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AUTOPHAGY AND VPS34 AS TARGETS IN ANTI-CANCER THERAPY

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Autophagy and VPS34 as targets in anti-cancer therapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family and friends for their endless love and support

"The world if full of wonders, but they become more wonderful, not less wonderful when science looks at them."

David Attenborough

POPULAR SCIENCE SUMMARY OF THE THESIS

Cancer is estimated to affect 1 in 3 people during their lifetime. During recent years tremendous advances in cancer prevention and care have been made. As a result, life expectancy has increased significantly for many cancer types. However, further research efforts are still urgently needed as many patients experience regrowth of their cancer after treatment while others do not even respond to treatment at all. Studying the biology of cancers lays the foundation for the development of better drugs and effective treatment combinations. It is, therefore, crucial to understand the entire process from cancer development, growth, and spreading in the body. In this way, unique vulnerabilities of cancer cells can be discovered that may be targeted with novel medicines and minimize side effects.

Tumors form by uncontrolled cell division. Such rapid cell growth creates an environment characterized by harsh conditions such as lack of space and/or blood vessels resulting in low availability of nutrients and oxygen. Besides, once a patient is given treatment, cancer cells start facing therapy-induced stress. To cope with such harsh conditions, cancer cells hijack a clever survival strategy: they initiate "self-cannibalism" to feed their metabolism while also clearing cellular waste. This process is called **autophagy** and essentially describes how cells can consume their components to promote survival. The discovery of this cellular "recycling" process and the genes controlling it by Yoshinoro Ohsumi was awarded the Nobel Prize in 2016. The last decade of autophagy research has shown that by preventing cancer cells from activating autophagy, tumor growth can be slowed significantly or stopped entirely. Therefore, blocking autophagy has become a promising strategy in cancer research.

This thesis aimed to study the impact of blocking **VPS34**, a protein involved in kickstarting the autophagic process in cancer cells. To do this we have developed a small molecule drug that can directly block VPS34 activity, termed **SB02024**. We observed that SB02024 treatment prevents cancer cell autophagy and slows down tumor growth in mice that have been implanted with human breast cancer cells. To find a suitable combination treatment with SB02024, we further identified several known cancer drugs, for example, Sunitinib and Erlotinib, that can trigger autophagy activation in cancer cells. A combination treatment using these drugs together with SB02024 successfully decreased the growth of cultured breast cancer cells.

Besides being a survival mechanism, autophagy has also been shown to play a key role in hiding cancer cells from the surveillance of the immune system. Why is this important? The recent introduction of cancer therapies that can activate the immune system's capability to fight cancer have proven to be very effective for selected cancer types including melanoma, colorectal, and kidney cancer. One of the hurdles to make these immunotherapies applicable to more cancer patients is that many tumors lack the presence of immune cells. We discovered, that by blocking VPS34, using either SB02024 treatment or genetic methods, we can increase immune cell infiltration resulting in decreased tumor growth in mice. The tested tumor types include melanoma, colorectal and kidney cancer. This effect was dependent on the release of signaling molecules from cancer cells that can attract immune cells into the tumor much like a

mosquito that is attracted by human scent. We show that blockade of VPS34 in cancer cells activates a cellular mechanism (termed cGAS-STING pathway) which leads to the production and the release of these immune cell-attracting molecules. Combination of VPS34 inhibitor with currently tested or approved immunotherapies showed promising effects in reducing melanoma and colorectal cancer growth in mice even further than each of these treatments alone. These results provide a basis for further investigation of VPS34 blockers for the treatment of cancer and demonstrate their promising benefits as combination therapy with existing anti-cancer drugs.

ABSTRACT

Autophagy (Greek for self-eating) is a cellular pathway that breaks down cytoplasmic components to fuel metabolism. In this way, autophagy maintains cellular homeostasis and sustains survival during stress conditions. In cancer, autophagy is often dysregulated and has been described to contribute to malignancy and therapy resistance. Recently, autophagy has also been identified to play a key role in immunosuppression. Strategies to target autophagy for anti-cancer treatment have therefore generated renewed interest. The general aim of this thesis was to evaluate whether inhibiting VPS34, an essential protein in the autophagic pathway, impacts cancer growth and survival and represents a promising target for anti-cancer therapy.

Autophagy has been consistently reported as a mechanism to enable cancer progression and to drive treatment resistance. In **Paper I,** we, therefore, set out to identify cancer drugs that may trigger autophagy induction in cancer cells. Using the classical cellular GFP-LC3 reporter assay, we performed a high-throughput screening of the FIMM compound library containing approved or clinically relevant anti-cancer drugs. Among the discovered autophagy inducers in the screen, we selected two receptor tyrosine kinase inhibitors, Sunitinib and Erlotinib, to evaluate whether simultaneous autophagy inhibition may increase their anti-cancer efficacy. As a potent autophagy inhibitor, we used SB02024, a small molecule VPS34 inhibitor (VPS34i) developed using a fragment-based drug design approach by Sprint Bioscience. We demonstrate that SB02024 significantly decreased tumor growth in two breast cancer xenograft models. Combination treatment with SB02024 further increased sensitivity to both RTK inhibitors in breast cancer cells *in vitro*. Based on these results, VPS34 represents a promising target for anti-cancer therapy and as a combination therapy may improve the clinical efficacy of autophagy-inducing drugs.

Immunotherapies such as immune checkpoint blockade (ICB) have revolutionized cancer medicine but many cancer patients are either not eligible for treatment or fail to respond. Autophagy by controlling inflammation and immunity has been proposed as a critical immune evasion mechanism. In **Paper II**, we, thus, investigated the impact of inhibiting VPS34 in immunocompetent mice bearing syngeneic mouse tumors. Using melanoma or colorectal cancer (CRC) tumor-bearing mice, we showed that either genetic or pharmacological VPS34 inhibition significantly decreased tumor growth in a T- and NK-cell dependent manner. The anti-tumor response was further characterized by an increase of the chemokines CCL5, CXCL10, and the cytokine IFNγ in the tumor microenvironment resulting in the recruitment and activation of immune effector cells. Mechanistically, we demonstrate that VPS34i treatment increased secretion of CCL5 and CXL10 by tumor cells through activation of IRF7/STAT1 signaling. Finally, VPS34i treatment increased sensitivity to anti-PD-1/-PD-L1 therapy resulting in decreased tumor growth *in vivo* and prolonged survival of mice. These data demonstrate that VPS34 plays a crucial role in cancer immune evasion and targeting VPS34 may improve clinical responses to ICB in melanoma and CRC cancer.

Deepening the knowledge of how autophagy inhibition unleashes anti-tumor immune responses may enable patient stratification and identify rational treatment combinations with immunotherapeutic agents. Based on the discovery of a VPS34i-mediated anti-tumor immune response in Paper II, we sought out to identify underlying signaling mechanisms of the observed chemokine release **in Paper III**. To do this, we treated Renca tumor-bearing mice, a syngeneic mouse model for renal cell carcinoma (RCC), with VPS34i and profiled tumor mRNA expression using a NanoString panel focused on immune-oncology gene signatures. As previously shown for melanoma and CRC tumors, VPS34i treatment induced immune effector cell infiltration and chemokine secretion in Renca tumors. We further identified that VPS34i treatment triggered type I Interferon signaling, an important innate immunity pathway to trigger inflammation in the TME. Using both mouse and human RCC and melanoma cells, we demonstrated that VPS34i-mediated type I interferon signaling is dependent on cGAS-STING pathway activation. Combination treatment of VPS34i with the STING agonist ADU-S100 further increased type I interferon signaling *in vitro*. Finally, SB02024 + ADU-S100 combination treatment potently reduced melanoma tumor growth and increased mice survival *in vivo*. In summary, these data identify VPS34 as a key regulator of cGAS-STING signaling in cancer cells. Pharmacological VPS34 inhibition thereby presents an attractive approach to increase immune cell infiltration in the TME and may provide clinical benefits for patients treated with immunotherapies including emerging drugs targeting the STING pathway.

LIST OF SCIENTIFIC PAPERS

I. Dyczynski M*, **Yu Y***, Otrocka M, Parpal S, Braga T, Henley AB, Zazzi H, Lerner M, Wennerberg K, Viklund J, Martinsson J, Grandér D, De Milito A, Pokrovskaja Tamm K. Targeting autophagy by small molecule inhibitors of vacuolar protein sorting 34 (Vps34) improves the sensitivity of breast cancer cells to Sunitinib.
Cancer Letters. 2018; 435:32-43.

II. Noman MZ, Parpal S, Van Moer K, Xiao M, **Yu Y**, Arakelian T, Viklund J, De Milito A, Hasmim M, Andersson M, Amaravadi RK, Martinsson J, Berchem G, Janji B. Inhibition of Vps34 reprograms cold into hot inflamed tumors and improves anti-PD-1/PD-L1 immunotherapy.
Science Advances. 2020; 6(18):eaax7881.

III. **Yu Y*,** Noman MZ*, Parpal S, Kleinendorst SC, Bilgrav Saether K, Alexeyenko A, Viklund J, Andersson M, Martinsson J, Pokrovskaja Tamm K, De Milito A, Janji B. VPS34 inhibition activates cGAS-STING signaling and sensitizes tumors to STING agonist.
Manuscript.

*Equal contribution

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS:

I. Kolosenko I, **Yu Y**, Busker S, Dyczynski M, Liu J, Haraldsson M, Palm Apergi C, Helleday T, Pokrovskaja Tamm K, Page BDG, Grandér D. Identification of novel small molecules that inhibit STAT3-dependent transcription and function.
PLoS ONE. 2017; 12(6):e0178844.

CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Explanation
ADU-S100	STING agonist developed by Aduro Biotech
ALK	Anaplastic lymphoma kinase
AMBRA1	BECN1-regulated autophagy protein 1
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate (AMP)-activated protein kinase
APC	Adenomatous polyposis coli proto-oncogene
ARG1	Arginine-degrading enzyme arginase I
ATGs	Autophagy-related genes
ATP	Adenosine triphosphate
BafA1	Bafilomycin A1
BECN1	Beclin-1
BRAF	B-raf proto-oncogene
BRCA1	Breast cancer type 1
CCL5	Chemokine (C-C motif) ligand 5
ccRCC	Clear-cell renal cell carcinoma
cGAS	cyclic GMP-AMP synthase
CMA	Chaperone-mediated autophagy
CML	Chronic myelogenous leukemia
CQ	Chloroquine
CR	Calorie restriction
CRC	Colorectal cancer
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL10	C-X-C motif chemokine ligand 10
DFCP1	FYVE domain-containing protein 1
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum

ER	Estrogen receptor
ERGIC	ER-Golgi intermediate compartment
ERK	Extracellular signal-regulated kinase
FAO	Fatty acid oxidation
FIP200	Family 200-kD interacting protein
FLT3	FMS-like tyrosine kinase 3
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GEMM	Genetically engineered mouse model
GIST	Gastrointestinal stromal tumors
HBV	Hepatitis B virus
HCQ	Hydroxychloroquine
HOPS	Homotypic fusion and protein sorting
HRAS	Harvey Rat sarcoma virus, proto-oncogene
ICB	Immune checkpoint blockade
ICD	Immunogenic cell death
ICI	Immune checkpoint inhibitors
IFN	Interferon
IFNAR	Interferon-alpha/beta receptor
IHC	Immunohistochemistry
IRF	Interferon regulatory factor
IT	Intratumorally
KRAS	Kirsten rat sarcoma virus proto-oncogene
KU	Ku-0063794, mTOR inhibitor
LAP	LC3-associated phagocytosis
LC3	Microtubule-associated protein 1 light chain 3
LIR	LC3-interacting region
LKB1	Liver kinase B1
MAP1LC3	Microtubule-associated protein 1 light chain 3
MAPK	Mitogen-activated protein kinase
MCS	Multicellular spheroids
MDSCs	Myeloid-derived suppressor cells

MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MLL-AF9	Fusion gene associated with de novo AML
MMTV-PyMT	Mammary specific polyomavirus middle T antigen overexpression mouse model
mTORC1	Mechanistic target of rapamycin complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NBR1	Neighbor of BRCA1 gene 1
NDP52	Nuclear dot protein 52 kDa
NEA	Network enrichment analysis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOX2	NADPH oxidase-2
NRBF2	Nuclear receptor binding factor 2
NRF2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung cancer
NSG	NOD *scid* gamma
p62/SQSTM1	Sequestosome-1
PALB	Partner and localizer of BRCA2
PanNET	Pancreatic neuroendocrine tumor
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed death-ligand 1
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI3P	Phosphatidylinositol-3-phosphate
PIK3C3	Phosphoinositide 3-kinase class III
PIK-III	VPS34 inhibitor developed by Novartis
PPT1	Palmitoyl-protein thioesterase 1
PSC	Pancreatic stellate cells
PTEN	Phosphatase and tensin homolog

RAB7	Ras-related protein 7
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcoma virus
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RUBCN	Run domain Beclin-1-interacting and cysteine-rich domain-containing protein
SAR405	VPS34 inhibitor developed by Sanofi
SB02024	VPS34 inhibitor developed by Sprint Bioscience
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptor
STAT1	Signal transducer and activator of transcription 1
STING	Stimulator of interferon genes
TCGA	The Cancer Genome Atlas
TK	Tyrosine kinase
TME	Tumor microenvironment
TRP53	Transformation-related protein 53, p53
ULK1/2	Unc-51 like autophagy-activating kinase 1/2
UVRAG	UV radiation resistance-associated
VEGFR	Vascular endothelial growth factor receptor
VPS34i	VPS34 inhibitor
WHO	World health organization
WIPI	WD-repeat protein interacting with phosphoinositide
WNT1	Wnt Family Member 1

1 INTRODUCTION

1.1 CANCER

Cancer is an umbrella term for a group of more than 100 diseases showing abnormal, uncontrolled cell growth and is often referred to as a malignant tumor. Unlike a benign tumor, a malignant tumor is defined by its ability to invade the surrounding tissue and spread to local or distant organs in the body. The spreading of the primary tumor to a secondary site (metastasis) often occurs via the blood or lymphatic system, and it is the metastatic disease, and not the primary tumor, which is the major cause of cancer death.

According to statistics published by the WHO International Agency for Research on Cancer an estimated number of 19.3 million people were diagnosed with and 10 million people died of cancer in 2020 worldwide (Sung et al., 2021). The cancer incidence and mortality worldwide and in Sweden are summarized in **Figure 1** below.

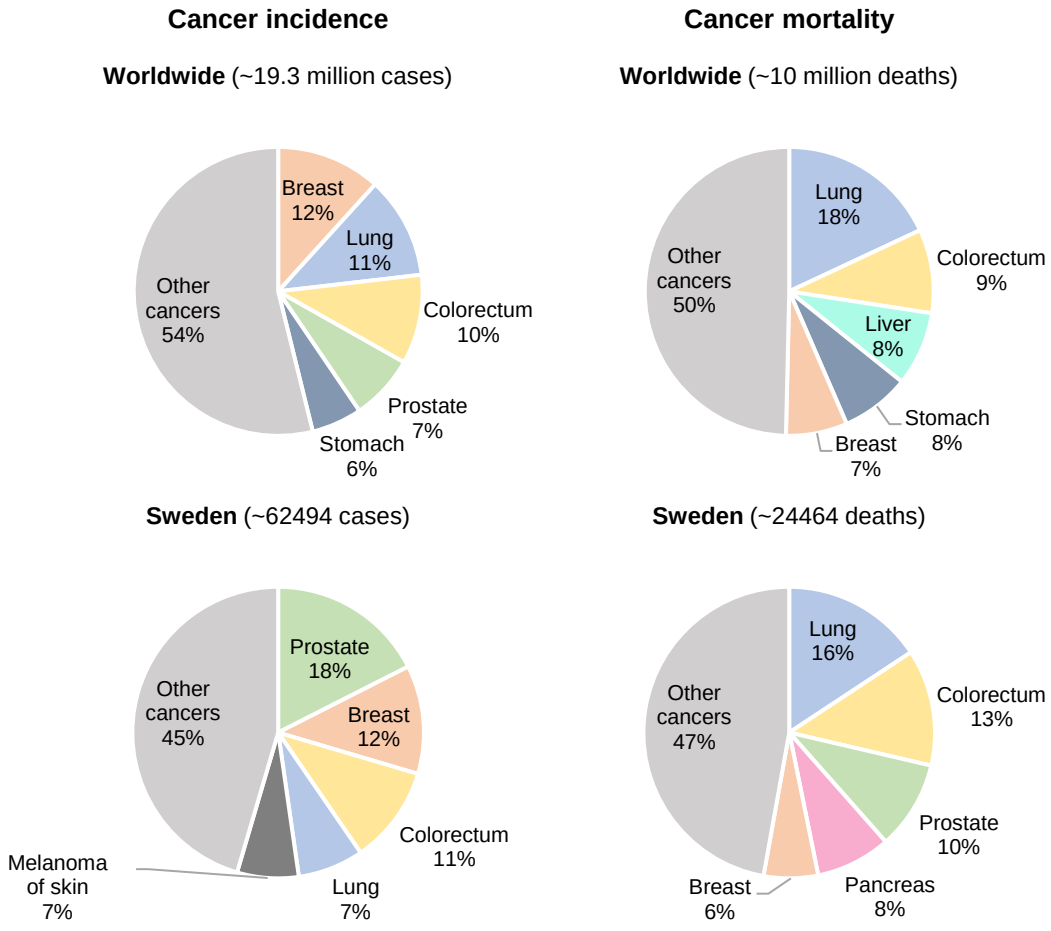


Figure 1. The Top 5 most common cancer types diagnosed (left) and causing deaths (right) worldwide and in Sweden in 2020. Data was extracted from the Global Cancer Observatory gco.iarc.fr/today, accessed 2021-08-10.

