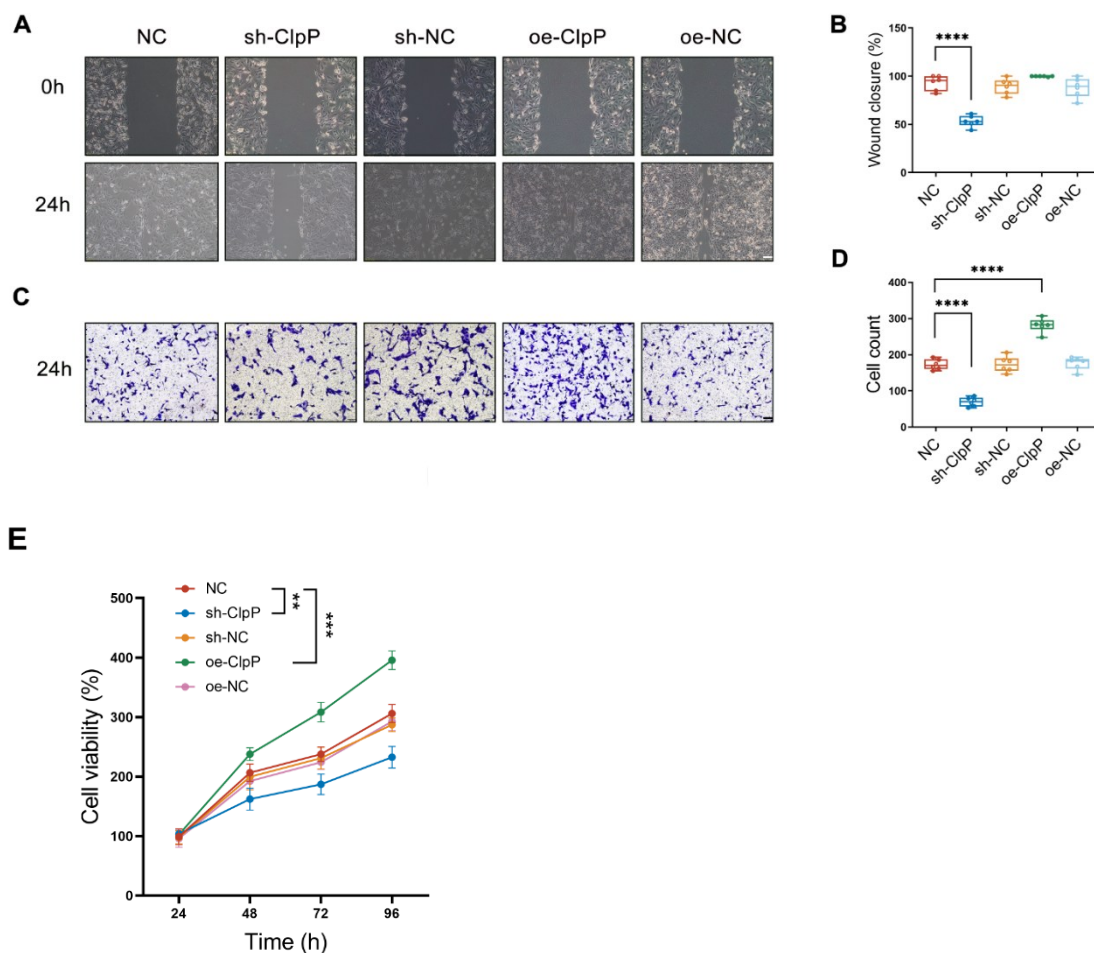


Figure 5-3. Effects of ClpP modulation on migration, invasion, and viability of RCC cells both *in vivo* and *in vitro*.

A, B) Analysis of RCC cell migration influenced by ClpP overexpression and silencing. (sh-, short hairpin RNA; oe-, overexpression. ****p < 0.0001)

C, D) Transwell assay for assessing RCC cell invasion capabilities with ClpP overexpression and downregulation. (****p < 0.0001)

E) Proliferation activity measured using cell counting kit-8 (CCK-8) in RCC cells transfected with ClpP-shRNA or control (NC) and cultured for 24, 48, 72, 96 hours. (**p < 0.01 and ***p < 0.001).

5.2.2 In Vivo Analysis: ClpP's Role in Tumour Progression Using an RCC Xenograft Model

To further investigate ClpP's impact on tumour growth and progression in an *in vivo* context, we utilized a renal cell carcinoma (RCC) xenograft model in BALB/c nude mice. RCC cells transfected with either oe-ClpP, oe-ClpP-Blank, sh-ClpP, or sh-ClpP-Blank, along with a PBS control group, were subcutaneously injected into the mice (**Figure 5-4A, B**).

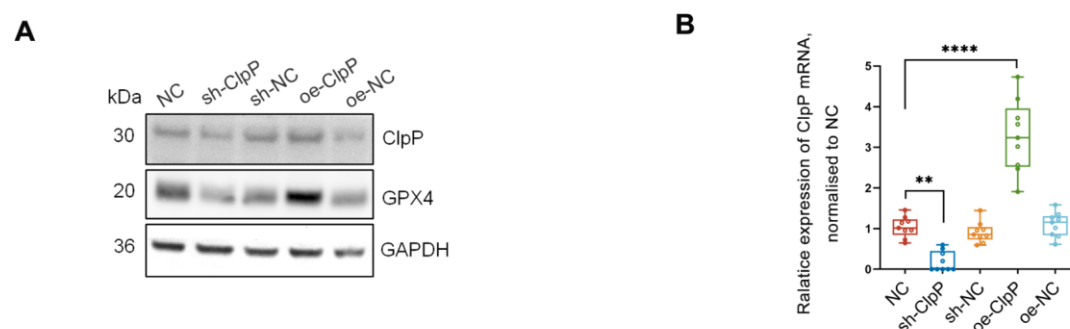


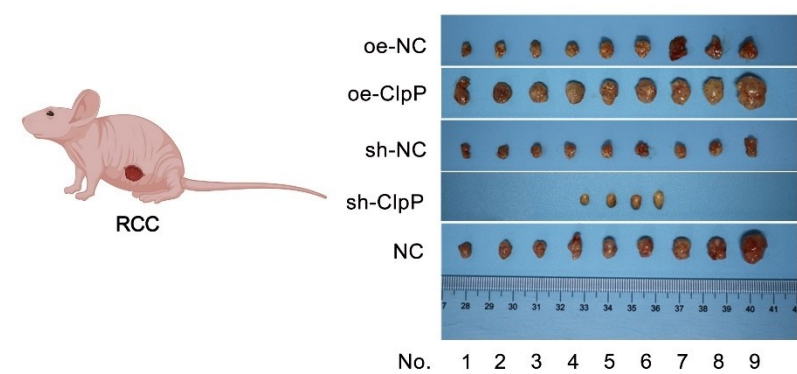
Figure 5-4. The RNA and protein expression levels in renal cell carcinoma (RCC) cells were analyzed to illustrate the effects of lentiviral transfection.

A) The ClpP and GPX4 expression of tumor tissues transfected with sh-ClpP, sh-NC, oe-ClpP, and oe-NC was analyzed through Western blotting.

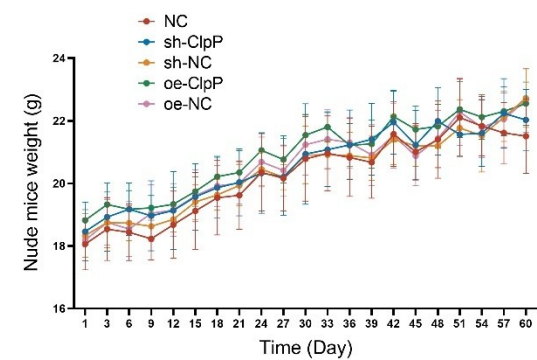
B) Analyzed ClpP mRNA levels in tumor tissues transfected with sh-ClpP, sh-NC, oe-ClpP, and oe-NC.

Analysis of tumour growth revealed that mice injected with ClpP-overexpressing RCC cells (oe-ClpP group) developed significantly larger tumours compared to the control groups (n=9/group). In contrast, the sh-ClpP group, wherein ClpP expression was downregulated, exhibited a notable reduction in tumour mass, with successful tumour formation observed in only 4 out of 9 mice, indicating a substantial inhibition of tumour growth due to ClpP knockdown (**Figure 5-5A-D**). These results suggest that ClpP overexpression drives tumour progression, whereas ClpP knockdown effectively suppresses tumour growth, underscoring the functional importance of ClpP in RCC tumourigenesis.

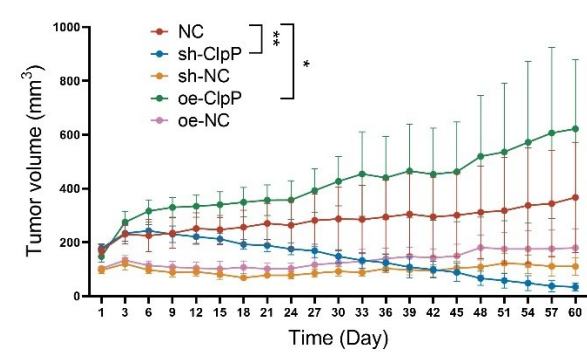
A



B



C



D

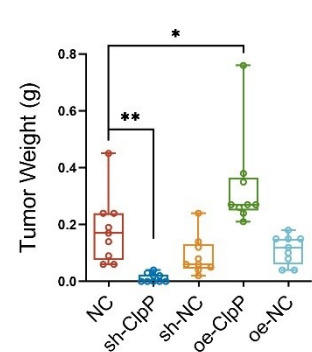


Figure 5-5. The Effect of Altered ClpP Expression on RCC Cell Behavior *in Vitro* Analysis
Representative data in mice bearing RCC xenografts at day 60 post-injection, including images (A) of changes in nude mouse weight (B), tumor growth curves (C), and tumor weights (D) in cell-derived xenografts developed from RCC cells with ClpP overexpression and downregulation.

5.2.3 ClpP Expression and its Association with Ferroptosis and Mitophagy Markers in Tumour Tissues

To gain further insight into the relationship between ClpP expression and key cellular processes such as ferroptosis and mitophagy, we conducted double immunofluorescence staining on the xenograft tumour tissues. The staining analysis revealed a high degree of co-localization and synchronous expression of ClpP with BNIP3 and GPX4, both of which are crucial markers for mitophagy and antioxidative defense, respectively. In contrast, ClpP exhibited a poor correlation with ACSL4, a pro-ferroptosis enzyme, indicating an inverse relationship between ClpP and ferroptosis induction (Figure 5-6).

