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CONNECTING VIRAL ONCOPROTEINS TO MICRORNA, AUTOPHAGY AND METABOLISM IN MERKEL CELL CARCINOMA

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Connecting viral oncoproteins to microRNA, autophagy and metabolism in Merkel cell carcinoma
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"When you stay on purpose and refuse to be discouraged by fear, you align with the infinite self, in which all possibilities exist."

Wayne Dyer

To my family, especially my parents, and friends

ABSTRACT

Merkel cell carcinoma (MCC) is an aggressive type of skin cancer. Around 80% of MCCs harbor an integrated Merkel cell polyomavirus (MCPyV) genome with a mutation in the large T antigen (LT) gene, leading to expression of truncated LT. It is evident that the viral truncated LT and small T antigen (sT) play important roles in MCC tumorigenesis. Yet, the molecular mechanisms how these viral oncoproteins contribute to MCC development remain unclear. The studies presented in this thesis aim to further our understanding of the functional role of these MCPyV oncoproteins in MCC tumorigenesis.

In **Paper I**, we demonstrated that MCPyV T-antigens induce *miR-375*, *miR-30a-3p* and *miR-30a-5p* through the DnaJ domain of the viral T-antigen. These miRNAs could target multiple autophagy genes (*ATG7*, *SQSTM1* and *BECN1*) and suppress autophagy in MCC cells. Additionally, we showed that both sT and truncated LT also suppress autophagy, but not the full-length LT. Inhibition of autophagy, but not pan-caspases, could rescue cell death induced by the mTOR inhibitor Torin-1, suggesting that suppression of autophagy is crucial for cell survival in MCC.

In **Paper II**, we found paranuclear dot-like staining of c-KIT in MCPyV positive (MCPyV+) MCC cell lines and tumor samples. Mechanistically, we showed that MCPyV truncated LT induces paranuclear retention of c-KIT through its Vam6p binding site. Our results also revealed that c-KIT interacts with BECN1. Silencing of c-KIT increased autophagy and apoptosis, and decreased LT expression. Inhibition of autophagic degradation in c-KIT depleted cells restored the LT expression, suggesting the importance of autophagy suppression to sustain the expression of viral oncoprotein and cell survival.

In **Paper III**, we showed that overexpression of *miR-375* suppressed cell growth and migration in MCPyV- MCC cell lines, while suppression of *miR-375* decreased cell growth and increased apoptosis in MCPyV+ MCC cell lines. The expression of LDHB, a target of *miR-375*, was inversely correlated with *miR-375*. Silencing of LDHB decreased cell growth in MCPyV- MCC cells, but rescued cell growth suppression resulted from *miR-375* inhibition in MCPyV+ MCC cells. Our findings suggest that *miR-375* regulation of LDHB plays distinct roles in MCPyV+ and MCPyV- MCC.

In **Paper IV**, we demonstrated that MCPyV T-antigens reduce LDHB expression and promote glycolysis in MCC. Overexpression of LDHB reduced cell viability and increased apoptosis in MCPyV+ MCC cells. Ectopic expression of LDHB reversed the growth-promoting effect of MCPyV oncoproteins. Inhibition of glycolysis reduced cell growth in MCPyV+ MCC cells, while inhibition of mitochondrial respiratory activity inhibited cell growth in MCPyV- MCC cells. Our findings suggest that MCPyV+ and MCPyV- MCC cells are dependent on different energy metabolism for cell growth.

Overall, this thesis work highlights the diverse functions of MCPyV oncoproteins and their involvement in regulating miRNA expression, autophagy and energy metabolism.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Satendra Kumar**, Hong Xie, Hao Shi, Jiwei Gao, Carl Christofer Juhlin, Viveca Björnhagen, Anders Höög, Linkiat Lee, Catharina Larsson, Weng-Onn Lui. Merkel cell polyomavirus oncoproteins induce microRNAs that suppress multiple autophagy genes.
Int J Cancer, 2019 Jun 10. doi 10.1002/ijc.32503.

II. Hao Shi, Linkiat Lee, Jiwei Gao, **Satendra Kumar**, Hong Xie, Vladana Vukojevic, Filip Farnebo, Viveca Björnhagen, Anders Höög, Carl Christofer Juhlin, Catharina Larsson, Weng-Onn Lui. Merkel cell polyomavirus oncoprotein induces paranuclear retention of c-KIT suppressing autophagy through interaction with Beclin-1.
Manuscript

III. **Satendra Kumar**, Hong Xie , Patrick Scicluna, Linkiat Lee, Viveca Björnhagen, Anders Höög, Catharina Larsson, Weng-Onn Lui. *MiR-375* regulation of LDHB plays distinct roles in polyomavirus-positive and -negative Merkel cell carcinoma.
Cancers (Basel), 2018 Nov 14, 10: E443.

IV. **Satendra Kumar**, Patrick Scicluna, Hao Shi, Jiwei Gao, Viveca Björnhagen, Anders Höög, Catharina Larsson, Weng-Onn Lui. Merkel cell polyomavirus oncoproteins suppress LDHB and promote glycolysis for cell growth.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acid
AGO	Argonaute protein
ALTO	Alternate frame of the large T open reading frame
CDS	Coding sequence
CK20	Cytokeratin 20
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
ECAR	Extracellular acidification rate
Endo H	Endonuclease H
ER	Endoplasmic reticulum
GFP	Green fluorescence protein
GIST	Gastrointestinal stromal tumor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HPV	Human papilloma virus
IF	Immunofluorescence
IP	Immunoprecipitation
LSD	Large T antigen stabilization domain
LT	Large T antigen
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
miRNA	microRNA
MUR1	MCPyV unique region 1
MUR2	MCPyV unique region 2
NCCR	Non-coding control region

NFP	Neurofilament protein
NLS	Nuclear localization signal
OCR	Oxygen consumption rate
ORF	Open reading frame
ORR	Objective response rate
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PNGase F	Peptide:N-glycosidase F
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
RFP	Red fluorescence protein
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription-quantitative PCR
SCF	Stem cell factor
SCLC	Small cell lung carcinoma
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
sT	Small T antigen
UTR	Untranslated region
UV	Ultra-violet

1 INTRODUCTION

1.1 MERKEL CELL CARCINOMA

Merkel cell carcinoma (MCC) is a neuroendocrine skin cancer, which was first described by Toker in 1972 [1]. About 90% of MCCs arise in sunlight-exposed areas, mainly head and neck, while some are also found on the trunk and genitals [2]. MCC mainly occur in the older age group (average age, 77 years) and in immunosuppressed patients. The incidence increased almost four times in the last two decades in the United States [3–5] and almost doubled in Sweden from 1993 to 2012 [6]. MCC is a highly metastatic disease; it has approximately 40% disease-specific mortality [4]. Miller *at al* reported that around half of the patients from a cohort of 179 patients with metastatic disease had a median survival of approximately 9 months [7].

MCC is believed to arise from Merkel cells, a type of mechanoreceptor cell present in the stratum basal areas of the epidermis, because they share some common immunophenotypes (such as cytokeratin 20, synaptophysin and several endocrine markers) and the presence of neurosecretory granules [8,9]. However, several pieces of evidence argue against this. For example, neural cell adhesion molecule L1 (CD171) and tyrosine kinase receptor c-KIT are detected in most MCCs but not in Merkel cells [10]. Furthermore, Merkel cells are differentiated and post-mitotic, while MCCs are highly proliferative [11]. Interestingly, MCCs commonly express pre-/pro-B specific expression markers (TdT and PAX5) and immunoglobulins, suggesting that early B cells may represent putative progenitors of MCC [12].

About 80% of MCCs are infected with Merkel cell polyomavirus (MCPyV), which was discovered by Moore and Chang in 2008 [13]. Despite that MCPyV is widespread in the general population, integration of mutated viral DNA into the host genome and expression of viral T-antigens are only detected in MCCs [14]. Importantly, the viral T-antigens are required for neoplastic transformation and maintenance of MCC cell growth [15], supporting their important roles in the pathogenesis of MCC. An introduction of MCPyV T-antigens and their involvement in MCC tumorigenesis is described in Chapter 1.2. Besides MCPyV, ultra-violet (UV) exposure is another etiology of MCC. MCPyV and UV-associated MCCs are molecularly distinct (for details, please refer to Chapter 1.1.2).

1.1.1 Diagnosis, prognosis and treatment of MCC

Three types of MCC are recognized based on histological features: intermediate, small cell and trabecular [16]. The trabecular type is a well-differentiated form with a better prognosis, while the small cell type is undifferentiated with worse prognosis. The intermediate type has more mitotic activity [4,17].

MCC is generally characterized by expressions of cytokeratin 20 (CK20) with a paranuclear dot-like staining pattern and neuroendocrine markers such as neuron-specific enolase (NSE), synaptophysin and chromogranin A [18]. Most MCCs are MCPyV positive (MCPyV+), therefore immunostaining of MCPyV LT can be used to identify virus-associated

MCCs. However, the virus-negative MCCs are hard to be diagnosed and differential diagnosis is necessary to distinguish MCC from other common neuroendocrine tumors, such as small cell lung carcinoma (SCLC), melanoma and cutaneous lymphoma (Table 1).

Table 1. Immunohistochemical staining used for differential diagnosis of MCC

	CK20	CK7	NSE	TTF1	S100	LCA
Merkel cell carcinoma	+	-	+	-	-	-
Melanoma	-	-	-	-	+	-
Small cell lung carcinoma	-	+	+	+	-	-
Cutaneous lymphoma	-	-	-	-	-	+

CK20, cytokeratin 20; CK7, cytokeratin 7; NSE, neuron-specific enolase; TTF-1, thyroid transcription factor 1; LCA, leucocyte common antigen. + and – refer to presence or absence of the staining, respectively. Adapted from Jaeger *et al* (2012) [19].

Based on the American joint committee on cancer (AJCC) and the European consensus-based interdisciplinary guidelines, clinical/ histological features, distant tissue involvement and staging system should be employed for prognostic classification of MCC [17,20,21]. Larger primary tumor size is associated with an increased risk of metastasis and sentinel lymph node biopsies are required for all the patients with primary tumor [22]. According to Surveillance, epidemiology and end results (SEER) data from 3870 MCC cases, 10-years survival rate was less in male (50%) than female (65%) patients [4]. Patients with local disease had 64% of five years survival rate, as compared to 40% in regional nodal disease and 18% in metastatic disease [23,24]. However, there were no significant differences observed in survival rate between different age groups [4]. Till date, no robust prognostic marker has been reported for MCC patients, however some reports showed that lack of lymphatic vessels invasion and T-cell infiltration can be used as a good prognosis indicator for MCC patients [20,25,26].

AJCC classified MCC staging based on three important indicators: tumor size and invasiveness of the primary tumor (T), degree of lymph node involvement (N) and metastatic state (M). Yiengpruksawan *et al* provided the first staging system for MCC, which defined tumor restricted to the skin as stage I, nodal metastasis as stage II, and distant metastasis as stage III and stage IV [27]. The overall staging for MCC is shown in Table 2.

Table 2. MCC staging and prognosis based on national cancer database

Stage	T	N	M	5-year survival
0	TIS	N0	M0	
IA	T1	pN0	M0	79%
IB	T1	cN0	M0	60%
IIA	T2/T3	pN0	M0	58%
IIB	T2/T3	cN0	M0	49%
IIC	T4	N0	M0	47%
IIIA	Any T	N1a	M0	42%
IIIB		N1b/N2	M0	26%
IV		Any N	M1	18%

T = primary tumor, N = degree of lymph node involvement, M = metastatic state, TIS = in situ primary tumor, pN0 = nodes negativity by pathologic examination, cN = nodes clinically detectable. Adopted from Lemos *et al* (2012) [17].

The choice of treatments for MCC depends on the stage of the tumor and health of the patient. Generally, wide surgical excision with 1-2 cm margins, followed by radiotherapy is used for treatment of the primary tumor. Chemotherapy regimens such as platinum-based, taxanes, anthracyclines and etoposide are considered as systemic therapeutic agents for treatments of advanced MCC. However, these drugs have less durable responses and no significant effects on survival rate. Chemotherapy is now considered to play a palliative role [28]. In the past few years, several new targeted therapies have been investigated as treatment options for treatment of advanced and metastatic MCC. For examples, YM155, a small molecule inhibitor targeting survivin, induces cell death in MCPyV+ MCC cell lines and xenograft [29,30]. ABT-263 is a potent inhibitor for the anti-apoptotic Bcl-2 family members, which induces apoptosis in MCC cell lines and xenograft [31,32]; however, this drug did not show any significant effect in a phase II trial [33]. Although *KIT* mutations are rarely found in MCC, its overexpression has been observed in MCC tumors [34,35]. Several MCC patients have been successfully treated with imatinib, a small molecule tyrosine kinase inhibitor that blocks c-KIT activation [36–38]. However the results from a phase II trial of imatinib in MCC were disappointing since a majority of patients progressed within 1 to 2 cycles of treatment, indicating its limited clinical benefit for MCC patients [39]. Furthermore, Pazopanib, which can inhibit several receptor tyrosine kinases including, FGFR, PDGFR, VEGFR and c-KIT, has demonstrated some clinical beneficial effects in a single MCC patient [40].