Cancer is among the most common causes of death worldwide. Due to the continuous growth and aging of the world population and increase of risk factors, it is projected that cancer

incidence will increase by 47% in the next twenty years, highlighting the importance of investments into cancer prevention and care to improve life expectancy (Sung et al., 2021).

What are the key features that distinguish cancer cells from normal cells? In efforts to summarize characteristics that define cancer cells, Douglas Hanahan and Robert A. Weinberg published a landmark article already back in 2000 describing six "hallmarks of cancer": sustained proliferation, evading growth suppressors, induction of angiogenesis, activation of invasion and metastasis, resistance to cell death and replicative immortality (Hanahan and Weinberg, 2000). Following further research into the cross-talk of cancer cells with the tumor microenvironment (TME) including stroma, this review was later revised in 2011 to include two additional hallmarks: deregulation of cellular energetics and avoiding immune destruction; as well as two enabling characteristic: tumor-promoting inflammation and genome instability and mutation (Hanahan and Weinberg, 2011) (all depicted in **Figure 2**).

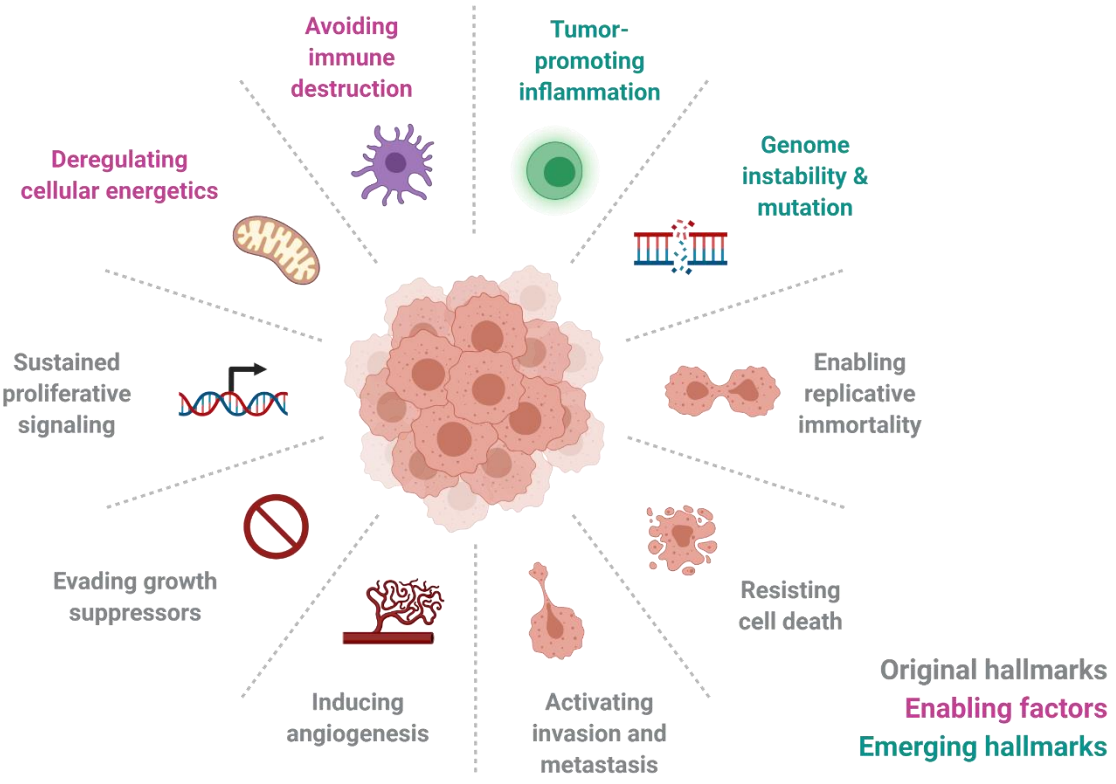


Figure 2. The hallmarks of cancer (adapted from Hanahan and Weinberg 2011).

To acquire these cancer cell properties, normal cells must undergo many genetic and epigenetic changes. Cancer cells may even acquire new features to adapt to changes in the tumor microenvironment, escape immune surveillance, and finally spread and metastasize to new sites. Due to this multistep process, the development of cancer typically takes time and cancer has been considered a disease of the elderly. However, several risk factors including lifestyle habits (e.g. tobacco use, obesity, alcohol consumption), exposure to carcinogenic agents or radiation, and inherited genetic predispositions can all contribute to carcinogenesis. Most often, the exact cause of cancer remains unknown.

1.1.1 Cancer treatment

What are the major challenges to treat cancer?

The pillars of anti-cancer therapy include **surgery, radiation therapy**, and **chemotherapy**. All these interventions have limited clinical efficacy as they often lack specificity. For example, surgery and radiation therapy can cause quite extensive damage to tissue surrounding the tumor. In addition, microscopic lesions and metastasis are not targeted. Systemic chemotherapy has severe side effects and often triggers drug resistance. This led subsequently to the combination of several chemotherapies with complementary mechanisms of action and the optimization of treatment regimens (DeVita et al., 1980; Hryniuk and Bush, 1984). However, successes achieved with so-called polychemotherapy have plateaued (Vasan et al., 2019) calling for more effective treatment options that may target tumor cells specifically while sparing normal cells thus causing less general toxicity. Technical and scientific advances have allowed extensive profiling of common genetic or molecular features of cancer cells that enable tumor growth and spread and can be exploited for the development of targeted therapies. The hope is that it will be possible to tailor treatment for each patients' tumor based on its genetic and molecular landscape, an approach also referred to as **precision medicine**.

This has led to the development of **targeted therapies** as a new anti-cancer treatment modality. Targeted therapies are mainly comprised of monoclonal antibodies, hormonal agents, and small molecule inhibitors that interfere with a specific protein implicated in tumorigenesis. In this way, targeted therapies generally confer more cancer-cell-specific cytotoxicity as compared to chemotherapy and are better tolerated. One of the success stories of targeted therapies is the tyrosine kinase (TK) inhibitor Imatinib which targets the constitutively active fusion protein BCR-ABL in chronic myelogenous leukemia (CML) (Palumbo et al., 2013). In the following years, several small molecule therapies targeting TKs in cancer have been developed including epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), vascular endothelial growth factor receptor (VEGFR) (Palumbo et al., 2013).

Two prominent TK inhibitors utilized as anti-cancer treatments in this thesis are Erlotinib and Sunitinib. Erlotinib is an EGFR inhibitor used for the treatment of non-small cell lung cancer (NSCLC) and pancreatic cancer harboring an EGFR mutation (Rosell et al., 2012). Sunitinib is a multi-TK inhibitor that targets VEGFR, platelet-derived growth factor receptor (PDGFR), FMS-like tyrosine kinase 3 (FLT3), and c-KIT leading to inhibition of angiogenesis and cell proliferation. Sunitinib treatment has been FDA-approved for renal cell carcinoma (RCC), Imatinib-resistant gastrointestinal stromal tumors (GIST), and pancreatic neuroendocrine tumors (PanNET) (Goodman et al., 2007; Raymond et al., 2011).

The addition of targeted therapies along with the decrease of risk factors, better screening, and diagnosis practice, has played a crucial part in decreasing mortality rates for many cancer types (Henley et al., 2020a). It has been estimated for the US population that the overall cancer death rate has fallen by about 15% between the years 2007-2017 (Henley et al., 2020b). The largest decrease in mortality rate has been achieved for melanoma of the skin (-6.1% in males and -

6.3% in females) and lung cancer (-4.8% in males and -3.7% in females) (Henley et al., 2020b). The falling mortality rate in these two cancer types in the last decade also reflects the addition of a new pillar of targeted therapy: immunotherapy.

Cancer immunotherapy attempts to activate, modulate, or enhance the immune system's ability to attack cancer cells. It comprises several therapeutic approaches such as cytokines, cancer vaccines, oncolytic viruses, cell therapy, and immune checkpoint inhibitors (ICI) - the latter being the most widely used one. ICIs include antibodies targeting CTLA-4, PD-1, or PD-L1 and have been a breakthrough therapy for many cancer patients with FDA approvals for as many as 50 cancer types (Robert, 2020). This has sparked enormous global investments into the cancer immunotherapy field with an estimated 2/3 of all ongoing clinical trials in 2019 testing immune-oncology drugs (Xin Yu et al., 2019).

Paradigm shifts for cancer therapy seem to happen at a higher pace now, especially given the promising advances in the field of immune-oncology (Hegde and Chen, 2020) which gives hope for finding "the cure" for cancer. However, like conventional chemotherapy, the clinical efficacy of targeted therapies is limited by drug resistance. Established mechanisms and possible solutions for drug resistance will be addressed in the next chapter.

1.1.2 Resistance against cancer therapy

Despite the beforementioned additions of targeted treatment modalities to clinical practice in recent years, resistance and relapse frequently occur in clinical practice. Drug resistance is often

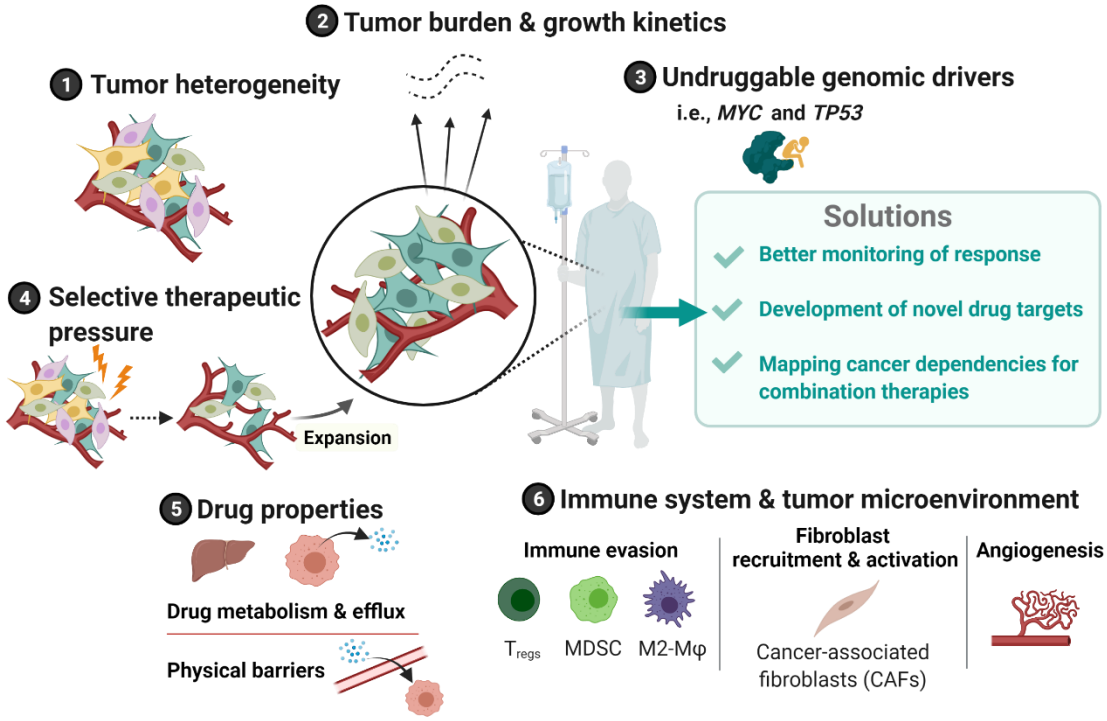


Figure 3. Mechanisms and solutions of drug resistance in anti-cancer therapy (adapted from Vasan et al. 2019).

attributed to a combination of several mechanisms which may already pre-exist (intrinsic) or occur during treatment (acquired). Key mechanisms limiting drug efficacy include unfavorable tumor features (e.g. tumor heterogeneity, high tumor burden, slow growth kinetics, undruggable driver mutations), poor drug properties (e.g. metabolism and/or efflux of the drug, physical barriers preventing drug penetration), selective therapeutic pressure (genetic and epigenetic changes, inductions of cellular survival mechanisms), and an unfavorable surrounding tumor environment (immunosuppression, activation of stroma and vasculature). An illustration of common drug resistance mechanisms and suggested approaches to prevent drug resistance (Vasan et al., 2019) can be found in **Figure 3**.

Unfortunately, drug resistance is often diagnosed with some delay in clinical practice. Undetected drug resistance may cause unnecessary toxicities due to side effects and allows cancer progression due to a lack of drug sensitivity. For this reason, improvement of diagnostic tests and discovery of biomarkers allowing for early detection/monitoring of resistance, development of novel treatments that address resistance mechanisms, and identification of cancer cell dependencies to find rationale combination strategies are imperative (Vasan et al., 2019).

In this thesis, we have addressed several of these proposed solutions to combat drug resistance. Firstly, we have investigated autophagy as a pathway that sustains cancer growth and progression and contributes to therapy resistance. Secondly, we have introduced VPS34 inhibitors as a novel small molecule therapy to inhibit autophagy in cancer therapy. Lastly, we have investigated the potential of VPS34 inhibitors as a combination therapy with a specific focus on cancer immunotherapy.

1.2 AUTOPHAGY

Autophagy is an evolutionarily conserved degradation process in eukaryotes that enables cells to eliminate or recycle cytoplasmic components by delivery to the lysosome (or the vacuole in yeast or plants) (Boya et al., 2013; He and Klionsky, 2009; Mizushima and Komatsu, 2011). Autophagy is a dynamic process that can occur both in physiological conditions and in adaptation to stress conditions such as starvation, infections, lack of oxygen or energy ensuring cellular and tissue homeostasis (White et al., 2015). There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy involves the isolation and degradation of cytoplasmic material in a double-membrane vesicle, termed autophagosome. In contrast, microautophagy and CMA do not require autophagosomes for cargo delivery to the lysosome but act via direct engulfment through lysosomal inward invagination or via protein translocation involving chaperone binding to lysosomal Lamp-2A, respectively (Mizushima and Komatsu, 2011).

Macroautophagy (hereafter referred to as autophagy) is the main type of autophagy and the subject of this doctoral thesis.

1.2.1 Mechanisms of autophagy

Detailed studies of autophagy regulation in the model system of yeast by Yoshinori Ohsumi led to the discovery of the core autophagy-related genes (ATGs) (Ohsumi, 2014) awarded with the Nobel Prize in Physiology or Medicine in 2016 (Tooze and Dikic, 2016). Homologs of the discovered yeast ATG proteins can be found in various organisms making this multi-step pathway highly conserved in eukaryotes. The following paragraph will describe mammalian autophagy and use the corresponding nomenclature.

Autophagy is a multi-step pathway that can be divided into five key stages which are depicted in **Figure 4** (Hansen et al., 2018):

1. **Induction** of autophagy
2. **Nucleation** or formation of the phagophore
3. **Expansion** of the phagophore leading to the **closure** of the autophagosome
4. **Fusion** of the autophagosome with the lysosome leading to the formation of the autolysosome
5. **Degradation** and recycling of the sequestered cargo by lysosomal hydrolases and permeases

The molecular mechanisms regulating these different steps of autophagy both positively and negatively have been under extensive research in the last decades.