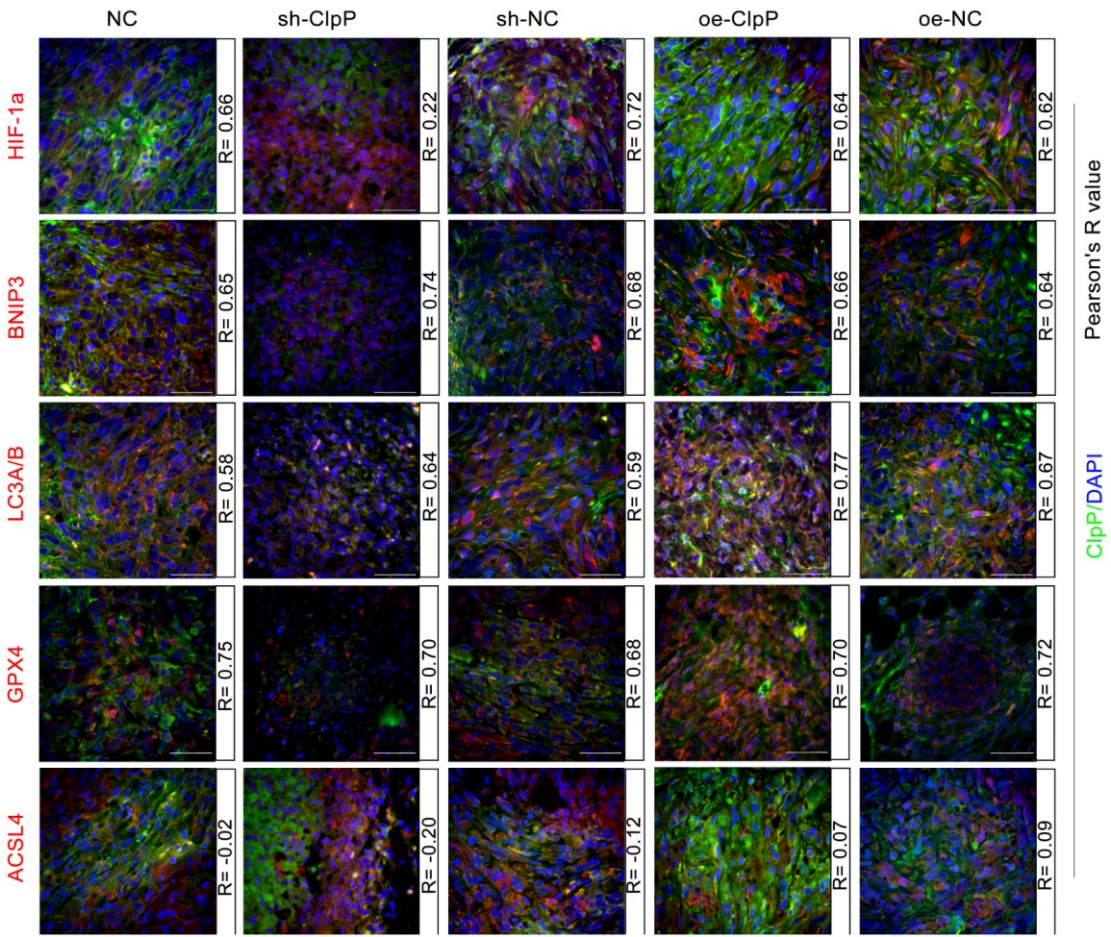


Figure 5-6. Double immunofluorescence staining revealed the co-localization and expression synchronicity between ClpP and Bnip3, GPX4, LC3A/B, ACSL4 in tumor tissues transfected with sh-ClpP, sh-NC, oe-ClpP, and oe-NC.

To explore this further, we assessed ferroptosis activity in the tumour tissues by measuring levels of malondialdehyde (MDA), an indicator of lipid peroxidation, and Fe^{2+} , a key contributor to ferroptosis. Predictably, tumour tissues from the sh-ClpP cohort demonstrated significantly elevated Fe^{2+} and MDA levels, suggesting enhanced ferroptosis activity, whereas tumours from the oe-ClpP group exhibited reduced Fe^{2+} and MDA levels, indicative of diminished ferroptosis (**Figure 5-7A and B**). These results collectively support the notion that ClpP overexpression enhances mitophagy while reducing ferroptosis susceptibility, promoting tumour progression. Conversely, ClpP knockdown facilitates ferroptosis and suppresses mitophagy, thereby hindering tumour growth.

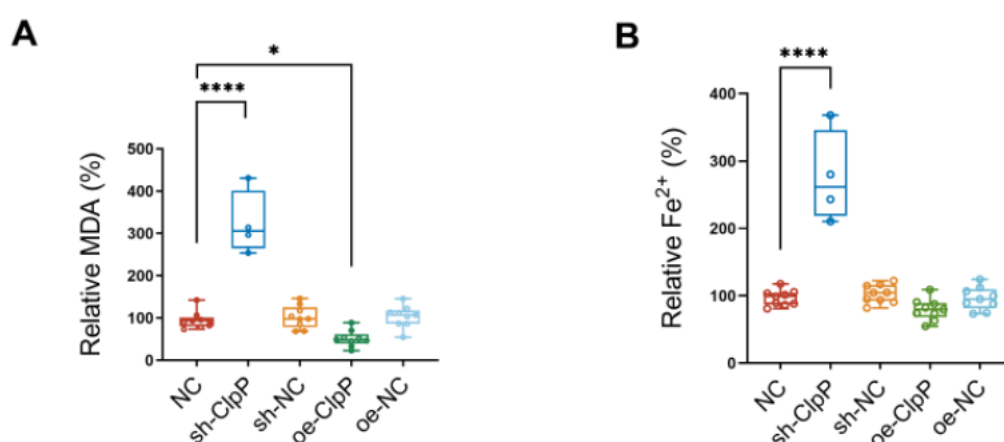


Figure 5-7. MDA (A) and Fe^{2+} (B) content in tumour tissues.

Our *in vitro* and *in vivo* findings underscore the significance of ClpP in regulating tumour growth and renal cell carcinoma behaviour. Inhibiting ferroptosis, increased ClpP expression enhances the migratory and invasive abilities of RCC cells, promotes tumour progression, and facilitates mitophagy. Conversely, the depletion of ClpP indicates that it is a crucial regulator in the adaptability and survival mechanisms of RCC, as it undermines these cellular functions.

5.3 DISCUSSION

This study emphasises the significant function of caseinolytic protease P (ClpP) in altering renal cell carcinoma (RCC) cell behaviour and tumour advancement.^{51,304} We demonstrated that ClpP expression is a crucial factor influencing RCC cell motility, invasiveness, tumour growth, and the equilibrium between mitophagy and ferroptosis by a comprehensive technique

encompassing both *in vitro* and *in vivo* experiments.^{210,305} These findings provide significant insights into the molecular pathways underlying RCC formation, highlighting ClpP as a potential therapeutic target and biomarker for RCC treatment.

5.3.1 ClpP and RCC Cell Behavior *in Vitro*

Our *in vitro* investigations demonstrated a definitive and strong link between ClpP expression levels and the aggressive behaviour of RCC cells. The overexpression of ClpP (oe-ClpP) significantly improved the migratory and invasive abilities of RCC cells, as demonstrated by quantitative data from wound healing and transwell migration tests. The data indicate that ClpP promotes the motility and invasiveness characteristic of metastatic development. Conversely, the suppression of ClpP expression (sh-ClpP) markedly diminished these capacities, highlighting its critical role in sustaining these aggressive characteristics.

These findings align with other research highlighting the significance of mitochondrial proteostasis in facilitating cancer cell adaptation to fluctuating metabolic and environmental stressors. ClpP's role in preserving mitochondrial function corresponds with its function in proteolytic quality control, facilitating the destruction of impaired mitochondrial proteins, essential for cellular adaptability and survival. The observed increase of BNIP3, a crucial mitophagy marker, in ClpP-overexpressing cells underscores a mechanistic connection. BNIP3's function in facilitating mitophagy, a critical mechanism for the removal of impaired mitochondria and sustaining cellular energy production during stress, seems to be modulated by ClpP.²⁶⁵ In contrast, ClpP knockdown diminished BNIP3 expression, hindering mitophagy and possibly leading to the noted decrease in cellular motility and invasiveness. These findings highlight ClpP's importance in maintaining the functional integrity of RCC cells via mitochondrial quality control.

5.3.2 ClpP and Tumour Progression *In Vivo*

The *in vivo* xenograft experiments further validate the essential role of ClpP in RCC tumourigenesis. Mice injected with ClpP-overexpressing RCC cells (oe-ClpP) exhibited significantly larger tumour volumes compared to controls, while those injected with ClpP-silenced cells (sh-ClpP) displayed markedly attenuated tumour growth. In fact, tumour formation in the sh-ClpP cohort was incomplete, with only a subset of mice developing tumours. These findings highlight the dependency of RCC tumour cells on ClpP for efficient growth and adaptability within the tumour microenvironment.³⁰⁶

The inability of sh-ClpP-transfected cells to sustain robust tumourigenesis may be attributed to a failure to maintain mitochondrial homeostasis. ClpP plays a critical role in proteostasis by ensuring the elimination of dysfunctional proteins, which is vital for sustaining high levels of energy production required for tumour proliferation.³⁰⁷ The loss of ClpP likely disrupts this balance, rendering RCC cells vulnerable to metabolic and oxidative stresses inherent in the tumour microenvironment.