Both MCPyV+ and MCPyV- MCC tumor cells express programmed cell death ligand 1 (PD-L1), with PD-L1 expression being more frequently detected in MCPyV+ tumor cells than MCPyV- MCC tumor cells [41]. The PD-L1 expression pattern in MCC tumor cells supports the ideas to use PD-1 signaling pathways for targeted therapy in advanced MCC patients. Several phase II trials are ongoing to use anti-PD-1 and/or anti-PD-L1 checkpoint inhibitors for MCCs patients. These inhibitors showed durable response in advanced MCC patients pre-treated with chemotherapy [41–43]. For example, avelumab (anti-PD-L1 antibody) was used to treat 88 MCC patients with metastatic disease and found that 32.8 % had objective response rate (ORR), including complete and durable response in 9.1 % patients,

which were also associated with improved quality of life [43]. In another study with pembrolizumab (anti-PD-1 antibody) as a first line therapy, 56 % achieved ORR, including complete response in 15.4 % of the patients [41]. Presently, these two checkpoint inhibitors (avelumab and pembrolizumab) are approved by the Food and Drug Administration (FDA) to treat MCC.

1.1.2 Mutations, cancer pathways and immune profiles

MCPyV is an established etiology for MCC, and the remaining 20% of MCPyV- MCCs are regarded as having a UV radiation etiology. These two groups are also known to display different genomic mutation profiles. While MCPyV+ tumors exhibit less somatic mutations and copy number alterations, MCPyV- tumors have higher mutational burden. MCPyV+ tumors have around 25-fold less mutation frequency compared to UV-associated tumors[44–46]. Notably two key tumor suppressor genes, *RB1* and *TP53*, are frequently mutated in UV-associated MCCs, but not in MCPyV+ MCCs; however, their functions are inactivated by MCPyV T-antigens in MCPyV+ tumors [45,47–50]. In addition, MCPyV- MCCs carry inactivating mutations in genes involved in several cancer pathways, such as PI3K/Akt/mTOR, Notch, Wnt signaling, DNA damage repair and chromatin modifying pathways [44,46,48,51].

Based on global mRNA profiles, gene expression patterns in MCC are distinct from other primary cutaneous carcinomas [52]. As compared to squamous cell carcinoma, MCCs had higher levels of *CK20*, chromogranin A, synaptophysin, *AKT3*, *FYN*, *RAB3B*, *JUND*, *FEV*, *SOX2*, *BCL2*, *MYCL1*, *VEGFA*, *GPC3*, *ATOH1*, *HIP1* and *c-KIT*. On the other hand, genes involved in Hedgehog signaling, including *GL1*, *GLI2*, *PTCH1* and *PTCH2*, were higher expressed in basal cell carcinoma compared to MCC. Notably, the gene expression signatures of MCPyV+ and MCPyV- MCCs were also distinct [52]. Among the differentially expressed genes, MCPyV- tumors had higher expressions of transcripts involved in Notch signaling (such as *DLL1*, *CTBP2*, *HES1*, *JAG2* and *JAG1*) and receptor tyrosine kinase signaling (*e.g. FGFR2*, *VEGFA*, *PDGFA*, *FGF14*, *FGF11* and *FGF9*). In addition, increased expression of immune response genes were also observed in MCPyV+ as compared to MCPyV- MCCs [52]. Importantly, the immune response gene signature, especially genes associated with cytotoxic CD8 lymphocytes, is associated with a good prognosis in MCC [53]. In line with the transcriptome analysis, tumor-infiltrating immune cells are associated with favorable survival in MCC [26,53–55]. Although several immune response genes are higher expressed in MCPyV+ than MCPyV- MCCs, both MCPyV+ and UV-associated MCCs are immunogenic [56]. It is now clear that MCC can evade the immune response using the PD-1/PD-L1 immune-checkpoint pathway. Most MCC tumors express PD-L1, which binds to its receptor PD-1 on T cells [48,57], leading to inhibition of T cell killing of tumor cells. Therefore, targeting the PD-1/PD-L1 immuno-checkpoint has become a promising therapy for MCC patients.

1.2   MERKEL CELL POLYOMAVIRUS (MCPyV)

MCPyV is a circular double-stranded DNA (dsDNA) virus, which belongs to the Polyomaviridae family. The genome is about 5.4 kb and harbors early and late regions separated by a non-coding control region (NCCR). The early region contains the T-antigen gene locus, the late region encodes the capsid proteins and the NCCR contains the bipartite origin of replication. Besides viral proteins, MCPyV also contains gene for a single microRNA (*mcv-miR-M1*) which is located in an antisense direction to the early region that negatively regulates early transcripts and limits viral DNA replication [58].

1.2.1 MCPyV T-antigens

The T-antigen locus generates multiple alternatively spliced transcripts, namely large T (LT), small T (sT), 57 kT and alternate frame of the large T open reading frame (ALTO). Domain structures of various T-antigens are shown in Figure 1, and their functions are summarized in the following sections.

1.2.1.1   Large T-antigen

The LT of MCPyV includes several conserved features (such as DnaJ, Rb binding motif, origin binding domain (OBD), zinc finger and ATPase/helicase) and two unique regions called MCPyV unique regions 1 and 2 (MUR1 and MUR2). The DnaJ domain comprises of the CR1 (LXXLL) and the Hsc70 binding (HPDKGG) motifs, which is required for viral replication [58] and growth promoting activity [59]. The Rb binding motif (LXCXE) is flanked by MUR1 and MUR2. This motif is highly conserved across polyomaviruses and is required for cell cycle progression through up-regulation of E2F target genes and survivin [60,61]. MUR1 has a Vam6p binding site, in which the interaction disrupts lysosomal trafficking by sequestration of Vam6p to the cell nucleus [62]. The functional role of nuclear sequestration of Vamp6 remains unclear, however it is known that Vam6p can regulate MCPyV replication *in vitro* [63], and it is not essential for promoting cell growth [60]. MUR2 has a nuclear localization signal (NLS, RKRK motif) [64]. The NLS was initially found to be conserved in MCC tumors and regarded as functionally important in MCC pathogenesis, but recent findings showed that this motif is not always preserved in the MCC-specific truncated LT and is not required for the growth promoting activity of LT [60]. Several key domains in the carboxyl-terminal of LT are required for viral replication. The OBD recognizes and binds the viral origin of replication in the NCCR, the zinc finger motif and the ATPase/helicase domains are required for replication initiation. Although the helicase domain of SV40 directly binds to p53, this domain in MCPyV cannot interact with p53 [64,65].

1.2.1.2   Small T antigen

MCPyV LT and sT share exon 1 of the T antigen, therefore they have the common DnaJ domain in the amino-terminal. The carboxyl-terminal of sT carries unique features, including LT-Stabilization Domain (LSD), and protein phosphatase 2A and 4C (PP2A and PP4C) binding sites. As aforementioned, the DnaJ domain of LT is required for viral replication, however mutation of this domain in sT does not affect MCPyV replication [59]. The function of

this domain remains elusive. A major feature of MCPyV sT is the LSD, which has been shown to inhibit Fbw7 ubiquitin ligase, thereby stabilizing LT, c-Myc and cyclin E. Stabilization of these proteins is important for viral replication and transformation [66]. The LSD also targets 4E-BP1 and prevents the turnover of its hyperphosphorylated form, which increases eIF4F activity and in turn, mitotic-dependent protein translation [15,66].

The PP2A domain is known to be the primary function of sT in other polyomaviruses such as SV40, in which sT binds PP2A by competing with B subunit (one of the subunits in the PP2A heterotrimeric complex) and inhibits Akt-mTOR signaling, leading to cell cycle progression, survival and differentiation [67–69]. However, PP2A binding of MCPyV sT is not required for transformation activity [32,70]; this is likely due to limited B subunit targeting in the MCPyV sT compared to the sT in SV40 and other polyomaviruses. PP4C and/or PP2A Aβ of MCPyV sT interact with NF-kB essential modulator (NEMO) protein and disrupt host cell inflammatory signaling mediated by NF-kB [71]. The PP4C site is also important for promoting cell motility and migration by destabilization of microtubules through inhibition of stathmin phosphorylation, a key microtubule binding protein [72,73]. MCPyV sT induces cell motility through Rho-GTPase, which induces filopodium formation in MCC [74]. This effect may explain the highly metastatic nature of MCC tumors. Additionally, sT induces aerobic glycolysis through induction of monocarboxylate transporter (MCT1) that regulates MCC cell proliferation and viability [75]. sT can also induce motility and invasiveness by inducing Cl^--channel proteins CLIC1 and CLIC4 [74] or cellular sheddase proteins ADAM 10 and ADAM 17 [76].

1.2.1.3 57 kT and ALTO

The 57 kT is an alternatively spliced T-antigen of 432 amino acids. It shares the first 332 and the last 100 amino acids with LT, including the DnaJ domain, Rb binding site, MURs and nuclear localization signal, but lacks the OBD and most of the helicase domain. Although the functional role of the 57 kT is still unclear, Cheng *et al* showed that the full-length 57 kT and the carboxyl-terminal of 100 residues encoded from exon 3 of the 57 kT has a growth inhibition function [77]. ALTO is encoded by an alternative ORF of LT, which is evolutionarily related to the middle T antigen of murine polyomavirus. ALTO is expressed during viral replication, however it is not essential for MCPyV genome replication *in vitro* [78] and cell growth in MCC cells [60].
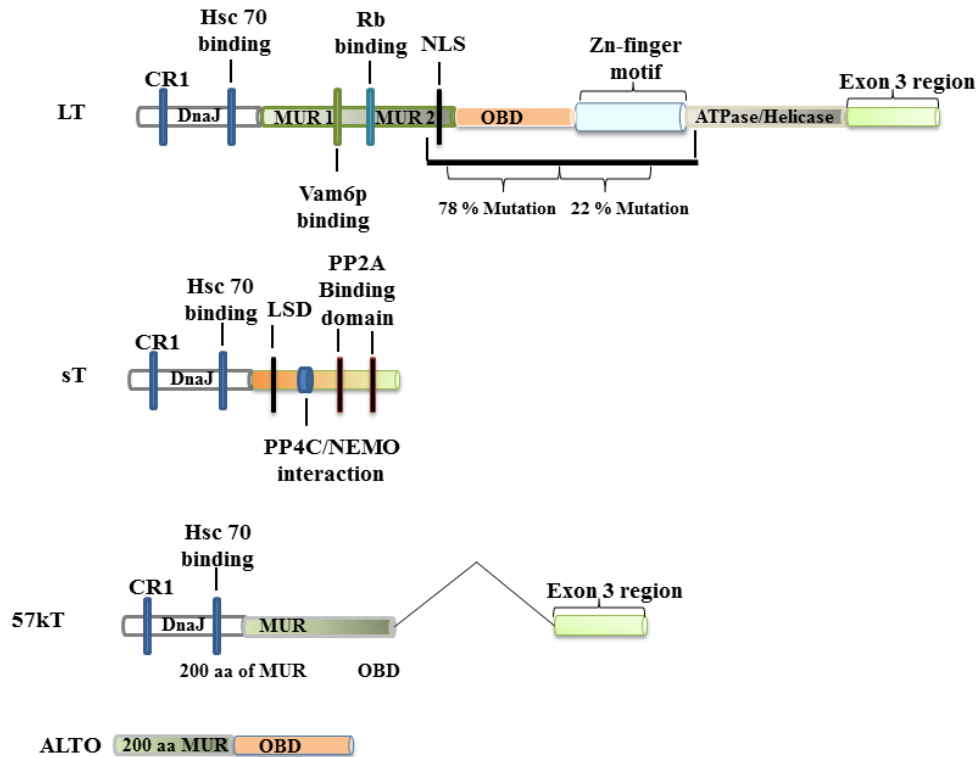
Figure 1. Functional domains of various MCPyV T-antigens. The T-antigen locus of MCPyV encodes four spliced transcripts. LT is 816 amino acids in size, containing DnaJ domain, retinoblastoma (Rb) binding motif, MCPyV unique regions 1 and 2 (MUR1 and MUR2), origin binding domain (OBD), zinc finger motif and ATPase/helicase. LT mutations are commonly found in MCC tumors, in which the mutation sites are located downstream of the Rb binding site. sT, 186 amino acids in length, shares the same DnaJ and Hsc70 binding domain of LT, and carries unique features LT-Stabilization Domain (LSD), protein phosphatase 2A and 4C (PP2A and PP4C) binding sites at its carboxyl-terminal. 57 kT, 432 amino acids in length, also shares the DnaJ domain with LT and sT, and contains the MUR1 region, Rb binding domain and exon 3 of LT. ALTO encodes 200 residues of MUR region of LT and OBD, which is closely related to middle T antigen of murine polyomavirus.

1.2.2 MCPyV integrations, mutations, expressions and functions in MCC

As aforementioned, MCPyV infection is widespread in the general population. However, the tumorigenic MCPyV can be distinguished from the non-tumorigenic ones based on the presence of viral DNA integration and LT mutations. MCPyV has been shown to monoclonally integrate into the genome of MCC tumors [14,79,80]. The viral integration sites have been identified within or in the vicinity of several cellular genes, including *PTPRG*, *GMDS*, *DENND1A*, *SNAT1*, *IL20RA*, *SRD5A2L2* and *MYC*; however no recurrent viral integration site has been identified.

All MCPyV+ MCC tumors harbor LT mutations (point mutations or small indels) that lead to expression of truncated LT (Figure 1). These mutants retain Rb binding but eliminate

viral replication capacity. Besides the truncated LT, most MCPyV+ MCC tumors also express intact sT [15], and some has truncated ALTO [78]. Despite the functional role of truncated ALTO remains to be characterized, both truncated LT and sT are required for cellular transformation and tumorigenesis *in vitro* and *in vivo* [15,32,77,81–83]. Furthermore, these T-antigens are also necessary for the maintenance of cell growth in MCPyV+ MCC cells [84].

