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REGULATION OF CELLULAR DEGRADATION PATHWAYS BY VIRAL ONCOPROTEINS AND MICRORNAS

Hao Shi

师 昊

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REGULATION OF CELLULAR DEGRADATION PATHWAYS BY VIRAL ONCOPROTEINS AND MICRORNAS

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By

Hao Shi

Principal Supervisor:
Associate Professor Weng-onn Lui
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):
Professor Catharina Larsson
Karolinska Institutet
Department of Oncology-Pathology

Professor Filip Farnebo
Karolinska Institutet
Department of Molecular Medicine and Surgery

Opponent:
Professor Johan Jakobsson
Lund University
Department of Experimental Medical Science

Examination Board:
Professor Maria Masucci
Karolinska Institutet
Department of Cell and Molecular Biology

Professor Stig Linder
Linköping University
Department of Biomedical and Clinical Sciences

Docent Yvonne Ceder
Lund University
Department of Laboratory Medicine

Karolinska Institutet

Institutionen för Onkologi-Patologi

Regulation of cellular degradation pathways by viral oncoproteins and microRNAs

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av

Hao Shi

Huvudhandledare:
Docent Weng-Onn Lui
Karolinska Institutet
Institutionen för onkologi-patologi

Bihandledare:
Professor Catharina Larsson
Karolinska Institutet
Institutionen för onkologi-patologi

Professor Filip Farnebo
Karolinska Institutet
Institutionen för molekylär medicin och kirurgi

Fakultetsopponent:
Professor Johan Jakobsson
Lunds Universitet
Institutionen för experimentell medicinsk vetenskap

Betygsnämnd:
Professor Maria Masucci
Karolinska Institutet
Institutionen för cell-och molekylärbiologi

Professor Stig Linder
Linköpings Universitet
Institutionen för biomedicinska och kliniska vetenskaper

Docent Yvonne Ceder
Lunds Universitet
Institutionen för laboratoriemedicin

Stockholm 2020

To my family

致我的家人

ABSTRACT

The cellular degradation system actively participates in cell homeostasis. Despite ongoing efforts and much progress in recent years, the underlying mechanisms of these pathways are not entirely understood. This thesis aims to contribute further insights how microRNAs and viral oncoproteins regulate key genes involved in cellular degradation pathways.

In **Paper I**, we found overexpression of *miR-223-3p* in testicular germ cell tumors (TGCTs), in which its expression was negatively correlated with the mRNA level of the *FBXW7* ubiquitin E3 ligase. Overexpression of *miR-223-3p* suppressed, while its inhibition increased, FBXW7 protein level in TGCT cell lines, suggesting FBXW7 as a target of *miR-223-3p*. Using both gain- and loss-of-function experiments, we showed that *miR-223-3p* induced cell growth and reduced apoptosis. Ectopic expression of the *FBXW7* open reading frame could reverse the effect of *miR-223-3p*. In conclusion, we suggest the oncogenic role of *miR-223-3p* – FBXW7 regulation in TGCT.

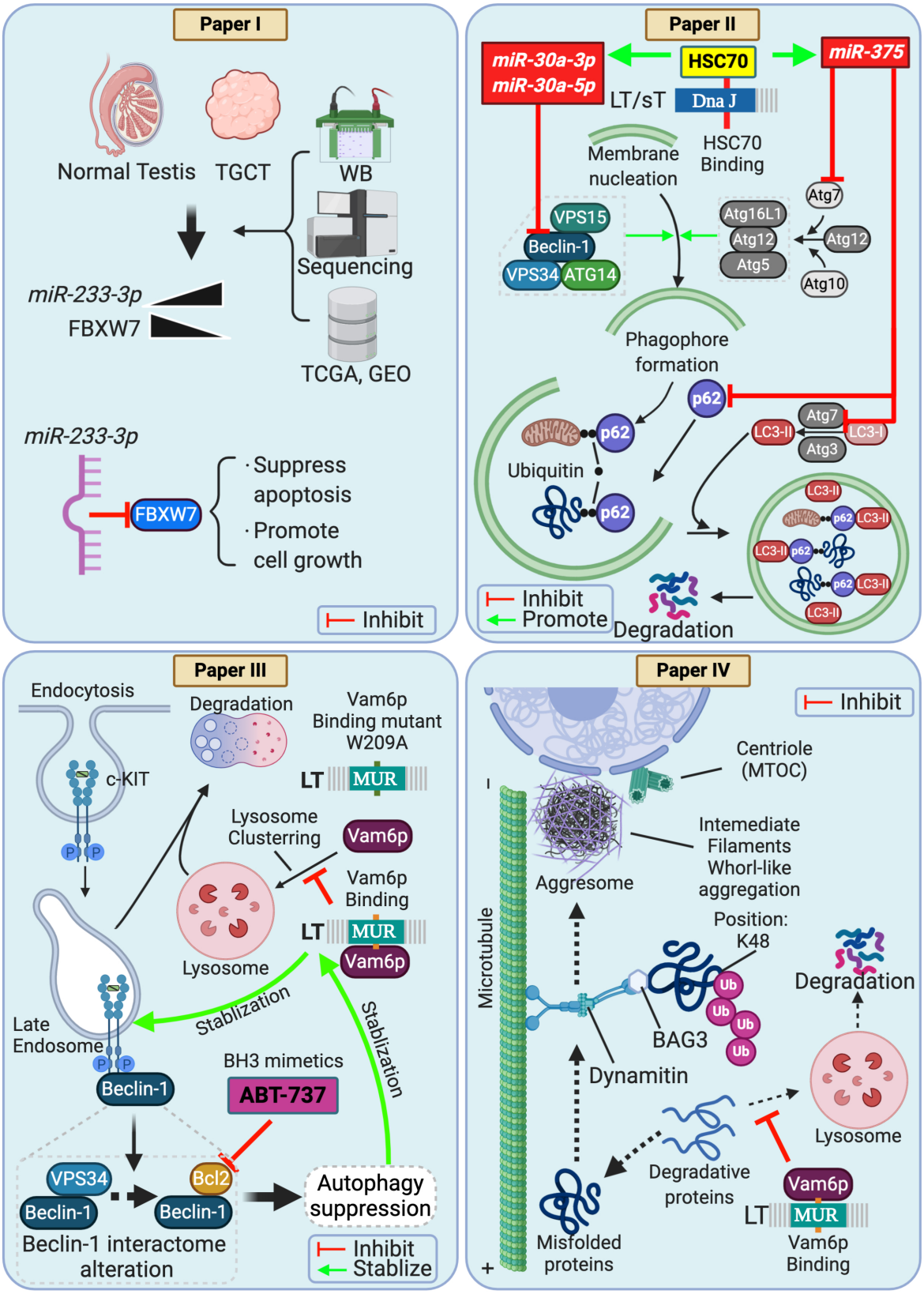
In **Paper II**, we demonstrated that *miR-375*, *miR-30a-3p* and *miR-30a-5p* are regulated by Merkel cell polyomavirus (MCPyV) T-antigens through the DnaJ domain. These miRNAs could suppress autophagy and target ATG7, SQSTM1 (p62) and BECN1. Lower protein levels of ATG7 and p62 are associated with MCPyV-positive MCC tumors. Additionally, we showed that ectopic expression of MCPyV oncoproteins could suppress autophagy and blockage of autophagy rescued Torin-1 mediated cytotoxicity in MCC cells. This study provides evidence that MCPyV oncoproteins induce microRNAs and suppress autophagy in MCC, suggesting the importance of autophagy suppression in protecting MCC cell survival.

In **Paper III**, we uncover a function and mechanism of MCPyV oncoprotein in autophagy evasion through c-KIT receptor tyrosine kinase. We show that the viral oncoprotein promotes c-KIT retention in late endosomes through its Vam6p binding site, which promotes c-KIT binding to Beclin-1 and enhances Beclin-1-BCL2 interaction. Silencing of c-KIT induces autophagy, which leads to degradation of the viral oncoprotein. Thus, MCPyV has developed a strategy to hijack cellular degradation system to sustain the viral oncoprotein expression.

In **Paper IV**, we identify CK20 paranuclear dot as a part of aggressome in MCC, in which these structures are associated with MCPyV status, localized at microtubule-organizing center and dependent on BAG3 expression and dynein-mediated microtubule transport. Additionally, we show that the MCPyV truncated large T-antigen promotes aggresome formation through its Vam6p binding site. This study suggests a model of BAG3-dependent aggresome formation contributed by viral oncoprotein in MCC.

Overall, this thesis work has demonstrated the involvement of microRNAs and viral oncoproteins in regulation of cellular degradation pathways, which contributes to the understanding of the molecular interplays between cellular degradation system and cancer development in TGCT and MCC (as illustrated in the Graphical Abstract).

GRAPHICAL ABSTRACT



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. Jikai Liu[#], **Hao Shi**, Xidan Li, Gang Chen, Catharina Larsson, and Weng-Onn Lui[#]. *miR-223-3p* regulates cell growth and apoptosis via FBXW7 suggesting an oncogenic role in human testicular germ cell tumors.
International journal of oncology, 2017, 50(2): 356-364.

II. 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.
International journal of cancer, 2020, 146(6): 1652-1666.

III. **Hao Shi**, Jiwei Gao, Satendra Kumar, Hong Xie, Ziqing Chen, Harri Sihto, Virve Koljonen, Vladana Vukojevic, Filip Farnebo, Viveca Björnhagen, Anders Höög, C. Christofer Juhlin, Linkiat Lee, Catharina Larsson, and Weng-Onn Lui[#]. Merkel cell polyomavirus oncoprotein induces paranuclear retention of c-KIT suppressing autophagy through interaction with Beclin-1.
Submitted manuscript, under review.

IV. **Hao Shi**[#], Jiwei Gao, Linkiat Lee, Hong Xie, Harri Sihto, Catharina Larsson, Weng-Onn Lui[#]. Merkel cell polyomavirus oncoprotein promotes BAG3-mediated aggresome formation.
Manuscript.

[*]Equal contribution
[#]Corresponding author

CONTENTS

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Baf. A1	Bafilomycin A1
BAG3	Bcl-2-associated athanogene 3
BECN1	Beclin-1
CK20	Cytokeratin 20
CLL	Chronic lymphocytic leukemia
CMA	Chaperone-mediated autophagy
co-IP	Co-immunoprecipitation
EBV	Epstein-Barr virus
EE	Early endosome
EGFR	Epithelial growth factor receptor
ER	Endoplasmic reticulum
FBXW7	F-box/WB repeat-containing protein 7
FFPE	Formalin-fixed paraffin-embedded
GIST	Gastrointestinal stromal tumor
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC6	Histone deacetylase 6
HPV	Human papillomavirus
HSC70	Heat-shock cognate protein 70
HSP70	Heat-shock protein 70
IF	Immunofluorescence
IHC	Immunohistochemistry
kDa	Kilodalton
LAMP1	Lysosome-associated membrane protein 1
LAMP2	Lysosome-associated membrane protein 2
LE	Late endosome
LIMP2	Lysosome integral membrane protein 2
LN	Lymph node

LSD	Large T antigen-stabilization domain
LT	Large T-antigen
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MCPyV-	Merkel cell polyomavirus negative
MCPyV+	Merkel cell polyomavirus positive
miRNA	Micro-ribonucleic acid
mRNA	Messenger ribonucleic acid
MTOC	Microtubule-organizing center
mTOR	Mammalian target of rapamycin
MVB	Multivesicular body
NG	Neurosecretory granular
NLS	Nuclear localization signal
nts	Nucleotides
NT	Normal testis
PARP-1	Poly (ADP-ribose) polymerase-1
PI3K	Phosphatidylinositol 3-kinase
PM	Plasma membrane
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
Rb	Retinoblastoma
RISC	RNA-induced silencing complex
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SCLC	Small cell lung cancer
shRNA	Short-hairpin RNA
siRNA	small interfering RNA
SLNB	Sentinel lymph node biopsy
SQSTM1	Sequestosome 1
sT	Small T
TGCT	Testicular germ cell tumor

TGN	Trans-Golgi network
TIL	Tumor-infiltrating lymphocyte
TMA	Tissue microarray
TTF-1	Thyroid transcription factor 1
Ub	Ubiquitin
ULK	Unc51-like kinase
UPS	Ubiquitin-proteasome system
VP	Viral capsid protein
UV	Ultraviolet
WT	Wild type

1 INTRODUCTION

The cellular degradation system is one of the essential biological processes to keep cellular homeostasis. It acts as a catabolic mechanism to interplay with multiple cellular processes, including but not limited to energy balance, macromolecule clearance and recycling, modification of regulatory proteins and expression control, and immunity against pathogen invasions. Therefore, the cellular degradation system is highly important in various pathophysiology processes, especially in cancer. This thesis partly contributes to the understanding of how cellular degradation pathways are regulated by microRNAs and viral oncoproteins.

1.1 EUKARYOTIC PROTEIN DEGRADATION SYSTEMS

In order to maintain normal intracellular homeostasis, eukaryotic cells require the cellular degradation system to break down and recycle cellular proteins for quality control. Two pathways have been extensively studied, i.e. the lysosome-mediated and the ubiquitin-proteasome degradation systems. Dysfunction of these two systems could contribute to pathological situations, such as neurodegenerative diseases and carcinogenesis.

1.1.1 Lysosome-mediated protein degradation

The lysosome is a single-layer round-membrane cytoplasmic vesicle mainly containing acid hydrolases that are active in acidic pH environment (De Duve, 1963). It was first described in 1955 as a "serendipity". When De Duve and colleagues explored the insulin action mechanisms in rat liver tissues, they surprisingly found distinct granular structures containing hydrolases (De Duve et al., 1955). Later in 1956, the electron-dense lysosome particles were first illustrated by electron microscopy, and the acid phosphatase activity in liver tissue was confirmed to be located in a vesicle called lysosome (Essner and Novikoff, 1961; Novikoff et al., 1956). Nowadays, it is known that the lysosome contains more than 50 different hydrolases involved in catalytic functions. These hydrolases can digest the macromolecules that are engulfed by lysosome, including nucleic acids, proteins, carbohydrates and lipids (Saftig and Klumperman, 2009). These molecules are generally delivered to lysosomes through endocytic or autophagic pathways.

The endocytic degradation pathway starts from the plasma membrane (PM) and ends at lysosomes. The cargos designated for degradation, as well as the recycling lysosomal proteins, begin their transportation from the PM to the early endosome (EE) and multivesicular body (MVB, also called intermediate or late endosome, LE), which is ultimately merged with a lysosome. Besides the PM-mediated endocytic pathway, the newly synthesized proteins can be directly transferred from the *trans*-Golgi network (TGN) to the

endosomal components such as MVB and EE, followed by delivery to lysosomes for degradation (Figure 1) (Saftig and Klumperman, 2009).

There are several lysosomal-membrane proteins with specific functions in lysosomes. Lysosome-associated membrane protein 1 (LAMP1) is involved in maintenance of lysosome stability, and both LAMP1 and lysosome-associated membrane protein 2 (LAMP2) guide the lysosomal fusion processes with autophagosomes and phagosomes (Andrejewski et al., 1999; Huynh et al., 2007). Lysosome integral membrane protein 2 (LIMP2, also known as SCARB2) helps the transportation of newly synthesized β-glucocerebrosidase (Reczek et al., 2007). The vacuolar-type ATPase proton pump in the lysosome membrane is necessary to maintain the low pH environment for hydrolases (Marshansky and Futai, 2008). Due to the crosstalk of the endocytosis-lysosome network (as shown in Figure 1), the lysosome and LE share some common membrane protein markers. For example, LAMP1, LAMP2 and LIMP2 are preferably located in lysosomes, and CD63 is mainly located in LE (Saftig and Klumperman, 2009). These markers can be applied to distinguish different cellular vesicles, as exemplified in **Paper III**.

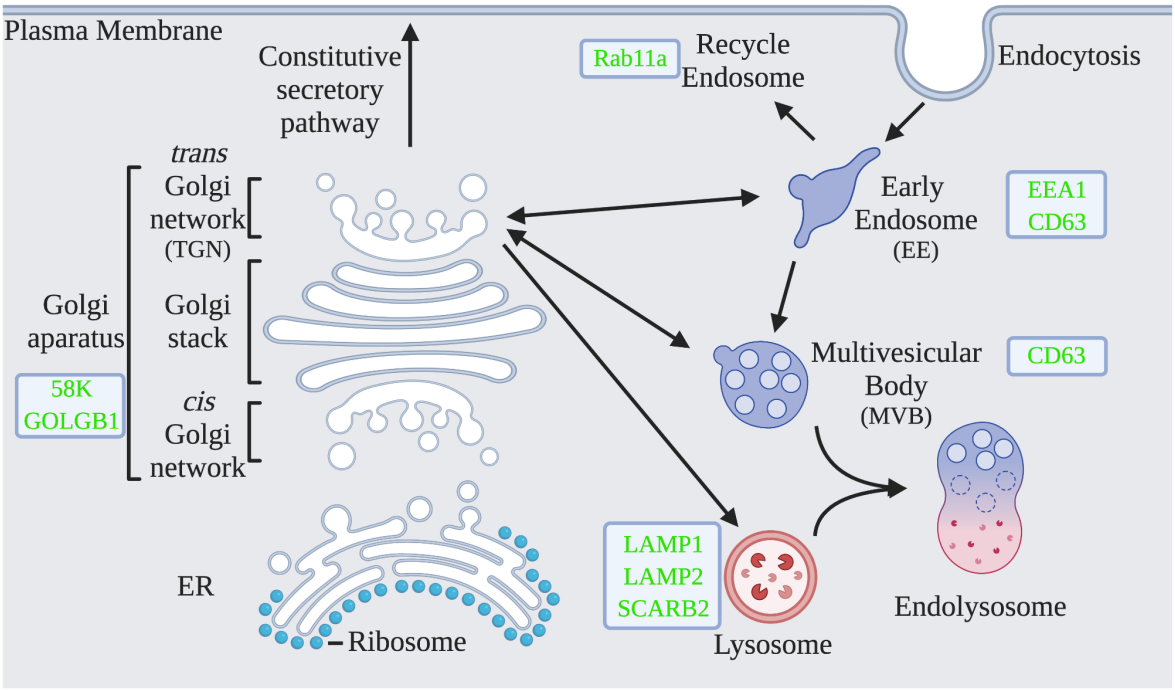


Figure 1. Schematic illustration of the interactive network between biosynthetic and endocytic pathways. Newly synthesized proteins are transported from the endoplasmic reticulum (ER) to the Golgi apparatus. From the *trans*-Golgi network (TGN), the proteins can be transported to the plasma membrane through the constitutive secretory pathway and subsequently delivered to lysosomes through endocytosis. Alternatively, the lysosomal targeting proteins can be directly delivered to the endosome-lysosomal system. Adapted and modified from (Saftig and Klumperman, 2009).