5.3.3 ClpP as a Regulator of Mitophagy and Ferroptosis

The most important finding of this work is the dual function of ClpP in controlling ferroptosis and mitophagy, two opposing cellular processes greatly influencing RCC development. Increased co-localization of ClpP with BNIP3 shown by tumour tissues from ClpP-overexpressing cells confirmed its ability in promoting mitophagy. This mechanism helps defective mitochondria to be recycled, therefore ensuring continuous ATP production and reducing the accumulation of reactive oxygen species (ROS). ClpP improves mitophagy, so permitting the metabolic flexibility of RCC cells and their survival in hypoxic and nutrient-starved conditions usually found in tumours.³⁰⁸

On the other hand, ClpP displayed an inverse relationship with ferroptosis as indicated by its negative association with ACSL4, a pro-ferroptosis biomarker. Iron-dependent lipid peroxidation defines ferroptosis, a new target for cancer treatment since it preferentially kills cancer cells. Reduced levels of lipid peroxidation markers, including malondialdehyde (MDA) and iron (Fe^{2+}), showed that ClpP-overexpressing cells essentially prevent ferroptosis. Two By shielding RCC cells from oxidative damage and hence increasing their invasive and proliferative capacity, this inhibition could offer a survival advantage.³⁰²

On the other hand, the suppression of ClpP (sh-ClpP) raised vulnerability to ferroptosis, as seen by higher MDA and Fe^{2+} in tumours. The interaction of reduced mitophagy and elevated ferroptosis probably compromises cellular homeostasis, which causes the observed decrease in cancer growth. These results identify ClpP as a molecular switch controlling the equilibrium between sensitivity to oxidative stress and mitochondrial quality control, hence fostering RCC cell survival and development.

5.3.4 Clinical Implications and Future Directions

ClpP's dual regulatory action in increasing mitophagy and lowering ferroptosis makes a strong argument for its therapeutic targeting in RCC. ClpP's pro-tumourigenic activities may be disrupted by pharmacological inhibition, sensitising RCC cells to ferroptosis and therefore compromising their mitochondrial quality control systems. Small-molecule inhibitors aiming at ClpP have shown promise in preclinical models of cancer and demand more research in the framework of RCC.³⁰⁹

Furthermore, the regulatory interaction between ClpP and BNIP3 emphasises the larger part mitochondrial dynamics play in the course of cancer. Targeting downstream effectors of ClpP, such BNIP3, or combining ClpP inhibitors with ferroptosis-inducing drugs, may present a synergistic therapeutic approach. Such combinations could take advantage of RCC cells' two weaknesses: ferroptosis could be induced while their mitochondrial activity is disrupted.³¹⁰

Clarifying the upstream signalling pathways controlling ClpP expression and activity in RCC should be the main emphasis of next studies. Development of focused treatments depends on an awareness of the molecular processes controlling its interactions with mitophagy and ferroptosis paths. Moreover, the function of ClpP in various cancer forms should be investigated to ascertain its general importance as a therapeutic target.

5.3.5 Conclusion

Overall, our work identifies ClpP as a fundamental control of RCC cell behaviour and tumour development. ClpP helps RCC cells to adjust to metabolic and oxidative stressors by changing mitophagy and ferroptosis, hence preserving tumour development and invasiveness. ClpP's dual function emphasises its possible therapeutic focus and provides fresh paths of action for RCC treatment. Disturbing ClpP function could compromise mitochondrial quality control and raise RCC cell sensitivity to ferroptosis, hence stopping tumour development. These results provide the foundation for next studies on the molecular network of ClpP and its application in the creation of focused cancer treatments.

CHAPTER SIX

CORRELATION BETWEEN CLPP EXPRESSION AND CLINICAL PROGNOSIS IN RCC PATIENTS

6. 1 INTRODUCTION

6.1.1 Clinical Study Data on Renal Clear Cell Carcinoma

Comprising around 70–80% of all RCC cases, renal clear cell carcinoma (ccRCC) is the most often occurring histological subtype of renal cell carcinoma (RCC).³¹¹ Originating from the epithelial lining of the proximal renal tubules, this malignancy is recognised by its typical transparent cytoplasm, a property ascribed to the accumulation of intracellular glycogen and lipids.³¹² ccRCC presents unique molecular and clinical attributes that separate it from other RCC subtypes, offering both distinct challenges and opportunities in the domains of diagnosis, treatment, and disease management.³¹³

6.1.2 Epidemiology and Clinical Presentation

Renal clear cell carcinoma is a worldwide health issue with somewhat high morbidity and death rates.³¹⁴ About thirty to forty percent of patients with ccRCC show metastatic disease at diagnosis, which adds to their poor prognosis.³¹⁵ With incidence rates rising rapidly, a development partly ascribed to improvements in imaging technologies allowing early identification and lifestyle-related risk factors including obesity, hypertension, and smoking. RCC accounts for 2–3% of all malignancies globally.^{316,317} ccRCC exhibits a notable gender disparity, affecting males approximately twice as often as females, and has a median age of diagnosis around 60 years.³¹⁸

Early on, ccRCC is sometimes asymptomatic and a significant number of cases are found inadvertently during imaging tests done for other disorders. Advanced instances marked by symptoms could show the characteristic triad of haematuria, flank pain, and a palpable abdominal mass. Less than 10% of patients, though, have this trio.³¹⁹ Furthermore, highlighting the systemic character of the disease are systematic symptoms including paraneoplastic syndromes like hypercalcemia, anaemia, and erythrocytosis, which are often linked with ccRCC.

6.1.3 Molecular Characteristics and Pathogenesis

With mutations in the von Hippel-Lindau (VHL) gene playing a crucial role, the aetiology of

ccRCC is intimately related with genetic and molecular changes. About 90% of sporadic ccRCC cases have VHL mutations, which cause the VHL protein to inactivate and thereby stabilise hypoxia-inducible factors (HIFs).³²⁰ These stabilised HIFs induce metabolic reprogramming, cellular proliferation, and overexpression of angiogenesis-related genes including vascular endothelial growth factor (VEGF), hence advancing tumour progression. A trademark resulting from this molecular dysregulation, the hypervascularity of ccRCC provides a therapeutic target for anti-angiogenic drugs.³²¹

Beyond VHL, other genetic and epigenetic changes include mutations in PBRM1, SETD2, and BAP1 help to explain the variation of ccRCC.³²² These changes affect tumour biology, rates of development, and therapy responses, therefore underscoring the complexity of the disease. The interaction of these genetic factors with the tumour microenvironment (TME)—defined by hypoxia, immunological suppression, and metabolic stress—further accelerates ccRCC progression and complicates therapy intervention.^{323,324}

6.1.4 Clinical Staging and Prognosis

The TNM classification system guides the staging of ccRCC in evaluation of tumour size (T), regional lymph node involvement (N), and the existence of distant metastases (M).³²⁵ Originally evaluated using the Fuhrman nuclear grading system, the International Society of Urological Pathology (ISUP) grading system has replaced it for a more thorough assessment of nuclear shape. These methods give a basis for risk categorisation and tailored treatment planning together with patient-specific elements including comorbidities and performance status.

The stage upon diagnosis affects the survival results in CCRCC greatly. Patients with localised disease obtain five-year survival rates over 90%; those with metastatic disease have survival rates less than 10%. Particularly in intermediate- and high-risk situations, the risk of recurrence stays great even after curative resections of localised tumours.

6.1.5 Current Therapeutic Landscape

Targeted treatments and immune checkpoint inhibitors have caused a paradigm change in the therapeutic scene for ccRCC. The cornerstone of ccRCC treatment has been 1,7 anti-angiogenic drugs aiming at the VEGF and mTOR pathways: sunitinib, pazopanib, and everolimus.³²⁶ In advanced illness stages, these treatments provide clinical advantages since they stop

angiogenesis and tumour growth.

More recently, immunotherapy has transformed ccRCC therapy. Often used with VEGF inhibitors, immune checkpoint inhibitors including nivolumab (anti-PD-1) and pembrolizumab (anti-PD-L1) have shown consistent responses and enhanced survival outcomes in a subgroup of patients.³²⁷ Now accepted as first-line treatments for metastatic cancer, combination regimens including nivolumab with ipilimumab (anti-CTLA-4) have synergistic effects addressing both tumour vascularity and immune evasion.³²⁸

6.1.6 Unmet Clinical Needs and Future Directions

Despite these advancements, the management of ccRCC continues to pose significant challenges.³²⁹ Two major obstacles that continue to impede the efficacy of current treatments are tumour heterogeneity and the development of resistance to systemic therapy.³³⁰ The absence of reliable biomarkers for assessing long-term prognosis and treatment response underscores the necessity for further research in this domain.^{331,332} Enhancing outcomes for ccRCC patients is fundamentally reliant on the discovery of novel molecular targets and the incorporation of biomarker-driven strategies into therapeutic interventions.³³³

6.1.7 Expanded Academic Content

Among the innovative areas of research in ccRCC is the focus on mitochondrial proteostasis and its implications for tumour biology. Mitochondria, as central regulators of energy production and cell death pathways, play an indispensable role in cancer progression.^{318,334} Ferroptosis, a regulated form of cell death driven by iron-dependent lipid peroxidation, has garnered attention for its dual role in tumour suppression and resistance. Tumour cells frequently develop mechanisms to evade ferroptosis, allowing survival within the hypoxic and nutrient-deprived conditions of the TME.³³⁵

6.1.8 Wax section assessment

Immunohistochemical images were assessed using a two-stage scoring method, where the total immunohistochemical score is calculated as the percentage of positive cells multiplied by cell staining intensity. Five high-magnification fields (×400) were randomly selected from each section to count positive cells. ClpP protein expression was considered positive if brownish-yellow or brown particles were present in the cell membrane and/or cytoplasm. The percentage of positive cells was scored as follows: ≤5% = 0 points, 6%–25% = 1 point, 26%–50%

= 2 points, >50% = 3 points. Staining intensity was scored as follows: no staining = 0 points, light yellow = 1 point, brown = 2 points, dark brown = 3 points. The total score is determined by multiplying the two individual scores. The resulting total score is classified as follows: 0 is considered negative (-), 1-2 is weakly positive (+), 3-4 is moderately positive (++), and 6-9 is strongly positive (+++). Total scores of 0-2 are defined as low expression, whereas scores of 3-9 are defined as high expression. These scores were independently assessed by two senior pathologists.

Caseinolytic protease P (ClpP), a mitochondrial serine protease, has emerged as a critical player in maintaining mitochondrial quality under cellular stress. Although extensively studied in bacterial systems, the role of ClpP in cancer, particularly in ccRCC, remains poorly understood. Preliminary findings suggest that aberrant ClpP expression may facilitate tumour progression through mechanisms such as metabolic reprogramming and ferroptosis resistance.³³⁶ This study systematically examines ClpP's expression in ccRCC and its associations with clinical outcomes, aiming to elucidate its potential as a prognostic biomarker and therapeutic target.