1.3 MICRORNA

microRNA (miRNA) was first discovered in *Caenorhabditis elegans* (*C. elegans*) by the Ambros and Ruvkun groups [85,86]. They found that *lin-4* binds to its complementary sequence in the 3´ untranslated region (UTR) of *lin-14* and down-regulate its translation. However, the miRNA field only started to emerge after the discovery of the highly conserved miRNA called *lethal-7 (let-7)* in 2000 [87]. *Let-7* was detected in a wide range of animal species, including human [88]. This finding inspired several groups to search for miRNAs in different organisms. In 2001, about 100 miRNAs had been discovered in fly, worm and human [89–91].

1.3.1 Biogenesis and function

The biogenesis of miRNAs starts from the transcription of the miRNA-encoding gene by RNA polymerase II, this primary transcript is called primary miRNA (pri-miRNA) [92,93]. This pri-miRNA is self-complementary and forms a hairpin structure with some imperfect base pairing [94], which is captured by an enzyme complex of Drosha (RNase type-III) and DiGeorge syndrome critical region gene 8 (DGCR8) that processes the pri-miRNA into the precursor miRNA (pre-miRNA) with a 2-nucleotide overhang at the 3´end; this complex is also termed as microprocessor complex [92,95–97]. After that, the pre-miRNA is transported from nucleus to the cytoplasm via exportin-5 (XPO5) in association with RAN-GTP. In the cytoplasm, the pre-miRNA is further processed by another dsRNase type-III enzyme called Dicer [95,98]. Dicer has N-terminal helicase activity, which helps in the processing of a double-stranded miRNA duplex. This miRNA duplex is actively incorporated into RNA induced silencing complex (RISC), resulting in the formation of RISC loading complex (RLC) [99]. Subsequently one strand of the miRNA duplex, called guide strand, is incorporated into the complex, while the other strand (passenger strand) is removed from the complex [100]. The miRNA biogenesis is schematically illustrated in Figure 2.
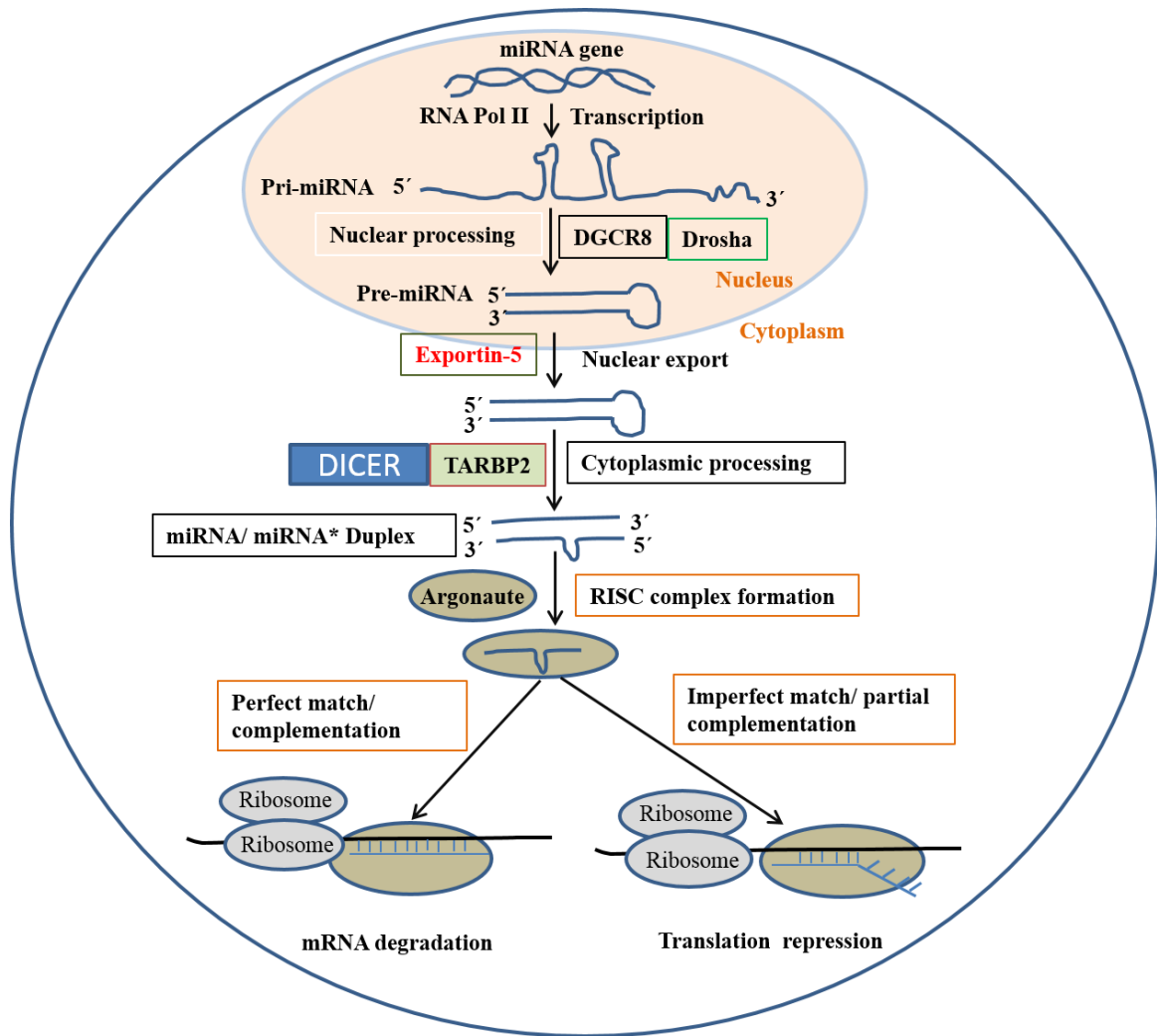
Figure 2. An overview of miRNA biogenesis and its mode of action. miRNA is transcribed from the host miRNA gene by RNA polymerase II and formed pri-miRNA, which is further processed by Drosha and DGCR8 protein complex to form pre-miRNA and then transported to the cytoplasm from nucleus by exportin 5. DICER and TARBP2 protein complex further processes the pre-miRNA into mature miRNA, which is then loaded into the RISC complex. miRNA binds to specific mRNA and degrades its target via translation repression or mRNA degradation depending on the degree of sequence complementarity between miRNA and mRNA.

miRNAs mostly functions by complementary base pairing with 3'UTR, but occasionally in the 5´UTR or coding sequence of the target mRNAs [101–104]. These interactions can either lead to degradation of target mRNA or inhibition of translation, depending upon the degree of base pairing. If there is more base-pairing or complete base pairing to the target mRNA, it is more likely to be degraded; in case of less number of base pairing or incomplete base pairing, it will lead to translational inhibition [105–107].

1.3.2 miRNAs in virus-induced tumorigenesis

Several human viruses are known to cause cancers, such as Epstein-Barr virus (EBV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human T-lymphotropic virus I (HTLV-I), human papillomavirus (HPV), Kaposi's sarcoma herpesvirus (KSHV) and MCPyV [108–115]. Among these, some are known to express viral oncogenes that directly contribute to cancer development through regulation of tumor suppressor pathways (such as Rb and p53), as well as other common targets that play important roles in tumorigenesis, including NF-κB, AKT-mTOR, autophagy and immune evasion. In this section, several examples of how miRNAs are involved in viral oncogenesis are described.

1.3.2.1 Hepatitis B/C (HBV or HCV)-associated hepatocellular carcinoma

Chronic infection with HBV and/or HCV is one of the known factors that contribute to hepatocellular carcinoma development. Although the mechanisms by which the viruses induce cellular transformation and tumorigenesis remain unclear, several pieces of evidence indicate the involvement of miRNAs in the pathogenesis of this tumor type. Several cellular miRNAs are known to regulate HBV and HCV gene expression and replication. For example, *miR-199a-3p*, *miR-210*, *miR-92a*, *miR-20a* and *miR-224* directly regulate HBV genes that reduce viral replication [116–118], while some miRNAs (*miR-1*, *miR-372/373*, *miR-141* and *miR-122*) can modulate HBV replication by targeting the cellular genes that regulate viral replication, such as farnesoid X receptor α, nuclear factor I/B, peroxisome proliferator-activated receptor alpha and cyclin G1 [119–121]. For HCV, *miR-122* binds directly to HCV RNA, which enhances HCV translation and replication [122]. Inhibition of *miR-122* effectively blocks HCV replication in human and chimpanzee [123; 124]. Additionally, *miR-141*, *miR-199a*, *miR-29*, *let-7b*, *miR-130a*, *miR-27a*, *miR-196* and *miR-448* can also regulate HCV replication through direct interaction of HCV RNA or indirectly via cellular targets [125–131].

Besides viral replication, viral proteins have also been shown to regulate miRNA expression that promotes tumorigenesis. For example, hepatitis B virus protein X (HBx, one of the transactivators that plays a crucial role in the hepatocellular carcinogenesis) represses *miR-148a*-mediated suppression of HPIP (a key regulator of tumorigenesis) that enhances tumorigenesis in a mouse model of hepatocellular carcinoma [132]. HBx also modulates multiple miRNAs, including *miR-216b*, *miR-373*, *miR-15b* and *miR-205*, while HBV preS2 (another transactivator of HBV) represses *miR-338-3p* that lead to the development or progression of hepatocellular carcinoma [133–138].

1.3.2.2 Human papillomavirus (HPV)-associated cervical cancer

Persistent infection with high-risk human papillomavirus (HPV), such as HPV 16 and 18, is a cause of cervical cancer [139]. This virus encodes two key oncoproteins, *i.e.* E6 and E7, which promote tumorigenesis by inhibiting the p53 and RB tumor suppressor, respectively. These two viral oncoproteins are also known to regulate miRNAs that play crucial roles in oncogenic pathways [139].

The high-risk HPV E6 has been shown to suppress several miRNAs resulting from the E6-mediated destabilization of p53, such as *miR-34a*, *miR-23b* and *miR-145* [139–141]. These miRNAs are transcriptionally activated by p53 and play an important role in p53 tumor suppression. The HPV E7 interacts with Rb and inhibits its binding to E2F, thereby promoting cell cycle progression through E2F-mediated transcription [142]. Similar to the E6-mediated effect of p53-regulated miRNAs, HPV16 E7 has been shown to regulate *miR-15b* [143], which is a transcriptional target of E2F1 [144]. Additionally, HPV16 E7 also induces *miR-27b* that regulates cell proliferation and apoptosis in cervical cancer cells through polo-like kinase 2 [145], and suppresses *miR-203*-mediated repression of p63 upon epithelial differentiation [146]. A few miRNAs have been implicated to regulate HPV DNA replication or gene expression. *miR-203*, *miR-145* and *miR-125b* can inhibit HPV genome amplification [146–148], while *miR-375*, *miR-875* and *miR-3144* directly suppress HPV transcripts [149].

1.3.2.3 *MCPyV-associated MCC*

As aforementioned, MCPyV plays an important role in MCC tumorigenesis. Our group previously identified specific miRNAs associated with MCPyV status, tumor metastasis and disease-specific survival in MCC patients [150], suggesting the importance of miRNAs in MCC development and progression. Importantly, *miR-375* is highly abundant and specific for MCC and its serum levels correlate with tumor burden in MCC [151]. However the functional role of miRNAs in MCC pathogenesis is still poorly understood. To date, only two miRNAs, *miR-203* and *miR-375*, have been functionally characterized in MCC. *miR-203* can inhibit cell growth and induce cell cycle arrest in MCPyV- MCC cells [150], while *miR-375* can regulate neuroendocrine differentiation, cell growth, autophagy and glycolysis in MCC cells [152–154]. In addition, Theiss *et al* demonstrated that *mcv-miR-M1* (a miRNA encoded by the MCPyV) suppresses early viral transcripts and limits DNA replication of viral episomes [155].

1.4 AUTOPHAGY

About 50 years ago, the term "autophagy" was given for the first time by Duve *et al* [156]. It is a Greek word, meaning self-eating property. Autophagy is a cellular degradation process required for development, differentiation, survival and maintaining the homeostasis of the cells [157]. Autophagy is mainly divided into three types, namely macro-autophagy, micro-autophagy and chaperone-mediated autophagy (CMA) [158], which differ on the basis of transportation of cytoplasmic material to the lysosomes. Macro-autophagy is the most common type of autophagy, which involves double membrane vesicles called autophagosomes containing cellular proteins and damaged organelles which are subsequently delivered to lysosomes for degradation [159]. Micro-autophagy involves projections of the lysosomal membrane that capture the cargo molecules, and CMA involves chaperone proteins that recognize the cargo proteins, then unfold and translocate them directly across the

lysosomal membrane [160,161]. As my thesis work mainly focused on macro-autophagy, a brief description of this process is given below.

1.4.1 Molecular mechanisms of autophagy regulation

Autophagosome formation requires autophagy related genes called ATGs [162]. The autophagy process is divided into three main steps: initiation, nucleation and expansion (Figure 3).

Initiation requires the unc-51-like kinase 1 (ULK1) complex, which contains ULK1 and ATG13. Induction of autophagy, due to any stress condition or activation signal by signaling molecules, releases the ULK1 complex from mTORC1. ULK1 translocates to the autophagy initiation sites and activates recruitment of a second kinase complex containing VPS34, BECN1, VPS15 and ATG14 [163], which leads to production of PI3P at the autophagosome initiation site. PI3P recruits another protein DFCP1 to start nucleation of autophagosome. The third step requires the ATG16L1-ATG5-ATG12 conjugation machinery for lipidation of light chain 3-II (LC3-II), which initiates the expansion and also fusion between autophagosome and lysosome [164–166].