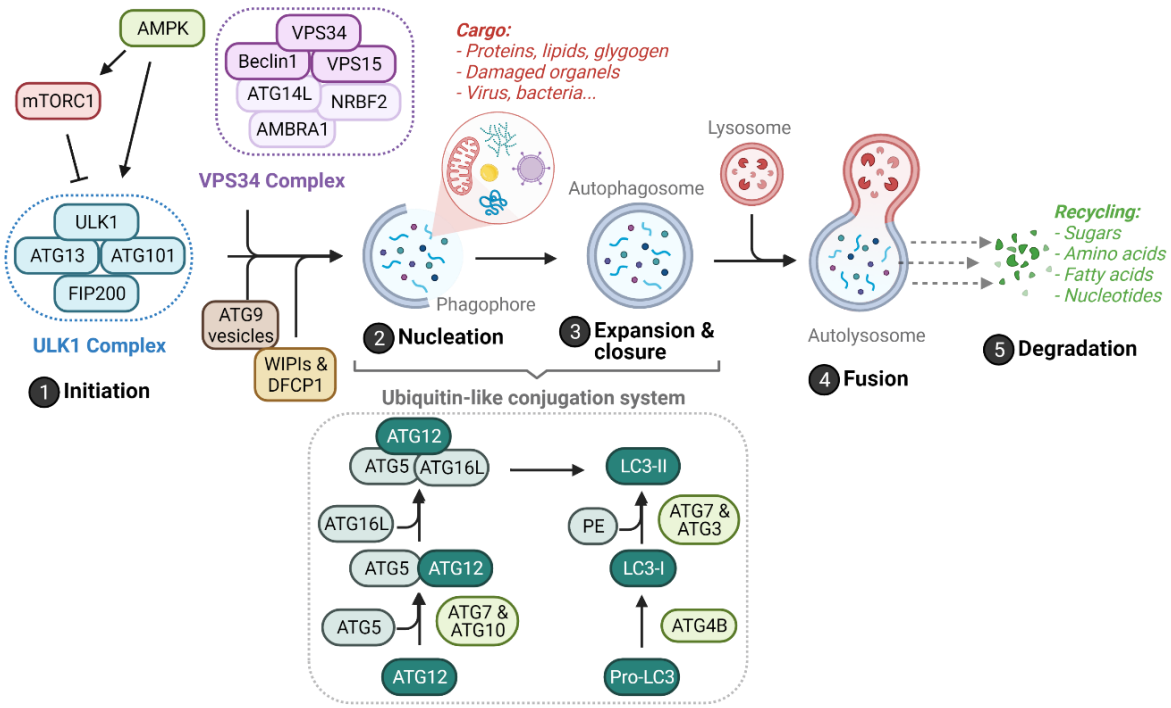


Figure 4. The autophagic pathway and its regulation (adapted from Hansen et al., 2018).

1.2.1.1 Induction of autophagy

Autophagy is induced when repression by the mechanistic target of rapamycin complex 1 (mTORC1) is released. The mTORC1 complex can sense nutrient and growth factor levels, and negatively regulates autophagy by directly phosphorylating the initiating kinase complex comprised of Unc-51 like autophagy-activating kinase 1/2 (ULK1/2), autophagy-related protein 13 (ATG13), ATG101, and family 200-kD interacting protein (FIP200) (Kim et al., 2018; Laplante and Sabatini, 2012). The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is an important upstream regulator of mTORC1 and can detect energy shortage through sensing decreasing intracellular ratio of ATP/AMP; but then again AMPK itself can also activate autophagy directly through phosphorylating the ULK1 kinase complex (Mihaylova and Shaw, 2011).

1.2.1.2 Nucleation: formation of the phagophore

Once activated, the ULK complex phosphorylates the vacuolar protein sorting 34 (VPS34, also referred to as phosphoinositide 3-kinase class III or PIK3C3) complex which is constituted of three key proteins: the lipid kinase unit VPS34, the regulatory subunit Beclin-1 (BECN1), and serine-threonine kinase VPS15. This "core" VPS34 complex further binds and interacts with different proteins regulating its function and localization. For example, depending on the binding of ATG14L or UV radiation resistance-associated gene (UVRAG), the VPS34 complex is referred to as complex I and II, respectively. While VPS34 complex I is involved in autophagy initiation (Itakura et al., 2008), VPS34 complex II was shown to regulate various processes including the formation of the autolysosome, endocytosis, LC3-associated phagocytosis (LAP), and cytokinesis (Klionsky et al., 2021). Further bound members of the VPS34 complex that have been identified are activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) (Maria Fimia et al., 2007) and nuclear receptor binding factor 2 (NRBF2) modifying both its activity and architecture (Young et al., 2016).

Upon complex activation, VPS34 phosphorylates phosphatidylinositol (PI) to phosphatidylinositol-3-phosphate (PI3P), a crucial step for the nucleation of the phagophore membrane (Backer, 2016). Several phospholipid sources for the phagophore membrane have been suggested, including the endoplasmic reticulum (ER), Golgi, ER-Golgi intermediate compartment (ERGIC) as well as mitochondria (Lamb et al., 2013). While sources might differ, the *de novo* autophagosome formation seems to be observed near the ER membrane (Nishimura et al., 2017). In yeast, it has been demonstrated that ATG9-containing lipid vesicles act as seeds for phagophore formation (Sawa-Makarska et al., 2020). In mammalians, the transmembrane protein ATG9 has two homologs, ATG9A and ATG9B, but their distinct roles have not been extensively studied. ATG9A-vesicles seem to be present close to phagophores but are only transiently associated with them (Nishimura and Tooze, 2020). PI3Ps clusters generated by VPS34 form a cytosol-facing platform that in turn triggers the recruitment of PI3P-binding proteins such as the WD-repeat protein interacting with phosphoinositide (WIPI) family members acting as effectors for the phagophore expansion (Proikas-Cezanne et al., 2015). Another PI3P-binding protein termed double FYVE domain-containing protein 1 (DFCP1)

which is localized at the ER has shown to be an effector protein during phagophore initiation but might not play a crucial role such as the WIPIs (Proikas-Cezanne et al., 2015).

1.2.1.3 Expansion of the phagophore and sealing of the autophagosome

During the expansion phase, two parallel ubiquitin-like conjugation reactions are essential. In the first step the E1-like ATG7 and the E2-like ATG10 enzymes and conjugate ATG5 to ATG12. The covalently bound ATG5-ATG12 then forms a complex with ATG16L1; and ATG5-ATG12-ATG16L together act as an E3-like enzyme in the second conjugation step (Bento et al., 2016). In the second step the E1-like enzymes ATG7 and the E2-like ATG3 enzyme, conjugate microtubule-associated protein 1 light chain 3 (MAP1LC3 or LC3, yeast Atg8) to phosphatidylethanolamine (PE) anchoring the cytosolic LC3 to the autophagosomal membrane (Bento et al., 2016). Preceding LC3 conjugation, the carboxyl terminus of pro-LC3 is cleaved by the cysteine protease ATG4B (Satoo et al., 2009). It is important to note that the LC3 family of proteins includes several isoforms, namely LC3A (two splice variants), LC3B, LC3B2, and LC3C, and a second family termed gamma-aminobutyric acid receptor-associated proteins (GABARAPs) including GABARAP, GABARAPL1, and GABARAPL2/GATE16 (Grand et al., 2011), yet their distinct functions are not well studied. The cytosolic/non-lipidated and membrane-bound/lipidated forms are referred to as LC3-I and LC3-II or GABARAP or GABARAP-PE, respectively (Klionsky et al., 2021). LC3-I to LC3-II conversion has been widely used as a marker for detecting autophagy by immunofluorescence or immunoblotting (Klionsky et al., 2021). Indeed, the lipidation of LC3 is also reversible as ATG4B is also responsible for recycling LC3 from the autophagosome (de-conjugation) (Satoo et al., 2009). Up till now, the last steps in the autophagosome maturation and its closure remain poorly studied.

*1.2.1.4 **Fusion** of the autophagosome with the lysosome*

The final step of autophagy is the fusion of the mature autophagosome with the lysosome. The outer membrane of the autophagosome and the single membrane of the lysosome fuse mediated by different tethering proteins including homotypic fusion and protein sorting (HOPS) complex, Ras-related protein 7 (RAB7), and N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes (Yu et al., 2018). This is then followed by degradation of the inner membrane of the autophagosome and subsequently the degradation of the exposed cargo by lysosomal hydrolases. Finally, the degraded cargo materials (e.g. sugars, amino and fatty acids, and nucleotides) are released back into the cytoplasm as recycled substrates for metabolic and biosynthetic pathways (White et al., 2015).

1.2.2 Selective autophagy

Originally, autophagy was assumed to be a random non-selective process of bulk degradation activated upon stress signaling. While bulk autophagy usually follows the induction of starvation, it is now clear that highly regulated selective autophagy can occur as well. The different forms of selective autophagy are named after the cargo that is engulfed (e.g., mitochondria, mitophagy; ribosome, ribophagy; lipid droplets, lipophagy; bacteria,

xenophagy) (Gatica et al., 2018). Cargo targeted for selective autophagy is recognized by binding of specific autophagy receptors and scaffolding proteins, e.g. sequestosome-1 (SQSTM1 or p62), neighbor of BRCA1 gene 1 (NBR1), nuclear dot protein 52 kDa (NDP52), or optineurin, due to a sequence containing LC3-interacting region (LIR) motifs (Gatica et al., 2018). The LIR motifs on these different cargo receptors are ubiquitin-binding and thus can recognize ubiquitinated substrates as cargo (Shaid et al., 2013). Upon cargo delivery to the autophagosome cargo receptors such as p62 and NBR1 are also degraded together with their substrates, making them useful autophagy markers besides LC3 (Klionsky et al., 2021).

1.2.3 Non-canonical forms of autophagy

There is increasing evidence of "autophagy-like" pathways that can lead to the formation of autophagosomes but only requires some components of the canonical autophagy machinery (Codogno et al., 2012). These include, for example, autophagic processes independent of ULK1, Beclin 1, VPS34, VPS34/VPS15, or different components of the ubiquitin-like conjugation system (Dupont et al., 2017). Indeed, these non-canonical forms of autophagy might capture different variations of autophagy depending on stimuli and thus question the essentiality of some of the core autophagic proteins. However, non-canonical autophagy forms might also present functionally distinct pathways and are underestimated due to shared components with canonical autophagy. One such mechanism, which has recently been more extensively defined, is LC3-associated phagocytosis (LAP). LAP is distinct from canonical autophagy in conjugating LC3 family proteins to phagosome membranes instead of *de novo* autophagosomes. While LAP shares several components with canonical autophagy, ULK complex (including ULK1/2, FIP200, ATG13, and ATG101), AMBRA1 and ATG14 are dispensable for LAP (Heckmann and Green, 2019). Instead, it depends on run domain Beclin-1-interacting and cysteine-rich domain-containing protein (RUBCN) and the production of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2 (NOX2) which are not required for canonical autophagy (Heckmann and Green, 2019).

Due to the complex involvement of such a multitude of proteins and the dynamic nature of autophagy, defining the key factors involved in the process of autophagy has been challenging. This complexity of autophagy also makes it difficult to define accurate tools/markers to capture either steady-state autophagy or the cargo turnover, also referred to as the "autophagic flux", experimentally. Thus, it is generally advised to monitor autophagy using several markers and methods (Klionsky et al., 2021). More efforts to comprehend the regulation mechanisms of the different steps of the process could be crucial to identify reliable markers of different types of autophagy including non-selective (bulk), selective, and non-canonical autophagy.

1.3 THE ROLE OF AUTOPHAGY IN CANCER

Autophagy is essential in maintaining cellular and tissue homeostasis and thus disturbance of autophagy has been implicated in several pathological conditions including neurodegenerative

disorders, diabetes and obesity, infectious disease, inflammation, and cancer (Choi et al., 2013; Rubinsztein et al., 2012; Wong and Cuervo, 2010). The role of autophagy in cancer has been evaluated extensively in the last decade with sometimes conflicting results and is thus thought to be dependent on multiple factors including the cancer stage and type, genetic background, and influence of the surrounding tumor microenvironment. Consequently, this highly context-dependent role has ignited an ongoing debate whether autophagy should be induced or inhibited for cancer therapy. This chapter aims to summarize key evidence in the literature examining the complex role of autophagy in cancer using different experimental settings.

1.3.1 Mouse models examining autophagy deficiency in cancer initiation and progression

A comprehensive study of genetic alterations of core autophagy genes across human cancers in The Cancer Genome Atlas (TCGA) database revealed that mutations in autophagy-related genes are uncommon with only a few cancer types displaying recurrent mutations of *FIP200*, *ULK4*, and *ATG7* (Amaravadi et al., 2016; Lebovitz et al., 2015). Despite the rarity of these mutations in human cancers, knock-out studies of essential autophagy genes in mice have been performed to elucidate the role of autophagy in cancer development and progression (Amaravadi et al., 2016; Mainz and Rosenfeldt, 2017). Experimental evidence of autophagy disturbance in genetically engineered mouse models (GEMMs), both sole autophagy deletion and in the background of cancer models, is summarized in **Table 1.**

1.3.1.1 Evidence from sole autophagy gene deletion

One of the first pieces of evidence of autophagy as a potential tumor-suppressive mechanism came from a study of human breast and ovarian cancers describing the monoallelic loss of *BECN1* encoding for Beclin 1 as a frequent event (Liang et al., 1999). This finding was followed up by studies in mice showing that monoallelic *Becn1* loss results in spontaneous tumor development (Qu et al., 2003; Yue et al., 2003) and increased tumorigenesis in Wnt1-driven breast cancer (Cicchini et al., 2014), indicating a tumor-suppressive function of Beclin 1. However, mice in these studies maintained a functional *Becn1* allele and displayed functional although reduced autophagy compared to controls. Furthermore, an analysis of monoallelic loss of *BECN1* in databases such as TCGA showed that it is accompanied by loss of the nearby breast and ovarian cancer tumor suppressor gene breast cancer type 1 (*BRCA1)* and thus questions the contribution of *BECN1* to tumorigenesis (Laddha et al., 2014). Nevertheless, it was later shown that decrease *BECN1* expression is an independent predictor of survival in estrogen receptor (ER)-negative breast cancer subtypes (Tang et al., 2015) indicating that *BECN1* expression levels might play a subtype-dependent tumor-suppressive role in breast cancer. In contrast, mice with systemic mosaic deletion of *Atg5* developed neoplastic changes in the liver (Takamura et al., 2011). This study suggested a tissue-dependent role of autophagy in tumor initiation but not in tumor progression as the lesions were shown to be benign (Takamura et al., 2011). This hypothesis was confirmed with a liver-specific homozygous deletion of *Atg7*, which also presented with benign hepatomas (Takamura et al., 2011).