6.1.8 Specific objectives include (hypothesis):

Objective 1: Evaluate the prevalence and distribution of ClpP expression in renal cell carcinoma (RCC) patients and analyze its relationship with clinicopathological characteristics.

Objective 2: Investigate the correlation between ClpP expression and key clinical indicators associated with aggressive RCC phenotypes.

Objective 3: Assess the prognostic significance of ClpP expression on overall survival (OS) in RCC patients.

6.2 RESULTS

6.2.1 Correlation Between ClpP Expression and Clinical Prognosis in RCC Patients

In light of our comprehensive findings indicating ClpP's critical role in RCC tumour biology and its influence on cell survival, we further explored the potential of ClpP expression as a prognostic marker. To assess this, we examined ClpP expression levels in pathological samples from a cohort of 437 patients diagnosed with ccRCC. Through immunohistochemical analysis, we categorized ClpP expression into three levels—weak, moderate, and strong. Notably, more than half of the cohort, 55.1% (241 out of 437 patients), displayed high levels of ClpP expression, underscoring the prevalence of elevated ClpP in RCC (**Figure 6-1**).

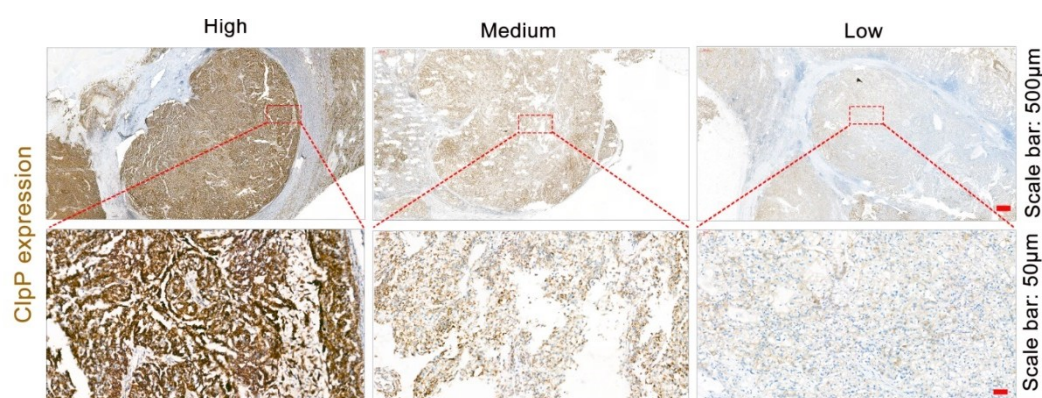


Figure 6-1. Association of ClpP expression with clinical outcomes in RCC patients. Hematoxylin and Eosin (H&E) staining of patient tissues showing varying ClpP expression levels from dark to light. (Scale bars: 500, 50µm)

Our analysis revealed a significant inverse correlation between ClpP expression and intracellular iron accumulation. Specifically, patients with high ClpP expression showed markedly lower levels of intracellular Fe^{2+} (**Figure6-2**). This observation aligns with ClpP's hypothesized role in ferroptosis regulation, as reduced intracellular iron levels are associated with decreased susceptibility to ferroptosis. Thus, elevated ClpP may contribute to ferroptosis resistance, which could support RCC cells' survival and growth within the iron-limited environment of the tumour microenvironment.

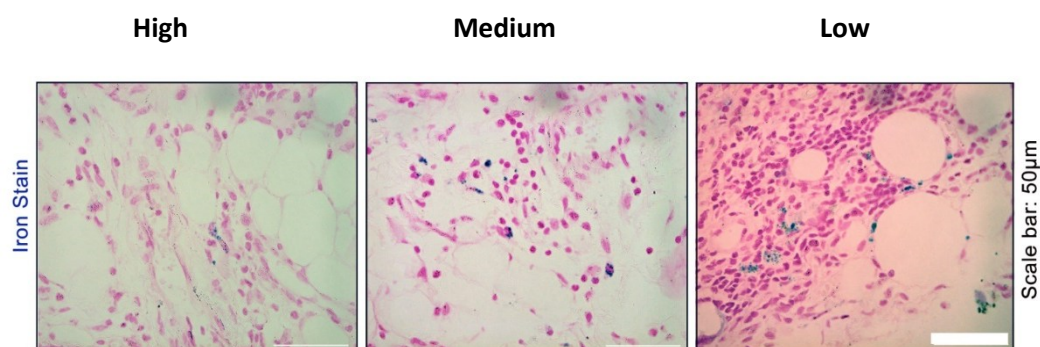


Figure 6-2 The expression changes of iron corresponding to different ClpP expression levels. Blue: Fe immunohistochemical staining (Scale bars, 50µm).

Further analysis examined the relationship between ClpP expression and various demographic characteristics, such as age, gender, and tumour size, among the RCC patients. Interestingly, no significant correlations were found between ClpP expression and these demographic factors ($p > 0.05$), suggesting that ClpP levels are independent of these general patient characteristics. However, ClpP expression was significantly associated with several clinical indicators of more aggressive RCC phenotypes. For instance, higher ClpP expression was correlated with advanced Fuhrman grade (Chi-square = 27.95, $p < 0.0001$), indicating a more aggressive tumour cell morphology. Furthermore, increased ClpP levels were associated with greater tumour invasion (Chi-square = 34.37, $p < 0.0001$), lymph node metastasis (Chi-square = 6.636, $p = 0.01$), distant metastasis (Chi-square = 13.25, $p = 0.0003$), and a higher TNM stage (Chi-square = 38.13, $p < 0.0001$) (**Table 6-1**). These findings underscore ClpP's association with aggressive disease characteristics in RCC.

Table 6-1 Clinical data in patients with different ClpP expression levels.

Characteristic	n	ClpP expression		χ^2	P
		High (n=241)	Low (n=196)		
Gender				0.063	0.802
Male	258	141	117		
Female	179	100	79		
Age (years)				0.168	0.682
≥60	245	133	112		
<60	192	108	84		
Tumor diameter (cm)				0.744	0.389
≥4	278	149	129		
<4	159	92	67		
Fuhrman grade				27.95	<0.0001
G ₁ -G ₂	189	77	112		
G ₃ -G ₄	248	164	84		
Tumor invasion				34.37	<0.0001
T ₁ -T ₂	247	106	141		
T ₃ -T ₄	190	135	55		
Lymph node metastasis				6.636	0.01
N ₀	409	219	190		
N ₁ -N ₂	28	22	6		
Distant metastasis				13.25	0.0003
M ₀	355	181	174		
M ₁	82	60	22		
TNM stage				38.13	<0.0001
I and II	225	92	133		
III and IV	212	149	63		

To further substantiate ClpP's value as a prognostic marker, we conducted a multivariate Cox regression analysis to identify factors independently associated with postoperative overall survival (OS) among RCC patients. The analysis demonstrated that high ClpP expression, along with high Fuhrman grade, deep tumour invasion, lymph node metastasis, distant metastasis, and advanced TNM stage, emerged as independent risk factors associated with poorer postoperative survival outcomes (**P < 0.05, Figure 6-3, Table 6-2**).

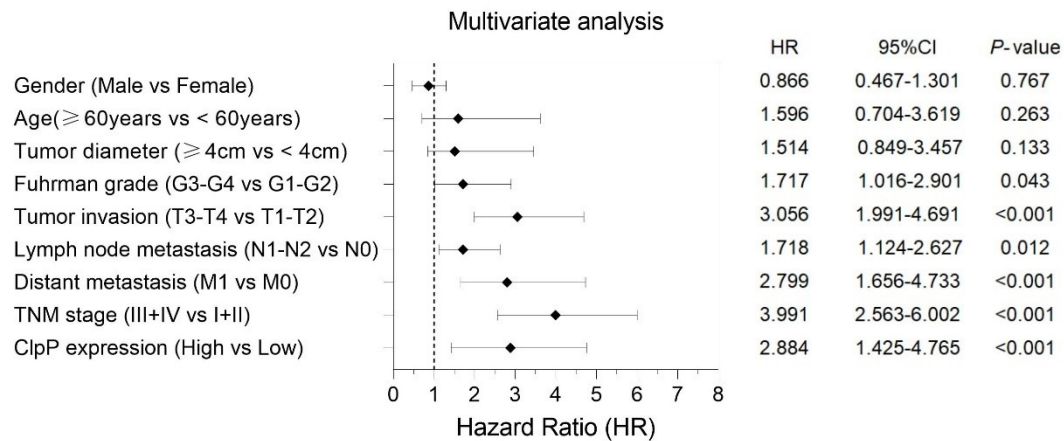


Figure 6-3 Multivariate Cox regression analysis of OS (overall survival) in RCC patients

Table 6-2. Univariable and Multivariable Cox regression analysis of OS (overall survival) in RCC patients

Variable	Univariable		Multivariable	
	HR (95%CI)	P	HR (95%CI)	P
Gender (Male vs Female)	0.924 (0.576-1.336)	0.608	0.853 (0.432-1.312)	0.735
Age(≥ 60 years vs < 60 years)	1.621 (1.035-2.632)	0.027	1.463 (0.693-3.536)	0.235
Tumour diameter (≥ 4 cm vs < 4 cm)	1.823 (1.435-2.547)	<0.001	1.536 (0.767-3.635)	0.145
Fuhrman grade (G3-G4 vs G1-G2)	2.592 (1.632-4.343)	<0.001	1.689 (1.157-2.792)	0.038
Tumour invasion (T3-T4 vs T1-T2)	2.752 (1.243-3.963)	<0.001	3.182 (1.872-4.873)	<0.001
Lymph node metastasis (N1-N2 vs N0)	2.865 (1.474-5.562)	<0.001	1.562 (1.303-2.468)	0.015
Distant metastasis (M1 vs M0)	4.351 (2.852-6.369)	<0.001	2.682 (1.382-4.494)	<0.001
TNM stage (III+IV vs I+II)	3.635 (2.456-5.763)	<0.001	3.862 (2.745-6.124)	<0.001
ClpP expression (High vs Low)	2.536	<0.001	2.678 (1.567-	<0.001

(1.493-4.812)

4.836)

Importantly, RCC patients with high ClpP expression exhibited significantly lower 5-year OS rates compared to those with lower ClpP levels ($p = 0.0005$) (**Figure 6-4**). This association highlights the prognostic relevance of ClpP, indicating that higher ClpP expression correlates with reduced survival likelihood.