Two distinct ubiquitin-like protein conjugation systems are involved in autophagy. The first system is ATG5-ATG12 interaction and conjugation, and the second is LC3 processing. The first step involves activation of ATG12 by ATG7 (E1 ubiquitin enzyme) through binding to its C-terminal glycine in an ATP-dependent manner. After that, the ATG12 protein is transferred to ATG10 (an E2 ubiquitin carrier protein) that makes covalent bond of ATG5 to ATG12. The ATG5-ATG12 complex then interacts with ATG16 dimer to form a multimeric ATG5-ATG12-ATG16 complex that fuses with the newly formed extending phagophore. The complex also induces swirl into the growing phagophore through recruitment of processed LC3 [159,167]. The second step is similar to the ubiquitin-mediated protein degradation systems involved in autophagosome. LC3 is present as full-length cytosolic proteins in most cell types. Activation of autophagy, induces proteolytic cleavage of LC3 by ATG4, a cysteine protease to generate LC3B-I. The synthesis and processing of LC3 is increased after induction of autophagy [158]. The activation of the PI3K3 or PI3K is required for formation of autophagosome, which mainly depends on the formation of a multiprotein complex including BECN1 [168].
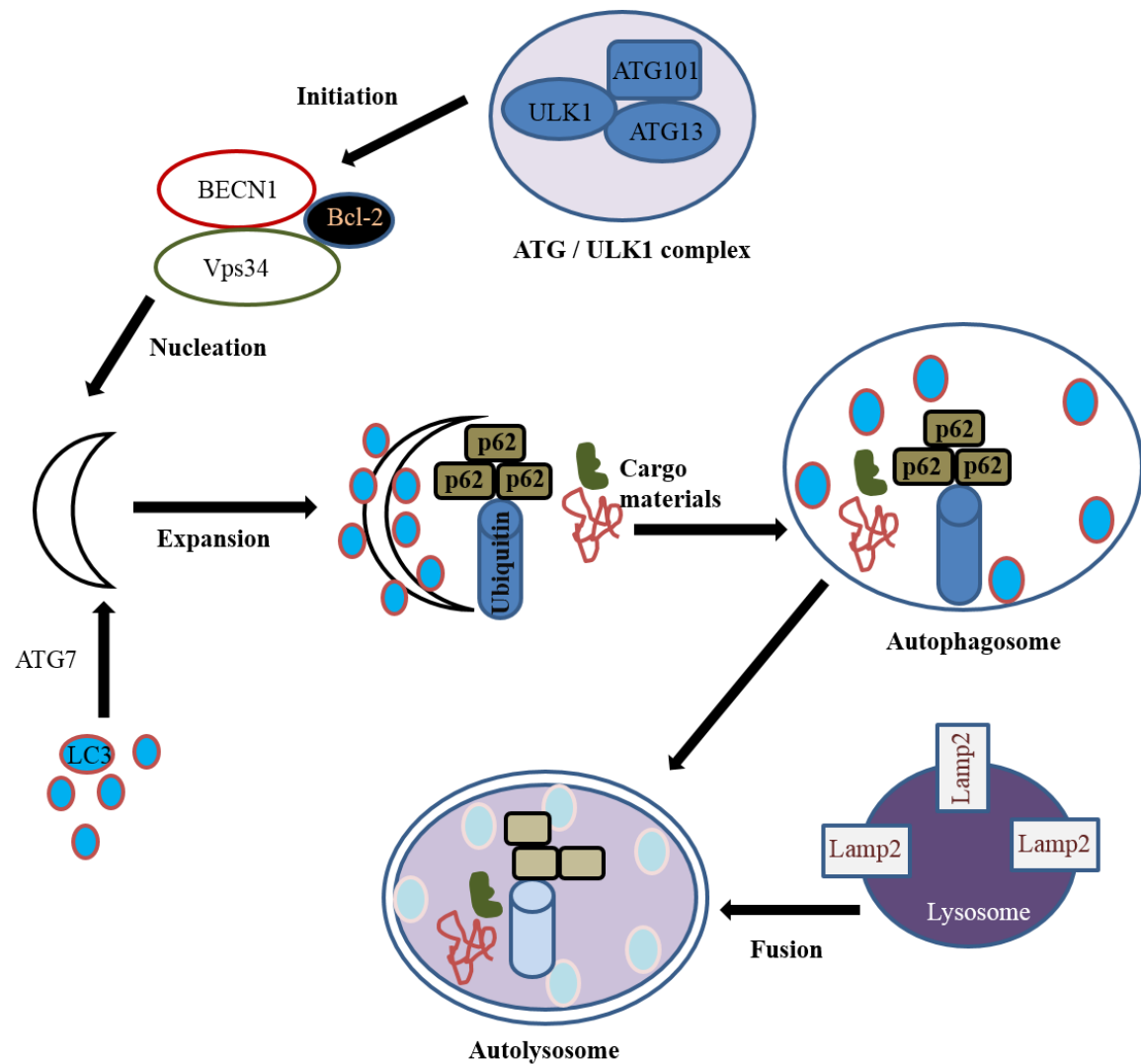
Figure 3. Different stages of autophagosome and autolysosome formation. Autophagosome formation starts with activation of the ATG/ULK1 complex, which recruits Beclin1 (BECN1) to induce nucleation with VPS34. VPS34 produces phosphatidylinositol-3-phosphate (PI3P), which helps to recruit other ATGs and light chain 3 (LC3). LC3 activation and phosphorylation leads to conjugation with phosphatidylethanolamine (PE). PE acts as a receptor for p62, which binds to ubiquitinated proteins and starts degradation once autophagosome fuses with lysosome and forms autolysosome.

1.4.2 Role of autophagy in tumorigenesis

Autophagy can play a dual role in cancer development. Autophagy genes are commonly deleted in tumors [169,170], leading to increased oxidative stress and DNA damage, which subsequently leads to genomic instability and tumor progression [170–173]. As such, autophagy plays a suppressor role. However, autophagy is induced upon cellular stress such as starvation, oxidative stress and DNA damage [174–177], which protect cancer cells for survival.

13

Autophagy can be suppressed by activation of the PI3K/Akt through activating mutations in PI3K, PTEN loss and AKT amplification, which subsequently leads to activation of mTOR pathway [178,179]. Bcl2 amplification and overexpression inhibit autophagy through BECN1 [180,181]. Nutrient deprivation or genotoxic stress induces p53 expression, which leads to activation of autophagy, due to inhibition of mTOR pathways [182,183]. p53 can suppress autophagy at the basal levels [184]. Ectopic overexpression of p62 inhibits autophagy and leads to tumorigenesis through activation of NF-κB signaling pathway and DNA damage response [170]. Autophagy promotes tumor cell survival under hypoxia or nutrient deprivation [171], as well as type II programmed cell death [185].

1.5   c-KIT IN CANCER

Besmer *et al* first discovered v-KIT in sarcoma virus [186], and one year later, cellular c-KIT was found by Yarden *et al* [187]. c-KIT is a receptor tyrosine kinase (RTK) and a transmembrane protein. It contains an extracellular domain, a transmembrane domain and an intracellular domain (also called kinase domain since most of the phosphorylation sites are present in this domain (Figure 4) [188].
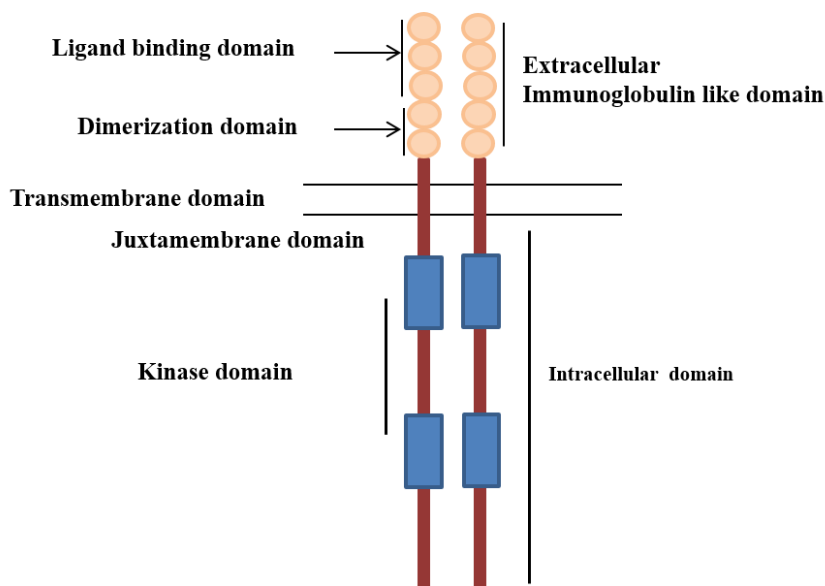
Figure 4. Domain structure of c-KIT. c-KIT is composed of three main domains: (i) The extracellular domain, which consists of five immunoglobulin-like domains, three of them bind to its ligand stem cell factor (SCF), and the fourth is known to help in dimerization; (ii) The transmembrane domain is present in the cell membrane; and (iii) The intracellular domain, which contains the tyrosine kinase domain.

c-KIT is expressed in several cell types such as hematopoietic progenitor cells, germ cells, melanocytes and mast cells. It plays important roles in organogenesis, development, cell differentiation, proliferation, viability and other cellular physiological functions [189–191]. Activation of c-KIT has been observed in several human diseases, including cancer [192–194].

1.5.1 Activation and function

In normal cells, c-KIT is activated after binding of its ligand, called stem cell factor (SCF), to the extracellular domain of c-KIT. SCF induces homodimerization of the intracellular domain or kinase domain of the c-KIT and activates Src-homology2 (SH2), which leads to signal transduction (Figure 5) [190,195]. It can activate several pathways depending on the cellular context, including Ras-MAP kinase, PI3K-Akt-mTOR pathways, Ras-Erk pathways, and PIP3-dependent PKC pathways [196,197]. After activation, c-KIT is then ubiquitinated, which leads to internalization of the receptor and is then transported into lysosomes for degradation [190].
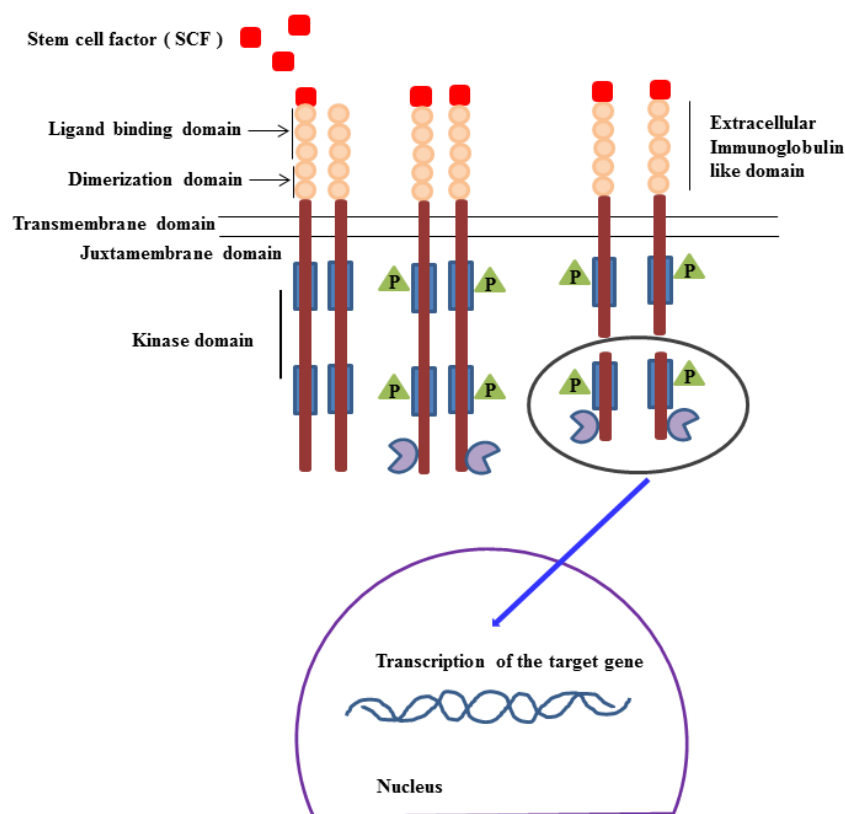
Figure 5. Activation and function of c-KIT. Once stem cell factor (SCF) binds to extracellular ligand binding domain of c-KIT, the dimerization domain is activated and starts dimerization, leading to activation of the kinase domain by phosphorylation. Its signal is then transferred to the nucleus to perform its functions.

c-KIT overexpression has been found in several human cancers, such as gastrointestinal stromal tumor (GIST), mast cell leukemia, testicular germ cell tumors and MCC [188]. Activating mutations of *c-KIT* are frequently observed in human cancers. Till now around 500 different types of *c-KIT* mutations had been reported in human tumors, most of the reported mutations are passenger but some of them are also driver mutations [188]. As an example, in GIST, more than 80% of the tumors contain activating mutations of *c-KIT* [193].

These mutations lead to ligand-independent activation of c-KIT [198–200], which activates PI3K-Akt, STATs and Erk-MAP kinase pathways that promote GIST development [201,202].

1.5.2 Paranuclear c-KIT dot in cancers

As aforementioned, c-KIT is generally expressed on the plasma membrane, where it is activated after binding with its ligand SCF, leading to activation of signaling cascade. However, c-KIT is also present as paranuclear dot-like structure in some cancer types, such as GIST, mast cell leukemia and testicular germ cell tumor [203–205]. For GIST and mast cell leukemia, it is now known that mutations of the *c-KIT* gene contribute to the paranuclear localization [204,206]. However, the exact subcellular localization of paranuclear c-KIT is different between GIST and mast cell leukemia.