Besides the interaction with endocytosis, the lysosome also interacts with chaperone-mediated autophagy (CMA), macro- and micro-autophagy (Figure 2) (Dunn, 1994). In CMA, the trafficking of cytosolic proteins towards the lysosome relies on the chaperone protein

heat-shock cognate protein 70 (HSC70) and other co-chaperones (Kaushik and Cuervo, 2012). Microautophagy is a lysosome-self engulfment process that resembles the endosomal-sorting complex for MVB formation (Sahu et al., 2011). Macroautophagy is thought to be the major type of autophagy, which deploys the double membrane vesicles called autophagosomes to lysosomes. Generally, macroautophagy is referred to as autophagy (Mizushima and Komatsu, 2011).

Macroautophagy is initiated under starvation or other stress conditions. Once macroautophagy starts to occur, the degradation substrates are sequestered into phagophores to form the autophagosome. This autophagosome formation includes three steps: initiation, nucleation and expansion (Lamb et al., 2013). The initiation step requires the unc51-like kinase (ULK) complex, consisting of ULK1 or ULK2, ATG13, FIP200 and ATG101. The activity of the ULK kinase is suppressed by mammalian target of rapamycin complex 1 (mTORC1). AMP-activated protein kinase (AMPK) can restore the initiation via inhibition of mTORC1 and directly facilitate the activation of ULK complex (Alers et al., 2012). Besides, the PI3K-AKT signaling pathway can activate the mTOR pathway that leads to autophagy suppression (Saiki et al., 2011). Once the ULK complex is phosphorylated and activated, it promotes phosphorylation of VPS34 and triggers the formation of the Beclin-1 (BECN1)-PI3K-VPS34 complex for autophagosome formation (Russell et al., 2013). The BECN1 complex assists in the binding to the E3-like ATG16L. Subsequently, the ATG16L1-ATG12-ATG5 complex is recruited by an ubiquitin-like reaction that promotes the autophagosomal elongation and lipidation of LC3. The lipidated LC3/ATG8 complex can bind to Sequestosome 1 (SQSTM1, also known as p62) to form autophagosomes (Pankiv et al., 2007). When the autophagosome has been assembled, it fuses with lysosomes to become autolysosomes. Finally, the cargos are degraded by lysosomal hydrolases for recycling of cellular constituents (Mizushima et al., 2002).

1.1.2 Ubiquitin-proteasome degradation system

The lysosome pathway was regarded as the major protein degradation system until 1977, when a new lysosome-independent and adenosine triphosphate (ATP)-dependent proteolytic system was identified in rabbit reticulocytes (Etlinger and Goldberg, 1977). Thereafter, this degradation system is described as a highly selective turnover of proteolytic substrates and named as the ubiquitin-proteasome system (UPS), which is distinguished from the lysosomal degradation system that is used under stress conditions (Ciechanover, 1994).

The UPS includes two major steps: the protein “tagging” step and the 26S proteasome complex proteolytic step. The accuracy of the UPS selection is based on a poly-ubiquitin chain tagging towards the degradation candidates. Ubiquitin (Ub) is a small and conserved protein with 76 amino acids. It acts as a post-translational modification called ubiquitination at lysine residues. Proteins designated for degradation will be covalently bound with one Ub molecule by the ubiquitin-activating enzyme (E1) along with ATP (Hershko et al., 1980). The

ubiquitin-conjugating enzyme (E2) acts as a carrier transferring the ubiquitinated substrate to another group of ubiquitin ligases called E3. E3 recognizes the mono-ubiquitin-tagged-protein and E2 complex, ligates additional Ub to elongate the Ub-tagged chain. The minimum requirement for downstream signal is tetra-ubiquitin, which is needed for effective proteasomal targeting (Thrower et al., 2000). The diversity of E1 and E2 is limited, but the E3 group contains a large number of proteins, which reflects the highly specific system for orchestrating protein homeostasis (Ardley and Robinson, 2005).

Proteasomes in mammals are cylindrical structures, containing the cytosolic 26S trimeric complex of 2,000 kilodalton (kDa). The 26S complex consists of one 20S catalytic core particle and two 19S cap regulatory subunits (Ciechanover, 2005). The 19S subunit contains ATPase active sites and one ubiquitin-binding site. During the degradation process, the 19S subunit recognizes the ubiquitin chain, cleaves the ubiquitin tag, partially unfolds the protein and translocates the protein into 20S (Finley, 2009; Matyskiela et al., 2013). The 20S core particle consists of four subunits (two outer α subunits and two inner β subunits), which are seven-base-units ring structures. The β subunit-ring accounts for the ATP-independent proteolytic chamber and releases the substrate-derived short polypeptides (Ciechanover, 2005). An overview of the UPS is illustrated in Figure 2.

1.1.3 Misfolded protein and aggresome

The correct linear amino acid sequence and subsequent folding pattern warrant the unique three-dimensional protein structure, which determine the appropriate protein functions. Amino acid mutations or abnormal protein folding can result in misfolded proteins (Ellis and Pinheiro, 2002). Misfolded proteins are under surveillance of the molecular chaperones system for correction (Ellis, 2006). However, when the critical accumulated level of misfolded proteins is reached, aggregated protein complexes and amyloid-like structures can be formed. Accumulated misfolded proteins can lead to enormous deleterious outcomes. For example, in cystic fibrosis, the F508 deletion mutation leads to misfolding of the cystic fibrosis transmembrane conductance regulator (CFTR), abolishing its function (Rubenstein et al., 1997). In neurodegenerative diseases, the accumulation of misfolded protein aggregates, such as Amyloid β peptide in Alzheimer's disease, Huntingtin aggregates in Hungtinton's disease and α-Synuclein forming Lewy bodies in Parkinson's disease disrupts the cellular homeostasis and contributes to the pathogenesis of the disease (Chiti and Dobson, 2006).

Cells employ the aforementioned diverse protein degradation systems to clear the misfolded proteins, including the UPS and the autophagy-lysosome systems. If the misfolded protein clearance has failed, the accumulated misfolded proteins will generally aggregate to form paranuclear inclusion bodies termed aggresomes (Kopito, 2000). An aggresome is a cytoplasmic structure formed by the intermediate filaments at the microtubule organizing center (MTOC), which sequesters misfolded proteins for subsequent degradation by autophagy (Johnston et al., 1998). Two pathways are known to contribute to aggresome

formation. The first involves the microtubule-associated histone deacetylase 6 (HDAC6), which has been identified to bridge between poly-ubiquitin tagged proteins and the dynein motor complex. This binding contributes to the transportation of misfolded proteins towards the MTOC via the dynein-motor complex (Kawaguchi et al., 2003). The second pathway involves the co-chaperone Bcl-2-associated athanogene 3 (BAG3). This protein interacts with both the HSP70-substrates and the microtubule-motor dynein, which loads misfolded protein onto the dynein motor complex. Unlike HDAC6-mediated pathway, substrate ubiquitination is not essential for BAG3-mediated aggresome formation (Gamerdinger et al., 2011). Commonly, both pathways rely on the microtubule and dynein-motor complex to deliver the misfolded proteins to the MTOC.

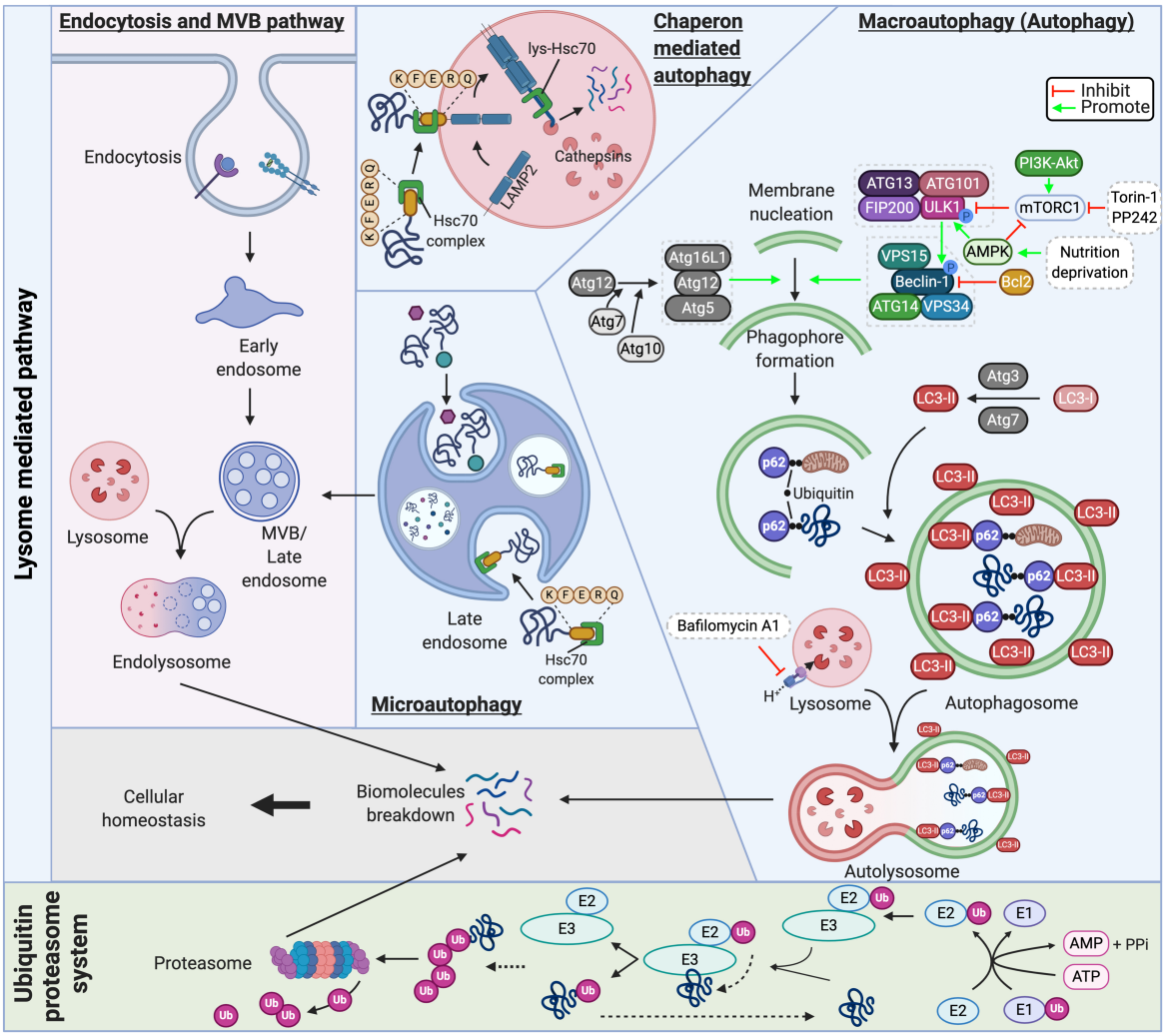


Figure 2. An overview of the two mammalian cellular degradation systems. The lysosome-mediated pathways, which include endocytosis, chaperone-mediated autophagy, microautophagy and macroautophagy are shown at the top and the ubiquitin-proteasome system is illustrated below.

1.1.4 Protein degradation systems in cancers

The protein degradation systems involved in cancer are important from several aspects related to: (1) Modulation of the expression level of key cancer regulators, (2) Contribution to the pathophysiological signaling pathways involved in tumor development and progression, and (3) Their possible use as targets in therapeutic interventions.

Besides the involvement in neurodegenerative disorders (Goldberg, 2003), the lysosome-autophagy system has also been extensively studied in cancer (Levine and Kroemer, 2008). However, autophagy is still heavily debated concerning its possible dual-role in tumor promoting as well as suppressing functions (Jin and White, 2007; Kroemer et al., 2010; Levine and Kroemer, 2008; Levy et al., 2017). There is strong scientific evidence that autophagy is a *bona fide* tumor suppressor system. For example, heterozygous deletion of Becn1 and homozygous deletion of Bif-1 (an interacting protein of Becn1, which is required for autophagosome formation) promotes spontaneous tumorigenesis (Qu, X. et al., 2003; Takahashi et al., 2007). Additionally, autophagy deficiency can also jeopardize its surveillance function and promote genomic instability by accumulation of p62 that inhibits the RNF168 E3 ligase activity for histone H2A ubiquitination, leading to impaired DNA repair (Eliopoulos et al., 2016; Wang et al., 2016). Alternatively, autophagy can also favor tumor cell survival by sustaining stress conditions, such as nutrient deprivation, misfolded protein accumulation and pathogen invasion (Kroemer et al., 2010). For example, silencing of macroautophagy can induce apoptosis and sensitize cells under nutrient starvation (Boya et al., 2005). Nowadays, the majority of the clinical trials for targeting autophagy in cancer are based on autophagy inhibition (Levy et al., 2017).

As a key protein degradation system, it is not surprising that the UPS plays a vital role in cancer. The UPS system particularly regulates the protein turnover of several key players involved in tumorigenesis, such as the cell cycle family members p53, c-Jun, c-Myc, and NF-κB (Adams, 2003, 2004; Ciechanover et al., 1991). For instance, the NF-κB pathway requires the UPS 26S complex to degrade its inhibitor IκB in order to regulate the pro-survival pathways (Palombella et al., 1994). Besides, the expression level of the tumor suppressor p53 is under the balance of UPS degradation (Maki et al., 1996). E3 ligases from the UPS also participate in cancer development via its ubiquitination function. The MDM2 oncogene has been confirmed as an E3 ligase that promotes ubiquitination of p53 for degradation via its RING ligase domain (Haupt et al., 1997; Honda and Yasuda, 2000; Maki et al., 1996). Other examples include the VHL and BRCA1 E3 ligases. Loss-of-function mutations of VHL leads to degradation failure of hypoxia-inducible factor (HIF) proteins in renal clear cell carcinoma (Gunaratnam et al., 2003), while the E3 ubiquitin ligase activity of BRCA1 provides signals for DNA repair (Wu et al., 2008).

The UPS has also been shown to be involved in virus-induced carcinogenesis, such as the F-box and WD repeat domain-containing 7 (FBXW7) E3 ligase that promotes degradation of viral oncoprotein in Merkel cell carcinoma, which will be discussed in more details at Section 1.2.4. Besides viral oncoprotein, FBXW7 also regulates several oncogenic

substrates, such as c-Myc and cyclin E, supporting its tumor suppressor function in human cancers (Welcker and Clurman, 2008; Yeh et al., 2018). The UPS has been reported to have higher activity in many cancer types (Arlt et al., 2009; Chen and Madura, 2005; Kumatori et al., 1990), which might be due to altered protein requirement for abnormal proliferation, apoptosis status and stress conditions. Therefore, protein degradation systems could provide multiple potential therapeutic targets in cancer medicine.

1.2 MERKEL CELL POLYOMAVIRUS AND MERKEL CELL CARCINOMA

1.2.1 Merkel cell carcinoma origin and diagnostic markers

MCC is a rare but aggressive type of skin cancer, which was first described as ”trabecular tumor of the skin” by Cyril Toker in 1972 (Toker, 1972). This tumor type is believed to originate from the mechanoreceptor Merkel cells, based on three cellular features: the presence of neurosecretory granular (NG) structures; the sharing of identical neuroendocrine markers, e.g. chromogranin A and synaptophysin (Tang and Toker, 1978); and expression of Merkel cell-like cytoskeleton markers, e.g. cytokeratin 20 (Moll, 2006). Examples of NG and whorl-like intermediate filament structure are shown in Figure 3. Several arguments have been put forward questioning whether the Merkel cell is the origin of MCC. Firstly, Merkel cells are located in the base of the epidermis, while MCC typically develops in the dermis (Sibley et al., 1985). Secondly, some immunohistological markers are expressed in MCC, such as the neural cell adhesion molecular L1, the mucin-like adhesion protein CD24 and receptor tyrosine kinase c-KIT, but which are not detected in Merkel cells (Deichmann et al., 2003; Su et al., 2002a). Thirdly, numerous mitoses are found in MCC, which is opposed to the terminally differentiated and post-mitotic nature of Merkel cells (Moll et al., 1996). Lastly, different mouse models using Merkel cell-specific drivers fail to generate MCC (Shuda et al., 2015; Spurgeon et al., 2015; Verhaegen et al., 2017; Verhaegen et al., 2015).

On the other hand, Zur Hausen *et al*. proposed early B-cells as the cellular origin of MCC based on the expression of early B-cell lineage markers such as the terminal deoxynucleotidyl transferase and paired box gene 5 (PAX5), and the evidence of clonal immunoglobulin chain rearrangement (Zur Hausen et al., 2013). Furthermore, chronic lymphocytic leukemia (CLL) is frequently associated with MCC (Tadmor et al., 2011), and concurrent development of MCC and CLL in the same patients has also been reported (Barroeta and Farkas, 2007; Khezri et al., 2011; Pan et al., 2014; Popovic et al., 2014). Interestingly, Merkel cell polyomavirus (MCPyV) is present in 27% of CLL, and mutation of the viral large T-antigen and viral integration into the host DNA has also been detected in a subset of CLL (Pantulu et al., 2010).

Intriguingly, Liu et al. demonstrated that the dermal fibroblast is the target of MCPyV infection, suggesting its putative origin for MCPyV-positive MCC (Liu, W. et al., 2016). Hitherto, the MCC cell origin is still uncertain, and viral-positive and -negative MCC may have different cells of origin.