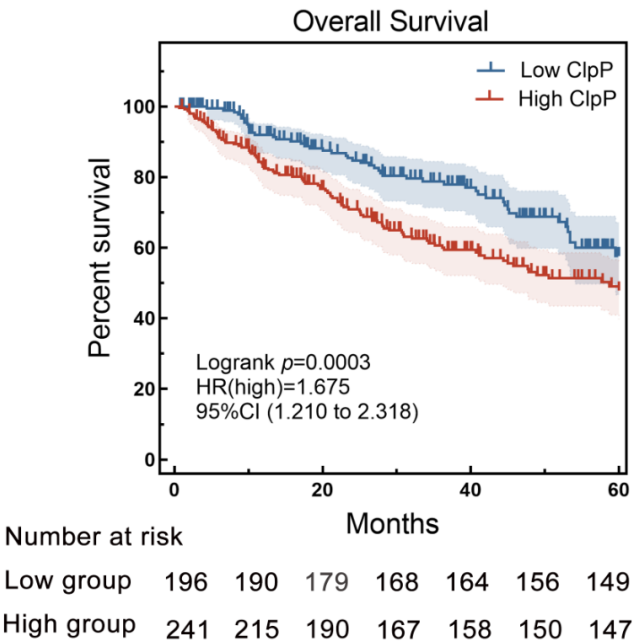


Figure 6-4. ClpP expression levels and 5-year overall survival rate.

In summary, these findings provide compelling evidence that ClpP expression is not only a critical driver of RCC progression but also a valuable prognostic biomarker. High levels of ClpP expression are associated with advanced disease stage, aggressive tumour characteristics, and decreased overall survival, positioning ClpP as a potential target for therapeutic interventions. Further studies could explore the therapeutic potential of targeting ClpP to counteract its effects on RCC progression and improve clinical outcomes for patients with renal cell carcinoma.

6.3 DISCUSSION

6.3.1 Overview of ClpP's Role in RCC Biology

Our study highlights the significant role of caseinolytic protease P (ClpP) in the biology of renal cell carcinoma (RCC),³²³ identifying it as both a contributor to tumour aggressiveness and a prognostic biomarker.³³⁷ ClpP, a mitochondrial protease involved in protein quality control and metabolic regulation,³³⁸ was highly expressed in 55.1% of the RCC cohort, suggesting its pivotal involvement in RCC pathophysiology.^{326,339} This study establishes ClpP as a key player in ferroptosis regulation, tumour progression, and overall patient survival, which opens new avenues for its clinical application as a biomarker and therapeutic target.^{71,340}

6.3.2 ClpP and Ferroptosis Resistance

One of the critical findings of this research is the inverse correlation between ClpP expression and intracellular iron (Fe^{2+}) levels.^{325,341,342} Elevated ClpP expression was associated with reduced intracellular Fe^{2+} accumulation, a hallmark of ferroptosis resistance.³³³ Ferroptosis, an iron-dependent form of regulated cell death, is increasingly recognized for its potential to suppress cancer progression.^{329,343} RCC cells' ability to evade ferroptosis may confer a survival advantage, particularly in the hypoxic and iron-limited tumour microenvironment.^{335,344,345} Our findings indicate that ClpP supports this evasion, potentially through its regulatory influence on mitochondrial metabolism and oxidative stress pathways.

These results align with prior studies indicating that ferroptosis resistance is a feature of aggressive cancers. ClpP's role in this process suggests it acts as a molecular safeguard against ferroptotic cell death, thus promoting tumour survival.³⁴⁶ Future research should aim to delineate the molecular mechanisms by which ClpP mediates ferroptosis resistance, potentially uncovering vulnerabilities that could be therapeutically targeted.

6.3.3 Correlation of ClpP Expression with Aggressive RCC Phenotypes

The association between ClpP expression and aggressive RCC phenotypes is a notable aspect of our study.^{347,348} Elevated ClpP expression was significantly correlated with clinical markers of tumour aggressiveness, including higher Fuhrman grade, increased tumour invasion, lymph

node metastasis, distant metastasis, and advanced TNM stage.^{328,348} These correlations suggest that ClpP may influence processes such as epithelial-to-mesenchymal transition (EMT), invasion, and metastasis, all of which are key drivers of tumour progression.³⁴⁹

ClpP's involvement in mitochondrial function might play a central role in facilitating these aggressive behaviors.^{314,350} As a regulator of mitochondrial proteostasis, ClpP ensures efficient mitochondrial function under stress conditions. Enhanced mitochondrial activity supports the energy demands of tumour cells, particularly during processes like invasion and metastasis.³¹⁴ Additionally, ClpP may modulate signalling pathways involved in cell migration and matrix remodeling, contributing to the invasive phenotype observed in high-ClpP RCC cases.

6.3.4 Independent Prognostic Value of ClpP

Our study further establishes ClpP as an independent prognostic marker for RCC. Multivariate Cox regression analysis revealed that high ClpP expression, along with established clinical parameters such as Fuhrman grade, tumour invasion, and metastatic status, is significantly associated with poorer postoperative overall survival (OS). Patients with elevated ClpP expression had markedly lower 5-year OS rates, reinforcing its role as a predictor of poor prognosis.^{338,351}

From a clinical perspective, these results are really significant. Monitoring ClpP expression levels in RCC patients might help to stratify risk and direct therapy decisions.²¹⁹ To increase their survival chances, patients with high ClpP expression could need more forceful treatment plans and closer observation. Furthermore, combining ClpP evaluation with other molecular and clinical indicators can improve the accuracy of RCC prognosis and treatment customising.²⁶⁶

ClpP presents a potential therapeutic target since it both promotes tumour development and ferroptosis resistance.⁷⁴ Reducing ClpP activity may be able to reverse ferroptosis resistance, hence increasing RCC cell vulnerability to oxidative stress and cell death. When paired with current treatments causing oxidative damage, including tyrosine kinase inhibitors (TKIs) or immune checkpoint inhibitors (ICIs), this kind of strategy could be especially successful.^{264,352}

Moreover, aiming at ClpP could have synergistic effects with newly developed drugs causing

ferroptosis.^{3,167} Recent developments in the generation of ferroptosis inducers show promise in preclinical cancer models; hence, combining these medicines with ClpP inhibitors should increase their therapeutic effect. Furthermore, knowledge of the interaction between ClpP and metabolic pathways in RCC could guide the development of combination treatments leveraging RCC cell metabolic weaknesses.

6.3.5 Broader Implications and Future Directions

Targeting ClpP for therapy has consequences beyond RCC. ClpP's wider relevance in oncology is suggested by its involvement in the pathophysiology of several cancers. Examining ClpP expression and activity in several cancer types could shed important light on its universal target status in cancer treatment.³⁵³

Development of specific ClpP inhibitors and evaluation of their efficacy in preclinical RCC models should take front stage in future studies. Given ClpP's critical importance in mitochondrial function, studies should also investigate the safety profile of such inhibitors. Furthermore, improving patient selection and treatment results could be looking at biomarkers that indicate response to ClpP-targeted treatments.

6.3.6 Limitations and Considerations

Although this work provides significant evidence of ClpP's impact on RCC development and prognosis, some restrictions should be mentioned. First, the study's retroactive method can induce selection bias. To verify our findings and create causal relationships, we need prospective study. Second, although immunohistochemical research presents significant fresh angles on ClpP expression, merging transcriptome and proteome studies might provide a more whole knowledge of its functional relevance. More experimental study is therefore required as finally it is still unknown mechanistically how ClpP affects ferroptosis and cancer development.

6.3.7 Conclusion

This work defines ClpP as a strong prognostic biomarker and a fundamental driver of RCC development. High ClpP expression emphasises its importance in RCC pathophysiology since

it is linked to ferroptosis resistance, aggressive tumour features, and lowered general survival. This work prepares the stage for new approaches targeted at bettering RCC management and patient outcomes by orienting ClpP as both a biomarker and a therapeutic target.

Future efforts should focus on translating these findings into clinical applications, including the development of ClpP-targeted therapies and their integration into personalized treatment regimens. As our understanding of ClpP's role in cancer biology deepens, it holds the potential to transform RCC therapy and contribute to broader advancements in oncology.

CHAPTER SEVEN

Final Discussion

7.1 Rationale and Significance

Renal cell carcinoma (RCC) presents a persistent clinical challenge due to its significant molecular heterogeneity and resistance to conventional therapies.³⁵⁴ Elucidating the intricate molecular mechanisms driving RCC progression is critical for identifying novel therapeutic targets and improving patient outcomes.³⁵⁵ By combining RNA sequencing (RNA-seq), proteomics, and bioinformatics, this work provides a thorough investigation of the molecular terrain of RCC aimed at identifying important pathways and molecular players underlining RCC pathogenesis.^{356,357}

7.1.1 Integration of Multi-Omics Approaches

This work methodically defines the genomic and proteomic changes separating RCC tissues from non-cancerous equivalents by means of a multi-layered analytical approach combining RNA-seq, proteomics, and bioinformatics.⁸ Important pathways crucial to RCC survival and advancement were found by this integrated method including ferroptosis, mitophagy, and hypoxia adaption.³⁵⁸ The consistency between RNA-seq and proteomic data emphasises the strength of these results and supports the dependability of the analytical approach.³⁵⁹

7.1.2 Role of ClpP in RCC Pathophysiology

ClpP, a mitochondrial protease, emerged as a pivotal regulator in RCC, orchestrating the interplay among mitophagy, ferroptosis suppression, and hypoxia adaptation.⁷¹ Elevated ClpP expression in RCC tissues, alongside its co-localization with key proteins such as BNIP3, LC3A/B, GPX4, and HIF-1 α , highlights its multifaceted role in cellular quality control and survival under stress conditions.²⁹⁹ The consistent upregulation of ClpP and associated proteins in both *in vitro* and *in vivo* models further reinforces its central role in RCC pathogenesis.