Table 1. Autophagy impairment modulates tumor initiation and progression in genetically engineered mouse models (GEMM). *Phenotypes supporting a role of autophagy in cancer that is tumor-suppressive are marked in red and tumor-promoting blue.*

Tissue	Genetic target	GEMM model	Impact of autophagy disturbance	Reference
Whole-body	***Becn1*$^{+/-}$** *(monoallelic)*		Lymphoma, lung/liver carcinoma, increased hepatitis B virus–induced premalignant lesions, increase tumorigenesis of *Wnt1*-driven breast cancer	(Cicchini et al., 2014; Qu et al., 2003; Yue et al., 2003)
Whole-body (mosaic deletion)	***Atg5*$^{F/F}$**		Benign hepatomas	(Takamura et al., 2011)
Liver	***Atg7*$^{F/F}$**		Benign hepatomas	(Takamura et al., 2011)
Bone marrow	***Atg5*$^{F/F}$**	MLL-AF9-driven transplantation model	Delayed acute myeloid leukemia (AML) onset	(Liu et al., 2016)
Breast	***Fip200*$^{F/F}$**	PyMT	Blocked tumor development, progression, and metastasis	(Wei et al., 2011)
	***Becn1*$^{F/-}$** *(monoallelic)*	*Palb*$^{-/-}$	Delayed tumor progression	(Huo et al., 2013)
Glial cells	***Atg7, Atg13* or *Ulk1*** (shRNA)	*Kras*^{G12D}	Delayed (shRNA-*Atg7*) or blocked (shRNA-*Atg13*, shRNA-*Ulk1*) tumor development	(Gammoh et al., 2016)
Intestine	***Atg7*$^{F/F}$**	*Apc*$^{F/+}$	Decreased tumor development	(Lévy et al., 2015)
Lung	***Atg7*$^{F/F}$**	*Braf*^{V600E} *Braf*^{V600E}, *Trp53*$^{F/F}$	Initial stage: Increased tumor development Later stage: Decreased tumor growth, conversion to benign oncocytomas, improved mice survival (irrespective of *Trp53*)	(Strohecker et al., 2013)
	***Atg7*$^{F/F}$**	*Kras*^{G12D} *Kras*^{G12D}, *Trp53*$^{F/F}$	Decreased tumor growth, conversion to oncocytomas, improved mice survival (**only in absence of *Trp53***)	(Guo et al., 2013)
	***Atg5*$^{F/F}$**	*Kras*^{G12D} *Kras*^{G12D}, *Trp53*$^{F/F}$	Initial stage: Increased tumor development Later stage: Decreased tumor growth, improved mice survival (**only in presence of *Trp53***)	(Rao et al., 2014)
	***Atg7*$^{F/F}$**	*Kras*^{G12D}, *Trp53*$^{frt/frt}$	Decreased tumor growth, conversion to benign oncocytomas (autophagy was ablated systemically in mice with pre-existing tumors)	(Karsli-Uzunbas et al., 2014)
	***Atg7*$^{F/F}$**	*Kras*^{G12D}, *Lkb1*$^{F/F}$	Decreased tumor development and progression, improved mice survival	(Bhatt et al., 2019)
Melanocytes	***Atg7*$^{F/F}$**	*Braf*^{V600E}, *Pten*$^{F/F}$ *or* *Pten*$^{F/+}$	Delayed tumor progression, improved mice survival	(Xie et al., 2015)
Pancreas	***Atg5*$^{F/F}$** *or* ***Atg7*$^{F/F}$**	*Kras*^{G12D} *Kras*^{G12D}, *Trp53*$^{F/F}$ *Kras*^{G12D}, *Trp53*$^{F/+}$	Initial stage: Increased premalignant pancreatic lesions Later stage: Blocked tumor progression and improved mice survival **in presence of *Trp53*** or **hemizygous Trp53 deletion**, accelerated onset in **absence of *Trp53*** (but this does not model human disease)	(Rosenfeldt et al., 2013; Yang et al., 2014, 2011)
	***Atg7*$^{F/F}$**	*Kras*^{G12D}, *Pten*$^{F/F}$ *or* *Pten*$^{F/+}$	Reduced mice survival (only for **hemizgous *Pten* loss**)	(Rosenfeldt et al., 2017)
	***Atg4B*^{C47A}** (inducible, dominant negative)	*Kras*^{G12D}, *Trp53*$^{lox/+}$	Tumor regression but increased pancreatic metaplasia resulting in no survival benefit, intermittent *Atg4B*^{C47A} expression prolonged survival with reduced metaplasia-related deaths	(Yang et al., 2018)
Prostate	***Atg7*$^{F/F}$**	*Pten*$^{F/F}$	Delayed tumor progression and decreased tumor growth	(Santanam et al., 2016)

1.3.1.2 Evidence from GEMMS of cancer

Results from these sole autophagy gene deletions proposing that proficient autophagy prevents tumor development prompted the investigation of autophagy deficiency in multiple GEMMs of cancer driven by oncogenes (e.g. *Kras*, *Braf, Apc*) and/or loss of tumor suppressor genes (e.g. *Trp53, Pten, Palb2*). Tissue-specific deletions of *Atg7* or *Atg5* in *Kras*^{G12D}-driven lung and pancreatic cancer models increased the development of premalignant lesions but blocked tumor progression to malignant disease (Guo et al., 2013; Rosenfeldt et al., 2013; Yang et al., 2014, 2011). Similar results were also obtained upon deletion of *Atg7* in *Braf*^{V600E}-driven lung cancers (Strohecker et al., 2013). These sets of studies propose different roles of autophagy during different stages of cancer: a tumor-suppressive role of autophagy in tumorigenesis but being required for later progression from benign to malignant disease.

The pro-tumorigenic role of autophagy during tumor progression is supported by several studies including *Atg7* deletion in melanoma and prostate cancer (Santanam et al., 2016; Xie et al., 2015), *Atg5* deletion in a transplantation model of acute myeloid leukemia (AML) (Liu et al., 2016), and monoallelic *Becn1* loss in breast cancer (Huo et al., 2013). Furthermore, conditional systemic deletion of *Atg7* in mice with already established lung tumors, decreased tumor growth and converted the lesions to benign oncocytomas (Karsli-Uzunbas et al., 2014), indicating a therapeutic benefit of autophagy inhibition in cancer.

However, some studies indicate a pro-tumorigenic role of autophagy even in tumor initiation. For example, intestinal-specific *Atg7* deletion in APC$^{+/-}$ mice decreased colorectal cancer (CRC) development (Lévy et al., 2015) and shRNA-mediated knockdown of *Atg7*, *Atg13* or *Ulk1* impaired Kras-driven glioblastoma development (Gammoh et al., 2016). Similarly, *Fip200* deletion in a polyoma middle T antigen (PyMT)-driven breast cancer model suppressed both tumor initiation as well as progression and metastasis (Wei et al., 2011). Finally, *Atg7* ablation in a *Kras*-driven lung cancer model that was also liver kinase B1 (*Lkb1*)-deficient resulted in decreased tumor initiation and progression, indicating an interesting dependency on this commonly mutated tumor suppressor gene (Bhatt et al., 2019).

Indeed, the role of autophagy in tumor progression may depend on specific oncogene activation and/or loss of tumor suppressor genes. For example, *Atg7* deficiency decreased mouse survival in *Kras*-driven pancreatic cancer with hemizygous but not with homozygous *Pten* loss (Rosenfeldt et al., 2017). Similar observations have been made for the tumor suppressor p53, the most frequently mutated gene (*TP53*) in human cancer. Simultaneous deletion of *Trp53* negatively impacts anti-tumor effects of autophagy deficiency in some GEMMs (Guo et al., 2013; Rao et al., 2014; Rosenfeldt et al., 2013). It has thus been proposed activation of p53 following autophagy disturbance may play an important role and contribute to the pro-tumorigenic function of autophagy in tumor progression (White, 2016). In contrast, other models showed decreased tumor progression upon autophagy disturbance independent of *Trp53* (Strohecker et al., 2013). Also, hemizygous *Trp53* deletion in a GEMM of pancreatic cancer, which more closely models the slow onset of human disease as compared to homozygous *Trp53* deletion, did not negatively impact the positive effects of Atg5 deficiency

(Yang et al., 2014). These paradoxical observations highlight the fact that recapitulation of human cancer is very difficult in mouse models and thus conclusions on the role of autophagy depending on selected oncogenic mutations/deletion in GEMMS are rather complex to make.

Taken together, experimental evidence from autophagy disturbance in GEMMs show a context-dependent role of autophagy in tumor initiation and progression. A great limitation of these models is that they all lead to an irreversible loss of autophagic capability in the target cells/tissue which does not mimic pharmacological inhibition. In addition, none of the studies, besides the study in lung cancer (Karsli-Uzunbas et al., 2014), addressed autophagy inhibition in a fully formed tumor, which is a more clinically relevant scenario. A landmark study by Yang et al. introduced an inducible mutant Atg4b^{C74A} variant in a GEMM of pancreatic ductal adenocarcinoma (PDAC) to address these limitations and to evaluate efficacy, durability, and toxicity of genetic autophagy inhibition in a tumor in a more "drug-like" setting. This model showed elegantly that continuous autophagy inhibition decreased led to tumor regression but did not improve mice survival due to toxicities (pancreatic metaplasia). However, intermittent autophagy inhibition led to both sustained tumor growth inhibition and prolonged mice survival (Yang et al., 2018). Moreover, Yang et al. also compared effects on tumor growth inhibition when autophagy was inhibited either in the tumor cells (cell-autonomous effects) or in the host (non-autonomous effects), showing that both can blunt tumor growth. In summary, the concept of autophagy being pro-tumorigenic in already established tumors seems to be valid in two independent studies, and most findings in GEMMs point towards autophagy being crucial during progression from benign to malignant disease. The cumulative evidence from mouse models thus suggests several autophagy proteins as promising drug targets for anti-cancer therapy.

1.3.2 Tumor-intrinsic autophagy

1.3.2.1 Autophagy suppresses cancer initiation

As outlined above, several studies in GEMMs support the concept that inhibition of autophagy can promote cancer initiation, indicating that proficient autophagy suppresses oncogenic transformation. Several mechanisms by which autophagy can restrict tumorigenesis have been suggested, which are all related to maintaining cellular and tissue homeostasis and thus avoiding genetic or genomic transformation (Galluzzi et al., 2015a). For example, accumulation of enlarged, dysfunctional mitochondria and protein aggregates [e.g. p62/SQSTM1 and nuclear factor erythroid 2-related factor 2 (NRF2)] upon autophagy blockade were shown to contribute to oxidative stress and genetic/genomic instability (Mathew et al., 2007, 2009; Takamura et al., 2011). Accumulating p62/SQSTM1 can further promote inflammatory gene expression via NF-κB (Mathew et al., 2009) and in this way may cause tumor-promoting inflammation, one of the hallmarks of cancer (Hanahan and Weinberg, 2011). An interesting example of how autophagy disturbance may cause tumor-promoting inflammation in humans is the ATG16L Thr300Ala polymorphism prevalent in Crohn's disease patients (Lassen et al., 2014). It was shown that this mutation causes an impaired anti-bacterial defense that may contribute to chronic inflammation and an increased risk of

colorectal cancer (Lassen et al., 2014). Another example of how intact xenophagy may suppress cancer initiation is the increased occurrence of hepatitis B virus (HBV)–induced premalignant lesions in mice upon heterozygous *Becn1* loss (Qu et al., 2003). Thus, autophagy may restrict tumorigenesis by preventing oxidative stress, DNA damage, tumor-promoting inflammation, and defending against carcinogenic bacteria/viruses (**Figure 5**).

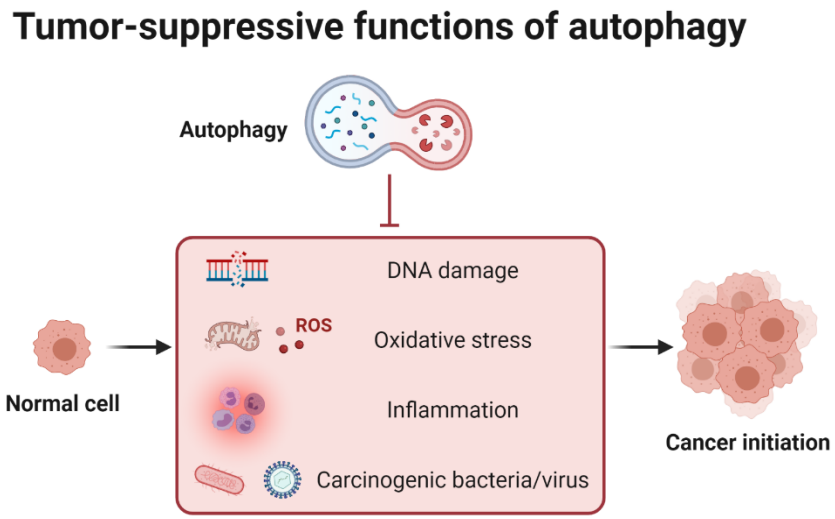


Figure 5. Mechanisms of autophagy contributing to tumor suppression.

1.3.2.2 Autophagy sustains tumor metabolism

Rapidly dividing cancer cells deregulate cellular metabolism which has been recognized as an enabling characteristic of tumorigenesis (Hanahan and Weinberg, 2011). It was discovered already back in 1926 by Otto Warburg that tumor cells require immense amounts of glucose as they undergo a metabolic switch from mitochondrial oxidative phosphorylation to aerobic glycolysis, known as "the Warburg effect" (Warburg et al., 1927). While this is an inefficient way to produce ATP, it also supplies nucleotide, lipid, and amino acid synthesis fueling cancer cell growth and proliferation (Heiden et al., 2009). It was thus long assumed that due to the glycolytic switch, mitochondrial metabolism might be dispensable or of lesser importance for tumor growth. In contrast, recent research has shown that mitochondrial metabolism is a crucial metabolic driver of cancer (Vasan et al., 2020) and that cancer cells exhibit extraordinary metabolic plasticity depending on various factors including oncogenes/tumor suppressor genes, TME, tumor stage, and the metabolism of the patient (Faubert et al., 2020).

A key function of autophagy in normal cells is the supply of substrates for cell metabolism. Given the high demand for nutrients combined with microenvironmental pressures (e.g. nutrient and growth factor limitation, hypoxia, and oxidative stress) in tumors, it is logical that autophagy plays a key role in tumor metabolism (Kimmelman and White, 2017). Due to a wide range of autophagic substrates (protein aggregates, lipids, and entire organelles), autophagy enables metabolic plasticity (Kimmelman and White, 2017). Autophagic deficiency has therefore been demonstrated to have several implications for tumor metabolism. For example, a reduction in glycolytic capacity in autophagy-deficient cells has been observed in several

studies (Guo et al., 2013; Lock et al., 2014; Wei et al., 2011). Another common finding is that autophagy disturbance results in the accumulation of defective mitochondria impairing *Kras*- and *Braf*-driven tumor progression, suggesting that autophagy sustains the mitochondrial quality and metabolic function (Guo et al., 2013; Rao et al., 2014; Strohecker et al., 2013). Indeed, a metabolic energy crisis in cancer cells caused by autophagy disturbance has been reported. For instance, metabolic tracing in *Kras*-driven lung cancer cells further revealed that autophagy is crucial in maintaining nucleotide pools during starvation (Guo et al., 2016). Moreover, in a *Kras*-driven, *Lkb1*-deficient lung cancer model *Atg7* deficiency caused excessive fatty acid oxidation (FAO) that depleted lipid reserves due to decreased amino acid supply (Bhatt et al., 2019).

Collectively, these studies suggest that autophagy inhibition may have detrimental effects on tumor metabolism. Thus, combining autophagy inhibition with therapeutic interventions that target tumor metabolism may hold clinical promise. An interesting therapeutic approach to further limit substrate supply to tumor cells may be to combine autophagy inhibition and calorie restriction (CR). CR is the reduction of calorie intake by around 30% and was shown to have beneficial effects in several preclinical cancer models and cancer patients when implemented before chemotherapy (Cozzo et al., 2020; O'Flanagan et al., 2017). CR in an *Atg5*-deficient *Hras*-transformed xenograft tumor model remarkably reduced tumor growth compared to controls, suggesting that limiting both extra- and intracellular nutrient availability may represent a promising therapeutic strategy (Lashinger et al., 2016).

1.3.2.3 Cytoprotective autophagy contributes to therapy resistance

As summarized in chapter 1.1.2, overcoming intrinsic or acquired drug resistance is a key challenge in the treatment of cancer patients. Cellular stress responses (e.g. energy stress, hypoxia, oxidative stress) trigger mTORC1 inhibition or AMPK activation further downstream inducing autophagy as a survival mechanism (White et al., 2021). While the rapid proliferation and hostile conditions in the tumor microenvironment may already induce these cellular stress responses, cytotoxic therapies can considerably amplify them. It is thus not surprising that induction of autophagy is a key resistance pathways in response to anti-cancer therapies (Amaravadi et al., 2016, 2019; Lim and Murthy, 2020).

Several reports have described a cytoprotective role of autophagy in chemotherapy. For instance, autophagy inhibition was shown to increase response to cyclophosphamide in Myc-driven lymphoma (Amaravadi et al., 2007). Autophagy inhibition increased sensitivity to chemotherapeutics vinblastine or cisplatin in $Braf^{V600E}$ mutated brain tumors (Levy et al., 2014). Equally, autophagy was shown to contribute to acquired cisplatin resistance (Schlütermann et al., 2017; Yu et al., 2014).

Autophagy also modulates response to radiation therapy. A recent report showed that autophagy inhibition increases responses to irradiation in immunocompetent breast cancer models (Yamazaki et al., 2020). Mechanistically, Yamazaki et al. demonstrated that autophagy

inhibition leads to accumulation of mitochondrial DNA and STING pathway activation which in turn triggers a systemic type I IFN signaling activating anti-tumor immunity.