In GIST, paranuclear c-KIT is mainly localized on Golgi apparatus [205]. The aberrant kinase activity of mutant c-KIT prevents its transport from the Golgi to the plasma membrane [205] and PKC-Φ promotes stabilization of the mutant c-KIT in the Golgi complex [207]. In mast cell leukemia, c-KIT is mainly localized on endolysosome and endoplasmic reticulum (ER) [204,208,209]. Similar to GIST, the kinase activity of mutant c-KIT is required for the accumulation of mutant c-KIT on endolysosomes via clathrin-mediated endocytosis [210]. Despite their different localizations, they share common oncogenic signaling; mutant c-KIT activates the PI3K-Akt pathway in both cancer types [205,210]. Besides PI3K-Akt pathway, mutant c-KIT can also activate STAT5 and Erk in GIST [205]. On other hand, in mast cell leukemia, mutant c-KIT activates STAT5 on the ER [210].

1.6 ENERGY METABOLISM

Energy metabolism is defined as the process that produces energy for performing all kind of cellular functions in living organisms. There are several processes or pathways in the cells to generate energy including glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathways and mitochondrial respiration. These are the major metabolic pathways in the cells to fulfill their energy requirement and maintain cellular homeostasis.

1.6.1 Glycolysis and mitochondrial metabolism

Glycolysis and mitochondrial energy metabolisms are the most commonly used processes to generate energy in the cells [211,212]. Glycolysis is a process by which glucose molecules are catalyzed by several enzymes and produce, pyruvate, 2 molecules of ATP and NADH each. It is a universal process used by almost all cells [213]. If cells are hypoxic, pyruvate is then converted into lactate [214]. In normal cells, pyruvate is converted into acetyl-coA, which enters the TCA cycles and is further catalysed by a series of enzymatic reactions to produce ATP, NADH and FADH2 as energy intermediates, and releasing CO_2 and H_2O as side products. The FADH2 and NADH produced from the TCA are then transported to the electron transport chain and produce ATP with the help of proton gradient through respiratory complexes I, II, III, and IV [215–218]. Mitochondria also regulate the energy

demand and supply balance by increasing the number of mitochondria whenever they are needed or decreasing the number of mitochondria through fission and mitophagy when the energy demand is less [217,219,220].

1.6.2 Metabolic reprogramming in cancer

Metabolic reprogramming is a hallmark of cancer cells [221]. In general, normal cells mostly rely on mitochondrial oxidative phosphorylation to produce energy to support their activities. However, most cancer cells depend on aerobic glycolysis to generate energy sources to support tumor growth [214,222,223] (Figure 6).
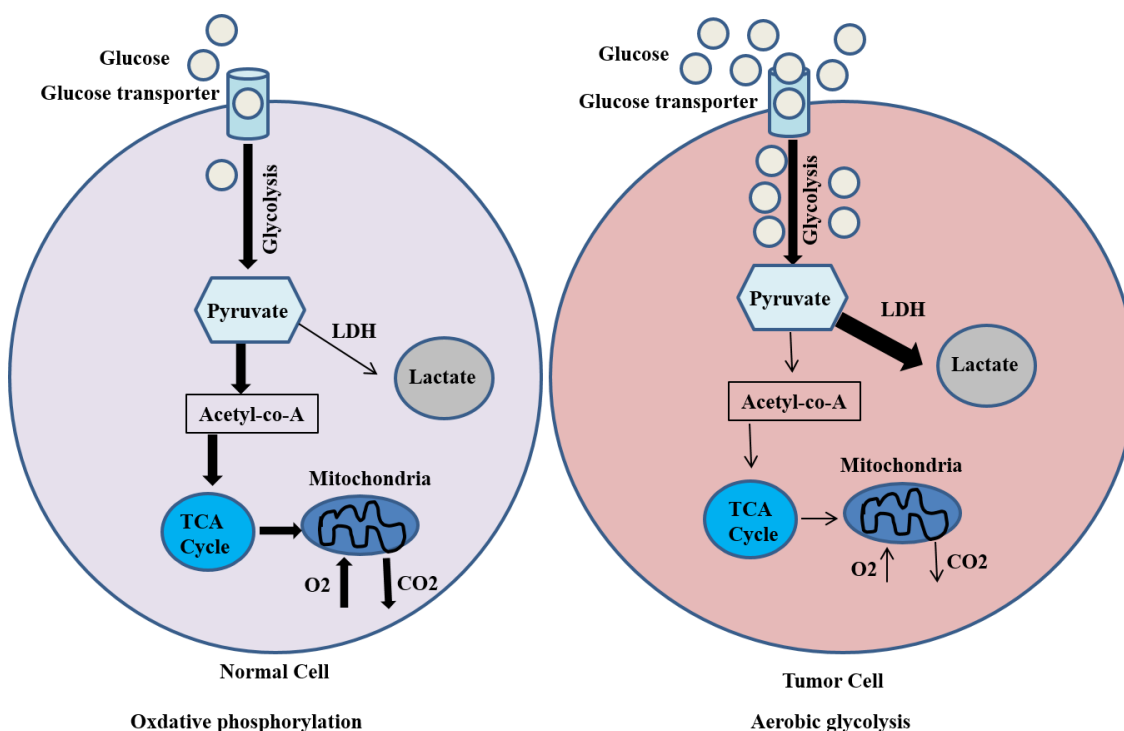
Figure 6. Energy metabolism in normal and tumor cells. Normal cells generally use mitochondrial respiration as a major process to produce energy. In this process glucose is converted into pyruvate by several enzymatic cascades to produce two ATP molecules. The pyruvate is further converted into acetyl-CoA and transported into tricarboxylic acid (TCA) cycle, which produces FADH2 and NADH. These molecules are further reduced in the electron transport chain to produce 32 molecules of ATP. Excessive pyruvate is then converted into lactate, by lactate dehydrogenase enzyme. Unlike normal cells, tumor cells preferentially use aerobic glycolysis and lactate as a major source of energy. This process is also called Warburg effect.

It is now known that several mechanisms can lead to metabolic reprogramming from oxidative phosphorylation to glycolysis in cancer cells. Among them, overexpression of glycolytic enzymes, such as GLUT1, HKII and LDHA, is frequently observed in various cancer cells [224,225]. Lactate dehydrogenases (LDHs) play an important role in the interconversion of pyruvate and lactate. LDHB is responsible for converting lactate to pyruvate that fuels the TCA, while LDHA converts pyruvate to lactate. The balance between

these two enzymatic activities can affect lactate homeostasis in the cells, which can switch between glycolysis and oxidative phosphorylation. Deregulations of these enzymes can lead to metabolic reprogramming, which has been observed in several cancer types, such as pancreatic cancer and lymphoma [226,227]. Additionally, lactate homeostasis also requires monocarboxylic acid transporters (MCTs) that control the excessive levels of lactate in the cells by transporting the lactate across the plasma membrane. High levels of these transporters have also been observed in several tumor types, including breast cancer, choriocarcinoma and head and neck cancer [228–230].

2   AIMS OF THE STUDY

The overall aim of this thesis work was to investigate the functional roles of MCPyV T-antigens in MCC tumorigenesis. The specific aims of the thesis were to:

• Determine the involvement of MCPyV T-antigens in regulation of miRNAs and autophagy (**Paper I**)

• Investigate the expression and function of c-KIT in MCC (**Paper II**)

• Characterize the functional role of *miR-375* and its target LDHB in MCPyV+ and MCPyV- MCC cells (**Paper III**)

• Study the role of MCPyV T-antigens in regulation of LDHB expression and glycolysis (**Paper IV**)

3 MATERIALS AND METHODS

3.1 CELL LINES AND MCC PRIMARY CULTURE

Eight human established cell lines and one primary culture (ME27) were included in this thesis, which are listed in Table 3.

Table 3. Human cell lines and primary culture used in this thesis

Name	Description	MCPyV status	Source	Paper
MCC13	Merkel cell carcinoma	negative	Cell Bank Australia, Westmead, NSW, Australia	I-IV
MCC14/2	Merkel cell carcinoma	negative	Cell Bank Australia, Westmead, NSW, Australia	I-IV
MCC26	Merkel cell carcinoma	negative	Cell Bank Australia, Westmead, NSW, Australia	I-III
WaGa	Merkel cell carcinoma	positive	Gift from Dr. JC Becker (Medical University of Graz, Austria)	I-IV
MKL-1	Merkel cell carcinoma	positive	Gift from Dr. NL Krett (Nortwestern University, IL, USA)	I-IV
MKL-2	Merkel cell carcinoma	positive	Gift from Dr. Roland Houben (University Hospital Würzburg, Germany)	II, III
ME27	Merkel cell carcinoma	positive	Shi et al., unpublished	II
GIST882	Gastrointestinal stromal tumor	n.a.	Gift from Dr. JA Fletcher (Brigham and Women's hospital, Boston, MA)	II
HEK293	Human embryonic kidney	n.a.	Gift from Dr. Yingbo Lin (Karolinska Institutet, Sweden)	II

n.a.=not analyzed

The authenticity of the six MCC cell lines and GIST882 had been confirmed by genotyping of short tandem repeat (STR), as shown in Table 4.

Table 4. Short tandem repeat (STR) profiles of the 6 MCC cell lines and GIST882 used in this thesis

Locus	MCC13	MCC14/2	MCC26	WaGa	MKL-1	MKL-2	GIST882
D8S1179	13, 14	10, 14	8, 10	10, 13	10, 10	11, 13	12, 12
D21S11	30, 31	29, 32.2	31, 32.2	28, 30	30, 30	31.2, 31.2	29, 31.2
D7S820	10, 10	8, 10	8, 9	10, 10	8, 11	10, 13	10, 15
CSF1PO	12, 12	10, 11	10, 11	12, 12	11, 12	10, 12	11, 11
D3S1358	16, 18	16, 16	17, 17	14, 14	16, 16	17, 17	15, 15
THO1	7, 9.3	6, 9.3	9.3, 9.3	9.3, 9.3	9, 9.3	7, 8	9, 9
D13S317	12, 12	13, 13	13, 14	8, 13	8, 11	12, 13	12, 12
D16S539	9, 11	13, 13	11, 13	11, 12	10, 12	10, 12	11, 11
D2S1338	19, 20	19, 19	23, 26	20, 23	17, 17	17, 23	18, 26
D19S433	15, 15	12, 14	16, 16	14, 16	14.2, 15.2	13.2, 14	13, 15
vWA	17, 17	17, 18	16, 18	16, 17	16, 18	16, 17	16, 17
TPOX	8, 8	8, 8	8, 8	8, 11	8, 8	8, 11	11, 11
D18S51	16, 17	15, 17	15, 18	10, 13	12, 18	14, 17	14, 14
AMEL	X, X	X, X	X, X	X, Y	X, Y	X, Y	X, Y
D5S818	9, 12	13, 13	12, 12	12, 12	11, 12	12, 14	12, 12
FGA	19, 20	21, 21	24, 25	19, 19	21, 25	19, 26	23, 23

3.2 MCC TUMOR SAMPLES

Forty-five formalin-fixed paraffin-embedded and 28 frozen MCC tumor samples were collected at Karolinska University Hospital or Stockholm South General Hospital. The diagnosis of MCC was established at the time of diagnosis by routine histopathological and immunohistochemical analyses. Forty-five FFPE samples were used in **Paper I**, 45 FFPE sections in **Paper II** and 28 frozen and 26 FFPE samples were used in **Paper III**. The clinical information of the patients is described in their respective studies.

3.3 RNA EXTRACTION AND RT-QPCR

3.3.1 RNA extraction

Total RNA was isolated by using mirVana miRNA isolation kit (Ambion/Thermo Fisher Scientific, Waltham, MA) and the concentrations were measured with a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington, DE).

3.3.2 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR is a reliable method used to detect and measure transcripts in a particular sample through PCR. There are two types of RT-qPCR methods: SYBR green and TaqMan assays. SYBR green is a relative cost effective and easy method to quantify target gene expression. It contains fluorescent dye that binds to dsDNA molecules. The amount of fluorescence measured at the end of each PCR cycle reflects the quantity of the amplified cDNA. This method was used to quantify mRNA expression of MCPyV T-antigens (**Paper I**).

The TaqMan assay requires specific primers and TaqMan probes. TaqMan probe is a sequence-specific DNA molecule with a fluorophore attached at the 5′-end and a quencher at the 3′-end. When the fluorophore and quencher are in close proximity it inhibits the fluorescence signal. During PCR amplification, the *Taq* DNA polymerase extends the primer and cleaves the TaqMan probe through its 5′-to 3′exonuclease activity. After cleaving the probe, the fluorophore is released from the quencher emitting a fluorescence signal for detection and quantification. This method was performed to detect *miR-375*, *miR-30a-3p*, *miR-30a-5p, miR-125a-3p*, *miR-16*, *pri-miR-375*, *pri-miR-30a*, *18S* rRNA and *RNU6B* in **Paper I**, *miR-375*, *LDHB*, *RNU6B* and *GAPDH* in **Paper III**, and *LDHB* and *β-actin* in **Paper IV**. All TaqMan assays were purchased from Applied Biosystems/Thermo Fisher Scientific (Foster City, CA).

3.4 PLASMIDS

Plasmid is a small circular dsDNA molecule that has the capacity to replicate independently from chromosomal DNA. It is widely used as a vector to transfer, multiply or express specific

gene(s) of interest. In this thesis, different plasmids were used to express MCPyV T-antigens, short-hairpin RNA (shRNA) targeting MCPyV T-antigens, miRNAs, miRNA sponges, cellular protein-coding genes and reporters.