7.1.3 Ferroptosis and Mitochondrial Dynamics in RCC

The results show that ClpP controls iron metabolism and lipid peroxidation to thus modify ferroptosis resistance.³⁶⁰ The inverse link between ClpP and ACSL4,³⁶¹ a pro-ferroptosis enzyme, emphasises ClpP's function in preserving redox balance. ClpP is a major modulator of RCC's

metabolic resilience since it reduces oxidative damage, therefore supporting RCC cell survival within the tumour microenvironment.^{362,363}

Emphasising its relevance in mitochondrial quality control, the study also shows ClpP's participation in mitophagy by means of its interaction with BNIP3 and LC3A/B.³⁶⁴ Following ClpP depletion, the inhibition of mitophagy-related processes emphasises its vital role in preserving mitochondrial integrity, which is necessary for RCC cell survival and adaptability to hypoxia-induced stress.³⁵³

7.1.4 ClpP as a Prognostic and Therapeutic Target

The clinical relevance of ClpP is underscored by its correlation with aggressive RCC phenotypes and overall poor survival (OS).²⁹¹ High ClpP expression is significantly associated with advanced tumour grade, invasion, metastasis, and higher TNM stage, establishing its role as a biomarker for disease progression.³⁶⁵ Multivariate analysis confirmed ClpP as an independent prognostic factor for RCC, reinforcing its potential utility in clinical decision-making.³⁶⁶

Targeting ClpP has major therapeutic ramifications. A potential approach to overcome RCC's natural resistance to oxidative stress and hypoxia is to disrupt ClpP-mediated pathways, therefore improving the sensitivity of RCC cells to ferroptosis and impairing their adaptive capacity. This work offers a thorough framework for comprehending the molecular foundations of RCC and places ClpP as a fundamental controller of RCC development. Future studies should concentrate on clarifying the particular pathways under control by ClpP and creating inhibitors or modulators to augment current treatments and defeat RCC's adaptive defences.

7.2 Summary of the Findings

Emphasising RNA sequencing (RNA-seq), proteomics, and bioinformatics, this thorough work used a multi-omics strategy to clarify the molecular foundations of renal cell carcinoma (RCC). Particularly the mitochondrial protease ClpP, which turned out as a critical modulator of RCC advancement, the results throw insight on important pathways, regulatory networks, and molecular players causing RCC pathogenesis. The following summarises important observations.

7.2.1 Molecular Landscape and Pathway Disruptions in RCC

Combining proteomics with RNA-seq revealed significant genetic and proteomic differences between RCC and related non-tumourous tissues. Emphasising strong experimental repeatability, hierarchical clustering and principal component analysis (PCA) identified unique chemical markers defining RCC. Suggesting their important roles in RCC pathogenesis, differential expression analysis found elevated pathways linked with ferroptosis, mitophagy, and hypoxia adaption.³⁶⁷

With important players like GPX4 and ACSL4 clearly engaged, pathway enrichment study highlighted disturbances in mitochondrial and lipid metabolism.^{368,369} These results imply, crucial to RCC cell survival, a dynamic interaction between ferroptosis resistance and mitochondrial adaptability.

7.2.2 ClpP as a Key Molecular Regulator

ClpP was identified as a pivotal regulator within RCC, with its expression significantly upregulated in tumour tissues compared to non-cancerous counterparts.³⁰⁵ Its co-localization with markers of mitophagy (BNIP3, LC3A/B),³⁷⁰ antioxidative defense (GPX4), and hypoxia adaptation (HIF-1 α) underscores its multifaceted role in RCC's cellular survival mechanisms.³⁷¹ Notably, ClpP exhibited an inverse correlation with ACSL4, a pro-ferroptosis enzyme, highlighting its involvement in suppressing ferroptosis through redox homeostasis.

7.2.3 Functional Role of ClpP in Ferroptosis and Mitophagy

Functional assays confirmed that ClpP modulates ferroptosis resistance by stabilizing GPX4 and mitigating lipid peroxidation.^{372,373} ClpP depletion led to heightened ROS production, increased lipid peroxidation, and reduced mitochondrial membrane potential, indicative of disrupted ferroptosis regulation.³⁷⁴ Concurrently, ClpP's interaction with BNIP3 and LC3A/B demonstrated its role in maintaining mitophagy flux and mitochondrial quality control under hypoxic stress, essential for RCC cell survival.^{375,376}

7.2.4 Impact of ClpP on RCC Cell Behavior and Tumour Progression

In vitro and *in vivo* studies established ClpP's role in enhancing RCC cell migration, invasion, and tumour growth. Knockdown experiments revealed impaired cell motility and reduced tumour progression, while overexpression promoted aggressive phenotypes. Xenograft models demonstrated that ClpP depletion significantly inhibited tumour growth and enhanced

ferroptosis susceptibility, further highlighting its therapeutic potential.^{377,378}

7.2.5 Mitophagy protects against ferroptosis

Ferroptosis is a form of autophagy-related cell death in which autophagy plays a central role by regulating intracellular iron homeostasis and reactive oxygen species (ROS) production.^{379,380} Mitochondria are essential for various critical cellular functions, including ATP synthesis, intracellular calcium balance, cell differentiation, and cell death, making them vital for cellular homeostasis.^{381,382} Consequently, regulating mitochondrial dysfunction or mitochondria-mediated signalling pathways represents an effective strategy for modulating cell death. Mitochondrial autophagy, the primary mechanism for clearing damaged mitochondria, has been implicated as a key factor in ferroptosis. Studies have shown that ferroptosis-induced mitochondrial autophagy is triggered by compounds such as Celastrol, a triterpenoid with potent anticancer activity.¹⁶⁷ The combination of Celastrol and Erastin significantly increases ROS production, disrupts mitochondrial membrane potential, and promotes mitochondrial damage.³⁸³ Additionally, this co-treatment induces ATG5-dependent autophagy and PINK1/Parkin-mediated mitochondrial autophagy.³⁸⁴

Mitochondrial autophagy also influences cellular sensitivity to ferroptosis by modulating lipid peroxidation and iron metabolism through ROS.¹⁷⁷ For example, in the nervous system, neurons rely on mitochondrial autophagy to clear damaged mitochondria.³⁸⁵ Loss of Parkin function results in defective mitochondrial autophagy, contributing to Parkinson's disease (PD), which is characterized by elevated iron, decreased reduced glutathione, and high lipid peroxidation in the midbrain. Iron chelation therapy can reduce iron accumulation and alleviate early PD symptoms.³⁸⁶ Conversely, in non-neurological diseases, mitochondrial autophagy may exacerbate ferroptosis. For instance, the mitochondrial respiratory chain complex I inhibitor BAY 87-2243 increases ROS through mitochondrial autophagy, leading to ferroptosis in melanoma cells, while PINK1 knockout suppresses this process.³⁸⁷ Similarly, pharmacological inhibition of mitochondrial autophagy using Mdivi-1 prevents Parkin activation by DOX, preserving mitochondrial biosynthesis and mitigating DOX-induced ROS production and mitochondrial dysfunction.³⁸⁸

Mitochondrial autophagy also plays a role in iron metabolism. For instance, cellular O-GlcNAcylation, a nutrient and stress receptor, increases under ferroptosis stress. Reduced O-GlcNAcylation leads to mitochondrial fragmentation and linear autophagy, releasing

mitochondrial iron and promoting ferroptosis.³⁸⁹ Thus, ferroptosis is closely tied to mitochondrial autophagy, although its precise mechanisms require further elucidation.

In summary, mitochondrial autophagy exerts diverse effects across different diseases. Fully understanding the specific role of mitochondrial autophagy in ferroptosis is critical for uncovering the dynamic regulation of mitochondria and ferroptosis. Targeted therapies, a conventional approach in cancer treatment, can induce both mitochondrial autophagy and ferroptosis in tumour cells. However, no studies to date have demonstrated a connection between ClpP-induced mitochondrial autophagy and ferroptosis in tumour cells. This study explored the association between ClpP-mediated regulation of mitochondrial autophagy and ferroptosis in RCC to elucidate a novel mechanism of ferroptosis induction through targeted therapies in cancer.

Mitophagy, the selective autophagy of damaged mitochondria, is mediated by several receptors, including BNIP3 and ClpP, and is important for mitochondrial homeostasis.³⁵² Mitochondria are also the major source of reactive oxygen species (ROS); mitochondrial ROS (mtROS) can give rise to lipid peroxides, which, in turn, induce ferroptosis, a specific type of cell death.³ In this study, Kanki and co-workers provide direct evidence that mitophagy mediated by BNIP3 and NIX protects cells against ferroptosis by reducing mtROS.^{390,391} The authors show that BNIP3 and NIX are essential for mitophagy as cells with double knockout of BNIP3 and ClpP are impaired in mitophagy and exhibit reduced mitochondrial function,³⁹² including reduced basal respiration and ATP production.³⁹³ Interestingly, these mitophagy-deficient cells are highly sensitive to ferroptosis, which can be rescued with expression of BNIP3 and NIX, or by treating cells with a mitochondrial superoxide scavenger.^{394,395} These findings point to an increase in mtROS levels, owing to the absence of mitophagy as an inducer of ferroptosis. This study identifies a crucial role for mitophagy in the regulation of ferroptosis that might be exploited under pathological conditions.³⁹⁶

7.2.6 Prognostic Value of ClpP

ClpP expression correlated with aggressive RCC phenotypes, including advanced tumour grade, invasion, metastasis, and higher TNM stage. Multivariate analyses confirmed high ClpP expression as an independent predictor of poor overall survival (OS), with patients exhibiting elevated ClpP levels showing significantly lower 5-year OS rates. These findings position ClpP as a robust prognostic biomarker for RCC.

7.3 Scientific and Translational Implications

The findings from this study underscore the pivotal role of ClpP in the progression and resilience of renal cell carcinoma (RCC).^{320,397} By integrating transcriptomic, proteomic, and bioinformatic analyses, we reveal the multifaceted functions of ClpP as a central regulator of ferroptosis resistance, mitochondrial quality control, and hypoxia adaptation. These insights have profound scientific and clinical implications, offering new opportunities for therapeutic interventions and prognostic strategies.³²³

7.3.1 ClpP as a Molecular Convergence Point in RCC Survival Mechanisms

ClpP operates as a critical nexus integrating mitochondrial homeostasis, ferroptosis suppression, and hypoxia-induced stress responses. Its interactions with key regulators, such as GPX4, BNIP3, LC3A/B, and HIF-1 α , enable RCC cells to withstand oxidative stress, nutrient deprivation, and low oxygen levels commonly encountered in the tumour microenvironment.