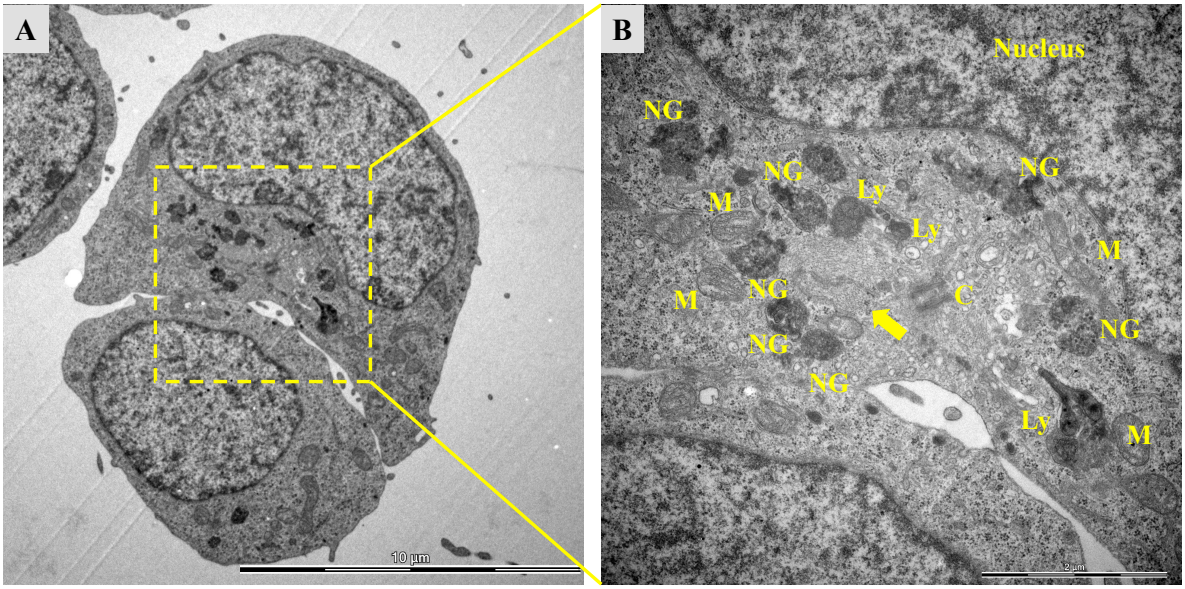


Figure 3. Electron microscopic image of WaGa MCC cell line. (**A**) A representative cell showing typical paranuclear whorl-like intermediate filament structures. (**B**) Enlargement of the yellow box in (**A**), illustrating the location of whorl-like intermediate filament structure (as indicated by arrow) adjacent to centriole (C) and surrounded by lysosomes (Ly), mitochondria (M) and neurosecretory granule (NG). Scale bar: 10 µm in (**A**) and 2 µm in (**B**).

MCC presents as a rapidly growing red-violet nodule or mass at cutaneous layers, including dermal and deeper region (Becker et al., 2017). Histopathological examination with hematoxylin-eosin staining typically shows a morphology with small round blue cells and a high number of mitoses (Harms, 2017). However this morphology is also present in other tumor types, such as small cell lung cancer (SCLC), and Ewing sarcoma. Hence further confirmation with immunohistochemical analyses of specific markers is essential (Bichakjian et al., 2018). The recommended panel of diagnostic markers is listed in Table 1, which include cytoskeletal keratins (CK20 and CK7), neuroendocrine markers (neuron-specific enolase, chromogranin A and synaptophysin), thyroid transcription factor 1, huntingtin-interacting protein 1 (HIP1), S100, leucocyte common antigen and the MCPyV large T-antigen (CM2B4) (Becker et al., 2017; Bichakjian et al., 2018; Lebbe et al., 2015).

Table 1. Immunohistochemical markers for differential diagnosis of MCC and other malignancies with small round blue cells.

	MCC	Lymphoma	Melanoma	SCLC
CK20	+	-	-	-
CK7	-	-	-	+
NSE	+	-	-	+/-
CgA	+/-	-	-	+/-
HIP1	+	+/-	-	-
TTF-1	-	-	-	+
S100	-	-	+	-
LCA	-	+	-	-
MCPyV LT	+	-	-	-

CK20, cytokeratin 20; CK7, cytokeratin 7; NSE, neuron-specific enolase; CgA, chromogranin A; HIP1, huntingtin-interacting protein 1; TTF-1, thyroid transcription factor 1; LCA, leucocyte common antigen. MCC, Merkel cell carcinoma; SCLC, small cell lung cancer. + = presence, - = absence, +/- = presence or absence of the staining marker. Adapted from Lebbe et al., 2015.

1.2.2 MCC incidence, risk factors and mortality

The incidence of MCC has increased more than 4-fold (1.5 to 7 per million) from 1986 to 2013 in the United States (Hodgson, 2005; Paulson et al., 2018). About 2,500 new cases are diagnosed each year in the United States and Europe (Paulson et al., 2018; Stang et al., 2018). In Sweden, the incidence has risen from 1.1 to 1.9 per million from 1993 to 2012, and 47 new cases were reported in 2012 (Zaar et al., 2016). A similar ascending trend has been reported worldwide, including Finland (Kukko et al., 2012), France and Australia (Fondain et al., 2018; Youlden et al., 2014).

The risk factors for MCC include age, gender, fair skin and ultraviolet (UV) exposure. MCC occurs slightly more common in male (more than 54%) than female in the United States, Finland and Sweden, especially in older population (Paulson et al., 2018; Sihto et al., 2011; Zaar et al., 2016). The average and median age of MCC diagnosis is above 75 years (Fondain et al., 2018; Paulson et al., 2018; Sihto et al., 2011; Zaar et al., 2016). The incidence (per million inhabitants per year) rises exponentially with age: from 1 in the age group 40-44 years, to 10 in the group 60-64 years and to 98 above 85 years of age (Paulson et al., 2018). MCC predominantly occurs in the white population (94.9%), which might be explained by the fact that skin pigmentation protect skin from UV damage (Albores-Saavedra et al., 2009). The association between UV radiation and MCC has been well documented. The MCC incidence is positively associated with increasing UVB (one of the UV rays with 280-315 nm) index in the United States (Agelli and Clegg, 2003). MCCs are commonly, but not exclusively, located in sun-exposed skin areas, such as the head, neck and extremities (Paulson et al., 2018; Sihto et al., 2009; Zaar et al., 2016). Youlden *et al.* reported that the

incidence of MCC was double in Queensland, Australia, as compared to elsewhere in the world (Youlden et al., 2014); this is likely due to the higher intensity of sunlight exposure.

In addition, immune deficiency has a crucial etiological role in MCC. The incidence of MCC increases sharply among individuals who received therapeutic immunosuppression after solid organ transplantation (Clarke et al., 2015). Acquired immune-deficiencies, such as leukemia, lymphoma and acquired immune deficiency syndromes, are also associated with higher risk of MCC development (Engels et al., 2002; Heath et al., 2008). The combination of aging, UV exposure and immune-deficiencies also indicate the key role of immune surveillance for the prevention of MCC. Interestingly, MCC bears remarkable similarities to other tumors with a viral etiology, such as Kaposi sarcoma, both of which commonly occur in older and immunosuppressed individuals (Engels et al., 2002; Sihto et al., 2009).

Staging of MCC is also based on the TNM classification of Malignant Tumors, in which "T" stands for the size of the primary tumor, "N" for regional lymph node involvement and "M" for distant metastasis. For MCC, primary tumors with a diameter of ≤ 2 cm, > 2 but ≤ 5 cm, and > 5 cm are classified as T1, T2 and T3, respectively. MCCs with deep invasion (such as bone and muscle) will be classified as T4 (Lebbe et al., 2015). Due to limited experience of the disease and the lack of early diagnostic markers, MCC patients are usually diagnosed at the advanced stage. The staging and 5-year overall survival of MCC is shown in Table 2 (Harms et al., 2016; Lebbe et al., 2015). Over 40% of MCC patients develop a recurrence within a median time of 9 months, and the median survival for patients with distant metastasis is approximately 9 months (Allen et al., 2005).

Table 2. MCC staging and corresponding 5-year overall survival

Stage	T	N	M	5-year overall survival (%)
O	TIS	N0	M0	
IA	T1	pN0	M0	79
IB	T1	cN0	M0	60
IIA	T2/T3	pN0	M0	55
IIB	T2/T3	cN0	M0	49
IIC	T4	N0	M0	47
IIIA	Any T	N1a	M0	40
IIIB		N1b/N2	M0	27
IV		Any N	M1	14

TIS, tumor *in situ*; T1, primary tumor diameter ≤ 2 cm; T2, between 2 cm and 5 cm; T3, > 5 cm; T4, deep invasion; N0, no regional node metastasis; cN0, nodes not clinically detectable but no pathologic examination, pN0, nodes negative both clinically and by pathologic examination; N1a, micrometastasis; N1b, macrometastasis; N2, in transit metastasis; M0, no evidence of distant metastasis; M1, metastasis beyond regional lymph nodes. Adapted from Lebbe et al., 2015; Harms et al., 2016.

1.2.3 Merkel cell polyomavirus and association with MCC

The Merkel cell polyomavirus (MCPyV) was first discovered in MCC by whole-transcriptome sequencing in 2008, and this virus was found integrated into the host genome

in 8 out of 10 MCC (Feng et al., 2008). The full-length genome is 5,387 base-pairs, and the genome map with corresponding transcripts are shown in Figure 4 (DeCaprio and Garcea, 2013). It harbors a non-coding control region (NCCR) where the transcription starts with corresponding early and late promoter. The genome is divided into the early region for viral antigens and the late region for viral capsid proteins (Topalis et al., 2013).

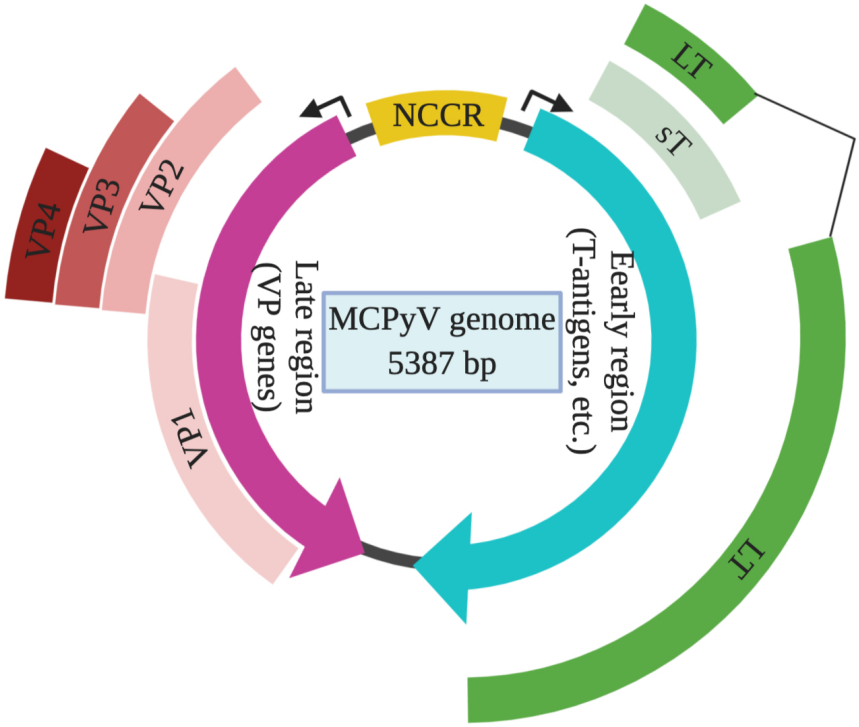


Figure 4. MCPyV genome map with different spliced variants from the early and late regions. NCCR, non-coding control region; sT, small T-antigen; LT, large T-antigen; VP, viral protein. Adapted from DeCaprio and Garcea, 2013.

Later, several studies with larger cohorts have confirmed that MCPyV is found in the majority of MCC, ranging from 75% to 89% (Becker et al., 2009; Feng et al., 2008; Foulongne et al., 2008; Kassem et al., 2008; Prieto Munoz et al., 2013). Worldwide, MCPyV+ MCC is also the dominant type except in Oceania areas (Kuwamoto, 2011).

Interestingly, MCPyV infection is also widespread in the general population. Substantial MCPyV antibody titers have been detected in newborns, children and adults (Chen et al., 2011; Martel-Jantin et al., 2013; Tolstov et al., 2009). An age-dependent prevalence of MCPyV infection has been observed based on sera antibody test. The prevalence is below 40% before 5 years of age, but linearly increases to above 80% for the group aged above 50 (Tolstov et al., 2009). However, MCPyV integration into the host genome is only found in MCC tumors (Feng et al., 2008; Sastre-Garau et al., 2009) and a subset of CLL (Pantulu et al., 2010). Numerous reports have suggested that the viral genome integration occurs during early tumorigenesis, which is based on the identification of the

same MCPyV mutated sequence and integration in primary tumor and distant metastasis of the same patient (Laude et al., 2010; Sastre-Garau et al., 2009; Shuda et al., 2009).

1.2.4 Role of MCPyV oncoproteins

Similar to other polyomaviruses, MCPyV encodes four different viral tumor antigens (T antigens), including large T, small T (sT), 57 kT and alternative T antigen open reading frame. Among these T-antigens, sT and LT are regarded as the main tumor-driven proteins (Kwun et al., 2009; Shuda et al., 2009). The functional domains of these two transcripts are shown in Figure 5.

The majority of MCC tumors harbor an integrated MCPyV genome with a mutation in the LT, leading to expression of truncated LT and intact sT. Mutations of LT are found in exon 2 of LT and downstream of the Rb-binding domain (Figure 5), which lead to elimination of the helicase domain of LT for viral replication (Shuda et al., 2008). Silencing of both LT and sT can lead to growth suppression and cell cycle arrest, indicating that both T-antigens are essential for MCC growth. Furthermore, both T-antigens can also promote neoplastic transformation and tumor development *in vivo* (Houben et al., 2010; Shuda et al., 2008; Shuda et al., 2015; Spurgeon et al., 2015; Verhaegen et al., 2015).

LT and sT share the common DnaJ domain at the amino-terminus, which comprises the conserved CR1 and HSC70 binding motifs for viral DNA replication and cell growth through induction of E2F target genes (Feng et al., 2008; Houben et al., 2015; Kwun et al., 2009). LT has a MCPyV unique region (MUR), which contains the retinoblastoma (Rb) binding, Vam6p binding and nuclear localization signal (NLS) motif. The Vam6p interaction disrupts cellular lysosome trafficking (Liu et al., 2011) and protein degradation system (**Papers III** and **IV**). The MUR also contains the LXCXE motif that binds Rb, which can sequester Rb from E2F, ultimately leading to cell cycle progression (Shuda et al., 2008). The NLS was initially thought to be preserved in the truncated LT of MCC (Nakamura et al., 2010); however it is now clear that this motif is not always preserved in the tumors and is not essential for promoting cell growth (Houben et al., 2015).

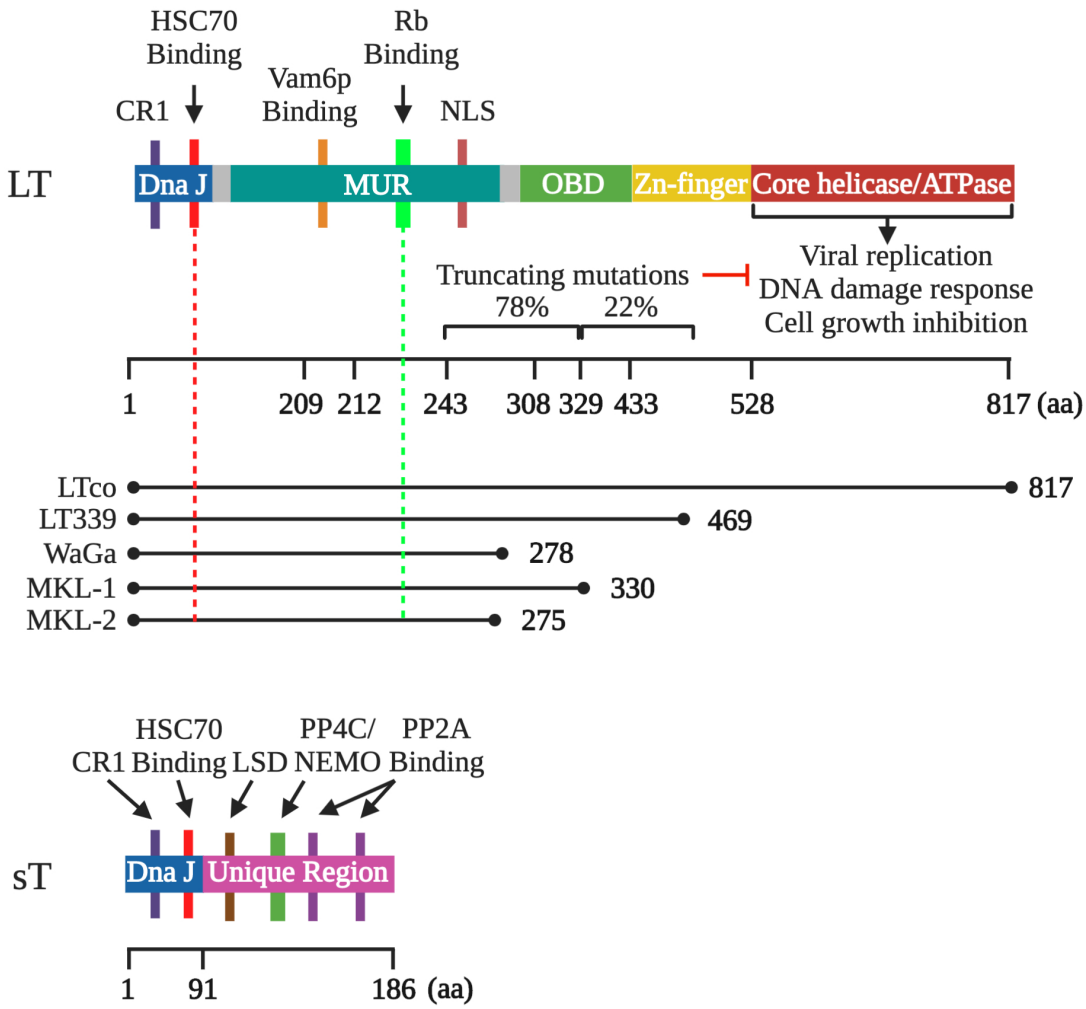


Figure 5. Functional domains of MCPyV LT and sT. At the top is an illustration of LT with different functional domains. The numbers below refer to the positions of amino acid. Mutations frequently occur downstream of Rb binding site. The truncated LT of the three MCPyV+ MCC cell lines (WaGa, MKL-1 and MKL-2) used in this thesis is illustrated in the middle panel of the figure. In addition, the two plasmids expressing full-length LT (LTco) and truncated LT (LT339) are also shown. At the bottom is the sT, which has the common DnaJ domain as the LT and a unique region. NLS, nuclear localization signal; MUR, MCPyV unique region; OBD, origin binding domain; LSD, LT-stabilization domain; PP2A/4C, protein phosphatase 2A/4C.