Autophagy was also found to contribute to resistance to several targeted therapies. Importantly, assessing autophagy dependencies in the context of targeted therapies may also pinpoint patient populations with specific mutations eligible for autophagy inhibition. One of the most studied targeted therapies in combination with genetic or pharmacological autophagy inhibition are inhibitors of the MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway). Autophagy inhibition was shown to overcome BRAF inhibitor resistance in BRAF$^-$mutant brain cancer (Levy et al., 2017, 2014) or melanoma (Ma et al., 2014). This was recently followed by several reports showing that pharmacological or genetic autophagy inhibition synergizes with BRAF, MEK, or ERK inhibitors in RAS-driven cancers including colorectal and pancreatic cancer or melanoma (Bryant et al., 2019; Kinsey et al., 2019; Lee et al., 2019). Indeed, this observation is in line with the evidence from GEMMs suggesting that in *Braf*- or *Kras*-driven tumors are susceptible to autophagy disturbance (see **Table 1**). Autophagy inhibition has further been shown to improve sensitivity to receptor tyrosine kinase (RTK) inhibitors in various cancer types including bladder (Chen et al., 2018; Kang et al., 2017), ovarian (DeVorkin et al., 2017), pancreatic (Wiedmer et al., 2017) and breast cancer (Dyczynski et al., 2018).

In conclusion, cytoprotective autophagy enables cancer cell adaptation to all pillars of anti-cancer therapy including chemotherapy, radiotherapy, and targeted therapy. Blocking autophagy induction may therefore represent a promising strategy to fight drug resistance and improve clinical responses.

1.3.2.4 Cancer cell autophagy contributes to immune evasion

The evasion of cancer cells from immunosurveillance by avoiding the recognition by immune cells or preventing immunological eradication has been established as a universal hallmark of cancer (Hanahan and Weinberg, 2011). Cancer immunogenicity may be improved as a "side effect" of conventional chemotherapy, targeted therapy, or radiation as dying cancer cells may release danger signals that trigger adaptive immune responses (Galluzzi et al., 2015b). This process is also referred to as immunogenic cell death (ICD) (Galluzzi et al., 2016). It was indicated that autophagy-deficiency may limit benefits of immunogenic cell death by decreasing ATP release (Michaud et al., 2011) suggesting that autophagy is crucial for immunogenic responses to cancer therapies. More recent work, however, suggests the opposite. Autophagy-deficiency in murine 4T1 or B16 tumors did not impair immune responses to chemotherapy doxorubicin (Starobinets et al., 2016). Furthermore, emerging evidence indicates that tumor cell autophagy may instead contribute to immune evasion and thus limit immunogenicity. Several immune evasion mechanisms have been proposed. Firstly, autophagy has been reported to restrain the secretion of proinflammatory signals and thus the recruitment and activation of innate and adaptive immunity (Deretic and Levine, 2018). This is achieved by degrading damaged proteins, organelles, or intracellular pathogens, which otherwise trigger activation of innate immunity pathways such as inflammasome activation or type I IFN

signaling (Deretic and Levine, 2018). Accordingly, several studies have demonstrated that autophagy deficiency activates an anti-tumor immune response by increasing cancer cell secretion of proinflammatory cytokines (Mgrditchian et al., 2017; Wei et al., 2011; Yamazaki et al., 2020). For instance, targeting *Becn1* resulted in increased secretion of chemokine CCL5 and subsequent recruitment of NK cells increasing tumor rejection in mouse models (Mgrditchian et al., 2017). Secondly, tumor-intrinsic autophagy has been shown to directly limit T and NK cell-mediated cytotoxicity (Baginska et al., 2013; Lawson et al., 2020; Noman et al., 2011). Mechanistically, Baginska et al. revealed that hypoxia-induced autophagy degraded Granzyme B released by NK cells. Finally, targeting of tumor cell autophagy also revealed a crucial role in limiting antigen presentation and thereby evade T cell-dependent immunity (Deng et al., 2021; Yamamoto et al., 2020). Mechanistically, autophagy inhibition was shown to increase expression of major histocompatibility complex (MHC) class I on the surface of pancreatic cancer cells (Yamamoto et al., 2020) and immunoproteasome activity in LKB1-mutant lung cancer cells (Deng et al., 2021). These studies suggest that inhibition of autophagy could be a potent strategy to increase both tumor recognition and immune cell infiltration which are crucial predictors of response to anti-cancer immunotherapies.

1.3.3 Tumor-extrinsic autophagy

Comparing the effects of inhibiting autophagy either in the tumor or systemically in preclinical models, highlights the tumor-supportive role of host autophagy. This chapter will summarize key studies investigating mechanistic insights into how tumor-extrinsic autophagy may contribute to tumor growth and progression.

1.3.3.1 Host autophagy provides nutrients to tumor cells

It has been shown by several studies that tumor metabolism is not only supported by tumor-intrinsic autophagy but also tumor-extrinsic autophagy. Transplantation of tumors from autophagy-deficient into autophagy-proficient *Drosophila melanogaster* (often referred to as fruit fly) changed tumor fate from dormant to proliferative, partly due to the nutrient supply of the autophagy-proficient host (Katheder et al., 2017). Similar observations have been made in mouse models. Host autophagy-deficiency resulted in arginine-degrading enzyme arginase I (ARG1) release from the liver decreasing circulating arginine levels and thereby blocking tumor growth (Poillet-Perez et al., 2018). This effect was rescued by supplying dietary arginine suggesting that host autophagy fuels tumor growth by providing arginine. Another type of autophagy-dependent “symbiosis” was described for pancreatic cancer cells and stroma-associated pancreatic stellate cells (PSCs) (Sousa et al., 2016). This work demonstrated that PSC autophagy supplies pancreatic cancer cell metabolism by secreting alanine. Co-injection of pancreatic cancer cells with PSCs (using both human xenografts in nude mice or a syngeneic model), further revealed that PSCs autophagy contributes to tumor growth and decreases mouse survival. Together, these studies show that host autophagy or the surrounding TME can supply tumor metabolism and growth by feeding amino acids such as arginine and/or alanine.

1.3.3.2 Autophagy in tumor-infiltrating immune cells

With the considerable success of immune-modulating agents such as anti-PD-1/PD-L1 therapy, activation or inhibition of cellular pathways that may enhance anti-tumor immune response or relieve immune suppression have gained significant research interest. A functional T cell response (recognition of tumor antigens, activation of effector function, and memory formation) is crucial for optimal ICB effectiveness (Jenkins et al., 2018). Several studies have shown that autophagy is critical for T cell development and function by regulating metabolism and mitochondrial maintenance (Deng et al., 2021; Parekh et al., 2013; Pua et al., 2009; Stephenson et al., 2009). Interestingly, impairing T cell autophagy during acute infection did not affect the effector function of T cells but was required for T cell memory formation (Puleston et al., 2014; Xu et al., 2014). The role of autophagy in the anti-tumor responses of T cells remains understudied. Autophagy was shown to be required for the survival of naïve T cells in the TME (Xia et al., 2017) and insufficient mitophagy levels contributed to the exhaustion of tumor-infiltrating $CD8^+$ T cells (Yu et al., 2020). T cell autophagy may thus play a positive role in promoting metabolic fitness and function required for anti-tumor responses. In contrast, anti-tumor T cell responses were not impaired in 4T1 breast cancer and B16 melanoma models upon treatment with chloroquine (Starobinets et al., 2016). In addition, conditional deletion of *Atg5* in $CD8^+$ T cells in mice was shown to enhance anti-tumor response (DeVorkin et al., 2019). Mechanistically, DeVorkin et al. demonstrated that autophagy-deficiency caused $CD8^+$ T cells to increase glycolysis while retaining oxidative phosphorylation, subsequently leading to transcriptional changes supporting an effector memory phenotype. Consequently, this study suggests that autophagy may restrain T cell glycolysis and thereby hampers an effective anti-tumor response (DeVorkin et al., 2019). Interestingly, also deletion of *Atg5* in T_{reg} cells in mice caused T_{reg} cell apoptosis resulting in an increased anti-tumor response as immunosuppression by T_{regs} was relieved (Wei et al., 2016). To conclude, interfering with T cell autophagy may have both pro- and anti-tumorigenic effects likely depending on T cell phenotype, timing, and duration of autophagy inhibition and requires further investigation.

Beyond T cell autophagy, autophagy in tumor-infiltrating myeloid cells (macrophages, monocytes, dendritic cells, and granulocytes) has also been investigated in several studies. Macrophages and dendritic cells shape the TME by secreting inflammatory cytokines and by a professional antigen presentation resulting in the activation of adaptive immunity. As outlined in Chapter 1.3.2.4, tumor-intrinsic autophagy was shown to limit the secretion of pro-inflammatory cytokines, and myeloid-cell autophagy may act similarly. Indeed, mice lacking GABARAP, the LC3 family member, exhibit a heightened anti-tumor immune response due to increased secretion of IL-1β, IL-6, and IL-2 by macrophages as well as IFN-γ by lymphocytes (Salah et al., 2016) suggesting that ablation of immune cell autophagy may increase inflammation in the TME. Inhibition of non-canonical autophagy by targeting *Beclin1*, *Atg5*, *Atg7*, or *Rubcn* (which are all involved in LAP) in myeloid cells decrease tumor growth in tumor-bearing mice (Cunha et al., 2018). The authors found that impairing LAP function shifted macrophage polarization towards an anti-tumorigenic M1 phenotype, which engages in

type I interferon production and T cell activation. Remarkably, the same mechanism of an M1 phenotypic shift underlying an increased anti-tumor T cell response was also reported for chloroquine treatment as a single agent (Chen et al., 2018b) or in combination with anti-PD-1 therapy (Sharma et al., 2020). The latter study by Sharma et al. also demonstrated that combination treatment decreased tumor-infiltrating myeloid-derived suppressor cells (MDSCs), a myeloid cell subset that was previously shown to depend on autophagy for their immunosuppressive function (Alissafi et al., 2018). In summary, targeting proteins involved in autophagy (and LAP) in myeloid cells may benefit anti-tumor immunity by several mechanisms including increasing inflammation, T cell activation as well as relieving immunosuppressive mechanisms.

To conclude, autophagy may benefit cancers by contributing to metabolic fitness, survival in stress conditions, drug resistance, and immune evasion by both tumor-intrinsic and tumor-extrinsic mechanisms. A graphical summary of these pro-tumorigenic functions of autophagy can be found in **Figure 6.**

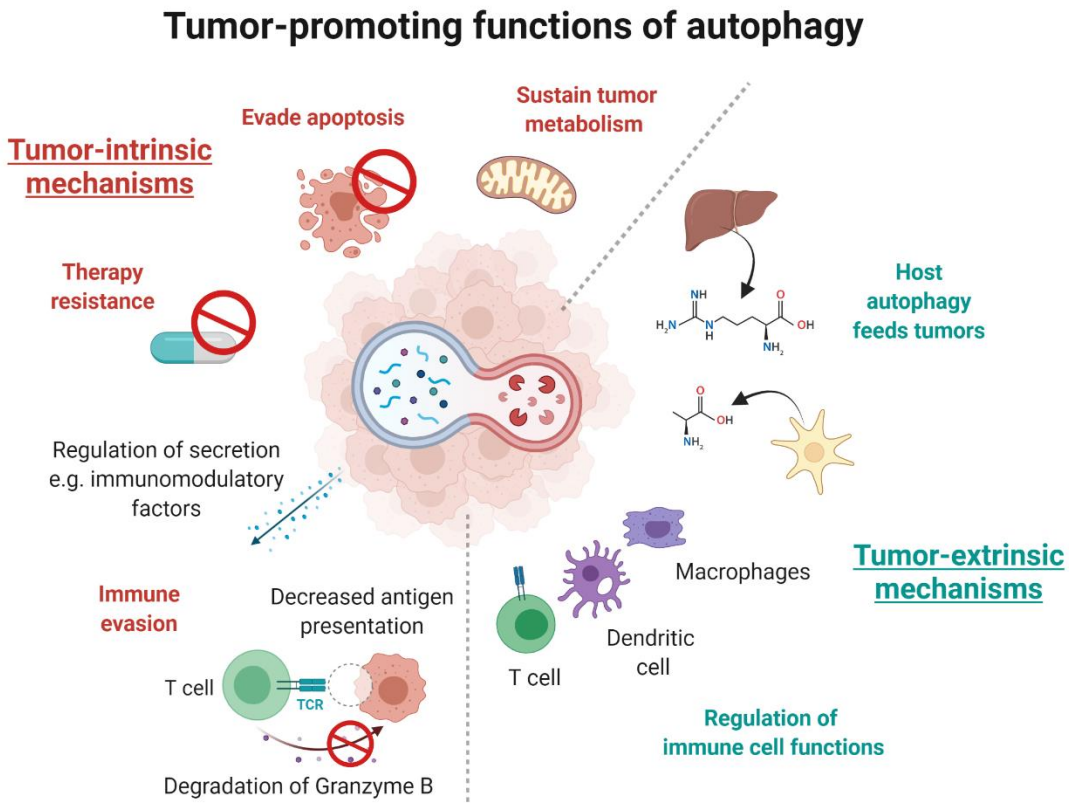


Figure 6. Autophagy promotes tumor growth and progression by intrinsic and extrinsic mechanisms.

1.4 PHARMACOLOGICAL TARGETING OF AUTOPHAGY FOR CANCER THERAPY

Given the increasing evidence of a tumor-promoting role of autophagy from studies suppressing core autophagy genes genetically, developing pharmacological autophagy

inhibitors for cancer therapy has received renewed attention. As described above, the regulation of autophagy is complex and involves several steps and a multitude of proteins. Many of the core autophagic proteins have other functions and/or are not easily druggable. Thus, so far only a limited number of targets have been selected for the development of autophagy inhibitors. The most prominent drugs including Chloroquine derivates, ULK1 inhibitors, and VPS34 inhibitors are described in more detail below. Other proposed approaches target the cysteine protease ATG4 (Fu et al., 2019; Kurdi et al., 2017), which has not yet lead to sufficiently selective and potent inhibitors, and more recently also the E1-like enzyme ATG7 (Huang et al., 2020).

1.4.1.1 Chloroquine and chloroquine derivates

To date, the only clinically tested autophagy inhibitors are the repurposed anti-malaria drug chloroquine (CQ) and its derivate hydroxychloroquine (HCQ). Recently completed Phase 2 clinical trials show promising results for HCQ treatment with regards to safety and preliminary anti-tumor efficacy in patients with advanced renal cell carcinoma and pancreatic cancer (Haas et al., 2019). However, it failed to extend overall survival in pancreatic cancer (Karasic et al., 2019). Despite its clinical availability, the limited potency of HCQ has fueled the pursuit of more potent derivates such as DC661 (Rebecca et al., 2017). The mechanisms of action of CQ are, however, not well understood. Contrary to the preceding notion that CQ increases lysosomal pH as a nonspecific weak base, it has been observed that CQ decreased the fusion of the autophagosome with the lysosome (Mauthe et al., 2018). CQ is thereby distinct from the vacuolar ATPase inhibitor Bafilomycin A1 (BafA1) which increases lysosomal pH and is commonly used in *in vitro* assay to block autophagy (Mauthe et al., 2018). Recently, lysosomal palmitoyl-protein thioesterase 1 (PPT1) has been discovered as a molecular target of CQ derivates (Rebecca et al., 2019) suggesting they may act as targeted therapies. Further investigations into the precise mechanisms of CQ derivates are needed to address the concerns regarding their specificity as autophagy inhibitors. Consequently, it cannot be excluded that observed anti-cancer effects of CQ derivates may also be attributed to other mechanisms than autophagy inhibition.

1.4.1.2 ULK1 inhibitors

In efforts to find more specific autophagy inhibitors, targeting the earlier steps of autophagy have been deemed promising. The first step in the autophagic cascade is the activation of the ULK1 complex followed by activation of the VPS34 complex. The development of kinase inhibitors has been proven as fruitful translating to many FDA-approved kinase-targeted cancer therapies. This makes the serine/threonine kinases ULK1 and the lipid kinase VPS34 very attractive drug targets. Several selective and potent small molecule ULK1 inhibitors have been reported including MRT68921 (Petherick et al., 2015); SBI-0206965 (Egan et al., 2015), and ULK-101 (Martin et al., 2018), all simultaneously targeting ULK2 due to the high degree of kinase similarity. Direct comparison in an *in vitro* kinase panel revealed superior potency and selectivity of ULK-101 as compared to SBI-0206965 (Martin et al., 2018). ULK1/2 inhibitors have shown *in vitro* anti-cancer efficacy in the context of pharmacological mTOR inhibition

and nutrient deprivation in KRAS-mutant lung cancer and FLT3-ITD-mutated acute myeloid leukemia (Egan et al., 2015; Hwang et al., 2020; Martin et al., 2018). Interestingly, MRT68921 was recently shown to activate an anti-tumor immune response in LBK1-mutant lung cancer and presented promising efficacy in combination with anti-PD-1 therapy (Deng et al., 2021). The unpublished inhibitor DCC-3116 developed by the company Deciphera Pharmaceuticals is the first-in-class ULK1 inhibitor entering the clinical stage. DCC-3116 as a single treatment and in combination with the MEK inhibitor Trametinib is currently being tested in a first-in-man Phase I clinical trial for the treatment of advanced or metastatic solid tumors with RAS or RAF mutations (NCT04892017). The design of this clinical study follows the literature describing a synergy of MAP kinase pathway inhibitor in combination with autophagy inhibition in mutant RAS-carrying cancers (Bryant et al., 2019; Kinsey et al., 2019; Lee et al., 2019), and it will be interesting to follow-up these studies in the future.