By stabilizing GPX4 and counteracting the pro-ferroptosis activity of ACSL4, ClpP helps maintain cellular redox homeostasis and mitigates lipid peroxidation, essential for RCC survival. ClpP's interaction with BNIP3 and LC3A/B supports mitochondrial quality control by facilitating mitophagy, ensuring mitochondrial functionality under hypoxic conditions. ClpP's positive correlation with HIF-1 α reflects its role in supporting hypoxia-responsive transcriptional programs, enabling metabolic reprogramming and tumour adaptation.³⁹⁸ These findings establish ClpP as an orchestrator of RCC's adaptive responses, safeguarding cellular viability under harsh microenvironmental conditions.

7.3.2 The functional importance of ClpP in RCC survival and progression identifies it as a promising therapeutic target

Disrupting ClpP's interaction with GPX4 could enhance lipid peroxidation and sensitize RCC cells to ferroptosis, particularly in combination with ferroptosis-inducing agents. Targeting ClpP could inhibit its interaction with BNIP3 and LC3A/B, compromising mitochondrial quality control and inducing mitochondrial dysfunction, ultimately impairing RCC cell survival. ClpP-targeted therapies may synergize with existing treatments, such as tyrosine kinase inhibitors or immune checkpoint inhibitors, to overcome treatment resistance.

The development of small molecule inhibitors or RNA-based therapeutics targeting ClpP

should be prioritized, with preclinical studies assessing their efficacy, specificity, and potential for integration into current treatment regimens.

7.3.3 ClpP as a Prognostic Biomarker

Elevated ClpP expression correlates strongly with aggressive RCC phenotypes, including advanced Fuhrman grades, greater tumour invasion, metastasis, and higher TNM stages. Moreover, patients with high ClpP levels exhibit significantly lower 5-year overall survival rates.³⁹⁹⁻⁴⁰¹

Since its initial proposal in 2012, ferroptosis has garnered significant attention. As research in this area expands, the biological mechanisms underlying ferroptosis in various diseases are becoming increasingly understood. A growing body of evidence indicates that ferroptosis, a newly recognized form of programmed cell death, can play a beneficial role in tumour suppression. Additionally, some clinically approved drugs have been found to influence ferroptosis. For instance, the FDA has approved Altretamine, Sorafenib, and artemisinin and its derivatives as adjuvant therapies that induce tumour ferroptosis, suggesting that ferroptosis-based tumour therapies hold considerable promise. However, many aspects of ferroptosis in cancer remain to be explored, including its relationship with the tumour microenvironment and immune-related clinical applications.

This study is the first to establish a significant association among ClpP, mitophagy, and ferroptosis in renal cell carcinoma (RCC).⁴⁰² Our findings demonstrate that ClpP enhances BNIP3-dependent mitophagy in RCC cells while suppressing ferroptosis, providing a survival advantage to cancer cells. Additionally, ClpP promotes the activation of nuclear HIF-1 α , a key regulator of cellular adaptation to hypoxic stress. These interconnected pathways collectively contribute to RCC progression and are strongly associated with poor clinical outcomes in patients. Our novel data position ClpP as a central player in RCC tumorigenesis, promoting cancer cell survival and disease progression through the concurrent activation of mitophagy and suppression of ferroptosis.⁶⁹

Tumour cells must adapt to inhospitable environments to survive, often activating stress response mechanisms that buffer cytotoxic stress. These adaptive processes enable cancer cells to thrive under stress conditions, contributing to therapeutic resistance, dormancy, and metastasis.⁴⁰³ Among these mechanisms, the removal of damaged or misfolded proteins

through autophagy is critical for maintaining mitochondrial integrity. Mitochondria, which house proteolytic systems conserved through evolution, play a pivotal role in cellular homeostasis and stress adaptation.⁴⁰⁴ The mitochondrial protease ClpP is central to mitochondrial protein quality control, as it degrades misfolded proteins and supports organelle function.⁴⁰⁵ Historically, research on ClpP has largely focused on its role in mitochondrial proteostasis, with studies showing that ClpP overexpression enhances the removal of misfolded proteins from the electron transport chain, increasing mitochondrial efficiency to support tumour survival and metastasis.

Emerging evidence highlights the therapeutic potential of targeting ClpP in cancer. Genetic ablation or pharmacological inhibition of ClpP increases mitochondrial ROS production, impairing cancer cell proliferation and migration.² While ClpP has been identified as a therapeutic target in leukemia, its role in solid tumours, including RCC, remains underexplored. Our findings address this gap by demonstrating that ClpP drives RCC tumorigenesis through mechanisms beyond its traditional role in proteostasis, such as augmenting BNIP3-mediated mitophagy and suppressing ferroptosis.

Ferroptosis, a recently identified form of regulated cell death, is characterized by iron-dependent lipid peroxidation and is modulated by key proteins such as GPX4 and ACSL4. GPX4 inhibits ferroptosis by reducing lipid peroxidation, whereas ACSL4 promotes it.³⁹⁴ In acute myeloid leukemia, ClpP overexpression has been shown to work synergistically with GPX4 to inhibit ferroptosis.^{97,106} Consistent with these findings, we identified a direct interaction between ClpP and GPX4 in RCC cells, further supporting the role of ClpP in protecting cancer cells from ferroptosis. This interaction highlights a novel mechanism by which ClpP facilitates ferroptosis resistance, contributing to RCC progression.¹¹⁰

Our study also demonstrates that ClpP enhances RCC malignancy through its modulation of both mitophagy and ferroptosis pathways. ClpP increases BNIP3-LC3A/B-mediated mitophagy, ensuring mitochondrial quality control and providing metabolic flexibility under stress conditions. Simultaneously, ClpP suppresses ferroptosis by interacting with GPX4, preventing lipid peroxidation and promoting cancer cell survival. The stimulation of nuclear HIF-1 α by ClpP emphasises even more its many functions in RCC aetiology. These processes were validated by *in vivo* and *in vitro* experiments; clinical data showed a clear link between poor prognosis in RCC patients and high ClpP expression.

7.4 Limitations and Future Works

7.4.1 Limitations

Though it is not without limits, this work offers important new understanding on the function of ClpP in renal cell carcinoma (RCC). First, just three RCC patients comprised the quite tiny sample size for the RNA-seq and proteomic study. Although the inclusion of TCGA and ICGC datasets helped corroborate our results, the limited experimental cohort might not completely reflect the heterogeneity of RCC throughout different patient populations. Improving the generalisability of the results in the next investigations depends critically on increasing the sample size.

Second, although this work used *in vitro* and *in vivo* models to evaluate the functional relevance of ClpP, these models might not entirely reflect the complexity of the tumour microenvironment. Crucially important in RCC pathogenesis, factors like immune infiltration, stromal interactions, and angiogenesis were not fully explored. Advanced models, including co-culture systems including immunological and stromal components or patient-derived organoids, might offer a more physiologically appropriate setting for next studies.

The reliance on knockdown and overexpression techniques to assess ClpP function adds still another constraint. These approaches clearly showed the function of ClpP in RCC cell survival and ferroptosis resistance, however off-target effects or compensating mechanisms might have affected the results that were seen. Small-molecule inhibitors unique to ClpP or CRISPR-Cas9 gene-editing technologies would offer a more exact assessment of its role.

Finally, the study did not investigate any resistance mechanisms RCC cells can create against treatments aiming at ClpP-targeting. The therapeutic use of these results depends critically on an awareness of how RCC cells adjust to ClpP inhibition and on the identification of combination approaches to overcome resistance.

7.4.2 Future Directions

Essential are thorough studies of the molecular interactions of ClpP with its important partners, GPX4, ACSL4, BNIP3, and HIF-1 α . High-resolution insights into ClpP's protein complexes made possible by structural studies employing cryo-electron microscopy or X-ray crystallography

might direct the creation of focused inhibitors.

First priorities should be the identification and development of RNA-based therapies aiming at ClpP or small-molecule inhibitors. These contenders must be carefully assessed for their safety, effectiveness, and pharmacokinetics in preclinical RCC models—including patient-derived xenografts.

Combining treatments that concurrently target ClpP and other key pathways (e.g., immunological checkpoints, tyrosine kinase receptors, or ferroptosis inducers) should be investigated given the multifarious function of ClpP in RCC survival. Synergistic treatments could reduce resistance and improve therapeutic efficacy.

Emerging data points to possible influence on immune cell activity inside the tumour microenvironment of mitochondrial and metabolic regulators including ClpP. Investigating ClpP's role in modulating immune evasion or immunotherapy response could provide novel insights into enhancing the efficacy of immune-based treatments.

Although this study focused on RCC, ClpP's regulatory roles in ferroptosis and mitophagy may extend to other malignancies characterized by hypoxia and oxidative stress. Comparative studies across different cancer types could uncover conserved mechanisms, potentially broadening the therapeutic relevance of ClpP inhibition. The utility of ClpP as a prognostic biomarker warrants further validation in larger, multi-center patient cohorts. Prospective studies should assess the correlation between ClpP expression and clinical outcomes, such as treatment response and overall survival, to solidify its prognostic and predictive value.

Future studies should investigate the feasibility of personalized therapeutic strategies based on ClpP expression levels. High-throughput screening platforms that integrate genetic, proteomic, and metabolic profiling could identify patient subgroups most likely to benefit from ClpP-targeted therapies.

7.5 Conclusions

This study comprehensively elucidates the pivotal role of ClpP in the pathophysiology, progression, and clinical outcomes of renal cell carcinoma (RCC). Leveraging an integrative

multi-omics approach combining RNA sequencing, proteomics, and bioinformatics, we identified ClpP as a critical regulator at the intersection of ferroptosis resistance, mitophagy, and hypoxia adaptation. The findings offer valuable insights into the molecular mechanisms driving RCC progression and highlight ClpP's potential as both a prognostic biomarker and a therapeutic target.

7.5.1 Key Molecular Functions of ClpP in RCC

ClpP turned out to be a major molecular hub organising RCC's adaptive responses to oxidative stress and hypoxia among environmental challenges. Increased mitophagy, enabled by interactions with BNIP3 and LC3A/B, was repeatedly linked to raised expression of ClpP in RCC tissues. Under hypoxic conditions, this mitophagy activity preserves mitochondrial integrity—necessary for preserving cellular bioenergetics. ClpP simultaneously reduces lipid peroxidation, counteracts the pro-ferroptosis effects of ACSL4, and stabilises GPX4, hence suppressing ferroptosis. This two-fold capacity guarantees RCC cell survival in the demanding tumour microenvironment.