The MCPyV sT has a special domain called LT-stabilization domain (LSD), which is responsible for inhibition of FBXW7 and thus stabilization of the expression of FBXW7-degradation substrates, such as LT, Myc and cyclin E (Kwun et al., 2013) and for induction of genomic instability (Kwun et al., 2017). Besides, the LSD domain also targets 4E-BP1 to preserve its hyperphosphorylation and release eIF4E for activation of cap-dependent protein translation (Shuda et al., 2011). sT also inhibits the NF-κB essential modulator related transcription via the direct interaction with PP4C (Griffiths et al., 2013). Unlike SV40 sT, the PP2A targeting by MCPyV sT is not required for cellular transformation (Kwun et al., 2015). Moreover, sT also participates in several biological processes, such as induction of cell motility and invasiveness through destabilization of microtubules (Knight et al., 2015) or

regulation of Rho-GTPase (Stakaityte et al., 2018), as well as increase aerobic glycolysis via monocarboxylate transporter for transforming activity (Berrios et al., 2016).

Interestingly, the full-length LT has distinct functional roles as compared to truncated LT. The carboxyl-terminal helicase-containing region of LT can activate cellular DNA damage response via the p53 pathway and suppress cell growth (Cheng et al., 2013; Li, J. et al., 2013). The inhibitory effect on proliferation can be rescued by a dominant-negative p53 inhibitor (Li, J. et al., 2013). These findings indicate that selection of truncated LT by elimination of the helicase domain is important for MCC tumor development.

1.2.5 Genetic differences between virus- and non-virus associated MCC

Generally MCC can be divided into two groups: MCPyV-positive (MCPyV+) and MCPyV-negative (MCPyV-). Genome-wide analyses have shown that MCPyV- MCC has much higher frequencies of somatic mutations than MCPyV+ MCC, and that the mutations in the MCPyV- tumors are associated with UV-mediated signatures (Harms et al., 2015; Starrett et al., 2017). The mutation signatures indicate dominant characteristics of C-to-T mutations, resulting from the UV-induced pyrimidine dimers. Besides, the high mutation burden in MCPyV- MCC is also in agreement with an UV etiology in MCPyV- MCC (Starrett et al., 2017).

Mutations of *RB1* and *TP53* are frequently found in MCPyV- MCC, while mutations of these two genes are uncommon in MCPyV+ MCC (Goh et al., 2016; Harms et al., 2015; Paulson et al., 2009). Yet, RB and p53 are inactivated by the viral LT (Park et al., 2019; Rodig et al., 2012; Starrett et al., 2017). Other pathogenic mutations are also detected in MCPyV- MCC, such as loss-of-function mutations of the tumor suppressor *PTEN*, and genes involved in chromatin-modification (*ASXL1*, *MLL2* and *MLL3*), DNA-damage checkpoint and repair systems (*ATM*, *MSH2*, *MLH1*, *BRCA1* and *BRCA2*) and WNT signaling (*APC*, *β-catenin* and *LEF-1*) (Goh et al., 2016; Harms et al., 2015; Paulson et al., 2009; Starrett et al., 2017). In addition, gain-of-function mutations in *JUN*, *EGFR* and *MYCL1* have been reported in MCPyV- MCC (Paulson et al., 2009; Starrett et al., 2017).

Gene expression profiling has revealed upregulation of several oncogenes in MCC, including *FYN*, *AKT1*, *AKT3*, *SOX2*, *BCL2*, *MYCL1*, *VEGFA*, *ATOH1*, *HIP1* and *c-KIT*, as compared to cutaneous squamous cell carcinoma, and downregulation of genes involved in Hedgehog signaling (*GLI1*, *GLI2*, *PTCH1* and *PTCH2*) as compared to basal cell carcinoma (Harms et al., 2013; Paulson et al., 2009).

Distinct gene expression profiles are associated with MCPyV status in MCC (Harms et al., 2013) (Harms et al., 2015; Starrett et al., 2017). MCPyV- tumors show upregulation of Notch family signaling genes (*NOTCH1*, *NOTCH2*, *DLL1*, *CTBP2*, *HES1, JAG2* and *JAG1*), receptor tyrosine kinases (*VEGFA*, *PDGFA*, *FGFR2*), DNA damage response genes (*MSH2* and *MLH1*) and *RB1*. On the other hand, upregulation of GABA receptor signaling genes

(*GABRB3*, *KCNN1*, *KCNN2* and *KCNQ3*) and cell cycle regulators (*CCNA1* and *CCND1*) are found in MCPyV+ MCC.

1.2.6 The immune escape of MCC

The immune profiles are also distinct between MCPyV+ and MCPyV- MCCs. In MCPyV+ MCCs, higher numbers of tumor-infiltrating immune cells are observed, concerning CD3+, CD8+, CD16+, FoxP3+ and CD68+ cells. However, high numbers of intratumoral CD3+ and CD8+ T-cells favor patient survival regardless of viral status (Sihto et al., 2012). Similarly, using transcriptomic profiling, Harms *et al*., identified higher levels of immune response genes, such as *CD3G*, *CD3D*, *ZAP70* and *IGHM*, in MCPyV+ MCCs, indicating the presence of tumor-infiltrating lymphocytes (TILs). Indeed, they verified higher numbers of CD8+ lymphocytes in MCPyV+ MCCs (Harms et al., 2013). However, impaired T-cell activation and T-cell exhaustion have been observed in MCC TILs, implicating immune evasion by MCC (Dowlatshahi et al., 2013) (Walsh et al., 2016). Importantly, tumor-specific T-cells can be stimulated for activation, expansion and antitumor function (Dowlatshahi et al., 2013) (Gavvovidis et al., 2018).

Together, these observations indicate immune escape in MCC, however the mechanisms of immune evasion in MCC remain unclear. Nevertheless, these findings have driven the rationale for using immune checkpoint inhibitors in MCC treatment (Nghiem et al., 2016; Topalian et al., 2020).

1.2.7 Clinical management of MCC

According to the newest National Comprehensive Cancer Network (NCCN) guidelines (Bichakjian et al., 2018), after physical examination of suspicious lesion on the skin, histopathological evaluation of the lesion, including the regional skin and lymph node (LN), will be performed using the immune-panel markers as described in Section 1.2.1 and Table 1. Sentinel lymph node biopsy (SLNB) is highly recommended for determination of subclinical nodal diseases, even if LN is negative. If LN and SLNB are negative, adjuvant radiotherapy treatment will be performed on the primary tumor site. If LN or SLNB is positive, comprehensive imaging studies, such as magnetic resonance imaging/ computed tomography (MRI/CT) with specific region or whole body positron emission tomography (PET)/CT scan, should be applied to detect regional or distant metastasis. Single therapy or a combination of surgical excision, radiation therapy, and systemic therapy such as chemotherapy and immune-checkpoint inhibitor (anti-PD1 or anti-PD-L1) may be applied. The surgical margin is still under discussion, but commonly 1-2 cm is recommended (Becker et al., 2017; Bichakjian et al., 2018). In those cases with distant metastasis, immunotherapy or other clinical trials with therapies shown to be effective for metastatic cancers will be preferred (Bichakjian et al., 2018).

1.3 CELLULAR DEGRADATION SYSTEMS AND VIRAL PATHOGENESIS

Since the viral genome is usually limited in size, viruses exploit the host cellular machineries to complete their life cycle, including viral entry, genome replication, translation, assembly and virion release. The cellular degradation systems, including the lysosome-autophagy and the UPS, are important regulators of viral pathogenesis.

1.3.1 Viral infection and cellular degradation systems

Virus and other pathogens can protect themselves by harnessing the host degradation systems during infection (Kirkegaard et al., 2004). Disruption of lysosome-autophagosome fusion and inhibition of proteasome for viral infection has been reported in several RNA viruses. For example, blocking lysosome with H^+-ATPase inhibitor bafilomycin A1 and inhibiting proteasome with MG-132 lead to higher infectivity of Human Immunodeficiency Virus Type 1 *in vitro* (Wei et al., 2005). Administrating the glycopeptide antibiotic to antagonize the Cathepsin L lysosomal hydrolase can block the viral entry for Ebola virus and two other Coronaviruses (MERS-CoV and SARS-CoV) (Zhou et al., 2016). Ding *et al*. illustrated that the Human Parainfluenza virus 3 can utilize the viral phosphoprotein to disrupt the host SNARE proteins, resulting in autophagy disruption and accumulation of viral productions (Ding et al., 2014). Similarly, DNA virus such as Hepatitis B virus (HBV) has also been shown that its replication is increased upon inhibition of autophagosome-lysosome fusion (Lin et al., 2019).

1.3.2 Viral oncogenesis and ubiquitin-proteasome system

Oncogenic viruses can manipulate multiple targets in the UPS for oncogenesis (Masucci, 2004). The first evidence was demonstrated in Human papillomavirus (HPV). HPV E6 oncoprotein can bind to E6AP, a cellular E3 ubiquitin ligase involved in the UPS, which directs E6AP to ubiquitinate p53 for proteasomal degradation (Scheffner et al., 1993; Scheffner et al., 1990).

Subsequently, several viral oncoproteins have also been demonstrated to hijack proteasomal degradation by targeting different components of the UPS (Masucci, 2004). In hepatitis B virus (HBV), its oncoprotein HBV X antigen (HBx) binds to the 20S subunit and the 19S subunit of proteasome that prevents degradation of c-JUN for cellular transformation (Zhang et al., 2000). In Epstein-Barr virus (EBV), the glycine-alanine repeat domain of the EBV nuclear antigens 1 (EBNA-1) can capture 19S proteasome subunit that blocks UPS substrate degradation (Levitskaya et al., 1995). In addition, EBNA-1 also competes for the binding of ubiquitin specific protease 7 (HSP7) to MDM2 that leads to stabilization of MDM2, which targets p53 for proteasomal degradation, resulting in B-cell immortalization and tumorigenesis (Boutell and Everett, 2003; Saridakis et al., 2005). In human adenoviruses, the viral E4rf6 and E1B55K proteins can recruit the Cullin-containing complex to induce

proteasome degradation of p53 (Querido et al., 2001). In MCC, the LSD domain of MCPyV sT directly disrupts the FBXW7 E3 ligase to stabilize the LT and other oncogenic substrates for promoting transformation (Kwun et al., 2013) and induces genome instability (Kwun et al., 2017).

1.3.3 Viral oncogenesis and aggresome-autophgay

Tumor viruses have also been reported to target the autophagy-aggresome system for tumorigenesis. All the seven known human oncogenic viruses are tightly linked to autophagy system (Vescovo et al., 2020), some of the examples are briefly described below.

Mattoscio et al. demonstrated that HPV E6 and E7 suppress autophagy by disrupting autolysosome formation that leads to upregulation of UBC9 for HPV-mediated tumorigenesis (Mattoscio et al., 2017). UBC9 is the key enzyme of the Small Ubiquitin-like Modifier (SUMO) pathway, in which its expression progressively increases during progression in both HPV-associated cervical and head and neck tumorigenesis (Mattoscio et al., 2015; Mattoscio et al., 2017).

As the major etiology for over 80% of hepatocellular carcinoma (HCC) (Zamor et al., 2017), both HBV and HCV can modulate autophagy for HCC development (Tarocchi et al., 2014; Vescovo et al., 2012). Although both viruses have been demonstrated to induce autophagy during infection, autophagy evasion has been observed in both HBV and HCV-associated cancers (Lan et al., 2014; Vescovo et al., 2012). Qu *et al.* provides the first evidence that autophagy suppression can promote HCC tumorigenesis, in which they show that heterozygous deletion of *Becn1* promotes tumorigenesis in HBV transgenic mice (Qu, X.P. et al., 2003), indicating the importance of autophagy evasion in HBV-mediated tumorigenesis. The viral oncoprotein HBx can impair lysosomal maturation, which results in inhibition of autophagic degradation (Liu et al., 2014). Inhibition of autophagic degradation will lead to accumulation of p62. Concordantly, accumulation of p62 level has been observed in HCC, including HCV-associated HCC, and that promotes tumorigenesis (Saito et al., 2016; Umemura et al., 2016).

In MCC, we demonstrate that the MCPyV T-antigens can suppress autophagy through miRNAs and an oncogenic receptor tyrosine kinase (RTK). The truncated LT and sT of MCPyV can induce *miR-375*, *miR-30a-3p* and *miR-30a-3p* levels through the viral DnaJ domain at the N-terminus, which directly targets ATG7, p62 and BECN1 (**Paper II**). The truncated LT can also promote intracellular retention and stabilization of c-KIT via its Vam6p interaction, which promotes the binding of c-KIT to BECN1 and recruitment of BCL2 complexes for autophagy suppression (**Paper III**). Induction of autophagy regulates cell growth and destabilizes the LT oncoprotein (**Papers II** and **III**), suggesting the importance of autophagy suppression in MCC tumorigenesis.

Some viral proteins can also promote aggresome formation during tumorigenesis. Such an example is found in adenovirus. The E1B-55K and E4orf3 proteins of the adenovirus can promote cellular transformation. Interestingly, these viral proteins can induce aggresome formation (Blanchette et al., 2013; Liu et al., 2005) and sequester different tumor suppressors into aggresomes, including p53 (Zhao and Liao, 2003), Mre11, a factor involved in DNA repair (Araujo et al., 2005; Liu et al., 2005) and SSBP2, a tumor suppressor in both leukemia and carcinoma (Fleisig et al., 2007), suggesting the importance of aggresomes in viral oncogenesis..

1.4 MICRORNAS

MicroRNAs (miRNAs) are a group of non-coding single-stranded ribonucleic acids (RNAs) with a length of about 22 nucleotides (nts). *lin-4* is the first miRNA described in *Caenorhabditis elegans* that control developmental timing through suppression of *lin-14* messenger-RNA (mRNA) (Lee et al., 1993; Wightman et al., 1993). Generally, miRNAs can affect gene expression by imperfect pairing to mRNA.

1.4.1 miRNA biogenesis and functional mechanisms

The primary transcript of miRNA (pri-miRNA) is generally transcribed by RNA polymerase II from miRNA gene in the nucleus (Lee et al., 2004). Pri-miRNAs can be several kilobases in length containing a stem-loop structure; the secondary structure navigates the pri-miRNA to anchor into the microprocessor complex formed by Drosha and DGCR8. In this complex, pri-miRNA is cropped to produce a hairpin precursor-miRNA (pre-miRNA) with a length of about 70 nts. The pre-miRNA is then transported to the cytoplasm via Exportin-5. After exporting out from the nucleus, pre-miRNA interacts with the Dicer RNase III enzyme and TARBP2 in which the loop region is cleaved, releasing a ~22 nts double-stranded RNA. This duplex is loaded onto Argonaute complex and forms RNA-induced silencing complex (RISC). Only one strand is preserved based on the thermodynamic stability, the other strand is named passenger strand and is usually degraded. Finally, the mature single-stranded miRNA is then guiding the RISC to search for its target mRNA (Lee et al., 2003; Lund et al., 2004; Miyoshi et al., 2005).

In human, the miRNA-mRNA interaction is commonly imperfect pairing, which can lead to translational repression or mRNA degradation (Bartel, 2004; Mortensen et al., 2011). The mechanistic details of how miRNAs inhibit translation are still unclear, but some studies have indicated that the repression step occurs at the translation initiation and/or elongation (Petersen et al., 2006; Pillai, 2005). In the case when miRNAs perfectly bind to mRNA, it normally leads to endonucleolytic cleavage of mRNA, however this mechanism is uncommon in mammals (Chen, 2004; Valencia-Sanchez et al., 2006; Yekta et al., 2004). miRNAs are generally negative gene expression regulators, however some miRNAs can

increase gene expression. For example, the HCV RNA expression could be positively regulated at multiple sites by the liver-specific *miR-122* (Jopling et al., 2008; Jopling et al., 2005; Li, Y. et al., 2013).

Besides the interaction between miRNA and mRNA, miRNAs can also interact with non-coding RNAs. For example, mouse *miR-709* directly binds to its recognization site of *mir-15a~16-1* to disturb the maturation (Tang et al., 2012). On the other hand, *let-7* can bind to its own primary transcript and promote autoregulation of its processing (Zisoulis et al., 2012). One miRNA can regulate multiple mRNAs, while multiple miRNAs can coordinately target a single mRNA (Bartel, 2004; Zhou et al., 2013), indicating the complexity of miRNA regulatory network.

1.4.2 miRNAs in cancer

The first cancer-related miRNA study was published in 2002, demonstrating the frequent deleted and down-regulated miRNAs (*miR-15a* and *miR-16*) in CLL (Calin et al., 2002). Calin *et al.* also revealed that many miRNA genes are located in fragile sites and/or cancer-related genomic region (Calin et al., 2004). Subsequently, numerous studies discovered many cancer-associated miRNAs in different cancer types (Calin and Croce, 2006).

The first oncogenic miRNA was discovered in B-cell lymphoma called *mir-17~92* cluster (He et al., 2005). This miRNA cluster is frequently amplified and overexpressed in hematological malignancies and solid tumors. He et al. provided the direct evidence that this miRNA cluster is oncogenic using a B-cell lymphoma mouse model (He et al., 2005). In addition, overexpression of this miRNA cluster can promote Notch1-induced T-cell acute lymphoblastic leukemia through targeting multiple genes, including Bcl2 family and PTEN (Mavrakis et al., 2010). Furthermore, this cluster can promote cellular proliferation in human lung cancer, and inhibits TGF-β pathway in neuroblastoma (Hayashita et al., 2005; Mestdagh et al., 2010).

miRNAs can act as oncogenes or tumor suppressors in different cancer types (Zhang et al., 2007). For example, *miR-223* has been demonstrated to function as an oncogene in several cancer types (Kurashige et al., 2012a; Laios et al., 2008; Li, J.H. et al., 2012; Petrocca et al., 2008a; Zhang et al., 2014), including testicular germ cell tumor in **Paper I**. However, this miRNA can also function as a tumor suppressor in lung cancer (Luo et al., 2019), nasopharyngeal cancer (Gao and Xiong, 2018) and prostate cancer (Kurozumi et al., 2016).

Furthermore, miRNA also participates in diverse oncogenic processes. A subset of miRNAs is under the regulation of p53. For example, expression of *miR-34* family (*miR-34a*, *miR-34b* and *miR-34c*) is transcriptionally regulated by p53 in response to DNA damage response (DDR), which plays important role in cell proliferation, apoptosis and epithelial mesenchymal transition (Hata and Lieberman, 2015). On the other hand, *TP53* is also directly

regulated by miRNAs, such as *miR-125b* and *miR-504* (Hu et al., 2010; Le et al., 2009), suggesting the importance of miRNAs in p53 network.