1.4.1.3 VPS34 inhibitors

As mentioned above VPS34, a lipid kinase phosphorylated upon ULK1 complex activation is a prominent drug target to inhibit autophagy at an early stage and the subject of this thesis. Over the years, several highly potent and selective VPS34 inhibitors (VPS34i) have been developed including SAR405 (Ronan et al., 2014), PIK-III (Dowdle et al., 2014), VPS34-IN1 (Bago et al., 2014), Compound 31 (Pasquier et al., 2015), and Autophinib (Robke et al., 2017). In this thesis, the preclinical characterization of SB02024, developed by Sprint Bioscience, will be presented (Dyczynski et al., 2018). The cellular functions of VPS34 and the use of VPS34i inhibitors for cancer therapy will be introduced in the following chapter.

1.4.2 VPS34 as a target in cancer therapy

The VPS34 complex I regulates the crucial initiating step of the autophagosome formation through generating PI3P on the nucleating phagophore. Due to this important function and a good "druggability", drug development efforts in the quest to discover autophagy inhibitors have been primarily focused on targeting the lipid kinase activity of VPS34.

Cellular characterization of the first small molecule VPS34i termed SAR405 revealed that VPS34 inhibition affects not only autophagy but also late endosome-lysosome compartments (Ronan et al., 2014). As outlined in chapter 1.2.1.2., VPS34 as part of VPS34 complex II has autophagy-independent functions in other vesicle trafficking processes such as endocytosis (Backer, 2016; Herman and Emr, 1990). Mechanistically, it has been shown that VPS34 regulates the maturation of Rab5-positive early and Rab7-positive late endosomes via the GTPase Armus (Backer, 2016; Jaber et al., 2016). Notably, genetic or pharmacological targeting of either VPS34 or the downstream PI3P-binding effector protein PIKfyve results in an accumulation of dilated and translucent endosomal structures; a phenomenon referred to as "aberrant vacuolation" which is reversible by V-ATPase inhibitor Bafilomycin A1 treatment (Ikonomov et al., 2018; Saveanu et al., 2016). The VPS34 complex II in association with Rubicon further participates in the non-canonical autophagy process LAP (Heckmann and

Green, 2019), cytokinesis (Thoresen et al., 2010), and autophagosome maturation (Liang et al., 2008). The structure and function of the VPS34 complexes are shown in **Figure 7**.

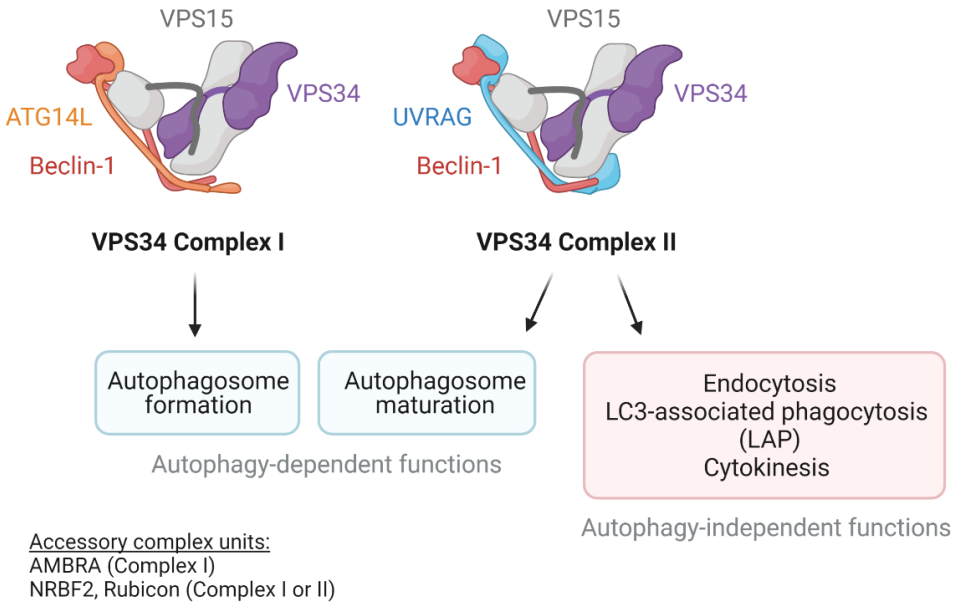


Figure 7. Structure and function of the VPS34 complex I and II (adapted from Ohashi et al. 2019).

Genetic knock-out of the *Pik3c3* gene coding for VPS34 in mice resulted in early embryonic lethality. For this reason, the physiological role of VPS34 has been quite extensively explored using different tissue-specific knockout models. For instance, VPS34 was found to play a key role in heart (Jaber et al., 2012), liver (Jaber et al., 2012), kidney (Bechtel et al., 2013; Grieco et al., 2018), neuron (He et al., 2019; Wang et al., 2011; Zhou et al., 2010), muscle (Reifler et al., 2014), blood platelet (Valet et al., 2017), T cell (Parekh et al., 2013; Willinger and Flavell, 2012), and dendritic cell function (Parekh et al., 2017).

Given the vast literature on the pro-tumorigenic role of autophagy, VPS34 inhibition poses a promising treatment strategy for cancer management. However, it remains poorly understood how exactly VPS34i could be used in a clinical setting. For this reason, several questions regarding the therapeutic use of VPS34i in cancer are addressed in this thesis. Firstly, it needs to be comprehended which cancer types may benefit from VPS34i treatment and secondly, which existing anti-cancer therapies may result in synthetic lethality when combined with VPS34i. A link between VPS34 and breast cancer has been proposed (Hirsch et al., 2010; Jiang et al., 2017). Furthermore, the initial publication of SAR405 revealed a synergism with mTOR inhibitor everolimus in lung and renal cell carcinoma cells (Ronan et al., 2014). Finally, and most importantly, based on the above-mentioned studies demonstrating the important physiological function of VPS34, side effects that may arise from VPS34i need to be properly investigated using preclinical toxicology models. Genetic knock-out models give only a limited insight into possible side effects of pharmacological VPS34 inhibition especially since VPS34 acts in complexes that are disrupted upon genetic knock-out. Indeed, this issue has been elegantly addressed in a heterozygous VPS34 kinase-dead knock-in mouse model which revealed a surprisingly limited pathology (Bilanges et al., 2017). Remarkably, these mice

displayed enhanced insulin sensitivity and altered mitochondrial metabolism in the liver, suggesting VPS34 as a potential target in metabolic disease (Bilanges et al., 2017). These studies motivate the evaluation of VPS34 as an anti-cancer therapy target and as a focus of this thesis.

1.4.3 Challenges to progress of autophagy inhibitors to the clinic

As described above, numerous studies investigating the manipulation of autophagy in established cancers and cancer progression provide strong evidence that autophagy inhibition might be beneficial for the treatment of cancer. However, most of the studies investigating the role of autophagy in cancer are somewhat limited to mouse biology. Future work in systems that can more closely mimic human biology and pathology such as patient-derived xenografts or *ex vivo* models will contribute to a deeper understanding of the relevance of autophagy inhibition or activation for the treatment of cancer patients.

Another limitation in the clinical pursuit of autophagy drugs is the lack of accurate biomarkers that may reveal the pharmacodynamics of autophagy modulation in a patient (Lim and Murthy, 2020). A reliable biomarker would be a useful tool both to determine autophagy levels at the beginning of treatment for patient stratification and during treatment to assess efficacy and disease progression. Yet, the dynamic nature of the autophagic flux makes it difficult to identify a single reliable biomarker and warrant simultaneous measurement of several markers and even the use of several methods (Klionsky et al., 2021).

Finally, autophagy is a rather complex pathway involving a multitude of proteins making it challenging to choose a proper target for the treatment of disease. Many promising autophagy drugs, including lysosomal inhibitors, ULK1, and VPS34 inhibitors, show autophagy-independent functions, which need to be considered carefully. Limited potency and selectivity as well as lack of *in vivo* efficacy have yet been obstacles to the clinical use of current autophagy inhibitors. Thus, the development of novel autophagy inhibitors is essential not the least as useful tool compounds to investigate the relevance of targeting different core-autophagy proteins. In this thesis, we present data showing that systemic use of VPS34i in combination with cytotoxic drugs or immunotherapy may present an effective therapeutic approach for anti-cancer therapy.

2 AIMS OF THIS THESIS

The general aim of this thesis was to evaluate the efficacy and impact of blocking autophagy using VPS34 inhibitors for the treatment of cancer.

SPECIFIC AIMS:

Paper I: To provide a complete biochemical and preclinical characterization of novel small molecule inhibitors of VPS34. To identify anti-cancer drugs that activate autophagy as a drug resistance mechanism and that in combination with VPS34 inhibitor treatment increase their anti-cancer efficacy.

Paper II: To examine whether the anti-cancer efficacy of VPS34 inhibition is mediated by the immune system and to further assess any effects of VPS34 inhibitors on the anti-tumor immune response.

Paper III: To reveal underlying cellular signaling mechanisms that trigger cytokine production and tumor inflammation upon VPS34 inhibitor treatment and to identify rational combination therapies for a synergistic anti-cancer response.

3 RESULTS & DISCUSSION

3.1 PAPER I

Targeting autophagy by small molecule inhibitors of vacuolar protein sorting 34 (VPS34) improves the sensitivity of breast cancer cells to Sunitinib

Background

Drug resistance is still a major problem in cancer management limiting responses to chemotherapy and targeted therapy (Vasan et al., 2019). Autophagy has been implicated as a key drug resistance mechanism (Amaravadi et al., 2016). As a result, the pharmacological targeting of autophagy to overcome tumor resistance and increase anti-cancer treatment efficacy has been considered a promising strategy (Levy et al., 2017).

Results & Discussion

High-content screening microscopy detects autophagy-inducing drugs

In this study, we aimed to perform a high-content cellular drug screening as an unbiased approach to identify oncology drugs that induce autophagy and may increase their cytotoxicity in combination with autophagy inhibition. To readily detect autophagic flux in cells, we used the osteosarcoma cell line HOS stably expressing a GFP-LC3 reporter and measured GFP-LC3+ puncta in the presence or absence of the lysosomal inhibitor Bafilomycin A1 (BafA1) at different time points. The mTOR inhibitor Ku-0063794 (KU) was used as a positive control for autophagy induction. To obtain results of translational significance, we chose to screen the FIMM drug library containing in total 306 preclinical and FDA-approved anti-cancer drugs available at physiologically relevant concentrations. The primary screening was performed at a set dilution of the drug library followed by a confirmation screening including three different concentrations. The results from the primary screening correlated well with the secondary screening with only 8 drugs failing to reproduce their effect on the autophagic flux. Taken together a total of 114 drugs were identified as autophagy inducers, making up more than a third of the drug library.

Cytotoxicity screen identifies drugs with diminished efficacy in autophagy-proficient cells

Next, we performed a cytotoxicity screen to test whether the identified drugs affect cell viability in an autophagy-dependent manner. We increased treatment time (48 and 72h) and pre-transfected the cells with either control scrambled siRNA or siRNA targeting ATG7 and VPS34, two core-autophagy genes. Inhibition of autophagy led to a decreased cell viability in cells simultaneously treated with 16 out of 116 drugs that induced autophagy in the first screen. This provided strong evidence that these 16 drugs induce cytoprotective autophagy.

Sunitinib and Erlotinib modulate autophagic flux in breast cancer cells

Autophagy has been described as a key mechanism of tumor progression and therapy resistance in breast cancer (Cook et al., 2014; Lefort et al., 2014; Maycotte et al., 2015; Sun et al., 2011;

Wei et al., 2011). We thus decided to further examine the efficacy of the identified autophagy inducers in breast cancer cells. To begin with, we validated the autophagy-inducing effect of the hits from the cytotoxicity screening in the triple-negative breast cancer cell line MDA-MB-231 stably transduced with GFP-LC3. We confirmed 6 out of the 16 drugs as autophagy inducers in the breast cancer cell line. Among those, we chose to further investigate Sunitinib and Erlotinib, two receptor tyrosine kinase inhibitors that demonstrated limited clinical efficacy in breast cancer patients (Elgebaly et al., 2016; Lluch et al., 2014) thus indicating the possibility of autophagy as a mechanism of resistance (Amaravadi et al., 2016). By treating at a larger dose range, we discovered that Sunitinib increased autophagic flux in a dose-dependent manner and to a higher extend than Erlotinib. In contrast to its autophagy-inducing effect at lower micromolar concentrations, Sunitinib blocked autophagy at the highest concentration of 20 µM. This dose-dependent effect was also confirmed in the breast cancer cell line MCF-7 stably expressing GFP-LC3 suggesting that high concentrations of Sunitinib may trigger lysosomal accumulation (Giuliano et al., 2015; Gotink et al., 2011) leading to autophagy inhibition.

Preclinical characterization of the novel VPS34 inhibitor SB02024 as an autophagy blocker

Based on the observed autophagy-inducing capabilities of Sunitinib and Erlotinib, we wanted to examine whether pharmacological autophagy blockade could benefit their anti-cancer efficacy in breast cancer. To block autophagy, we applied for the first time the potent and specific small molecule VPS34 inhibitor (VPS34i) SB02024, developed by Sprint Bioscience. Over the years several VPS34 inhibitors have been developed and tested preclinically in cancer cell lines, highlighting the relevance of the target (Pasquier, 2016; Robke et al., 2017). Treatment with SB02024 in breast cancer cells resulted in a blocked autophagic flux. Daily oral dosing of SB02024 further showed significant anti-tumor efficacy in MDA-MB-231 and MCF-7 xenograft models and good tolerability in mice. To our knowledge, this was the first study demonstrating the anti-tumor efficacy of VPS34i in a preclinical breast cancer model and supports VPS34 as a promising target in anti-cancer therapy.

VPS34 inhibition enhances Sunitinib efficacy in breast cancer cells

Combination with SB02024 significantly increased sensitivity of breast cancer cells to Sunitinib treatment as indicated by decreased cell viability and cell survival in a clonogenic assay. We confirmed the results by treating in parallel with PIK-III, a VPS34i developed by Novartis with a similar potency and selectivity profile, and Chloroquine (CQ), a lysosomal inhibitor currently tested in clinical trials as an autophagy inhibitor (Onorati et al., 2018). Our findings are in line with other studies demonstrating that targeting autophagy may increase the cytotoxicity of Sunitinib in clear cell ovarian cancer and pancreatic neuroendocrine tumors (DeVorkin et al., 2017; Wiedmer et al., 2017). One limitation of our study is the limited number of cell lines that we included in the experiments. On the other hand, it is interesting to note that the two breast cancer cell lines MDA-MB-231 and MCF-7, of triple-negative and luminal A subtype, respectively, displayed rather similar sensitivity to VPS34 inhibition *in vivo* and *in vitro,* and a similar pattern of response when a combination with Sunitinib was applied. This implies that (1) autophagy inhibition may benefit more subtypes of breast cancer than

previously implied, namely that triple-negative breast cancer cells are especially "autophagy-addicted" (Lefort et al., 2014; Maycotte et al., 2014); and (2) autophagy inhibition could be beneficial in combination with Sunitinib independently of a subtype. On the other hand, the effects of erlotinib in combination with VPS34i were significant only in MCF-7 cells in most of the assays used. We also recognize the fact that classical monolayer cell culture is a poor predictor of *in vivo* responses. In efforts to develop more translationally applicable *in vitro* systems, growing cancer cell lines as multicellular spheroids (MCS) in non-adherent conditions is more relevant. MSC recapitulate space, nutrient, and oxygen limitations that are inherent to rapidly forming tumors and thus give rise to a heterogeneous cell population (Hirschhaeuser et al., 2010; Weiswald et al., 2015). In addition, the 3D tumor-like architecture of MCS may impede drug penetration and can therefore predict *in vivo* drug resistance due to poor drug properties (Friedrich et al., 2009). We thus assessed the efficacy of the Sunitinib/VPS34i combination treatment in MDA-MB-231 and MCF-7 cells grown as MCS and could confirm its efficacy in this model system. Remarkably, no additional effect of the Sunitinib/CQ combination treatment as compared to CQ alone was observed in the MCS, which may indicate its lack of potency in acidic or hypoxic environments (Pellegrini et al., 2014).