3.4.1 MCPyV T-antigens expression plasmids

Five different MCPyV T-antigens expression vectors were used (Figure 7). LTco and sTco are codon-optimized vectors with deleted splicing donor and acceptor sites in the sequence, therefore they only express LT or sT, respectively. LT339 contains tumor-derived LT cDNA isolated from a MCC tumor, which encodes a truncated LT with 469 aa. Additionally, two LT mutants were also applied. $LTco^{D44N}$ expresses a full-length LT with a substitution mutation at residue 44 (D>N) in the HSC70 binding domain, which abolishes the interaction between HSC70 and LT. $LT339_{W209A}$ encodes a truncated LT with substitution of tryptophan for alanine at residue 209, disrupting the interaction between LT and VAM6P.
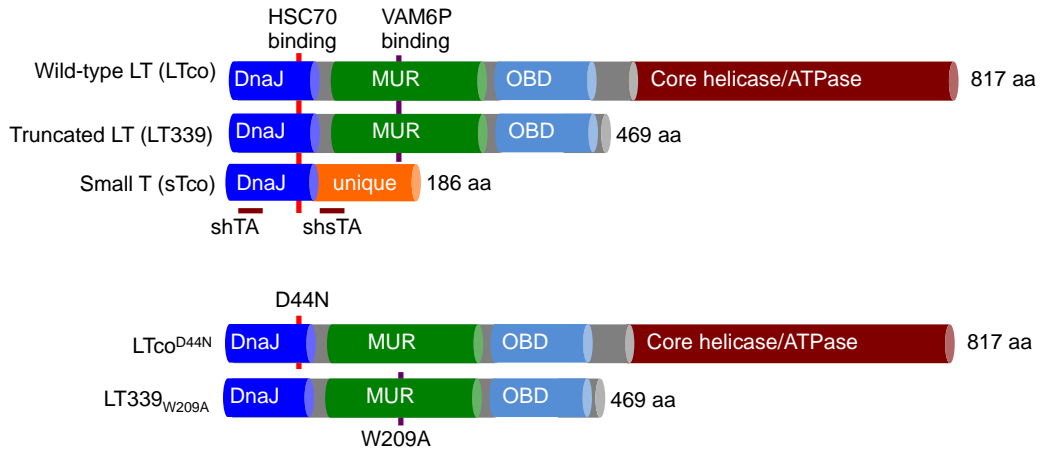
Figure 7. Plasmids expressing different MCPyV T-antigens and short-hairpin RNAs targeting T-antigens. LTco and LT339 express wild-type and truncated LT, respectively. sTco expresses only small T. $LTco^{D44N}$ has a substitution mutation of aspartic acid at residue 44 with asparagine, while the $LT339_{W209A}$ has alanine substituted for tryptophan at residue 209. shTA is a short hairpin RNA targeting the common exon 1 of both LT and sT, and shsTA only targets the unique region of sT.

3.4.2 Cellular protein-coding plasmids

In **Paper II**, pcDNA3-KIT-WT expressing full-length coding sequence of *c-KIT* was kindly provided by Dr. Lars Rönnstrand (Lund University), and in **Paper IV**, LDHB-FLAG plasmid expressing full-length coding sequence of LDHB and DYKDDDDK(FLAG)-tagged on its carboxyl terminus was purchased from GenScript (Piscataway, NJ).

3.4.3 Short hairpin RNA (shRNA), miRNA expression and sponges

shRNA vector is used to express a single-stranded RNA molecule with a hairpin structure, which can be used to silence a particular target gene by RNA interference (RNAi). Generally, shRNAs targeting specific genes of interest consist of sense and antisense sequences separated by a loop sequence, which folds back into a loop structure after being transcribed. This hairpin transcript is then exported to the cytoplasm and processed by DICER enzyme into ~21-nucleotide small interfering RNA (siRNA) duplexes that are loaded into RISC for gene silencing. In this thesis, shRNAs targeting MCPyV LT and/or sT were performed using shTA and shsTA, respectively (**Papers I** and **IV**). In **Paper II**, two shRNAs targeting common exon 1 of MCPyV T-antigens were used: shTA1 and shTA2. shTA2 targets the 5'UTR of LT/sT at nucleotide 173-193, while shTA1 (same as shTA in **Papers I** and **IV**) targets the nucleotide sequence 222-242 encoding the DnaJ domain of T-antigens (Figure 7). In **Paper II**, shRNA targeting exon 7 of *KIT* was also constructed to silence c-KIT expression.

miRNA expression vectors are similar to shRNAs, which express RNA transcripts with a loop structure. There are two ways to construct miRNA expression vectors. One way is to clone a specific mature miRNA sequence together with its respective complementary sequence and the loop sequence into a vector. Another way is to amplify or synthesize the precursor sequence of specific miRNA (that is known to have a natural hairpin structure after being transcribed) and clone it into a vector. Examples of these designs are illustrated in Supporting Information, Fig. S2 of **Paper I**.

miRNAs sponge vectors express RNA transcripts that contain multiple miRNA binding sites, which can sequester miRNAs from their endogenous targets, thereby inhibiting miRNA function. Description of miRNA sponge vectors for miR-375sp, miR-30a-3psp and miR-30a-5psp is given in Supporting Information, Fig. S1 of **Paper I**.

3.4.4 Reporter constructs

Two types of reporter constructs, mRFP-GFP-LC3 and luciferase reporters, were used in **Paper I**. The mRFP-GFP-LC3 reporter plasmid was used to detect autophagic flux. It is based on the concept of acidic lysosomal pH that quenches the GFP signal in autophagic substrate, which allows us to distinguish autolysosomes and autophagosomes. Autophagosomes are represented by yellow puncta (RFP+/GFP+), and autolysosomes are shown in red puncta (RFP+/GFP-). If both yellow and red puncta are increased, it indicates an increase of autophagic flux. If only yellow puncta are increased, it indicates that autophagic degradation is blocked.

Luciferase is a bioluminescence-producing enzyme commonly found in fireflies. This enzyme can react with a luminescent substrate, luciferin, to produce light emission. There are two commonly used luciferases: firefly and *Renilla* that generate light in the range of 550-570 nm and 480 nm, respectively. Typically, a luciferase gene is cloned upstream or downstream of a specific DNA sequence of interest that allows us to quantify the activity of the target

sequence by measuring the released light. In **Paper I**, luciferase reporters were used to verify specific miRNA binding sites and to determine the efficiency of miRNA sponges.

3.5 TRANSFECTION

Transfection is the most widely used method to introduce external DNA or RNA into the eukaryotic cells. This method is mainly based on opening the pores into the membrane by different mechanisms and delivers the external nucleic acid into the cells. In this thesis lipid based transfection and nucleofection were used.

3.5.1 Lipid-based transfection

Lipid based transfection is based on different charges between the lipid-based reagent and the nucleic acid. The positively charged head group of lipid reagents forms a complex with the negatively charged nucleic acid called liposome. The liposome then fuses with the cell membrane and delivers the nucleic acid into the cells by endocytosis. In this thesis Lipofectamine 2000 was used for transfection of plasmid DNAs and/or siRNAs (**Papers I-IV**). RNAiMAX was used to deliver miRNA mimics into the cells (**Paper III**). miRNA mimics are small chemically modified dsRNA molecules that mimic endogenous mature miRNAs. This transfection method was used for all, except MCPyV+, cell lines.

3.5.2 Nucleofection

For all MCPyV+ cell lines (**Papers I-IV**), nucleofection was used. This method is based on electroporation, which uses a combination of cell-type specific solutions and specific voltage parameters to deliver nucleic acid directly into the nucleus of the cells. This method is suitable for primary cell cultures, non-dividing cells and cell lines that have been difficult to transfect by lipid-based methods.

3.6 PROTEIN EXPRESSION AND MODIFICATION ANALYSES

3.6.1 Western blot

Western blot, also called immunoblot, is the most commonly used molecular biology technique to detect, quantify and identify specific proteins based on their respective molecular weight using a specific antibody. The first step of this method is to extract protein lysates from cells or tissue samples using lysis buffer. Subsequently the protein lysate is loaded onto an electrophoresis gel to separate proteins based on their respective sizes. The proteins are then transferred to a nitrocellulose or polyvinylidene fluoride membrane, and incubated with specific antibodies to the target proteins. After that, washing buffer is used to remove unbound or non-specific primary antibodies from the membrane, followed by incubation with species-specific horseradish peroxidase (HRP) or fluorescent-conjugated

secondary antibody. The results can be detected by chemiluminescence or fluorescence (depending on the type of secondary antibody) using an imaging system.

Besides detection and quantification of specific proteins, Western blot can also be used to detect protein modifications. Protein modification or posttranslational modification of proteins may occur after protein synthesis. There are several types of modification, mostly performed by catalytic enzymes that recognize a specific target sequence or amino acid of the specific proteins. The most common modifications are phosphorylation, acetylation, methylation and glycosylation. In **Paper II**, phosphorylation and glycosylation of c-KIT were analyzed. Protein phosphorylation is a post-translational modification of proteins where a phosphate is covalently bound to an amino acid residue by a protein kinase. Glycosylation is the modification where a carbohydrate is added to a protein. To detect the phosphorylation state of c-KIT, a phospho-specific antibody was used to recognize the phosphorylated amino acid. To assess the glycosylation status of c-KIT, two glycosidases (endonuclease H and peptide:N-glycosidase F) were used to digest high mannose or complex glycan structures, followed by detection of deglycosylated c-KIT using Western blotting.

3.6.2 Immunohistochemistry

Immunohistochemistry is widely used to identify expression levels, tissue distribution and subcellular localization of specific proteins on tissue sections. This method also requires specific antibody to recognize specific antigen in tissue samples, followed by detection of the antibody-antigen interaction using a secondary antibody conjugated with an enzyme or a fluorophore. This method has been applied to detect MCPyV LT, p62 and ATG7 in **Paper I**, and c-KIT in **Paper II**.

3.7   PROTEIN-PROTEIN INTERACTION

Protein-protein interactions play an important role in predicting the functions of specific target proteins. These interactions control a range of biological functions such as metabolic processes, cell-to-cell interactions and development. Around 80% of the proteins work in a group or cluster to perform their functions. There are several methods to identify these interactions. Two of them were used in this thesis, as described below:

3.7.1 Co-immunoprecipitation

Co-immunoprecipitation (co-IP) is a very popular technique to capture the primary target (i.e. antigen) and other proteins that are bound to the target in a protein lysate. Typically, the target protein and its associated protein complex are pulled down using immobilized antibody on beads. The isolated protein complexes are then separated and detected by Western blotting. Besides co-IP, co-localization of two proteins can also be assessed using immunofluorescence microscopy, as described below.

3.7.2 Immunofluorescence confocal microscopy

Immunofluorescence (IF) microscopy is a robust and reliable technique to assess expression and subcellular localization of a target protein. This technique can be used on cultured cells, single cell, and tissue sections. In **Paper II**, IF was used to detect the expression and subcellular localization of c-KIT. In addition, the interaction between c-KIT and BECN1 was also analyzed by co-localization of the two proteins using IF and confocal microscopy.

3.8 AUTOPHAGY ASSAYS

Autophagy is a dynamic process and multiple assays are recommended to verify an autophagic response. In this thesis, three different methods were applied to study the effect on autophagy.

3.8.1 Western blot detection of LC3-II

The LC3 protein is known to be present in all autophagic membranes from an early stage (i.e. phagophore) to a late stage (i.e. autolysosome). The total amount of LC3 is a good indicator of the autophagy index. Full-length LC3 is first cleaved by the ATG4 autophagy protein forming the cytoplasmic LC3-I. LC3-I is then conjugated with phosphatidylethanolamine by ATG7 and ATG3 to form LC3-II. Progression of autophagy can be detected by LC3-I to LC3-II conversion. Detection of LC3-II expression levels at a specific time point is not sufficient to estimate the actual autophagy flux because of its dynamic nature. Increased LC3-II expression levels can either reflect induction of autophagy or inhibition of autolysosme formation. To detect actual induction or inhibition of LC3-II levels, autolysosome formation can be blocked by specific chemical. In this thesis bafilomycin A was used to block autolysosome formation and then detection of the LC3-II level was evaluated with and without the treatment.

3.8.2 mRFP-GFP-LC3 reporter

Another method to evaluate autophagy flux is to measure the LC3 puncta from the mRFP-GFP-LC3 reporter by using confocal microscopy. These puncta correspond to the number of autophagosomes or autolysosomes (refer to Chapter 3.3.4). The principle of this reporter and the interpretation of the fluorescent puncta had been described in Chapter 3.3.4.

3.8.3 Transmission electron microscopy (TEM)

TEM is a powerful technique to study the ultrastructure of cells based on the image generated from a high-energy beam that passes through a very thin tissue section. This method is an important tool to identify autophagic vesicles in cells; however it may not be readily quantifiable. The LC3 immunoblot and reporter assays are more quantitative approaches for monitoring autophagy.

3.9 CELL VIABILITY OR GROWTH ASSAYS

Trypan blue exclusion and WST-1 assays were used to assess cell viability or cell growth in this thesis. These assays are briefly described in the following sections:

3.9.1 Trypan blue exclusion assay

The trypan blue assay is based on the principle that live cells possess a rigid and intact plasma membrane that prevents entry of trypan blue dye, whereas dead cells are not able to prevent the entry of the trypan blue dye. Dead and live cells can be distinguished and quantified using a bright field microscope or an automatic cell counter. This method was used to measure the number of live and dead cells after treatment with bafilomycin A1, torin-1, pan-caspase inhibitor (z-VAD-FMK) or camptothecin (**Paper I**). This assay was also used to assess the effect of *miR-375* and LDHB regulation on cell growth in MCC cell lines (**Paper III**).