Besides *miR-34*, *miR-24* and *miR-138* are regulated by DDR and both miRNAs target the histone H2A variant (H2AX) to recruit the DNA repair factors (Lal et al., 2009; Wang et al., 2011). In addition to DDR, some miRNAs are regulated by hypoxia. A well-known example is *miR-210*, in which its expression level is strongly induced by hypoxia (Ivan and Huang, 2014). This miRNA can shield tumor cells from apoptosis during hypoxia by targeting the caspase-8 associated protein-2 (CASP8AP2) and the BNIP3 (Kim et al., 2009; Wang et al., 2013), and promote angiogenesis by targeting the EFNA3 (Fasanaro et al., 2008). Moreover, *miR-210* is also induced by pro-inflammatory signals, which targets the NF-κB1 that suppresses Toll-like receptor 4 mediated pro-inflammatory cytokines (Qi et al., 2012).

1.4.3 miRNAs in virus-related cancers

In MCC, a miRNA profiling study performed by our group identified a list of miRNAs associated with MCPyV status, metastasis stage and clinical outcome (Xie et al., 2014). However, the functional evidence of miRNAs in MCC tumorigenesis is limited. To date, only a few miRNAs have been functionally characterized in MCC. *miR-203* can inhibit cell growth and survivin expression in MCPyV- MCC cells (Xie et al., 2014). On the other hand, *miR-375* was found to suppress neuroendocrine differentiation by targeting multiple Notch pathway genes (Abraham et al., 2016) and regulate cell growth via suppression of LDHB (Kumar et al., 2018). In **Paper II**, we demonstrate that *miR-375*, *miR-30a-3p* and *miR-30a-5p* can suppress multiple autophagy genes, including *ATG7*, *SQSTM1* and *BECN1*, leading to autophagy suppression. Importantly, *miR-375* is also the most abundant miRNA in MCC, whose serum level is correlated with tumor burden (Fan et al., 2018), suggesting its potential utility as biomarker for disease surveillance.

Involvement of miRNAs in other virus-related cancer has also been reported. A detailed miRNA regulation system has been characterized in hepatitis B or C virus-associated liver cancer (Wang et al., 2012). Several miRNAs can regulate viral gene expression level and oncogenic signaling in HBV- and HCV-related HCC. For example, *miR-122* can bind HCV RNA and promote HCV translation and replication. On the other hand, *miR-20a* and *miR-92a* from the *mir-17~92* cluster can bind HBV RNAs and suppress their expressions and viral replication (Jung et al., 2013). *miR-224* is upregulated in HCC through NF-κB pathways (Scisciani et al., 2012) and promotes cell growth, migration, invasion and tumor development (Lan et al., 2014; Li et al., 2010; Wang et al., 2008; Zhang et al., 2013). *miR-199a/b-3p* is consistently down-regulated in HCC, which suppresses tumor growth by targeting the PAK4/RAF/MEK/ERK signaling (Hou et al., 2011).

HPV is known to play important role in cervical cancer development. HPV16 and 18, the two most common HPV strains that promotes cervical carcinogenesis, can regulate cellular miRNAs through their viral oncoproteins E6 and E7 (Au Yeung et al., 2011). E6 has been

shown to suppress *miR-34a*, *miR-23b* and *miR-145* through destabilization of p53 (Shi et al., 2012; Wang et al., 2009; Yeung et al., 2011). E7 can induce *miR-15b* through E2F-mediated transcription, resulting from inhibition of Rb-E2F interaction (Myklebust et al., 2011b). Additionally, E7 also increases *miR-27b* expression, which promotes cell growth and suppresses apoptosis via polo-like kinase 2 (PLK2) in cervical cancer (Liu, F. et al., 2016; Myklebust et al., 2011a).

These observations highlight the interplay between viral oncoproteins and miRNA regulation. Further investigations are still needed to characterize the detailed regulatory network of these miRNAs in specific tumor types.

1.4.4 Virus-encoded miRNAs

Besides cellular miRNAs, some viruses can also encode miRNAs by harnessing host miRNA processing machinery. The first evidence of virus-encoded miRNAs was discovered in EBV-infected cells (Pfeffer et al., 2004). These EBV-encoded miRNAs can affect cell growth, apoptosis, metastasis, immune checkpoint, cytokine expression and signal transduction (Cai et al., 2015; Cristino et al., 2019; Lyu et al., 2018; Ma et al., 2016; Wang et al., 2019). Subsequently, many more miRNAs were found in other herpesviruses, including Kaposi sarcoma-associated virus, mouse gammaherpesvirus 68 and human cytomegalovirus (Pfeffer et al., 2005). Besides herpesvirus family, several other virus families, including polyomavirus, ascovirus, baculovirus and adenovirus, have also been reported to encode miRNAs (Grundhoff and Sullivan, 2011). To date, a total of 530 mature miRNAs from 34 different viruses are annotated in the miRBase database (release 22.1, October 2018). Their proposed function covers viral infection, viral replication, host apoptosis and transformation. MCPyV genome consists of a miRNA gene called *mcv-miR-M1* (also known as *mcpv-mir-P1*). This miRNA complementarily interacts with the viral LT sequence and controls its expression level (Seo et al., 2009). Additionally, this MCPyV miRNA can also suppress host immune response by targeting the SP100 innate immunity gene (Akhbari et al., 2018).

1.5 THE PATHOGENESIS OF C-KIT

c-KIT is a type III RTK, which was firstly described by Yarden and colleagues in 1987 (Yarden et al., 1987). This gene is the cellular homolog of the v-KIT oncogene of Hardy-Zuckerman 4 feline sarcoma virus (Besmer et al., 1986). Later, stem cell factor (SCF) was identified as the ligand of c-KIT (Zsebo et al., 1990). Upon ligand binding, c-KIT undergoes auto-phosphorylation and activates several downstream signaling pathways (Lennartsson and Ronnstrand, 2012). c-KIT is normally expressed in hematopoietic stem cells, germ cells, mast cell, melanoblast and interstitial cells of Cajal (Ikuta and Weissman, 1992; Matsui et al., 1990; Tsai et al., 1991). Its downstream signaling pathways affect cell death, proliferation and cellular development (more details in Section 1.5.1). c-KIT also acts as an oncogene in

different cancer types; the most well-known types are mast cell leukemia and gastrointestinal stromal tumor (GIST) (Lennartsson and Ronnstrand, 2012).

1.5.1 Molecular structure, post-translational modifications and activation of c-KIT

As a typical RTK, the protein structure of c-KIT includes an extracellular domain, a transmembrane domain, a juxtamembrane domain and an intracellular domain (Figure 6). The extracellular domain is consisted of five IgG-like motifs: the beginning three in the N-terminus accounts for SCF ligand binding, while the latter two helps the receptor dimerization. The SCF always performs as a dimer in order to induce c-KIT dimerization and activation. Once the c-KIT homodimeric status is initiated and stabilized by SCF, two tyrosine residues (Tyr568 and 570) in the juxtamembrane domain will be phosphorylated, continued by multiple phosphorylation modifications in the two kinase domains located in the intracellular region (including Tyr at 703, 719, 721, 730, 747 and 900) and the C-terminus (Tyr936) (Lennartsson and Ronnstrand, 2012; Serve et al., 1994).

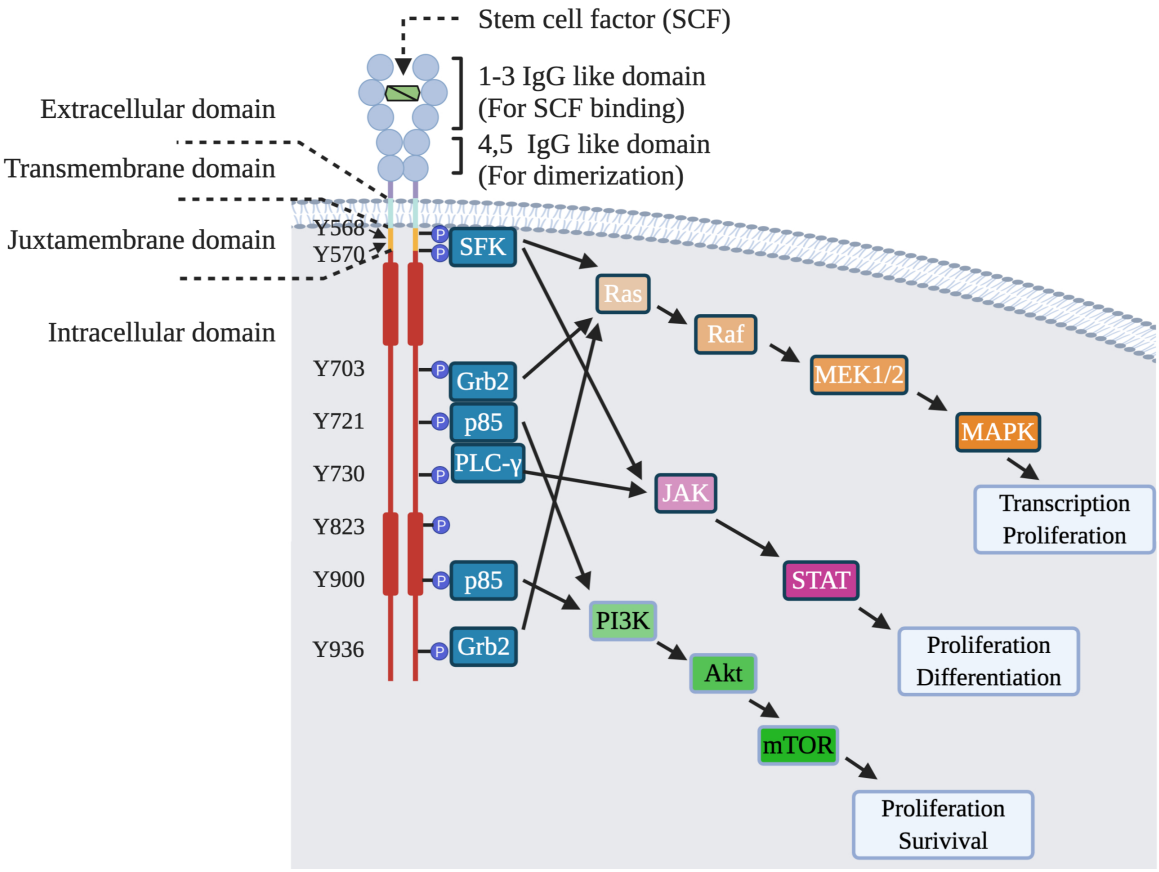


Figure 6. The protein structure and signaling pathways of c-KIT. The c-KIT protein consists of extracellular, transmembrane, juxtamembrane and intracellular regions. Upon binding with stem cell factor (SCF), multiple tyrosine residues (Y) of c-KIT are phosphorylated, which leads to activation of specific downstream targets for different signaling pathways.

The native c-KIT is a protein with a molecular weight of about 100 kDa. After synthesis, multiple glycosylation modifications occur in the ER and Golgi, in which glycosylations are added in the extracellular domain sites to form a high mannose form (120 kDa) and full complex glycosylated form (145 kDa) (Majumder et al., 1988; Qiu et al., 1988; Yarden et al., 1987). The complex glycosylated c-KIT is then transported to the PM for activation by its ligand. Upon binding of the SCF, the c-KIT is phosphorylated at multiple sites of tyrosine and/or serine residues, which can activate several signaling pathways, such as PI3K-AKT-mTOR, RAS-MAPK and JAK-STAT (Figure 6)(Blume-Jensen et al., 1994; Growney et al., 2005). After activation, the c-KIT is ubiquitinated for degradation by the endocytic pathway (Masson et al., 2006; Sun et al., 2007).

1.5.2 c-KIT in cancer

c-KIT activation and overexpression are involved in different cancer types, including GIST, SCLC, acute myeloid leukemia, testicular seminoma, melanoma and MCC (Lennartsson and Ronnstrand, 2012). GIST is one of the most studied tumor types for c-KIT oncogenic functions. About 85% of GISTs harbor *c-KIT* mutations (Corless et al., 2004). These mutations can occur in the transmembrane, juxtamembrane and kinase domains of c-KIT, which lead to ligand-independent activation (Hirota et al., 1998; Nishida et al., 1998). This activates several signaling pathways, including PI3K-AKT, JAK-STAT, RAS-RAF-MAPK and PLC-γ, for promoting tumor growth and apoptosis resistance (Ali and Ali, 2007; Bosbach et al., 2017).

RTK inhibitors can be used to attenuate the activation of downstream signaling of the RTK and inhibit tumor growth. Imatinib mesylate is an example of RTK inhibitor that blocks certain RTKs, including c-KIT, by binding in the ATP-binding site that blocks substrate phosphorylation (Demetri et al., 2002; Tuveson et al., 2001; Wang et al., 2000). The inhibition of c-KIT activation by imatinb can induce c-KIT internalization for degradation (D'Allard et al., 2013).

In normal cells, after ligand binding, c-KIT is rapidly internalized by endocytosis for recycling or lysosomal degradation. However, several studies indicate that mutated c-KIT proteins in GIST and mast cell leukemia are localized in specific organelles for oncogenic activities (Obata et al., 2017; Obata et al., 2014; Tabone-Eglinger et al., 2008). Interestingly, c-KIT can have different oncogenic signaling in different subcellular compartments. In mast cell leukemia, c-KIT proteins are accumulated in both ER and endolysosome. The immature c-KIT located in the ER activates STAT5 to promote proliferation, while the mature phosphorylated c-KIT in the endolysosome interacts with AKT-p85 that favors tumor cell survival (Obata et al., 2014). In GIST, the mutant c-KIT forms a paranuclear dot-like aggregation in the Golgi and activates STAT5, PI3K-AKT and ERK pathways to support tumor development.

In MCC, we also observed frequent c-KIT paranuclear dot staining in MCPyV+ MCC. Unlike GIST and mast cell leukemia, no activating mutation of c-KIT has been found in MCC (Waltari et al., 2011). In **Paper III**, we show that MCPyV LT oncoprotein promotes c-KIT retention in the late endosomes via its Vam6p interaction and c-KIT binds to BECN1 that enhances BCL2 interaction for autophagy suppression.

2 AIMS OF THE THESIS

The general aim of this thesis was to demonstrate how members of the protein degradation system are regulated in two different types of cancer, i.e. testicular germ cell tumor (TGCT) and Merkel cell carcinoma (MCC). The specific aims were to:

- Elucidate the functional role of *miR-223-3p* and its functional target in TGCT (**Paper I**)

- Characterize the functional role of the MCPyV-associated *miR-375*, *miR-30a-3p* and *miR-30a-5p* and their targets involved in MCC development (**Paper II**)

- Characterize the c-KIT expression pattern and determine its possible functional relationship with MCPyV T-antigens in MCC (**Paper III**)

- Elucidate the mechanism of paranuclear CK20 aggregation formation in MCC and whether MCPyV T-antigens are involved (**Paper IV**)

3 MATERIALS AND METHODS

3.1 CLINICAL SAMPLES

3.1.1 Testicular germ cell tumors and normal testes

Fifteen testicular germ cell tumors (TGCTs) and five normal testes (NT) were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute, USA. These samples were used in **Paper I**.

3.1.2 MCC tumor samples

Two cohorts were included in this thesis. The Karolinska cohort consists of 45 formalin-fixed paraffin-embedded (FFPE) MCC tumor samples collected at Karolinska University Hospital or Stockholm South General Hospital. The Helsinki cohort includes tissue microarray (TMA) slides containing 435 FFPE MCC tumors provided by Helsinki University Hospital. The clinical information of the patients has been described in previous studies (Kumar et al., 2020; Sihto et al., 2009, 2011; Xie et al., 2014). The Karolinska cohort was used in **Papers II** and **III**, and the Helsinki cohort was used in **Papers III** and **IV**.

3.2 ESTABLISHED CELL LINES AND MCC PRIMARY CULTURE

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

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

Name	Description	MCPyV status	Source	Paper
TCam-2	TGCT	n.d.	Kindly provided by Dr. Leendert Looijenga (Erasmus MC-University, Netherlands)	**I**
2102Ep	TGCT	n.d.	Kindly provided by Dr. Peter Andrews (University of Sheffield, UK)	**I**
MCC13	MCC	negative	Cell Bank Australia, Westmead, NSW, Australia	**II, III, IV**
MCC14/2	MCC	negative	Cell Bank Australia, Westmead, NSW, Australia	**II, III, IV**
MCC26	MCC	negative	Cell Bank Australia, Westmead, NSW, Australia	**II, III, IV**
WaGa	MCC	positive	Kindly provided by Dr. JC Becker (Medical University of Graz, Austria)	**II, III, IV**
MKL-1	MCC	positive	Kindly provided by Dr. NL Krett (Northwestern University, IL, USA)	**II, III, IV**
MKL-2	MCC	positive	Kindly provided by Dr. Roland Houben (University of Würzburg, Germany)	**II, III, IV**
ME27	MCC	positive	Shi H et al., Unpublished	**II, III, IV**
GIST882	Gastrointestinal stromal tumor	n.d.	Kindly provided by Dr. JA Fletcher (Brigham and Women's Hospital, Boston, MA, USA)	**III**
HEK293	Human Embryonic Kidney	n.d.	Originally purchased from American Type Culture ollection and kindly provided by Dr. Yingbo Lin (Karolinska Institutet, Sweden)	**III, IV**

TGCT = testicular germ cell tumor; MCC = Merkel cell carcinoma; n.d. = not done

The authenticity of the cell lines has been verified by short tandem repeat (STR) genotyping, as shown in Table 4.