7.5.2 Implications for Tumour Aggressiveness and Disease Progression

Functional experiments indicate that ClpP significantly affects RCC cell motility, invasion, and tumour development. The lowering diminished RCC cell viability and induced ferroptosis, while ClpP overexpression exacerbated these malignant behaviours. The xenograft model data corroborated *in vitro* findings, demonstrating that ClpP expedites tumour progression and diminishes *in vivo* cellular apoptosis. These findings underscore the role of ClpP in fostering an aggressive renal cell carcinoma phenotype.

7.5.3 Prognostic Value of ClpP

Clinically, advanced tumour stage, increased invasibility, and metastases were linked to higher ClpP expression. ClpP is shown by multivariate Cox regression analysis to be an independent predictor of poor overall survival (OS). Emphasising its use as a predictive biomarker, RCC patients with elevated ClpP expression showed notably reduced 5-year OS rates. Monitoring ClpP expression might give important information for patient risk assessment and guide therapy decisions.

7.5.4 Therapeutic Potential of Targeting ClpP

One intriguing approach for upsetting RCC's adaptive defences is targeting ClpP. Reducing ClpP

could help to increase tumour sensitivity to ferroptosis and other controlled cell death pathways, hence overcoming RCC's natural resilience to metabolic and oxidative stress. When paired with current medications, ClpP-targeted medicines could be very successful in meeting unmet clinical needs and enhancing therapeutic results.

7.6 Conclusion

ClpP is shown in this work as a crucial regulator of RCC survival and development. Its centrality in RCC's adaptive framework is shown by its dual function in ferroptosis resistance and mitophagy. Considered as a useful prognostic biomarker and therapeutic target, elevated ClpP expression links with aggressive tumour behaviour and poorer patient outcomes. By means of a fresh approach to destroy RCC's metabolic defences, targeting ClpP opens the path for creative therapies to enhance clinical results for RCC patients. Development of ClpP inhibitors and mechanistic studies should take front stage in future studies to completely clarify its therapeutic value in RCC and beyond.

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9. Resume and Publica on list during my PhD study

Enqiang Chang

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Driven by a vibrant passion towards medical sciences, I joined Imperial College London in 2017 to study MRes Biomedical Sciences and was awarded a master degree with distinction. Prior to joining Imperial College, I was trained as an anaesthetist in China and have been in the clinical practice for 10 years, during which I have taken a particular keen interest in the perioperative wellbeing and long-term recovery of cancer patients. On a daily basis, the sight of cancer patients coping with agonizing pain, treatment side-effects, surgical procedure and unfortunate recurrences, has consolidated my resolve to invest myself in the search for a more targeted, efficacious anti-cancer strategy. Such determination in improving cancer patient outcome coupled with my enthusiasm for biomedical sciences prompted me to excel in MRes degree, and continue to motivate me to pursue PhD study in cancer biology and therapy.

My research interest focuses on mitochondrial adaptation in cancer cells, and I dedicated my MRes project to investigate the mechanism of cancer cell mitophagy, which is the selective clearance of damaged mitochondria through autophagy pathway. Specifically, my aim was to establish the involvement of mitochondrial serine protease caseinolytic peptidase proteolytic subunit (ClpP) in cancer mitophagy process, and understand the interaction between ClpP and hypoxia-inducible factor-1 alpha (HIF-1 α) in regulating cancer cell mitophagy during hypoxia. Remarkably, I demonstrated that hypoxia increased ClpP expression, reduced ClpP/HIF-1A protein interaction, and concurrently enhanced mitophagy in cancer cells.

To summarise, my proposed PhD study aims to investigate the role of ClpP in cancer cell mitophagy, as well as establishing ClpP as cancer mitophagy-specific molecule that influences cancer biology and behaviour in patients. I strongly believe a PhD study in this direction will enrich the current understanding of mitophagy mechanism, and strengthen rationale for targeting mitophagy and ClpP in future cancer therapy. The insights gained from my PhD study encourage me to pursue my career development by fostering innovation in surgical techniques,

and paving the way for future advancements in Cancer research.

Publication list during my PhD study

- 1) **Chang, E.**, Wu, L., Li, X., Zhou, J., Zhi, H., Sun, M., Chen, G., Bi, J., Li, L., Li, T., Ma, D, Zhang J. (2024). Dexmedetomidine decreases cerebral hyperperfusion incidence following carotid stenting: A double-blind, randomized controlled trial. **Med, Cell press**. 10.1016/j.medj.2024.09.012.
- 2) **Chang, E.Q.**, Wang, Y., Zhu, R., Wu, L., Yang, Y., Zeng, S., Li, N., Ruan, X., Sun, M., Zhang, W., Ma, D, Zhang J. (2023). General anesthetic action profile on the human prefrontal cortex cells through comprehensive single-cell RNA-seq analysis. **iScience, Cell press**. 26, 106534. 10.1016/j.isci.2023.106534.
- 3) Yanjun Li, Wenke Yang, Chaojun Liu, Shengli Zhou, Xiaozhuan Liu, Tingting Zhang, Lingzhi Wu, Xinyi Li, Jiaqiang Zhang, **Enqiang Chang***, (2024). SFXN1-mediated immune cell infiltration and tumourigenesis in lung adenocarcinoma: A potential therapeutic target. **International Immunopharmacology**. 132(2024)111918.
- 4) **Chang, E.Q.**, Wu, L., Ruan, X., Lu, J., Lin, B., Soo, C.P., Li, T., Zhang, J., and Ma, D. (2020). Laryngeal mask position evaluated by ultrasonography and fiberoptic bronchoscopy along with 3D-CTR constructive images: A prospective observational study. **J Clin Anesth** 67, 109974. 10.1016/j.jclinane.2020.109974.
- 5) Wang, Y.Y. #, **Chang, E.Q.** #, Zhu, R.L. #, Liu, X.Z., Wang, G.Z., Li, N.T., Zhang, W., Zhou, J., Wang, X.D., Sun, M.Y., and Zhang, J.Q. (2022). An atlas of dynamic peripheral blood mononuclear cell landscapes in human perioperative anaesthesia/surgery. **Clin Transl Med** 12, e663. 10.1002/ctm2.663.
- 6) **Chang, E.Q.**, Ruan, X.G., Wang, Y.Y., , Zhu, R.L., Ma, D, Zhang, J.Q. (2021).Values of Single-Cell RNA Sequencing in Development of Cerebral Cortex. **Advances in Experimental Medicine and Biology**. 1255:231-247.
- 7) **Chang, E.Q.**, Wu, L., Masters, J., Lu, J., Zhou, S., Zhao, W., Sun, M., Meng, F., Soo, C.P., Zhang, J., and Ma, D. Iatrogenic subglottic tracheal stenosis after tracheostomy and endotracheal intubation: A cohort observational study of more severity in keloid phenotype. **Acta Anaesthesiol Scand** 63, 905-912. 10.1111/aas.13371.
- 8) Chen Q, Liu X, Liu Z, Zhang S, Chen L, Eguchi S, Alam A, Cahilog Z, Sun Q, Wu L, **Chang E**, Wang Z, Gu J, Zhao H, Ma D. Tackling regulated cell death yields enhanced protection in

- lung grafts. **Theranostics**. 2023 Jul 31;13(13):4376-4390. doi: 10.7150/thno.87375. PMID: 37649611; PMCID: PMC10465232.
- 9) Chen Q, Qin Z, Sun Y, Liu X, Pac Soo A, **Chang E**, Sun Q, Yi B, Wang DX, Zhao H, Ma D, Gu J. Dexmedetomidine Activates Akt, STAT6 and IRF4 Modulating Cytoprotection and Macrophage Anti-Inflammatory Phenotype Against Acute Lung Injury in vivo and in vitro. **J Inflamm Res**. 2022 Apr 26;15:2707-2720. doi: 10.2147/JIR.S357012. PMID: 35502244; PMCID: PMC9056075.
- 10) Chen Q, Sun J, Liu X, Qin Z, Li J, Ma J, Xue Z, Li Y, Yang Z, Sun Q, Wu L, **Chang E**, Zhao H, Zhang Y, Gu J, Ma D. Dexmedetomidine and argon in combination against ferroptosis through tackling TXNIP-mediated oxidative stress in DCD porcine livers. **Cell Death Discov**. 2024 Jul 11;10(1):319. doi: 10.1038/s41420-024-02071-7. PMID: 38992027; PMCID: PMC11239900.
- 11) Wu L, **Chang E**, Zhao H, Ma D. Regulated cell death in hypoxic-ischaemic encephalopathy: recent development and mechanistic overview. **Cell Death Discov**. 2024 Jun 11;10(1):277. doi: 10.1038/s41420-024-02014-2. PMID: 38862503; PMCID: PMC11167026.
- 12) Wu L, Zhao H, Zhang M, Sun Q, **Chang E**, Li X, Ouyang W, Le Y, Ma D. Regulated cell death and inflammasome activation in gut injury following traumatic surgery in vitro and in vivo: implication for postoperative death due to multiorgan dysfunction. **Cell Death Discov**. 2023 Nov 7;9(1):409. doi: 10.1038/s41420-023-01647-z. PMID: 37935670; PMCID: PMC10630406.
- 13) Sun Q, Kamath P, Sun Y, Liang M, Wu L, **Chang E**, Chen Q, Alam A, Liu Y, Zhao H, Ma D. Dexmedetomidine attenuates lipopolysaccharide-induced renal cell fibrotic phenotypic changes by inhibiting necroinflammation via activating α_2 -adrenoceptor: A combined randomised animal and in vitro study. **Biomed Pharmacother**. 2024 May;174:116462. doi: 10.1016/j.biopha.2024.116462. Epub 2024 Mar 20. PMID: 38513598.
- 14) Li X, Ma X, Liu Y, **Chang E**, Cui J, Ma D, Zhang J. Predictive value of Leukocyte ImmunoTest (LIT™) in cancer patients: a prospective cohort study. **Front Oncol**. 2022 Aug 1;12:897968. doi: 10.3389/fonc.2022.897968. PMID: 35978826; PMCID: PMC9376289.
- 15) Li X, **Chang E**, Cui J, Zhao H, Hu C, O'Dea KP, Tirlapur N, Balboni G, Zhang J, Ying L, Ma D. Bv8 mediates myeloid cell migration and enhances malignancy of colorectal cancer. **Front Immunol**. 2023 Apr 5;14:1158045. doi: 10.3389/fimmu.2023.1158045. PMID: 37090721; PMCID: PMC10113555.