3.9.2 WST-1 assay

WST-1 is a colorimetric based assay used for quantification of cell proliferation and viability. The principle of this assay is based on the conversion of tetrazolium salt WST-1 into a colored compound by dehydrogenase enzymes, which are produced by mitochondria. The amount of colored product in the medium is directly proportional to the amount of enzymes produced by the mitochondria; as a result it corresponds to the number of metabolic active cells. This method was used as the primary method for assessment of cell growth or viability in **Papers I-IV**.

3.10 APOPTOSIS ASSAYS

Apoptosis is a form of cell death, which plays an essential role to maintain homeostasis and in the development of organisms. Apoptosis can be induced by several external and internal factors like UV-radiation, DNA damaging chemicals or any kind of stress inside the cells. Apoptotic cells generally display morphological changes (e.g. membrane blebbing, nuclear fragmentation and chromatin condensation) and biochemical modifications (e.g. protein cleavage, DNA breakage). These features are used in the following methods to detect apoptosis.

3.10.1 Annexin V assay

Annexin V has a strong affinity binding specifically to phosphatidylserine (PS). When apoptosis is activated, changes occur in the membrane structure of the apoptotic cells causing PS flips from the inner to the outer membrane surface. This allows the binding of Annexin V to PS of the apoptotic cells. Typically, Annexin V is conjugated with a fluorophore that allows the detection using flow cytometry. Propidium iodide (PI) staining is also commonly used together with the annexin V detection. This stain does not stain viable cells or early apoptotic cells because of an intact plasma membrane, thus enables to distinguish late apoptotic cells from early apoptotic cells. This method was applied in **Papers I**, **III** and **IV**.

3.10.2 Cleaved PARP

Poly (ADP-ribose) polymerase-1 (PARP-1) is a nuclear protein physiologically involved in DNA repair mechanism by adding poly (ADP ribose) polymers in response to several types of cellular stresses. During apoptosis, the full-length PARP-1 (116 kDa) is cleaved by caspases to generate two fragments: 89 kDa and 24 kDa. The 89 kDa carboxyl-terminal fragments containing the catalytic domain has reduced DNA binding capacity and is released to the cytosol, while the 24 kDa fragment irreversibly binds to nicked DNA and inhibits DNA repair. These PARP-1 cleavage products can be detected by Western blotting using specific antibodies targeting these fragments, which had been applied in **Papers II** and **III**.

3.11 METABOLIC ASSAYS

Energy metabolism is the process of energy production from nutrients, which is required to maintain cellular homeostasis in the presence or absence of oxygen. Generally, normal cells rely primarily on mitochondrial respiration to generate energy and cancer cells preferentially use glycolysis. In this thesis, two metabolic assays were used to measure extracellular oxygen consumption and acid production, which monitor rates of glycolysis and respiration (**Paper IV**).

3.11.1 Extracellular Acidification Rate (ECAR)

Glycolysis is a catabolic metabolic process, which provides energy to the cells to maintain their growth, viability and perform all other functions for their survival. In glycolysis, glucose is converted to lactate and protons. As lactate is the source for glycolytic acidification, glycolytic activities can be measured by calculating the rates of extracellular acidification. This assay is based on the pH-sensitive reagent. This reagent is chemically stable and inert. When acidification is increased, it increases the signal for detection.

3.11.2 Oxygen Consumption Rate (OCR)

The second major pathway for cells to provide energy is oxidative phosphorylation. Oxidative phosphorylation uses tricarboxylic acid cycle (TCA), which requires the electron transport chain in the mitochondria using oxygen as a terminal electron acceptor. The Abcam extracellular oxygen consumption reagent is an oxygen-sensitive fluorescent dye, which measures the amount of oxygen in the assay medium. Normally, the dye is quenched by oxygen. During mitochondrial respiration, oxygen is depleted, which reduces the quenching of the fluorescent dye that leads to an increase of the fluorescence signal.

3.12 STATISTICAL ANALYSES

Student's paired t-test was used to compare two groups in transfection experiments (**Papers I-IV**). Multiple comparisons were evaluated using One-way ANOVA with post-hoc Tukey

test (**Paper I**) or Dunnett's test (**Paper II**). Associations of immunohistochemical staining and MCPyV status were assessed using Fisher's exact text (**Paper I**), while correlation between *miR-375* and *LDHB* expressions was evaluated using Spearman's rank order correlation (**Paper III**). All analyses were two-tailed and *p*-values <0.05 were considered as significant.

4 RESULTS AND DISCUSSIONS

4.1 INVOLVEMENT OF MCPYV T-ANTIGENS IN MIRNAS AND AUTOPHAGY REGULATION (PAPER I)

In our previous study, we identified a subset of miRNAs associated with MCPyV status in MCC clinical samples [150]. Two of these MCPyV-associated miRNAs (*miR-375* and *miR-30a-5p*) are known to target autophagy genes [231–233]. We therefore investigated whether MCPyV T-antigens could regulate these specific miRNAs and autophagy.

4.1.1 MCPyV T-antigens regulate miRNAs through the DnaJ domain of T-antigen

We first determined whether MCPyV T-antigens could regulate specific miRNA expression. Here, we had chosen to analyse three MCPyV-associated miRNAs, i.e. *miR-375*, *miR-30a-3p* and *miR-30a-5p*, identified from our previous study. Indeed, silencing of MCPyV LT and/or sT in MCPyV+ MCC cell lines decreased levels of *miR-375*, *miR-30a-3p* and *miR-30a-5p*. Concordantly, ectopic expression of MCPyV sT, full-length or truncated LT in MCPyV- MCC cell lines increased the expressions of these miRNAs. To demonstrate the specificity of the results, we also included two non-MCPyV-associated miRNAs (*miR-125a-3p* and *miR-16*), which did not show significant changes upon modulation of MCPyV T-antigen expressions. We further showed that silencing of LT and/or sT had no effect on the primary transcripts of *miR-375* and *miR-30a*, excluding the effect at transcriptional levels. On the other hand, silencing of LT and/or sT reduced the stability of these mature miRNAs, suggesting that the viral T-antigens regulate these miRNAs at post-transcriptional level.

We next sought to identify which domain of the viral T-antigens is required for miRNA regulation. We focused on the DnaJ domain because it is shared between the LT and sT. Here we applied a mutant in the DnaJ domain, D44N, and compared the effect with its wild-type. Our results revealed that the DnaJ mutant completely abolished the LT-induced miRNA expressions. Further investigations are now being performed to address the mechanism how MCPyV T-antigens regulate these miRNAs through the DnaJ domain.

4.1.2 MCPyV-regulated miRNAs targets multiple autophagy genes

miR-375 and *miR-30a-5p* are known to suppress *ATG7* and *BECN1*, respectively [231,232]. Here, we showed that these two miRNAs could also target these autophagy genes and suppress autophagy in MCC cell lines. Additionally, we discovered that *miR-375* could also target the autophagic receptor *SQSTM1* (also known as p62), which binds directly to LC3 to facilitate autophagic degradation. Similarly, we also observed that, besides *miR-30a-5p*, *miR-30a-3p* also regulates BECN1 expression. Using a computational approach, we identified a putative target site in the coding sequence of *SQSTM1* and *BECN1*. Further experiments validated the direct interactions between *miR-375* and *SQSTM1*, as well as between *miR-30a-3p* and *BECN1*, using luciferase reporter assays. *miR-30a-3p* was also verified to regulate autophagy using Western blot analysis of LC3-II and mRFP-GFP-LC3 reporter assays.

4.1.3 MCPyV sT and truncated LT regulate autophagy

Given that these three miRNAs were regulated by MCPyV T-antigens, we investigated whether MCPyV T-antigens could also regulate autophagy. Indeed, our results showed that silencing of MCPyV LT and/or sT in MCPyV+ cell lines increased LC3-II levels in the presence or absence of bafilomycin A1 by Western blotting, as well as the number of autophagosomes and autolysosomes by LC3 fluorescence reporter assay and transmission electron microscopy. Concordantly, ectopic expression of truncated LT and sT could suppress autophagy in MCPyV- MCC cell lines; however the wild-type LT did not have significant effect on autophagy. One plausible explanation for this effect is that the carboxyl-terminal of wild-type LT can induce autophagy by DNA damage response that counteracts the autophagy suppression effect.

To evaluate the clinical significance of our findings, we evaluated immunohistochemical staining of ATG7 and p62 in 45 MCC tumors. Our results revealed that both ATG7 and p62 expressions were lower in MCPyV+ than MCPyV- MCC tumors, suggesting the involvement of MCPyV oncoproteins in regulation of autophagy genes that leads to autophagy suppression. However, the viral oncoproteins can also suppress autophagy through other mechanisms, such as c-KIT in **Paper II**.

4.1.4 Inhibition of autophagy rescues cell death induced by Torin-1

Here, we hypothesized that suppression of autophagy could protect MCC cells from cell death in MCPyV + MCC cells. To address this question, we evaluated the effect on viability in cells treated with Torin-1 (an mTOR inhibitor) or a combination of Torin-1 and bafilomycin A1 (autophagy inhibitor) or z-VAD-FMK (pan-caspase inhibitor). Using WST-1 and Annexin V assays, we showed that inhibition of autophagy could rescue cell death induced by Torin-1, however inhibition of caspases could not rescue cell death. The results suggest that suppression of autophagy is important for MCC cell survival.

In summary, we describe a network that MCPyV oncoproteins and their-regulated miRNAs can hijack the autophagy machinery in MCC by directly targeting multiple autophagy genes. Suppression of autophagy could protect MCC cell survival. Our findings may shed light on a potential therapeutic strategy for this aggressive disease.

4.2 EXPRESSION AND FUNCTION OF C-KIT IN MCPYV+ MCC (PAPER II)

c-KIT is a receptor tyrosine kinase that is commonly overexpressed and activated in cancer [193,234]. Similar to other tumor types, c-KIT overexpression has been reported in MCC and is associated with poor prognosis [235]. However its functional role in MCC is yet to be determined.

4.2.1 MCPyV truncated LT induces paranuclear retention of c-KIT

We first investigated c-KIT expression in both MCPyV+ and MCPyV− MCC cell lines using immunofluorescence. Unexpectedly, we observed paranuclear dot-like staining of c-KIT in all three MCPyV+ MCC cell lines, but its expression was barely detectable in all three MCPyV- MCC cell lines. Similarly, the paranuclear dot-like staining was also more commonly observed in MCPyV+ than MCPyV- MCC tumors; On the other hand, membranous staining of c-KIT was more common in MCPyV- MCC tumors.

We then assessed whether MCPyV T-antigens play a role in paranuclear localization of c-KIT. Indeed, when we silenced MCPyV T-antigens in the MCPyV+ WaGa cells, the number of cells with paranuclear c-KIT dot structure was markedly reduced. On the other hand, we observed an increase number of cells with cytoplasmic distribution of c-KIT. To further evaluate which viral T-antigen(s) contribute to the paranuclear c-KIT dot, we ectopically co-expressed c-KIT and MCPyV sT, wild-type or truncated LT. The results showed that only the truncated LT could recapitulate the paranuclear dot of c-KIT. The LT is known to interact with Vam6p [62], which is involved in lysosome trafficking [236]. We therefore hypothesized that the interaction between LT and Vam6p results in paranuclear retention of c-KIT. Using the W209A mutant of LT339 (which is defective in Vam6p interaction), the paranuclear retention of c-KIT was abolished.

The next question was to address the subcellular localization of c-KIT. Using immunofluorescence, our results showed that the paranuclear c-KIT was partially overlapped with the Golgi marker (GOLGB1). As c-KIT proteins undergo a series of glycosylations, we assessed the glycosylation status of c-KIT in MCPyV+ MCC cells. The results revealed that c-KIT proteins in the MCPyV+ cell lines were mainly in the mature form (145 kDa) and complex glycosylated, as evidenced by digestion with the PNGase F but not with the endo H. Furthermore, we observed that all three MCPyV+ MCC cell lines had detectable level of phospho-KIT, as detected by immunoblotting. These results suggest that the complex glycosylated c-KIT has been transported to the plasma membrane and activated by its ligand, SCF. We therefore hypothesize that the endocytosed c-KIT is likely blocked from degradation by the LT-Vam6p interaction, leading to stabilization of c-KIT in the paranuclear compartment. In line with our hypothesis, using immunoblotting, we observed an accumulation of c-KIT in the truncated LT-expressing cells and very low c-KIT expression in cells expressing sT or full-length LT. Ongoing experiments are conducted to address whether c-KIT is accumulated on the Golgi from the early secretory or endocytic pathways.

4.2.2 c-KIT interacts with BECN1 in MCPyV+ MCC cell lines

In **Paper I**, we have shown that MCPyV truncated LT can inhibit autophagy and another tyrosine kinase receptor, epidermal growth factor (EGFR), has been demonstrated to suppress autophagy through BECN1 [237]. We therefore sought to address if c-KIT also interacts with BECN1 and suppresses autophagy in MCPyV+ MCC. Using co-immunostaining of c-KIT and BECN1, BECN1 and c-KIT were in very close proximity within the paranuclear dot-like

region. To further strengthen the results, the interaction between BECN1 and c-KIT was confirmed by co-immunoprecipitation and reciprocal immunoprecipitation assays.