Table 4. Short tandem repeat profiles of the established human cell lines involved in this thesis

Locus	TCam2	2102Ep	WaGa	MKL-1	MKL-2	MCC13	MCC14/2	MCC26	GIST882
D8S1179	14, 17	14, 15	10, 13	10, 10	11, 13	13, 14	10, 14	8, 10	12, 12
D21S11	30, 30	28, 29	28, 30	30, 30	31.2, 31.2	30, 31	29, 32.2	31, 32.2	29, 31.2
D7S820	8, 11	7, 7	10, 10	8, 11	10, 13	10, 10	8, 10	8, 9	10, 15
CSF1PO	9, 11	11, 12	12, 12	11, 12	10, 12	12, 12	10, 11	10, 11	11, 11
D3S1358	16, 17	16, 16	14, 14	16, 16	17, 17	16, 18	16, 16	17, 17	15, 15
THO1	6, 9	9, 9.3	9.3, 9.3	9, 9.3	7, 8	7, 9.3	6, 9.3	9.3, 9.3	9, 9
D13S317	8, 12	8, 8	8, 13	8, 11	12, 13	12, 12	13, 13	13, 14	12, 12
D16S539	9, 9	11, 12	11, 12	10, 12	10, 12	9, 11	13, 13	11, 13	11, 11
D2S1338	23, 23	17, 20	20, 23	17, 17	17, 23	19, 20	19, 19	23, 26	18, 26
D19S433	15, 15	13, 14	14, 16	14.2, 15.2	13.2, 14	15, 15	12, 14	16, 16	13, 15
vWA	18, 19	16, 17, 18	16, 17	16, 18	16, 17	17, 17	17, 18	16, 18	16, 17
TPOX	9, 9	8, 11	8, 11	8, 8	8, 11	8, 8	8, 8	8, 8	11, 11
D18S51	13, 13	15, 15	10, 13	12, 18	14, 17	16, 17	15, 17	15, 18	14, 14
AMEL	X, Y	X, Y	X, Y	X, Y	X, Y	X, X	X, X	X, X	X, Y
D5S818	11, 12	11, 11	12, 12	11, 12	12, 14	9, 12	13, 13	12, 12	12, 12
FGA	25, 25	22, 22	19, 19	21, 25	19, 26	19, 20	21, 21	24, 25	23, 23

3.3 DATA FROM PREVIOUS PUBLISHED STUDY AND PUBLIC DATABASES

We extracted *miR-223-3p* and *miR-16* expressions from the global TaqMan miRNA profiling data published in (Gillis et al., 2007), consisting of 49 TGCTs and three NTs. The *miR-223-3p* levels were normalized to *miR-16*.

For comparison of *FBXW7* mRNA levels between TGCTs and NTs, we extracted the microarray gene expression data deposited at the National Center for Biotechnology Information Gene Expression Omnibus (GEO accession no. GSE3218), which consists of 101 TGCTs and five NTs.

For correlation analysis between *FBXW7* mRNA and *miR-223-3p*, their expression data were extracted from The Cancer Genome Atlas. The details are described in **Paper I**.

3.4 RNA EXTRACTION AND REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

Total RNAs for both cell cultures and tissue samples were prepared using *mir*VANA™ miRNA Isolation Kit (Ambion, Thermo Fisher Scientific, CA, USA). The procedure includes the classical acid phenol and chloroform extraction, followed by solid phase column RNA immobilization, purification and elution. RNA purity and concentration was measured by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, DE, USA).

RT-qPCR assay is based on PCR reaction, which is commonly used to quantify RNA expression levels by measuring the amount of fluorescence signals released during PCR amplifications in real-time. The first step needs to convert the RNA into single-stranded complementary DNA (cDNA). The qPCR part contains two widely used methods: SYBR-green and TaqMan assays. SYBR-green assay uses the SYBR green dye, which only binds to double-stranded DNA formed during PCR step and this dye only give fluorescence signals

while binding. This character offers the possibility to detect the amplified product at each round of PCR. SYBR green assay was used to evaluate mRNA expression level of MCPyV T-antigens in **Paper II**.

The miRNA reverse transcription primers contain a special target-specific stem-loop design, which provides highly specific binding only towards the chosen mature miRNA sequence for cDNA synthesis. Besides, the TaqMan probe contains a fluorescence reporter dye at the 5' end and a quencher at the 3' end. When the probe is cleaved during PCR extension step, it releases the fluorescence signals. TaqMan assay can also be used for mRNA analysis, and it provides better specificity than the SYBR green assay by designing a probe spanning the exon-exon junction of the target mRNA. TaqMan assays were used to detect *miR-223-3p* in **Paper I**, and *miR-375*, *miR-30a-3p*, *miR-30a-5p*, *miR-125a-3p*, *miR-16*, *pri-mir-375*, *pri-mir-30a*, *18S rRNA* and *RUB6B* in **Paper II**.

3.5 PLASMIDS, MIRNA INHIBITORS, MIRNA MIMICS, SHRNAS AND SIRNAS

3.5.1 Plasmids and expression vectors

Plasmids are small circular extra-chromosomal DNA molecules, which can independently replicate in bacterial host cells. They have been widely used as an expression vector to introduce gene(s) of interest *in vitro*. Four types of plasmid expression vectors have been used in this thesis: protein expression, short-hairpin RNA (shRNA), miRNA sponge, miRNA expression and luciferase reporters.

Protein expression plasmid provides the DNA template for transcription and translation by the host machineries that lead to expression the transgene of interest. This type of plasmid has been applied to express FBXW7 in **Paper I**. MCPyV sT, full-length and truncated LT (sTco, LTco and LT339, respectively), D44N point mutant of LT (LT_{D44N}), mRFP-GFP-LC3 reporter in **Paper II**; wild-type and W209A point-mutant of truncated LT (LT339 and $LT339_{W209A,}$ respectively), wild-type c-KIT, FLAG-tagged full-length and deletion mutants of BECN1 in **Paper III**; wild-type and truncated MCPyV T-antigens and mCherry-tagged Dynamitin in **Paper IV**.

miRNA sponge is a vector that can express RNAs containing multiple miRNA binding sites, which sequesters the targeting miRNA from its cellular targets and suppresses the miRNA function. The design of miRNA sponge vector for targeting *miR-375*, *miR-30a-3p* and *miR-30a-5p* are described in **Paper II**.

Luciferase reporter is widely used to monitor gene expression based on the bioluminescence of the luciferase activity generated from the luciferase gene that is coupled to the gene of interest. We applied the pmirGLO luciferase reporter, which contains miRNA targeting sequence from different autophagy genes or the reverse complementary sequence of

mature miRNA at the 3' end of the luciferase gene for validation of miRNA targeting sites and the efficiency of miRNA sponge in **Paper II**.

Short hairpin RNA (shRNA) is used to express single-stranded RNAs with a loop structure. Once the shRNA has been transcribed in the host cell, it is processed by Dicer in cytosol to produce small interfering RNA (siRNA) duplexes. One strand of the siRNAs is then loaded into RISC for silencing the target gene. Several shRNAs were generated and applied in this thesis work, including: shTA, which targets the common exon 1 of both LT and sT; shsTA, which targets the unique sequence of sT in exon 2 (**Papers II** and **III**); shKIT, which targets the c-KIT (**Paper III**); shBAG, which targets the BAG3 (**Paper IV**). All plasmids used in this thesis are listed in Table 5.

Table 5. All plasmids used in the thesis

Plasmid	Source	Identifier*	Paper
pcCMV-Myc-FBXW7	Rajagopalan et al., 2004	RRID_Addgene_16652	**I**
pcDNA3-U6M2-miR-30a	This thesis	N/A	**II**
pcDNA3-U6M2-miR-30a-3p	This thesis	N/A	**II**
pcDNA3-U6M2-miR-30a-5p	This thesis	N/A	**II**
pcDNA3-miR-375sp	This thesis	N/A	**II**
pcDNA3-miR-30a-3psp	This thesis	N/A	**II**
pcDNA3-miR-30a-5psp	This thesis	N/A	**II**
pmirGLO-ATG7wt	This thesis	N/A	**II**
pmirGLO-ATG7mut	This thesis	N/A	**II**
pmirGLO-BECN1wt	This thesis	N/A	**II**
pmirGLO-BECN1mut1	This thesis	N/A	**II**
pmirGLO-BECN1mut2	This thesis	N/A	**II**
pmirGLO-SQSTM1wt	This thesis	N/A	**II**
pmirGLO-SQSTM1mut	This thesis	N/A	**II**
mRFP-EGFP-LC3 (or ptfLC3)	Kimura et al., 2007	RRID_Addgene_21074	**II**
pcDNA6.MCV.LTco.D44N	Kwun et al., 2009	N/A	**II**
pcDNA6.MCV.LTco	Shuda et al., 2011	RRID:Addgene_40200	**II-IV**
pcDNA6.MCV.LT339	Shuda et al., 2008	RRID:Addgene_28193	**II-IV**
pcDNA6.MCV.sTco	Shuda et al., 2011	RRID:Addgene_40201	**II-IV**
pcDNA3-U6M2-shTA	Xie et al., 2014	N/A	**II-IV**
pcDNA3-U6M2-shsTA	This thesis	N/A	**II-IV**
pcDNA3-c-KIT-WT	Agarwal et al., 2013	N/A	**III**
pcDNA4.Beclin1 (FL).3xFLAG	Sun et al., 2008	RRID:Addgene_24388	**III**
pcDNA4.Beclin1 1-150.3xFLAG	Sun et al., 2008	RRID:Addgene_24389	**III**
pcDNA4.Beclin1 151-241.3xFLAG	Sun et al., 2008	RRID:Addgene_24390	**III**
pcDNA4.Beclin1 243-450.3xFLAG	Sun et al., 2008	RRID:Addgene_24392	**III**
pcDNA4.Beclin1 Δ151-241.3xFLAG	Sun et al., 2008	RRID:Addgene_24393	**III**
pcDNA3-U6M2-shKIT	This thesis	N/A	**III**
pcDNA6.MCV.LT339.W209A	This thesis	N/A	**III, IV**
pcDNA3-U6M2-shBAG3	This thesis	N/A	**IV**
mCherry-Dynamitin	Shrum et al., 2009	N/A	**IV**
mCherry-C2	N/A	RRID:Addgene_54563	**IV**

*Research Resource Identifiers (RRID) at https://scicrunch.org/resources
N/A, not available

3.5.2 miRNA inhibitors, miRNA mimics and siRNAs

miRNA inhibitors are chemically modified single-stranded RNAs that bind and attenuate the function of specific endogenous miRNAs. miRNA mimics are chemically modified double-stranded RNAs that produce mature miRNA of interest, which can be loaded into RISC complex and execute similar function as the corresponding endogenous miRNA. The negative controls are RNA molecules with similar chemical modification as the miRNA inhibitors or mimics but with non-targeting random sequence. In **Paper I**, we applied miRNA mimic (Pre-miR-223), miRNA inhibitor (Anti-miR-223) and their respective controls (Pre-miR-NC and Anti-miR-NC) to evaluate the effect of *miR-223-3p* regulation on FBXW7 expression and functions.

siRNAs are chemically modified synthetic small RNA oligonucleotides mimicking the endogenous siRNA duplexes that can be loaded directly to the RISC for silencing target genes of interest. Two commercial siRNAs targeting different sites of HDAC6 were used in **Paper IV**.

3.6 TRANSFECTION

Transfection is a molecular technique that introduces the exogenous nucleic acid into the eukaryotic cells for regulation of gene expression *in vitro*. Two types of transfection methods have been used in this thesis, which are briefly described below:

3.6.1 Lipid-based transfection

This method is based on the lipid-based particles that can pack the nucleic acid in the complex, which fuse with the cellular membrane and deliver the nucleic acid into the cells. Examples of lipid-based transfection reagents are: siPORT-NeoFX and Lipofectamine 2000 in **Paper I**, Lipofectamine RNAiMAX in **Paper II**, and Lipofectamine LTX in **Papers II-IV**.

3.6.2 Electroporation

Electroporation is a method that directly uses high voltage pulse to create transient opening pores in both plasma and nuclear membranes, allowing the nucleic acid to enter targeted cells. This method is specifically useful for transfection of large DNA molecules or cells with low transfection efficiency such as primary cell cultures, non-dividing cells and stem cells. We used the Ingenio electroporation system (Mirus Bio, Madison, WI, USA) and the Nucleofector device (Lonza, Basel, Switzerland) in **Papers II-IV**.

3.7 WESTERN BLOT

Western blot is a well-established technique to detect and quantify protein of interest. The first step is to prepare protein lysate from cells or tissues using lysis buffer or homogenization extraction. Subsequently, the sample lysates will be loaded into a SDS polyacrylamide gel for protein separation by electrophoresis. Proteins will be separated by velocity according to their molecular weights and charges. The separated proteins are then transferred to a membrane (nitrocellulose or polyvinylidene difluoride), where proteins can be detected using specific antibodies. Western blot had been used to detect proteins of interest in all four papers in this thesis.

3.8 IMMUNOSTAINING

Immunostaining is a method to detect the expression and subcellular localization of specific protein in a cell or tissue. This method first requires the antigen (i.e. proteins of interest) exposure treatment from the biomaterial barriers, such as cellular membrane and adjacent tissues, followed by binding with specific primary and secondary antibodies sequentially. The secondary antibody can be conjugated with an enzyme (*e.g.* alkaline phosphatase and horseradish peroxidase) or a fluorophore. For the secondary antibody conjugated with an enzyme, a chromogen substrate is needed to generate color precipitate for visualization by the enzymatic activities, while the fluorescence signals can be visualized directly using the fluorescence microscopy. The immunostaining assays used in this thesis includes immunohistochemistry (IHC) for tissue samples and immunofluorescence (IF) for cell lines. IHC with chromogen substrate was used for ATG7 and p62 detection in **Paper II**, while IHC with double flourescence was used for detection of c-KIT and LT in **Paper III**, and CK20 and LT in **Paper IV**. IF with chromogen substrate was used to detect CK20 or MNF116 in MCC cell lines in **Paper IV**, while the fluorescence detection was used to detect the co-immunostaining of MCPyV LT and autophagy reporter mRFP-GFP-LC3 in **Paper II**, c-KIT, MCPyV T-antigens, GOLGB1, 58K, EEA1, CD63, LAMP1, BECN1, MNF116 and Rab11a in **Paper III**, CK20, MCPyV T-antigens, MNF116, BAG3, HDAC6, Ub(48K), γ-tubulin, and Vimentin in **Paper IV**. Cell nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) due to its strong binding to adenine-thymine region in DNA, which could be visualized by fluorescence microscopy (**Papers II-IV**).

3.9 CO-LOCALIZATION AND CO-IMMUNOPRECIPITATION ASSAYS

Protein-protein interactions can be detected by co-localization analyses of two different fluorescently labeled proteins and co-immunoprecipitations. Additionally, immunoprecipitation can also be used to pull down vesicles using organelle-specific antibody. A brief description of these methods are given below:

3.9.1 Immunofluorescence based co-localization assay

For co-localization analysis, the two proteins are detected by different host-specific primary antibodies and fluorescent-tagged secondary antibodies using IF. Pearson's correlation coefficient will be calculated for the co-localization pixels between the two fluorescence channels, which provides a quantitative measurement of their co-localization (Costes et al., 2004). During the image acquisition, the optimal optical setup for different fluorescence signals needs to be considered in order to get a constant point spread function (PSF) for correction of pixel intensities. PSF is calculated by laser wavelength lambda / (2 * N.A.), N.A. = numerical aperture value of the lens used for detection. In **Paper III**, Pearson's correlation coefficient was calculated using the Coloc 2 plugin of ImageJ software (version 1.52p, USA), which was used to evaluate the localization of c-KIT in different cellular organelles.

3.9.2 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) is a widely used approach to capture the target protein and its associated proteins in a protein lysate, based on antibody recognition and immobilization. Generally, specific antibody is used to capture the protein of interest and other proteins that bound to the target protein in protein lysates, in which the antibody-antigen complex is then immobilized on beads, and eventually pulled out from the total lysate. The isolated protein complexes are analyzed by protein analysis methods, e.g. western blot. Co-IP assay was used to analyze the interaction between c-KIT and BECN1 as well as BECN1-associated complexes in **Paper III**, and between LT and Vam6p interaction in **Paper IV**.

3.9.3 Vesicle immunoprecipitation

Based on the same principle as co-IP, cellular vesicles can be pulled down for analysis. The difference is the cell lysis procedures, which need to break down the plasma membrane but keep the intact of vesicle compartment. This is normally achieved by using hypotonic buffer that swells and breaks the cell membrane, but not the cellular organelles. The vesicles can be enriched by immunoprecipitation using organelle-specific antibody and the content of the vesicles can be detected by western blotting. Vesicle IP has been used to isolate the late endosome and lysosome/endolysosome vesicles using anti-CD63 and anti-LAMP1, respectively, in **Paper III**.

3.10 AUTOPHAGY ASSAYS

Multiple autophagy assays are recommended to evaluate the effect on autophagy, due to its highly dynamic process. The following methods were applied in this thesis:

3.10.1 Transmission electron microscopy

Transmission electron microscopy (TEM) is a direct method to evaluate the morphology of autophagosomes (organelle with double membrane, which is separated by a bright lumen) and autolysosomes (single membrane structure containing partially degraded or electron dense material due to acidic environment). TEM was applied for evaluating the effect of MCPyV T-antigens and miRNAs on autophagosomes and autolysosomes (**Paper II)**. Although this method is useful, it has limitation for quantification.

3.10.2 Western blot detection of LC3-II

LC3 (also known as ATG8) is a protein that has both non-lipidated (LC3-I) and lipidated (LC3-II) forms. The LC3-I form is found in the cytoplasm, while the membrane-bound LC3-II is converted from LC3-I and that is involved in membrane closure of autophagosome. After fusion with lysosome, LC3-II is degraded in autolysosomes. Due to this dynamic process, it is necessary to quantify the amount of LC3-II in the presence or absence of lysosome inhibitor treatment (e.g. Bafilomycin A1) in order to distinguish autophagy flux or autophagy formation. This method was used in **Papers II** and **III** to evaluate the effect of MCPyV T-antigenes, miRNA and c-KIT silencing on autophagy.

3.10.3 mRFP-GFP-LC3 autophagy tandem reporter assay

The mRFP-GFP-LC3 reporter is a very useful assay to quantify the number of autophagosomes and autolysosomes based on the fluorescence puncta. The principle of this assay is based on the acidic environment in autolysosomes that quenches GFP signal, but not mRFP. Therefore the red (mRFP only) puncta represent the autolysosome, while the autophagosome is represented by yellow (mRFP+GFP) puncta. This reporter was used in **Paper II**.

3.11 FUNCTIONAL ASSAYS

3.11.1 Cell viability/proliferation assays

Three different cell growth assays have been applied in this thesis: trypan blue exclusion, WST-1 and 5-ethynyl-2'-deoxyuridine (EdU) assays.

Trypan blue is a dye that can quickly check the percentage of live cells from the sample using bright field microscopy or automatic cell counting. The principle is that membrane-intact alive cells will not be stained with the dye, but the dead cell will be stained due to loss of membrane integrity. This assay was used in **Papers I** and **III**.