Significance

In summary, our study demonstrates anti-cancer efficacy of the novel VPS34i SB02024 in breast cancer models. We further show encouraging results suggesting that pharmacological VPS34 inhibition in breast cancer may further potentiate the anti-cancer effect of receptor tyrosine kinase inhibitors that induce autophagy as a resistance mechanism such as the tyrosine kinase inhibitor Sunitinib.

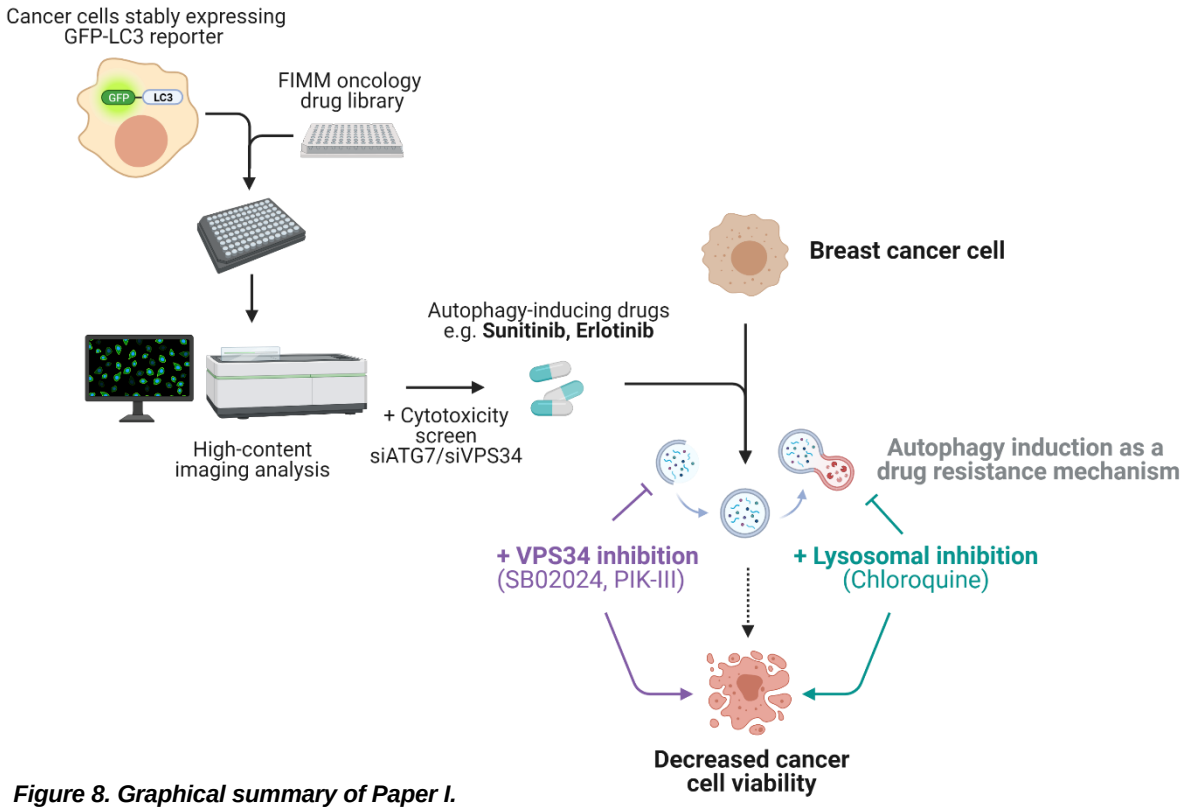


Figure 8. Graphical summary of Paper I.

3.2 PAPER II

Inhibition of VPS34 reprograms cold into hot inflamed tumors and improves anti–PD-1/PD-L1 immunotherapy

Background

The targeting of immune checkpoints PD-1 or PD-L1 using monoclonal antibodies has proven to be a highly effective type of cancer immunotherapy (Robert, 2020). Although some patients show remarkable therapeutic responses, most patients either fail to ever respond or develop resistance to ICB (Jenkins et al., 2018). The mechanisms that contribute to resistance to ICB are therefore under current investigation. The presence of functional T cells in the tumor microenvironment, also described as an inflamed or "hot" tumor, has been shown to be a major determinant of ICB response (Galon and Bruni, 2019). Targeting of autophagy has been demonstrated to control tumor growth by regulating cancer cell susceptibility to immune cell killing (Baginska et al., 2013; Noman et al., 2011) and by increasing NK cell infiltration (Mgrditchian et al., 2017). Treatment with small molecule VPS34i presents a novel pharmacological tool to block autophagy and shows promising anti-cancer efficacy in breast cancer models (Dyczynski et al., 2018; Ronan et al., 2014). The purpose of this study was therefore to investigate possible immunomodulatory effects of VPS34i and to examine a possible combination therapy approach with ICB.

Results & Discussion

Targeting VPS34 triggers an anti-tumor response dependent on T and NK cells

To evaluate the impact of VPS34 inhibition in immunocompetent mouse models, we targeted VPS34 both genetically and pharmacologically. To do this we applied shRNA-mediated knockdown (shVPS34) or oral dosing of either VPS34i SB02024 (Dyczynski et al., 2018) or SAR405 (Ronan et al., 2014) in B16-F10 melanoma or CT26 colorectal cancer (CRC) models. We show that genetic or pharmacological VPS34 inhibition resulted in a significantly decreased tumor growth and weight and improve mice survival. To understand if the observed anti-tumorigenic effects of targeting VPS34 could be due to an altered tumor immune response, we examined immune infiltrates using flow cytometry and immunohistochemistry (IHC). We found that either genetic or pharmacological targeting of VPS34 increased $CD45^+$ immune cell infiltration in B16-F10 tumor-bearing mice. Using flow cytometry, we further showed targeting VPS34 significantly increased the presence of immune effector subpopulations including NK cells, $CD8^+$ and $CD4^+$ T cells, dendritic cells, and M1 macrophages in B16-F10 tumors. In addition, NK cells, $CD8^+$ and $CD4^+$ T cells exhibited increased expression of activation marker CD69+ expression, suggesting that targeting VPS34 stimulates an adaptive immune response. The percentage of immunosuppressive M2 macrophages, T_{reg} cells, and myeloid-derived suppressor cells (MDSCs) remained unchanged in shVPS34 or VPS34i-treated tumors. All the above effects on the immune infiltrate in B16-F10 were confirmed in VPS34i-treated CT26 tumors except for a significant increase in T_{reg} cells which was not present in B16-F10 tumors. To examine the role of adaptive immunity in the anti-tumor

response, we targeted VPS34 in immunodeficient NOD *scid* gamma (NSG) mice lacking mature T, B, and NK cells. We observed that genetic or pharmacological VPS34 inhibition failed to affect B16-F10 tumor growth in NSG mice suggesting a dependency on a functional T and NK cell response. In addition, antibody-mediated depletion of $CD8^+$ T cells or NK cells in immunocompetent mice rescued the B16-F10 tumor growth inhibition by genetic or pharmacological VPS34 inhibition. This data implies that targeting VPS34 results in a T and NK cell-dependent anti-tumor response. Recently, similar data showing a $CD8^+$ and $CD4^+$ T cell-dependent anti-tumor response in *Atg7*-deficient tumors or hosts have been reported (Arensman et al., 2020; Poillet-Perez et al., 2020). Given the similarity between these and our data, using VPS34 genetic or pharmacological inhibition, this may suggest that it is autophagy inhibition that underlies VPS34-inhibitor-mediated immune response. Notably, liver *Atg7*-deficiency was sufficient to drive an effective anti-tumor immune response (Poillet-Perez et al., 2020), suggesting that systemic autophagy inhibition may be beneficial.

VPS34i treatment triggers CCL5 and CXCL10 release recruiting $CD8^+$ T and NK cells

What is the mechanism that leads to the attraction/activation of several immune cell subsets in the tumor upon inhibition of VPS34/autophagy? Targeting autophagy has been shown to stimulate cancer cell secretion of immunomodulatory factors including cytokines and chemokines (Kraya et al., 2014; Wei et al., 2011). We thus analyzed cancer cell expression of different chemokines known to attract cytotoxic T cells. Indeed, VPS34i treatment resulted in significantly enhanced expression and secretion of the chemokines CCL5 and CXCL10 in multiple human and murine cell lines. An increase of CCL5 and CXCL10, as well as IFNγ, was also detected in the interstitial fluid (or tumor plasma) of shVPS34 B16-F10 tumors or VPS34i-treated B16-F10 or CT26 tumors. Increased CCL5 and CXCL10 levels were further confirmed in the blood plasma of SB02024-treated tumor-bearing mice, suggesting a systemic proinflammatory response. Indeed, ablation of hepatic autophagy by targeting *Atg7* has also been shown to increase liver secretion of CCL5/CXCL10 and blood serum presence of CXCL10 in tumor-bearing mice (Poillet-Perez et al., 2020). However, we concluded that in our model the released chemokines must be largely tumor-derived as VPS34i-treated non-tumor-bearing mice showed no significant change of CCL5/CXCL10 levels. Antibody-mediated CCL5 neutralization restored tumor growth in VPS34i-treated mice and decreased NK and $CD8^+$ T cell infiltration, indicating a mechanistic link of the chemokine secretion with the effector immune cell recruitment. Mechanistically, the VPS34i-triggered chemokine response in tumor cells was shown to depend on the transcription factors STAT1 and IRF7. VPS34i treatment increased STAT1 and IRF7 expression and phosphorylation leading to the upregulation of CCL5 and CXCL10. In our next study (Paper III), we have examined the mechanism of cytokine/chemokine secretion and STAT1 activation in the VPS34i-treated cancer cells. Several other tumor-intrinsic or -extrinsic mechanisms of how autophagy may contribute to immune evasion have since been reported (Lim and Murthy, 2020). These include reports of regulating antigen presentation (Deng et al., 2021; Yamamoto et al., 2020), T cell metabolism (DeVorkin et al., 2019), and immune cell cytotoxicity (Lawson et al., 2020). It would be interesting to evaluate these newly suggested mechanisms in the context of VPS34i

treatment, specifically testing the function of isolated T or NK cells from treated tumors. Altogether, these data add to the emerging evidence of autophagy restricting tumor inflammation and immune cell function in tumors and provide a good basis for using VPS34i (or other autophagy inhibitors, see below) for turning "cold" tumors into "hot".

VPS34 inhibition increases sensitivity to immune checkpoint blockade

Given the impact on T cell infiltration and the induction of a proinflammatory TME by VPS34i, we wanted to examine a possible combination therapy approach using VPS34i together with ICB. To do this we used the B16-F10 and CT26 model, previously reported as poorly and moderately responsive to anti-PD-1/PD-L1 therapy, respectively (Curran et al., 2010; Kim et al., 2014). VPS34i treatment in combination with either anti-PD-1 or anti-PD-L1 resulted in increased tumor growth inhibition and significantly improved mice survival as compared to each single agent in both models. This is in line with the anti-tumor effects of HCQ treatment in combination with PD-1 blockade in the B16 melanoma model (Sharma et al., 2020). However, the authors did not observe any single agent activity on tumor growth like previously shown (Sharma et al., 2020; Starobinets et al., 2016). More recently, it was further demonstrated that pharmacological targeting of ULK1, a kinase upstream of VPS34, or PIKfyve, a kinase binding to VPS34-generated phosphatidylinositol 3-phosphate (PI3P), potentiates responses to anti-PD-1 therapy in *Lbk1*-mutant lung or prostate cancer, respectively (Deng et al., 2021; Qiao et al., 2021). This suggests that perhaps targeting the early steps of autophagy, such ULK1, PIKfyve, or VPS34, as opposed to the late stages, such as lysosomal inhibition, could be more beneficial from a therapeutic perspective, a highly debated question in the autophagy field urging further investigation.

Significance

In summary, this study provides evidence that VPS34i modulates ICB responsiveness by activating chemokine secretion which in turn contributes to increased immune cell infiltration in the tumor microenvironment. Targeting VPS34 pharmacologically may therefore present a prospective therapeutic strategy to increase inflammation in "cold", immune cell-deprived tumors known to display limited clinical responses to ICB.

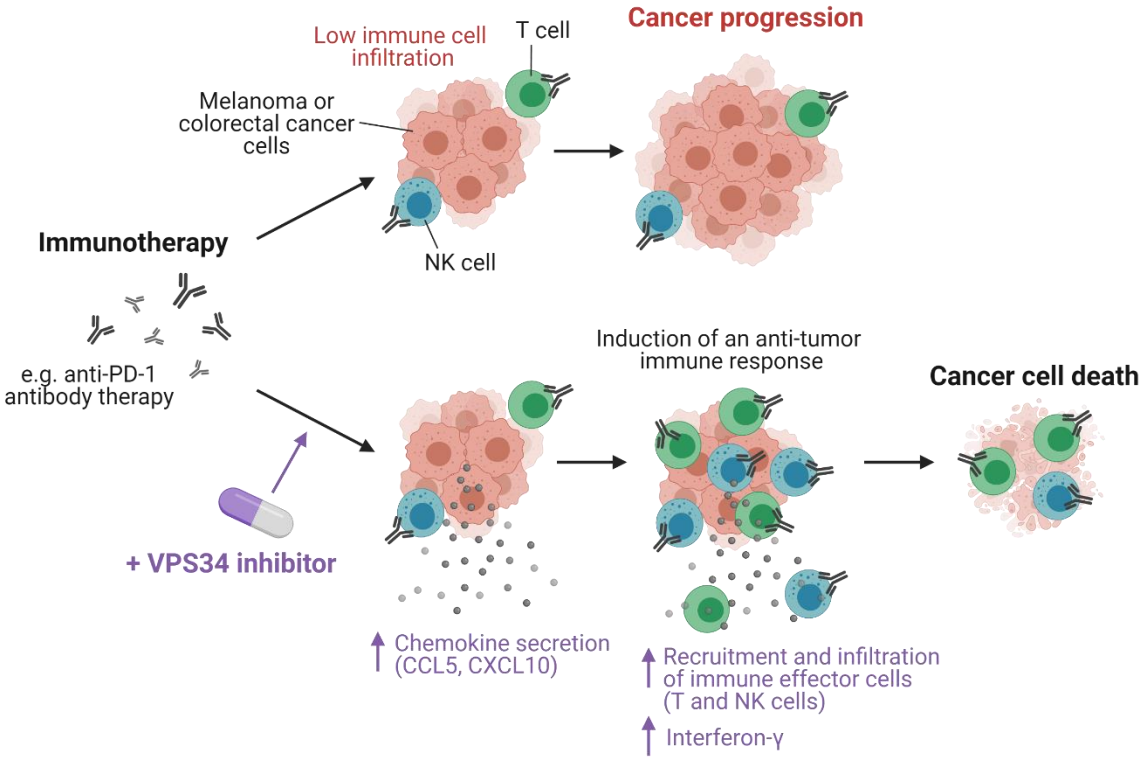


Figure 9. Graphical summary of Paper II.

3.3 PAPER III

VPS34 inhibition activates cGAS-STING signaling and sensitizes tumors to STING agonist

Background

As pointed out above, substantial evidence supports a key role of autophagy in immunosuppression (Thorburn and Towers, 2021; White et al., 2021). An immunosuppressive TME is one of the major challenges of cancer immunotherapy (Hegde and Chen, 2020), and therefore targeting autophagy has raised a renewed interest as a potential combination strategy with cancer immunotherapy. We have shown in Paper II that targeting VPS34 enhanced CCL5 and CXCL10 secretion leading to the recruitment of T and NK cells in the tumor microenvironment of melanoma or CRC models (Noman et al., 2020). However, the mechanisms of how autophagy may contribute to immune evasion require further investigation. The purpose of this study was, thus, to further investigate the underlying mechanisms of the VPS34i-induced chemokine secretion.