4.2.3 Silencing of c-KIT induces autophagy and apoptosis in MCPyV+ MCC

To address the involvement of c-KIT in regulation of autophagy, we silenced c-KIT in WaGa cells and evaluated the effect on LC3-II levels with and without bafilomycin A1 treatment using Western blotting. The results showed an induction of LC3-II in the absence or presence of bafilomycin A1. c-KIT can activate AKT-mTOR signaling, which can also suppress autophagy [238]. We therefore examined whether AKT-mTOR signaling contributes to c-KIT-mediated autophagy regulation in MCC. Our results showed that silencing of c-KIT led to subtle decreases in both phosphorylation statuses of mTOR and/or AKT, suggesting that c-KIT-mediated autophagy suppression may partly depend on the AKT-mTOR signaling pathways.

Besides autophagy regulation, we also observed an increase of apoptosis upon silencing of c-KIT, as indicated by increased expression of the 89-kDa cleaved PARP. The increased expression of cleaved PARP was also noted in the c-KIT-depleted cells with inhibition of autophagy, suggesting that the apoptosis was not induced by autophagy. Together, our results demonstrate that c-KIT is required for autophagy suppression and cell viability in MCPyV+ MCC cells.

4.2.4 Autophagy can degrade LT protein

Unexpectedly, we observed reduction of MCPyV LT in the c-KIT-depleted cells and its level was restored upon treatment with the bafilomycin A1 autophagy inhibitor. We therefore evaluated whether MCPyV LT is degraded by autophagy. Indeed, our results showed that LT expression was decreased upon induction of autophagy (by EBSS or Torin-1), and restored upon inhibition of autophagy (by bafilomycin A1). To strengthen the results, we are now silencing autophagy genes and assess the degradation rate of viral oncoproteins.

Collectively we uncovered a novel mechanism where the viral truncated LT can promote paranuclear retention of c-KIT, and a new role for c-KIT in autophagy regulation through interaction with BECN1.

4.3   FUNCTIONAL STUDIES OF *MIR-375* AND LDHB IN MCC (PAPER III)

In **Paper I**, we identified *miR-375* as one of the differentially expressed miRNAs between MCPyV+ and MCPyV- MCC tumors. This miRNA is highly abundant in most MCC tumors and its high levels in serum samples correlate with tumor burden in MCC [151]. *miR-375* has been demonstrated to promote neuroendocrine differentiation in MCPyV- MCC cell lines. However, its functional role in MCPyV+ MCC remains undefined. LDHB, one of the known targets of *miR-375*, has been detected in MCC tumors [239]. In this study, we investigated whether *miR-375* could regulate LDHB and their functional roles in MCC.

4.3.1 *miR-375* regulates LDHB levels in MCC

We first evaluated whether LDHB could be a target of *miR-375* in MCC. In consistent with higher levels of *miR-375* in MCPyV+ than MCPyV- MCC tumors observed in **Paper I**, *miR-375* levels were also higher in all three MCPyV+ MCC cell lines (WaGa, MKL-1 and MKL-2) compared to the three MCPyV- MCC cell lines (MCC13, MCC14/2 and MCC26). On the other hand, LDHB mRNA and protein levels were higher in the MCPyV- than MCPyV+ MCC cell lines. Furthermore, *LDHB* mRNA levels were also inversely correlated with *miR-375* in MCC tumor samples. Additionally, overexpression of *miR-375* in MCPyV- MCC cells reduced, and silencing of *miR-375* induced, LDHB mRNA and protein levels. These results indicate that LDHB is a target of *miR-375* in MCC.

4.3.2 *miR-375* overexpression suppresses cell growth and migration in MCPyV- MCC cell lines

To determine the functional role of *miR-375* in MCC cells, we overexpressed *miR-375* in MCPyV– MCC cells using *miR-375* expression plasmid or mimic and evaluated the effect on functional phenotypes. Our results showed that *miR-375* overexpression reduced cell growth, as assessed by WST-1 and trypan blue exclusion assays. Furthermore, over-expression of *miR-375* led to a subtle increase in G1 or G2 phases compared to the negative control. Using wound healing scratch assay, we observed a reduction in wound closure rate compared to the negative control in *miR-375*-overexpressing cells. In addition, overexpression of *miR-375* increased the expression of cleaved PARP, an apoptotic marker. Together, our results suggest that *miR-375* functions as a tumor suppressor in MCPyV- MCC cell lines, by inhibiting cell growth and migration, as well as inducing apoptosis.

4.3.3 Suppression of *miR-375* decreases cell growth and increases apoptosis in MCPyV+ MCC cell lines

In MCPyV+ MCC cell lines, inhibition of *miR-375*, using miRNA sponges, reduced cell growth in both WaGa and MKL-1 cell lines, as evaluated by trypan blue exclusion and WST-1 assays. Concomitantly, suppression of *miR-375* led to an increase of apoptosis, as evaluated by Annexin V and caspase-3 activity assays. The results indicate that *miR-375* is required for cell growth and viability in MCPyV+ MCC cells.

4.3.4 Silencing of LDHB reduces cell growth in MCPyV- MCC cells but rescues cell growth suppression mediated by *miR-375* inhibition in MCPyV+ MCC cells

We further investigated whether LDHB plays a role in *miR-375*-mediated cell growth in MCPyV+ MCC cell lines. We determined cell growth in cells transfected with miR-375sp together with and without siRNAs targeting LDHB, as well as cells transfected with miR-375sp or vehicles only. Inhibition of *miR-375* reduced cell growth and silencing of LDHB rescued the cell growth inhibition effect caused by *miR-375* suppression. On the other hand, silencing of LDHB decreased cell growth and increased apoptosis in MCPyV- MCC cell lines.

Collectively, our study suggests that LDHB functions as an oncogene in MCPyV- MCC, and a tumor suppressor in MCPyV+ MCC cell lines. Given that LDHB is a key enzyme involved in energy metabolism, we speculated that the differential role of LDHB could relate to different cellular metabolisms in MCPyV+ and MCPyV- cells, which led to the investigations in **Paper IV**.

4.4   MCPYV T-ANTIGENS AND LDHB IN GLYCOLYSIS IN MCC (PAPER IV)

In **Paper III**, we have shown that LDHB can regulate cell growth in MCC cells, and depending on virus status, LDHB can be an oncogene or a tumor suppressor. Given that LDHB is a metabolic enzyme involved in glycolysis and its expression level is associated with the virus status, we investigated whether MCPyV T-antigens could regulate LDHB levels and its effect on energy metabolism and cell growth.

4.4.1 MCPyV T-antigens regulate LDHB expression and glycolysis

Using RT-qPCR and Western blotting, we showed that silencing of MCPyV T-antigens increased LDHB expression at mRNA and protein levels in MCPyV+ MCC cells, while ectopic expression of MCPyV sT and truncated LT decreased LDHB expression in MCPyV- MCC cell lines. Silencing of MCPyV T-antigens also decreased glycolysis rates and ectopic expressions of sT and truncated LT increased glycolysis. Cells expressing wild-type LT did not have consistent effect in MCC13 and MCC14/2 cells; its glycolysis rate was similar to the vector control in MCC13 but increased in MCC14/2 cells. Together, our results showed that MCPyV sT and truncated LT could suppress LDHB expression and promote glycolysis.

4.4.2 LDHB overexpression reduces cell growth and induces apoptosis in MCPyV+ MCC cell lines

In **Paper III**, we demonstrated that silencing of LDHB rescued *miR-375*-mediated cell growth suppression in MCPyV+ MCC cell lines. To demonstrate the direct effect of LDHB as a suppressor in MCPyV+ cells, we ectopically expressed full-length coding sequence of LDHB in WaGa and MKL-1 cell lines. We observed that LDHB overexpression reduced cell growth and increased apoptosis in both cell lines, supporting its tumor suppressor role in MCPyV+ MCC cells.

4.4.3 Ectopic expression of LDHB reverses the growth-promoting effect of MCPyV oncoproteins

We next investigated whether the functional role of LDHB is dependent on MCPyV oncoproteins by comparing the effects of cells co-transfected with MCPyV T-antigen together with and without ectopic LDHB expression in MCPyV- MCC cell lines. Our results showed that sT and LT339 increased cell growth in both cell lines, which is consistent with the results reported in previous studies [15,240]. However, the full-length LT did not have significant effect on cell growth. Co-transfection of LDHB and sT or LT339 reversed the

growth-promoting effect of these viral oncoproteins. Similarly, cells transfected with LDHB and sT or LT339 had more apoptotic cells than the cells transfected with sT or LT339. No differences were observed for cell growth and apoptosis in cells expressing full-length LT with and without ectopic LDHB. These findings suggest that low LDHB level is important for cell growth and viability of viral oncoproteins expressing cells, due to reprogramming of energy metabolism in the cells. Cells expressing viral oncoproteins may rely on glycolysis for promoting cell growth. Increased LDHB expression promotes conversion of lactate to pyruvate for the TCA cycle that switches from glycolysis to oxidative phosphorylation. These observations led us to investigate whether MCPyV+ and MCPyV- MCC cells are dependent on different metabolic activities for cell growth.

4.4.4 MCPyV + and MCPyV- MCC cells rely on different energy metabolism for cell growth

To address this question, we treated MCC cells with oxamic acid (glycolysis inhibitor) or antimycin A (oxidative phosphorylation inhibitor). In MCPyV+ MCC cells, we found that oxamic acid-treated cells reduced glycolysis in both cell lines, while treatment with antimycin A increased glycolysis due to increased lactate production [226]. Inhibition of glycolysis reduced cell growth in MCPyV+ MCC cell lines, while inhibition of oxidative phosphorylation had no effect on cell growth. In MCPyV- MCC cell lines, antimycin A treatment reduced oxygen consumption rate (OCR), while oxamic acid treatment did not have any effect on OCR. Antimycin A treatment inhibited cell growth in MCPyV- cell lines, but not with oxamic acid.

In summary, our findings revealed that MCPyV oncoproteins suppress LDHB expression and induce glycolysis for promoting cell growth and viability in MCC. MCPyV+ and MCPyV- MCC cells are dependent on different energy metabolism for cell growth, which leads us to propose targeting metabolism as an alternative strategy for treating MCC patients.

5   CONCLUSIONS

MCPyV oncoproteins (sT and truncated LT) clearly play important roles in MCC tumorigenesis. This thesis explored the involvement of the viral oncoproteins in regulating miRNA expressions, autophagy and metabolism. The main findings of this thesis are summarized below:

- MCPyV T-antigens regulate a subset of miRNAs, including *miR-375*, *miR-30a-3p* and *miR-30a-5p*, through the DnaJ domain of viral T-antigen (**Paper I**).

- MCPyV oncoproteins can suppress autophagy via multiple mechanisms, including miRNA regulation of multiple autophagy genes and c-KIT interaction with BECN1 (**Papers I and II**).

- *miR-375* can regulate autophagy by targeting *ATG7* and *SQSTM1*, and metabolism by suppressing *LDHB* (**Papers I and III**).

- c-KIT can regulate autophagy and apoptosis in MCPyV+ MCC cells (**Paper II**).

- The Vam6p of MCPyV truncated LT is required for paranuclear retention of c-KIT (**Paper II**).

- *miR-375* and LDHB have different functions in MCC cells with and without MCPyV (**Paper III**).

- Low level of LDHB is required for cell growth and viability in MCPyV+ cells (**Paper IV**).

- MCPyV+ MCC cell lines are dependent on glycolysis and MCPyV- MCC cell lines rely on oxidative phosphorylation for cell growth and viability (**Paper IV**).

6 FUTURE PERSPECTIVE

This thesis work describes new functions of MCPyV T-antigens, *i.e.* autophagy suppression and reprogramming energy metabolism. Mechanistically, the viral oncoproteins could suppress autophagy through miRNA regulations and c-KIT interaction with BECN1. Suppression of autophagy is crucial to sustain MCPyV T-antigen expression and cell viability in MCC (**Papers I** and **II**). On the other hand, MCPyV T-antigens can reprogram energy metabolism through regulation of LDHB expression levels (**Paper IV**) or other glycolytic genes, such as MCT1 [75]. These findings reveal the involvement of autophagy and metabolism reprogramming in MCC tumorigenesis. Further studies investigating the functional impact of autophagy suppression and glycolysis may facilitate our understanding the role of the viral oncoproteins in MCC development.

In **Paper I**, we demonstrated that MCPyV T-antigens could regulate specific miRNAs at post-transcriptional level. Although we identified that the DnaJ domain of the viral T-antigens is crucial for miRNA regulation, the molecular mechanism of this regulation is still unclear. Within the DnaJ domain, there is an Hsc70 binding site. Hsc70 can bind to the AU-rich elements (AREs) of *Bim* mRNA and regulate its mRNA stability [241]. This leads us to hypothesize that the interaction between MCPyV T-antigens and Hsc70 can increase mRNA stability and/or translation of miRNA processing gene(s).

In **Paper II**, we have shown that c-KIT is mainly localized on Golgi apparatus; however it is still unclear whether c-KIT accumulated on the Golgi after the early secretory pathway or endocytosis from the plasma membrane. Although we showed that c-KIT interacts with BECN1, the interaction sites between c-KIT and BECN1 and the mechanism of this interaction that contributes to autophagy suppression remain to be determined.

In **Papers III and IV**, we demonstrated that MCPyV oncoproteins could regulate LDHB expressions and glycolysis; it is still unclear how these viral oncoproteins regulate LDHB expression, which leads to change of the energy metabolisms in MCCs.

Although recent studies revealed a positive outcome with anti-PD-1 or anti-PD-L1 treatment in advanced MCC patients, the long-term outcomes of these patients remain unknown; moreover, half of them are still refractory to these immune-checkpoint inhibitors. Identification of alternative treatment options for advanced MCC patients are still warranted. Delineating the functional roles of MCPyV oncoproteins in MCC may lead to identification of new avenues for developing novel therapeutic approaches for MCC treatment.

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