WST-1 assay is based on the cleavage of tetrazolium salt to color formazan by the dehydrogenase enzyme from mitochondria of live cells. The color formazan can be measured at 440 nm, and the intensity is directly proportional to mitochondria activity from live cells, therefore it corresponds to cell viability or growth. WST-1 assay was used to assess cell growth in **Papers I** and **II**.

Cell proliferation requires the replication of genomic DNA, therefore quantification of newly synthesized DNA is a direct method for measuring cell proliferation. EdU is a thymidine analog, which is incorporated into DNA during DNA synthesis. The EdU contains the alkyne and azide-Alexa Fluor dye, which can be used to catalyze a chemical reaction to incorporate a fluorescence dye. The fluorescently labeled live cells can be assessed by flow cytometry. This assay was used to evaluate the effect of *miR-223-3p* on cell proliferation in TCGT cells (**Paper I**).

3.11.2 Cell apoptosis assays

Apoptosis is a term describing programmed cell death. It is considered as vital process for homeostasis maintenance, embryonic development and normal function of immune system. Abnormal apoptosis is often linked to pathogenic conditions, such as carcinogenesis. Several experimental methods can be used to evaluate apoptosis, which are normally divided into two groups: morphology of apoptotic cells and apoptotic marker.

Morphology is based on the membrane integrity of apoptotic cells. Annexin V can bind to phosphatidylserine (PS). In healthy cells, PS stays in the inner membrane leaflet to maintain the cell membrane structure. When apoptosis is initiated, the cellular membrane is still intact, therefore the permeable dye (such as trypan blue and propidium iodide) cannot enter into the cells. However the membrane asymmetry is damaged and PS is translocated to the external side, where it is exposed for Annexin V binding. Thus, Annexin V, combined with propidium iodide, can detect early and late apoptosis, which was used in **Papers I** and **II**.

The poly (ADP-ribose) polymerase-1 (PARP-1) is a cleavable substrate by the proteolytic function of cellular caspases, including caspase 3 and caspase 7. Besides, PARP plays important role in DNA repair to preserve cell viability. During apoptosis, the 116 kDa PARP is cleaved at Asp214-Gly215 into two subunits (89 kDa and 24 kDa) by caspase 3 that prevents DNA repair. The amount of cleaved PARP can be utilized as an apoptosis marker. This assay was used in **Papers I** and **III**.

3.12 STATISTICAL ANALYSIS METHODS

All statistical methods in this thesis were performed by MS Office Excel 2007, SPSS 22.0 or GraphPad Prism 8. Pearson's correlation analysis was used to detect the relationship between

two continuous factors (*FBXW7* mRNA v.s. *miR-223-3p*) in **Paper I**. Mann-Whitney *U*-test was used to compare *miR-223-3p* or *FBXW7* mRNA in TGCT v.s. NT (**Paper I**), c-KIT staining dot pattern between MCPyV+ and MCPyV- MCC groups (**Paper III**), CK20 staining dot pattern between MCPyV+ and MCPyV- MCC groups (**Paper IV**). Student paired *t*-test was used to compare two experimental conditions in **Papers I-IV**. One-way ANOVA with *post hoc* Tukey test was used to compare multiple experimental conditions (**Paper II**). One-way ANOVA with *post hoc* Dunnett test was used to compare multiple experiment conditions v.s. a control (**Papers III** and **IV**). Two-way ANOVA with *post hoc* Dunnett test was used to detect interactions with two affecting factors in **Paper III**. χ^2 or Fisher's exact test was used to evaluate association of ATG7 or p62 protein levels with virus status in MCC tumors (**Paper II**), c-KIT dot/membrane staining pattern between MCPyV+ and MCPyV- MCC samples (**Paper III**) and CK20 dot/diffuse-cytoplasmic staining pattern between MCPyV+ and MCPyV- MCC groups (**Paper IV**). All analyses were characterized as two-tailed and *P*-value <0.05 was regarded as significant.

4 RESULTS AND DISCUSSIONS

4.1 *MIR-223-3P* REGULATES CELL GROWTH AND APOPTOSIS VIA FBXW7 SUGGESTING AN ONCOGENIC ROLE IN HUMAN TESTICULAR GERM CELL TUMORS (PAPER I)

As previously published by our group, a subset of miRNAs with dysregulated expression level has been identified in testicular germ cell tumors (TGCTs), by small RNA sequencing analysis (Ozata et al., 2017). *miR-223-3p* is one of the up-regulated miRNAs in TGCTs, but its functional role in TGCT development was still unknown. Therefore, this study was conducted to explore the functional role of *miR-223-3p* in TGCTs.

4.1.1 *miR-223-3p* regulates FBXW7 protein expression in TGCT

To further confirm the upregulation of *miR-223-3p* in TGCTs, we re-analyzed the miRNA profiling data from the study of Gillis *et al*., which showed increased expression of *miR-223-3p* in TGCTs (Gillis et al., 2007).

FBXW7 has been verified as a direct target of *miR-223-3p* in different cancer types (Kurashige et al., 2012b; Li, J. et al., 2012; Mavrakis et al., 2011), we therefore evaluated FBXW7 protein expression in our cohort and *FBXW7* mRNA in microarray gene expression data from a public database (GEO GSE3218). We found decreased expression of FBXW7 in TGCTs than normal testes (NTs) in both cohorts. Using the TCGA data, we found negative correlation between *FBXW7* mRNA and *miR-223-3p* in a series of 101 TGCTs and five NTs.

To further evaluate whether FBXW7 could be a target of *miR-223-3p* in TGCT, we suppressed (using anti-miR-223) or overexpressed (using pre-miR-223) in TGCT cell lines, which resulted in increased or decreased FBXW7 protein levels, respectively, suggesting that *miR-223-3p* negatively regulates FBXW7 in TGCT.

4.1.2 miR-*223-3p* regulates cell apoptosis and cell growth in TGCT cell lines

To address the biological consequence of *miR-223-3p* in TGCT, we modulated *miR-223-3p* expression and evaluated the effect on apoptosis and cell growth. We showed that suppression of *miR-223-3p* increased apoptosis, as evaluated by Annexin V assay and detection of cleaved PARP by immunoblotting. On the other hand, overexpression of *miR-223-3p* had an opposite effect.

For cell growth analysis, we applied both EdU and WST-1 assays. Inhibition of *miR-223-3p* led to decreased cell growth, while its overexpression promoted cell growth *in vitro*. These results suggest an oncogenic role for *miR-223-3p* in TGCT.

4.1.3 The *miR-223-3p* mediated apoptosis and cell growth could be restored by ectopic expression of FBXW7

To further assess whether FBXW7 contributes to the *miR-223-3p* mediated functional effects, we co-transfected pre-miR-223 or pre-miRNA negative control together with full-length coding sequence of *FBXW7* or empty vector control into TCam-2 cell line. The *miR-223-3p* mediated apoptosis suppression and cell growth promotion were reversed by the ectopic expression of FBXW7, suggesting that *miR-223-3p* controls cell growth and apoptosis through FBXW7.

Deregulation of *miR-223-3p* has been reported in various tumor types. It is known that *miR-223-3p* can function as an oncogene or a tumor suppressor gene in different cellular context (Fazi et al., 2007; Li et al., 2011; Petrocca et al., 2008b; Xu et al., 2013). Here, we show that *miR-223-3p* has an oncogenic role in TGCT. Given that most TGCTs are responsive to cisplatin treatment and *miR-223-3p* has been demonstrated to modulate cisplatin sensitivity in different cancer types, it is worthy to examine the relationship between *miR-223-3p* expression levels and cisplatin response in TGCT.

In addition, FBXW7 is an E3 ligase, which is known to function as a tumor suppressor by controlling the degradation of multiple oncoproteins involved in cell growth, apoptosis and cell cycle, such as c-Myc and Cyclin E (Welcker and Clurman, 2008). Thus, the functional phenotypes observed in this study are likely due to accumulation of FBXW7 substrates. Further investigations are still warranted to elucidate the regulatory network of *miR-223*-FBXW7.

In conclusion, we demonstrate dysregulation of *miR-223-3p* and FBXW7 in TGCT. Our data suggest an oncogenic role for *miR-223-3p*, which promotes cell growth and suppress apoptosis through suppression of FBXW7. This study provides further insights about the role of *miR-223-3p* in TGCT development.

4.2 MERKEL CELL POLYOMAVIRUS ONCOPROTEINS INDUCE MICRORNAS THAT SUPPRESS MULTIPLE AUTOPHAGY GENES (PAPER II)

Our group previously identified a subgroup of miRNAs associated with MCPyV status in MCC (Xie et al., 2014). Among the verified deregulated miRNAs, *miR-30a-3p*, *miR-30a-5p* and *miR-375* are overexpressed in MCPyV+ compared to virus-negative MCC tumors. Previous studies have reported that *miR-375* and *miR-30a-5p* target ATG7 and BECN1, respectively (Chang et al., 2012; Zhu et al., 2009). Therefore, we sought to investigate the relationship of these miRNAs with MCPyV and to determine whether these miRNAs could regulate autophagy in MCC.

4.2.1 MCPyV T-antigens regulates miRNAs through the viral DnaJ domain

We first determined whether the MCPyV T-antigens regulate the miRNA levels. We designed two short hairpin shRNAs, one targeting the common exon 1 of viral T-antigens (shTA) that silences LT and sT, and the other targeting the unique sequence of sT (shsTA) that silences sT only. Indeed, we observed reduced expression of *miR-375*, *miR-30a-3p* and *miR-30a-5p* upon silencing of LT and sT or sT only. Concordantly, overexpression of different T-antigens (sT, full-length LT and truncated LT) induced expressions of all three MCPyV-associated miRNAs.

Upon inhibition of transcription using actinomycin D, we did not observe any effect on RNA stability of the primary transcripts for both *miR-375* and *miR-30a*, but the mature miRNAs *miR-375*, *miR-30a-3p* and *miR-30a-5p* were decreased in cells with silencing of LT and sT or sT only. These results suggest that MCPyV T-antigens regulate miRNAs at post-transcriptional level. The DnaJ domain is the only common domain between LT and sT, and it has been proven to interact with HSC70 (Kwun et al., 2009). Given that HSC70 is linked to miRNA processing complex RISC (Iwasaki et al., 2010), we speculated that this domain could contribute to miRNA regulation. Indeed, mutation in the DnaJ domain, LT_{D44N} abolished the induction of *miR-375*, *miR-30a-3p* and *miR-30a-5p* by LT. These findings indicate that the DnaJ domain of MCPyV T-antigens is required for miRNA regulation.

4.2.2 MCPyV T-antigens suppress autophagy in MCC cell lines

We next investigated whether MCPyV T-antigens modulate autophagy in MCC cell lines. Three different autophagy assays were applied to evaluate the autophagy levels, i.e. immunoblotting detection of LC3-II, mRFP-GFP-LC3 reporter and transmission electron microscopy. Silencing of LT and sT or sT only significantly upregulated LC3-II level, as well as increased number of autophagosomes and autolysosomes evaluated by TEM and mRFP-GFP-LC3 reporter. Concordantly, ectopic expression of truncated LT and/or sT suppressed autophagy.

To further evaluate the association of MCPyV and autophagy suppression in clinical samples, we assessed the expression of two key autophagy factors ATG7 and SQSTM1 (also known as p62) using immunohistochemistry in our MCC cohort. The staining for both ATG7 and p62 were significantly lower in MCPyV+ compared to MCPyV- MCC tumors. These results provide evidence of autophagy suppression in MCPyV+ MCC.

4.2.3 MCPyV-regulated miRNAs suppress autophagy by targeting multiple autophagy genes

Using both gain- and loss-of-function experiments, we demonstrated that *miR-375*, *miR-30a-3p* and *miR-30a-5p* regulate autophagy, as evaluated by the three autophagy assays mentioned above. We also confirmed *miR-375* and *miR-30a-5p* directly suppresses *ATG7* and *BECN1*, respectively, in MCC. In addition, we also identified *SQSTM1* and *BECN1* are novel targets of *miR-375* and *miR-30a-3p,* respectively, in MCC. Their direct interactions were validated by luciferase reporter assays. Together, these results suggest that MCPyV oncoproteins suppress autophagy through induction of *miR-375*, *miR-30a-3p* and *miR-30a-5p* that target multiple autophagy genes.

4.2.4 Autophagy inhibition could rescue Torin-1 induced cytotoxicity in MCC cell lines

Since autophagy can regulate cell death (White, 2015), we hypothesized that suppression of autophagy by MCPyV T-antigens could favor MCC viability. We therefore used mTOR inhibitor Torin-1 to trigger autophagy in MCPyV+ MCC cells, together with and without autophagy inhibitor baf. A1 or pan-caspase inhibitor z-VAD-FMK, to assess the effect on cell viability. Using both WST-1 and Annexin V assays, we showed that Torin-1 induced cell death and reduced cell viability that could be rescued by the autophagy inhibitor baf. A1, but not the pan-caspase inhibitor.

Here, we uncovered a novel role of MCPyV T-antigens in autophagy suppression. As described in Section 1.3.3, some tumor viruses can modulate autophagy for transformation and tumorigenesis (Ding et al., 2014; Lin et al., 2019; Wileman, 2007). Even though the functional roles of autophagy in MCPyV infection and tumorigenesis remain unknown, it is tempting to postulate that autophagy evasion may help MCPyV to escape host immunity during viral infection and may protect oncoproteins that are degraded by autophagic mechanism. Indeed, we show that autophagy can degrade LT (**Paper III**).

Besides, autophagy has dual roles in different cancers. Generally, autophagy is regarded as pro-survival in tumors, in which the increased autophagic flux is required for tumor cell survival and growth under stressed conditions (Amaravadi et al., 2016; White, 2012). On the

other hand, our data suggest anti-survival role for autophagy in MCPyV+ MCC. In line with our study, autophagy suppression has also been reported in other viral related cancers (Ding et al., 2014; Lin et al., 2019). These data suggest that autophagy suppression might be essential for viral-related cancer. There are two published studies to suggest a link of autophagy in MCC development. Shuda *et al*. demonstrated that sT could activate mTOR and its downstream targets 4E-BP1 and S6K (Shuda et al., 2011). mTOR activation can lead to autophagy inhibition (Kim and Guan, 2015). Notably, mTOR activation and suppressed autophagy were also observed in MCC tumor samples (Lin et al., 2014). Here, we provided direct evidence that both sT and truncated LT can suppress autophagy. Interestingly, the full-length LT did not show consistent suppression effect. This could be explained by the carboxyl-terminus of LT containing the helicase domain, which can inhibit cell growth and induce DNA damage response (Cheng et al., 2013).

In summary, we reveal a network for MCPyV T-antigens mediated suppression of autophagy through miRNA activities. The viral oncoproteins induce *miR-375*, *miR-30a-3p* and *miR-30a-5p* to inhibit the expression of multiple autophagy factors, including SQSTM1, BECN1 and ATG7. Autophagy suppression protects MCC cells from Torin-1 cytotoxicity, suggesting that induction of autophagy could be a therapeutic strategy for MCC.

4.3 MERKEL CELL POLYOMAVIRUS ONCOPROTEIN INDUCES PARANUCLEAR RETENTION OF C-KIT SUPPRESSING AUTOPHAGY THROUGH INTERACTION WITH BECLIN-1 (PAPER III)

c-KIT (also known as CD117) is a type III receptor tyrosine kinase, which has been validated as an oncogene in different cancer types (Lennartsson and Ronnstrand, 2012). In MCC, overexpression and activation of c-KIT has been reported (Husein-ElAhmed et al., 2016; Krasagakis et al., 2009; Swick et al., 2013; Waltari et al., 2011). Yet, c-KIT activating mutations are rare in MCC (Rodig et al., 2012; Swick et al., 2013; Waltari et al., 2011), and the association of c-KIT expression level with virus status in MCC is unknown (Brunner et al., 2008; Kuwamoto, 2011; Waltari et al., 2011). Here, we describe a molecular mechanism how MCPyV oncoprotein blocks lysosomal degradation of c-KIT, leading to its stabilization and interaction with BECN1 for autophagy regulation.

4.3.1 The paranuclear c-KIT dot is associated with MCPyV+ MCC and is localized in late endosomes

We first characterized c-KIT expression pattern in MCC using IHC. Unexpectedly, we observed that c-KIT has a special paranuclear dot-like pattern in MCPyV+ MCC cell lines and tumor samples, while MCPyV- MCC cell lines had low or no expression of c-KIT and membranous or cytoplasmic staining pattern of c-KIT were more frequently found in MCPyV- MCC tumors.

The paranuclear c-KIT dot has also been observed in GIST and mast cell leukemia and their different subcellular localizations have been shown to execute different oncogenic signaling (Obata et al., 2017; Obata et al., 2014). These observations led us to investigate the subcellular localization of c-KIT. We applied co-staining of c-KIT together with different cellular organelle markers, including Golgi, early endosome, late endosome and endolysosome. Co-localization analysis using the Pearson's correlation coefficients showed that c-KIT had strongest co-localization with both late endosome marker CD63 and endolysosome marker LAMP1. To distinguish these two compartments, we performed vesicle-immunoprecipitation assays with anti-CD63 or anti-LAMP1 in MCPyV+ cells, which revealed that c-KIT was mainly localized in late endosomes. Our data indicate that the paranuclear retention of c-KIT is associated with MCPyV+ MCC, and c-KIT is aggregated in late endosomes.

4.3.2 c-KIT is fully glycosylated and phosphorylated, and it traffics from plasma membrane to late endosome via clathrin-dependent endocytosis

In normal cells, c-KIT undergoes a series of modification in ER and Golgi to become fully glycosylated form, which is then phosphorylated upon binding to its ligand on the plasma membrane (Majumder et al., 1988). Using biochemical approach, we found that c-KIT in the

MCPyV+ MCC cell lines was digested by PNGase F (which digests complex glycosylation form), but not with endo H (which digests the high-mannose N-glycosylation form), indicating that the c-KIT is fully glycosylated. To evaluate c-KIT phosphorylation status, we chose to analyze phosphorylation at tyrosine 719 residue, which accounts for the c-KIT kinase-dependent PI3K-AKT signaling (Hashimoto et al., 2003). We detected pTyr-719 of c-KIT in all three MCPyV+ MCC cell lines, indicating that c-KIT is activated in MCPyV+ MCC.

We next investigated how c-KIT was transported from plasma membrane to late endosomes. Generally, mammalian cells used either clathrin-mediated endocytosis (CME) or lipid raft-dependent non-clathrin endocytosis (NCE) to transport RTK for lysosomal degradation or recycling (Barbieri et al., 2016). We found that CME inhibition using Pitstop2 and hypertonic sucrose, but not with NCE inhibitor Filipin, led to accumulation of c-KIT on plasma membrane. Furthermore, the possible involvement of c-KIT receptor recycling was excluded by co-staining with the recycle endosome marker Rab11a.

4.3.3 MCPyV truncated LT contributes to the paranuclear retention of c-KIT by its interaction with Vam6p

The c-KIT paranuclear dot-like staining pattern is associated with MCPyV+ MCC, we therefore speculated that viral T-antigens could contribute to the intracellular retention of c-KIT. Indeed, we observed that knockdown of LT and sT could lead to cytoplasmic re-distribution of c-KIT. Notably, silencing of LT and sT decreased stability of c-KIT protein as compared to silencing of sT only or vector control. Additionally, we observed paranuclear aggregation of c-KIT only in cells transfected with the truncated LT (LT339) expression. Furthermore, the truncated LT also increased c-KIT protein level. As aforementioned, the truncated LT interacts with Vam6p to disrupt lysosome clustering (Liu et al., 2011), we therefore speculated that the Vam6p-interaction might be the explanation for c-KIT stabilization. We constructed the $LT339_{W209A}$ mutant, harboring the Vam6p binding site mutation, which disrupted its binding with Vam6p, as demonstrated by co-IP. The c-KIT protein stabilization and paranuclear aggregation of LT339 was abolished in the $LT339_{W209A}$ mutant. These results indicate that the MCPyV truncated LT promotes paranuclear retention and stabilization of c-KIT via Vam6p interaction.

4.3.4 c-KIT binds to BECN1 that enhances BECN1-BCL2 interaction in MCPyV+ MCC

Subsequently, we explored the functional role of c-KIT. Since we previously showed autophagy suppression in MCPyV+ MCC (**Paper II**) and another type of RTK, epithelial growth factor receptor (EGFR), has been shown to interact with BECN1 for autophagy suppression (Wei et al., 2013). We speculated that c-KIT could also target BECN1 for

modulating autophagy in MCPyV+ MCC. Indeed, we demonstrated the interaction between c-KIT and BECN1 by co-localization and co-IP. Using a series of deletion mutants of BECN1, we showed that c-KIT binds to evolutionarily conserved domain (ECD) of BECN1, which is also known to interact with VPS34 for induction of autophagy. We further revealed that c-KIT bound to BECN1 that enhanced its interaction with BCL2 and depletion of VPS34 interaction in MCPyV+ MCC cell lines. Additionally, the same results were obtained in the cells transfected with LT339, in which the effect was abolished in the $LT339_{W209}$ mutant. Together, these results demonstrate that MCPyV LT oncoprotein promotes c-KIT and BECN1 interaction that enhances BECN1-BCL2 interaction.

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

To address the functional role of c-KIT, we silenced c-KIT in MCPyV+ WaGa cells and observed increased autophagy and apoptosis. However, the increased autophagy was observed as early as 24 h, while the stimulation of apoptosis was more obvious at 48 h, indicating that the autophagy induction is not the consequence of cell death. We also noted that c-KIT silencing did not affect activation of the PI3K-AKT-mTOR signaling, suggesting that the activation of autophagy is independent of the signaling.

To assess the role of BECN1-BCL2 interaction, we treated MCPyV+ cells with ABT-737, a BH3 mimetic which competes with BCL2 to bind to BECN1 (Maiuri et al., 2007). The treatment increased apoptosis in a dosage-dependent manner, which could be partially rescued by autophagy inhibitor baf. A1.

4.3.6 MCPyV LT is degraded by autophagy

We noted down-regulation of LT level upon silencing of c-KIT, in which autophagy inhibitor baf. A1 could restore LT expression in c-KIT knocked down cells. This observation suggested that LT is degraded by autophagy. To strengthen this observation, we investigated the effect of autophagy modulation and proteasome inhibition using chemical approach on LT expression in WaGa cells. The results showed that LT was significantly downregulated upon autophagy stimulation, but restored upon autophagy inhibition. Moreover, silencing of the ATG7 autophagy gene led to LT stabilization. Together, out data support that MCPyV LT is degraded by autophagy.

c-KIT overexpression and no detectable activating mutation has been described in MCC (Rodig et al., 2012; Swick et al., 2013; Waltari et al., 2011). However, the functional role of c-KIT and its correlation with MCPyV status was unknown. In concordance with previous studies, we detected c-KIT activation in MCPyV+ MCC without activating mutations. Co-

expression of c-KIT and SCF has been reported in MCC tumor samples, and SCF-stimulated c-KIT is necessary for MCC cell growth *in vitro* (Kartha and Sundram, 2008; Krasagakis et al., 2011; Krasagakis et al., 2009). Together, these findings suggest that c-KIT is activated by its ligand.

One of the highlight points in this study is the observation of paranuclear dot-like aggregation of c-KIT in MCPyV+ MCC. Here we identify a mechanism by which MCPyV LT induces c-KIT retention via its Vam6p interaction. Given that the LT-Vam6p promotes lysosome disruption in MCC (Liu et al., 2011), it is tempting to speculate that MCPyV LT affects general lysosomal degradation, and c-KIT is one of them.

Activation of c-KIT regulates PI3K-AKT-mTOR pathway (Masson and Ronnstrand, 2009), and mTORC1 complex is involved as a negative regulator for autophagy (Schmelzle and Hall, 2000). However, we show that the c-KIT mediated autophagy suppression is independent of the PI3K-AKT-mTOR pathway. On the other hand, we showed that c-KIT directly interacts with BECN1.

BECN1 performs a central role for autophagy regulation. It associates with multiples cofactors (e.g. Atg14L, UVRAG, Rubicon, Ambra1) to dynamically regulate the BECN1-Vps34-Vps15 core-complex initiating autophagy flux (Kang et al., 2011). Besides, the BCL2, BCL-XL and other BCL2 family proteins could bind to the BH3 domain of BECN1 (Oberstein et al., 2007). This can lead to suppression or induction of autophagy, as well as a multiple crosstalk with apoptosis (Erlich et al., 2007; Kang et al., 2011; Maiuri et al., 2007).

In concordance with the mechanisms of autophagy suppression by EGFR in lung cancer (Wei et al., 2013), Mst1 in cardiac stress and Her2 in breast cancer (Maejima et al., 2013; Vega-Rubin-de-Celis et al., 2018), we identify c-KIT as a new BECN1 interacting partner for autophagy regulation. Similar to the EGFR-BECN1 mediated autophagy suppression, c-KIT binds to the BECN1 on its ECD domain. It is possible that c-KIT might modify the phosphorylation sites on BECN1 to alter the BECN1 dimerization for BCL2 binding. Therefore, BH3 mimetics (e.g. ABT-737, ABT-263) that can competitively bind to BECN1 or drugs targeting BECN1 core complex components, such as VPS34 inhibitor SB02024 (Dyczynski et al., 2018; Maiuri et al., 2007; Noman et al., 2020) can be therapeutic options for MCC. In conclusion, this study reveals a mechanism for autophagy regulation via c-KIT-BECN1 interaction, which could expand the potential for MCC treatment.

4.4 MERKEL CELL POLYOMAVIRUS ONCOPROTEIN PROMOTES BAG3-MEDIATED AGGRESOME FORMATION (PAPER IV)

Cytokeratin 20 (CK20), the typical diagnostic marker for MCC, is often reported as paranuclear dot-like staining pattern in MCC (Alvarez-Gago et al., 1996; Jensen et al., 2000; Kervarrec et al., 2019; Leech et al., 2001; Su et al., 2002b; Tanaka et al., 2004). However, the identity of this cytokeratin dot-like structure and its function in MCC are uncharacterized. Here, we sought to characterize this cytokeratin dot and its relationship to MCPyV T-antigens.

4.4.1 Paranuclear dot cytokeratin staining is more common in MCPyV+ MCC

To determine whether the CK20 dot is associated with MCPyV status in MCC, we first performed IHC staining of CK20 and another pan-cytokeratin maker MNF116 in three MCPyV- and three MCPyV+ MCC cell lines. All three MCPyV+ MCC cell lines harbored the paranuclear dot-like staining of CK20 and MNF116, while the cytoplasmic/membranous-staining pattern was found in all three MCPyV- MCC cell lines. The results were concordant with the immunofluorescence staining of MCPyV LT and CK20 in clinical samples, in which CK20 dot was more frequently found in MCPyV+ MCC tumors compared to the MCPyV- cases. Using the transmission electron microscopy, we also observed a whorl-like intermediate filament structure located adjacent to nucleus and surrounded by multiple mitochondria, which highly resembles the intermediate filament cage of aggresome (Johnston et al., 1998).

4.4.2 The cytokeratin paranuclear dot-like structure is an aggresome and its formation is BAG3-dependent

To demonstrate whether the cytokeratin paranuclear dot could be an aggresome, we applied immunofluorescence assays for co-localization analyses between pan-cytokeratin MNF116 and aggresome markers. The results showed strong co-localization between the cytokeratin dot and BAG3, Ub(K48) and γ-tubulin, but not with HDAC6. Aggresome formation requires the dynein motor complex for transporting the misfolded proteins along the microtubule track towards MTOC, which is marked with γ-tubulin (Olzmann et al., 2008). Upon depolymerization of the microtubule, the MNF116 paranuclear aggregation was disrupted. Additionally, overexpression of the dynamitin, which disturbs the dynactin complex (Shrum et al., 2009), also reduced number of cells with MNF116 aggregation. Together, our data support that the cytokeratin paranuclear dot-like structure in MCPyV+ MCC is aggresome.

Two mechanisms are known to contribute to aggresome formation, i.e. chaperone-BAG3 pathway or Ub-HDAC6 pathway (Gamerdinger et al., 2011; Kawaguchi et al., 2003). To further characterize which one is involved in the MCPyV-associated aggresome formation, we knocked down BAG3 or HDAC6 in WaGa cells and evaluated its effect on aggresome

formation. We observed that the aggresome was disrupted upon BAG3 silencing, while no effect was observed in the HDAC6 silencing group. These results indicate that the paranuclear cytokeratin dot-like structure in MCPyV+ MCC is a BAG3-dependent aggresome.

4.4.3 The Vam6p binding site of MCPyV LT oncoprotein is required for aggresome formation in MCPyV+ MCC

Since the aggresome structure is associated with MCPyV+ MCC, we wanted to determine if the MCPyV T-antigens were involved in aggresome formation. We showed that only silencing of both LT and sT, but not sT only, led to aggresome clearance in WaGa cells. On the other hand, only ectopic expression of truncated LT LT339 recapitulated the aggresome formation in MCPyV- cell line.

To explore whether the LT-Vam6p binding is involved in the aggresome formation, we transfected LT339 or $LT339_{W209A}$, Vam6p binding defective mutant, into the HEK-293 cells and evaluated the effect on aggresome formation. Indeed, the aggresome formation was markedly decreased in the $LT339_{W209A}$ mutant compared to both LT339 and vector control. These results indicate that the LT-Vam6p interaction is essential for aggresome formation in MCPyV+ MCC.

Herein, we revealed that the CK20 paranuclear dot is associated with MCPyV+ MCC, and this structure is BAG3-mediated aggresome, which is partly contributed by MCPyV truncated LT through its Vam6p interaction. CK20 expression pattern is distinct in both Merkel cell and MCC, in which diffuse cytoplasmic in Merkel cells and paranuclear dot in MCCs (Moll et al., 1992). Cytokeratin (CK) proteins constitute two major subgroups of intermediate filaments, which undertake cytoskeletal formation and mechanical basis for epithelial cells (Chang and Goldman, 2004). Given some cytokeratins are tissue-specific and their expressions are normally preserved during tumorigenesis, CKs are commonly used for diagnosis in many different cancer types, such as breast, colorectal and gastric (Braun et al., 2000; Maehara et al., 1996; Wong et al., 2009). As aforementioned, CK20 expression is used for diagnosis of MCC, regardless of the virus status (Lebbe et al., 2015). Notably, despite the CK20 dot is preferentially found in MCPyV+ MCC, around half of the MCPyV- MCC tumors (46/96) had CK20 dot, which hints the involvement of other mechanism for CK20 paranuclear aggregation beyond the viral oncoprotein. Nevertheless, the MCPyV truncated LT is partly responsible for aggresome formation via Vam6p.

Vam6p (also known as VPS39) is the key subunit of the Homotypic fusion and protein sorting (HOPS) complex, which is required for late endosome and lysosome fusion and protein aggregates clearance (Babazadeh et al., 2019; Pols et al., 2013). In MCC, Vam6p is bound with MCPyV LT, leading to disruption of lysosome clustering (Liu et al., 2011). Thus,

it is tempting to speculate that the aggresome formation in MCPyV+ MCC is a consequence of protein-degradation failure induced by LT-Vam6p interaction. Besides Vam6p, we also showed that this aggresome formation is dependent on BAG3 co-chaperone. BAG3 mediates the transportation of HSP70-bound targets via dynein-motor along the microtubule (Gamerdinger et al., 2011). The impairment of proteasome degradation can lead to BAG3 up-expression, which sequesters proteasomal clients into aggresome for autophagic degradation (Minoia et al., 2014). However, we did not observe any effect on BAG3 level upon modulation of MCPyV T-antigens or the interaction between BAG3 and LT by co-IP (data not shown). Whether LT affects BAG3 subcellular localization and/or its associated partners have yet to be investigated.

Although aggresome is a cellular defense response towards toxic misfolded proteins, this structure resembles viral factory that has been observed in other DNA viruses, such as adenovirus, herpes virus and African swine fever virus (Heath et al., 2001; Liu et al., 2005; Nozawa et al., 2004). Given the MCPyV in MCC tumors loses its replication ability due to the truncated mutations of LT eliminating the helicase domain required for viral replication (Shuda et al., 2008), it is unlikely that aggresome in MCPyV+ MCC serves as viral replication platform. Banerjee and colleagues reported that Influenza A could harness the host HDAC6-dynein aggresome system to assist its infection (Banerjee et al., 2014). Therefore, the aggresome formation may happen in the early stage of MCC or during MCPyV viral infection. On the other hand, aggresome could act as cytoprotective compensatory response against the cytotoxicity of accumulated misfolded proteins upon proteasome impairment (Bjorkoy et al., 2005; Takahashi et al., 2018). Our preliminary data showed that disrupting aggresome formation by dynamitin overexpression increases cytotoxicity in WaGa cells (data not shown), suggesting cytoprotective role for aggresome in MCPyV+ MCC.

In conclusion, we identify a BAG3-mediated aggresome in MCPyV+ MCC, in which the Vam6p binding site of the LT oncoprotein is required for promoting aggresome formation, which protects cell from toxic protein aggregates.

5 CONCLUSIONS

Protein degradation system is an essential networking that transfers the misfolded proteins and damaged organelles into elementary molecules for cellular homeostasis maintenance and recycling energy supply. Various factors could interfere with degradation system to regulate tumor development with diverse dimensions. This thesis partly contributed to the molecular understanding of miRNAs and viral oncoproteins involved in cellular degradation pathways. The general finding are summarized as the following:

- miRNAs can regulate key genes involved in cellular degradation pathways, such as *miR-223-3p* regulation of FBXW7 E3 ligase in TGCT and *miR-375*, *miR-30a-3p*, *miR-30a-5p* regulation of BECN1, SQSTM1, ATG7 in MCC (**Papers I** and **II**).

- MCPyV T-antigens suppress autophagy through miRNAs (i.e. *miR-375*, *miR-30a-3p*, *miR-30a-5p*) and c-KIT mediated BECN1-BCL2 interaction in MCC (**Papers II** and **III**).

- Autophagy induces MCPyV LT degradation and apoptosis in MCC (**Papers II** and **III**).

- c-KIT directly binds to the ECD of BECN1 that diminishes the BECN1-VPS34 interaction, while enhancing the BECN1-BCL2 interaction for autophagy suppression (**Paper III**).

- The Vam6p binding site of MCPyV truncated LT is required for paranuclear retention and stabilization of c-KIT (**Paper III**), as well as aggresome formation (**Paper IV**)

6 FUTURE PERSPECTIVE

As described in the introduction section and several studies in this thesis, it is not surprised that protein degradation system interplays with enormous aspects in cancer development and is regulated by multiple factors. One of such factors is miRNA. We show that *miR-223-3p* regulates the E3 ligase FBXW7 in TGCT (**Paper I**), while *miR-375*, *miR-30a-3p* and *miR-30a-5p* target multiple autophagy factors in MCC (**Paper II**). In **Paper I**, although we demonstrated that *miR-223-3p* regulates cell growth and apoptosis through FBXW7, it is still unclear whether *miR-223-3p* overexpression in TGCT increases protein stability of specific FBXW7 substrates that are required for TGCT development. Identification of the downstream targets of the *miR-223-3p*-FBXW7 regulation would be relevant to improve our understanding of this disease. In **Paper II**, we demonstrate that MCPyV T-antigens can induce miRNA expressions through the DnaJ domain of the viral T-antigens. Several outstanding questions have been raised from this finding. For example, how does the DnaJ domain of the viral T-antigens contribute to miRNA regulation? How does this regulation contribute to selective processing of specific miRNAs?

Another factor is the viral oncoprotein. In **Papers III** and **IV**, we found that the Vam6p binding site of MCPyV truncated LT can promote intracellular retention of c-KIT and aggresome formation. In **Paper III,** the functional consequences of the c-KIT-mediated BECN1 complex dynamics of autophagy suppression have yet to be determined in MCPyV+ MCC. Furthermore, c-KIT is a kinase receptor, it is unclear whether the kinase function is required for the BECN1 interaction. Further investigations are needed to determine the BECN1 phosphorylation and dimerization status and other BECN1 interactomes. Additionally, the importance of the c-KIT-BECN1-BCL2 interaction in MCC tumorigenesis has yet to be determined and the effect of BH3 mimetics on MCC xenografts. The findings in **Paper III** also raise a question whether c-KIT-BECN1 interaction happens in other tumor types. Indeed, our preliminary data also showed that c-KIT directly interacts with BECN1 in GIST (unpublished data).

In **Paper IV**, functional consequences of the aggresome formation have yet to be investigated in MCC. Besides, the aggresome in MCC could also be the treatment target. Similar strategy has been used in pancreatic cancer cells, in which they inhibit HDAC6 to disrupt the bortezomib-induced aggresome formation that sensitizes the aggresome-positive cell to apoptosis via ER stress (Nawrocki et al., 2006). Further understanding of aggresome formation in MCC might benefit the treatment for MCC patients.

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