Results and Discussion

SB02024 treatment activates an anti-tumor response associated with increased IFN signaling in renal cancer

To evaluate the impact on tumor immunity and chemokine secretion, we chose to take a closer look at the renal cell carcinoma (RCC) model Renca, one of the syngeneic mouse models shown to be sensitive to VPS34 inhibition (Noman et al., 2020). We could confirm that treatment with VPS34i SB02024 decreased tumor growth and increased CCL5/CXCL10 blood serum levels in the Renca model, similar to melanoma and CRC models (Noman et al., 2020). SB02024 or SAR405 treatment *in vitro* further resulted in a dose-dependent significant CCL5 increase in the human clear cell RCC (ccRCC) cells A-498 and 786-O. We thus hypothesized that VPS34 inhibition may trigger a similar cancer cell-intrinsic signaling mechanism leading to enhanced immune infiltration and anti-tumor response in several cancer types. To investigate immune cell subsets and signaling pathways involved in the anti-tumor response in VPS34i-treated Renca tumors, we performed gene expression analysis using a NanoString panel focused on immune-oncology. Gene expression analysis of immune cell markers indicated that SB02024 treatment induced a significant increase of $CD45^+$ immune cells, cytotoxic cells, T cells, NK cells, macrophages, and neutrophils in the tumors. A significant increase of NK cell infiltration upon SB02024 treatment was further confirmed by flow cytometry. This also validates our previous findings that VPS34i treatment activates an NK cell-dependent anti-tumor response in melanoma and CRC models (Noman et al., 2020). To assess altered signaling pathways, we applied network enrichment analysis (NEA) (Alexeyenko et al., 2012; Jeggari et al., 2018). NEA confirmed that cytokine and chemokine signaling is the most affected pathway in SB02024-treated Renca tumors. Furthermore, increased activation of interferon (IFN) signaling with SB02024 treatment was detected. This was of particular interest to us as the canonical type I IFN signaling is an upstream activator of STAT1 and IRF7, two transcription

factors previously shown as crucial for the VPS34i-mediated chemokine increase (Mogensen, 2019; Noman et al., 2020). Indeed, we found that VPS34i treatment significantly increased *IFNB1* expression and triggered IFN alpha/beta receptor (IFNAR)-dependent STAT1 phosphorylation in A-498 cells, clearly indicating induction of a type I IFN response.

VPS34 inhibition activates a type I IFN response through cGAS-STING signaling

The induction of Type I IFN signaling and pro-inflammatory cytokine secretion is regulated by the stimulator of interferon genes (STING) pathway. Previously, a cross-talk between autophagy and the STING pathway including its upstream activator cyclic GMP-AMP synthase (cGAS) has been suggested (Gui et al., 2019; Liang et al., 2014; Prabakaran et al., 2018). Our extensive experiments have indeed demonstrated that pharmacological or RNAi-mediated VPS34 inhibition activates a cGAS- and STING-dependent type I IFN response in RCC cells. A STING-dependent type I IFN signaling induction was also confirmed in VPS34i-treated melanoma cells. Taken together, we have determined that genetic or pharmacological inhibition of VPS34 leads to the activation of the cGAS/STING pathway leading to secretion of IFN-β and activation of type I IFN signaling.

VPS34 inhibitors potentiate the effect of STING agonist ADU-S100 on the proinflammatory cytokine response in RCC and melanoma cells

Activation of the STING pathway by STING agonists is currently pursued by the pharmaceutical industry due to its crucial role in activating anti-tumor immune responses through proinflammatory cytokine secretion (Le Naour et al., 2020). Yet, preliminary clinical data from Phase II indicates, that targeting STING is more challenging than assumed (Le Naour et al., 2020) and may thus require novel combination therapy approaches. Using RCC and melanoma cells *in vitro*, we found that combination treatment of STING agonist ADU-S100 with VPS34i results in increased expression of proinflammatory cytokines (IFNβ, CCL5, CXCL10) as compared to each of the single agents alone. This is in line with a recent study showing that irradiation, an indirect STING activator through cytosolic DNA accumulation, of *Atg5* or *Atg7*-deficient breast cancer cells enhances type I interferon secretion (Yamazaki et al., 2020). Yamazaki et al. further showed that increased mitochondrial DNA accumulation was the cause for an increased type I IFN response upon autophagy inhibition (Yamazaki et al., 2020). It would be interesting to study whether a similar mechanism may be underlying the VPS34i-mediated STING pathway activation in our experiments. This indeed could be the case given the dependency on cGAS sensing cytosolic DNA for its activation. Additional hypothetical sources of cytosolic DNA and cGAS-STING pathway activators are also micronuclei (including genomic DNA) or DNA from dying tumor cells.

Combination treatment with SB02024 improves ADU-S100 efficacy in B16-F10 tumors

This thesis focuses not only on the preclinical characterization of the novel VPS34i SB02024 and unraveling molecular mechanisms underlying its anti-cancer activity but also on identifying a rationale for combination therapies. Therefore, based on the increased

proinflammatory cytokines response *in vitro*, we wanted to test whether SB02024 treatment could improve the anti-tumor efficacy of STING agonist of ADU-S100 *in vivo*. To assess any direct cytotoxic effects on tumor cells we initially tested the SB02024/AUD-S100 combination treatment in murine RCC Renca and melanoma B16-F10 cells. Interestingly, the combination treatment did not affect the B16-F10 cells but caused a STING-dependent decrease in proliferation and induction of apoptosis in Renca cells. This may indicate a specific tumor-cell intrinsic vulnerability of the Renca cells to increased STING activation. As we wanted to primarily assess effects on the anti-tumor efficacy due to an increased STING-driven immune response, we thus focused on the B16-F10 model. Indeed, combined treatment of SB02024 with intra-tumoral (IT) ADU-S100 injections significantly decreased tumor growth and weight and prolonged survival of B16-F10 tumor-bearing mice. It will be very important to test a similar treatment combination on Renca tumor-bearing mice. This model system could help confirm the effects of ADU-S100/SB02024 on tumor growth and assess the contribution of tumor cell death vs. immune cell infiltration/activation to the anti-cancer activity. Our data in this study also strongly supports the conclusions made in previous studies and in Paper I and II, that VPS34i may represent a valuable tool in augmenting the anti-tumor response of several anti-cancer agents.

Significance

In summary, we show that VPS34 inhibition triggered type I IFNs and proinflammatory cytokines by activating cGAS-STING signaling. This study gives a deeper understanding of the VPS34i-mediated anti-tumor immune responses and may imply new combination treatment opportunities with STING-targeting therapies.

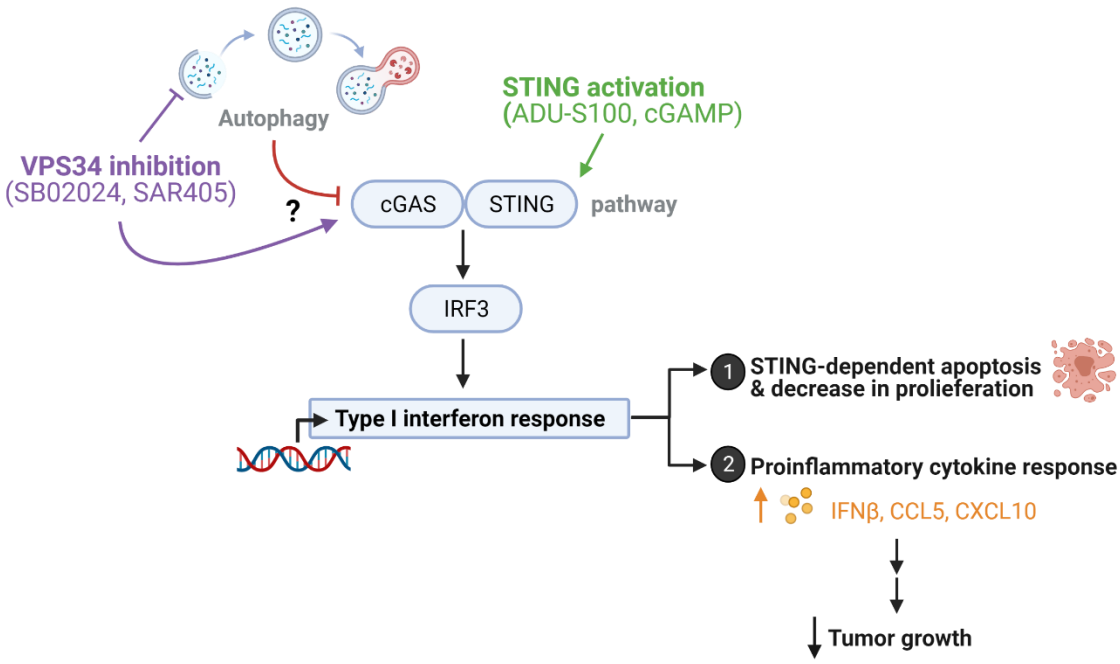


Figure 10. Graphical summary of Paper III.

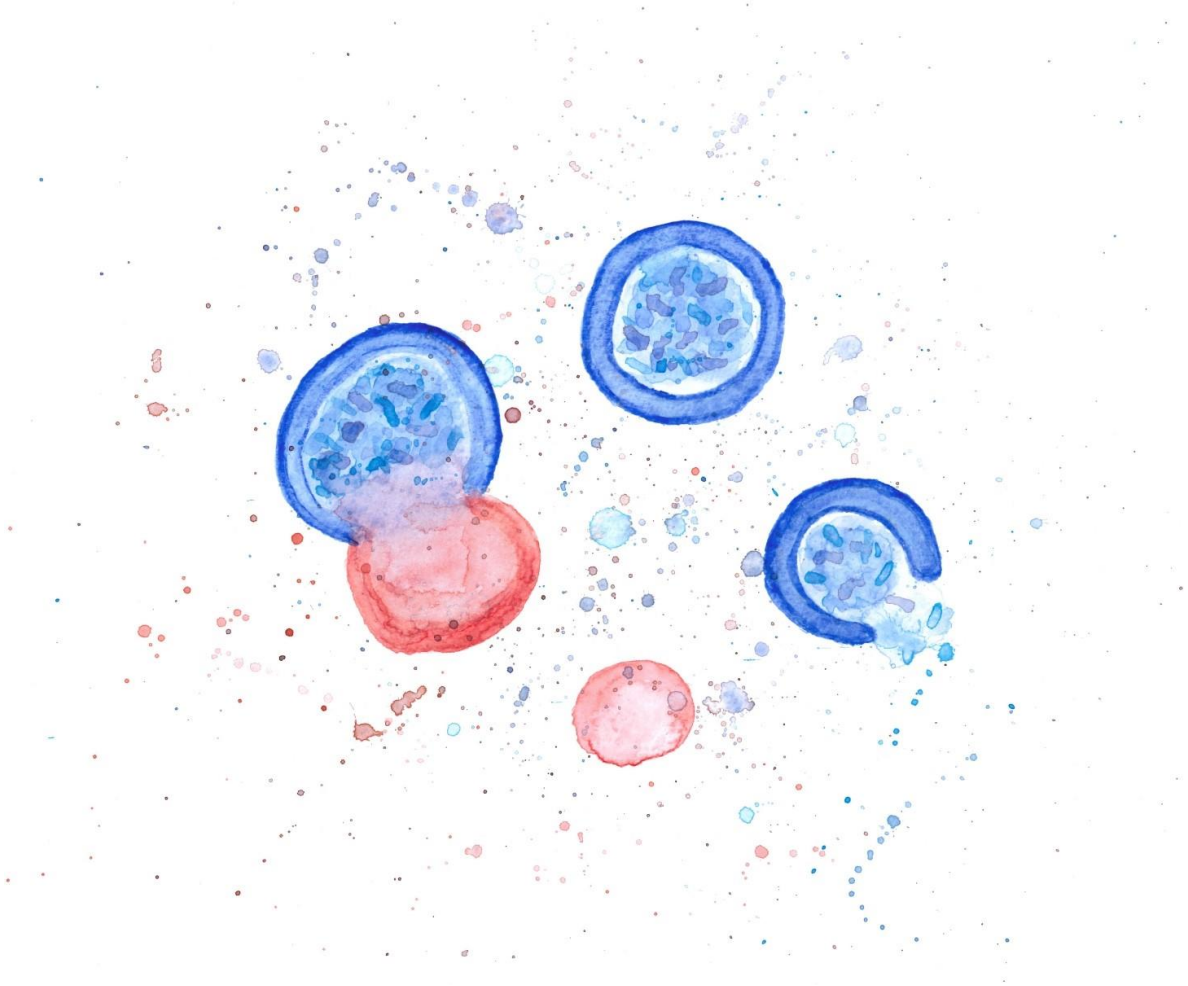
3.4 ETHICAL CONSIDERATIONS

This thesis includes experiments involving the use of mouse models. All experiments were reviewed and approved by independent ethics committees and have followed strict ethical guidelines to prevent unnecessary animal use or suffering as described in the Materials and Methods section of each study. Like humans, mice can experience pain and suffering and therefore their use for research purposes must be justified. In this thesis, studies using tumor-bearing and tumor-free mice needed to be undertaken to study the efficacy and safety of novel compounds or combination treatment for anti-cancer therapy. The results of these experiments may ultimately contribute to the development of life-saving therapies for cancer patients. In efforts to reduce the use of animals, we have further tested novel drug combinations in multicellular spheroids (MCS) in Paper I. MCS were shown to better predict *in vivo* responses as compared to monolayer culture and more closely resemble morphological and metabolic characteristics of tumors (Friedrich et al., 2009). However, MCS model presents a very simple system with many limitations including the lack of immune cells, stromal cells, and blood vessels. Yet, increasingly advanced *in vitro* models, e.g. patient-derived tumor organoid models retaining even a somewhat intact tumor microenvironment (Liu et al., 2021), are emerging and their use in cancer research will be crucial to decrease animal experiments in the future.

4 CONCLUDING REMARKS

Autophagy has a context-dependent role in tumors. However, accumulating evidence indicates a protumorigenic role of autophagy in already established tumors by fueling metabolic demands and contributing to stress tolerance. Autophagy-related stress tolerance further contributes to anti-cancer therapy resistance. Recently, discoveries in the field of immuno-oncology are shedding new light on the role of autophagy in cancer implying that autophagy promotes immune evasion. Targeting of autophagy has therefore raised renewed interest and may provide a strategy to increase the efficacy of and overcome resistance to cancer immunotherapies.

In **Paper I**, we identify several anti-cancer drugs, including the TK inhibitors Sunitinib and Erlotinib, that induce cytoprotective autophagy. We present a novel VPS34 inhibitor SB02024 that blocks the autophagic flux *in vitro* and demonstrates anti-cancer efficacy in breast cancer xenograft models. VPS34 inhibition can further increase the sensitivity of breast cancer cells to Sunitinib suggesting VPS34 as a potential therapeutic target to improve drug resistance in breast cancer. However, further work is needed to elucidate the therapeutic potential of VPS34 inhibitor in different breast cancer subtypes, especially in TNBC, the most aggressive form of breast cancer. Notably, metastatic TNBC was recently approved for ICB in combination with chemotherapy (Cortes et al., 2020) and may therefore present an interesting case to investigate potential immune-activating effects of VPS34 inhibition. In **Paper II and III**, we investigated the role of VPS34 inhibition on the anti-tumor immune response in melanoma, CRC, and RCC. We uncover that VPS34 inhibition leads to an anti-tumor immune response in an NK- and T cell-dependent manner. We further show that VPS34 inhibition creates a proinflammatory TME characterized by increased IFN signaling and CCL5/CXCL10 chemokine release leading to the recruitment of immune effector cells. We reveal that activation of a type I IFN response is dependent on the cGAS-STING pathway. Lastly, we show that combining cancer immunotherapy (anti-PD-1/PD-L1 therapy or STING agonists) with VPS34i is a promising approach to increase anti-tumor efficacy and improve survival in mouse models. Most of the ongoing clinical trials in oncology are currently focusing on combination approaches with ICB. However, limited translation of promising preclinical findings in mouse models to human immunity is a major challenge especially with regards to immune cell subsets and/or function (Hegde and Chen, 2020). Further work should therefore be done to elucidate individual effects of VPS34i on different human immune cell subsets and their specific functions in anti-tumor immune responses. This will be a crucial next step to define a therapeutic window of systemic VPS34i. Results from currently ongoing clinical trials combining HCQ with immunotherapy (Xia et al., 2021) may soon provide important insights into the role of autophagy inhibition in anti-tumor immune responses in humans. This will provide important guidelines also for further development of VPS34i for clinical use. In conclusion, this thesis suggests potential applications of VPS34i in anti-cancer therapy, especially in the emerging field of cancer immunotherapies, and provides a basis for the further preclinical and clinical development of these novel anti-cancer drugs.



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BioRender.com has been used to create Figure 2-10 present in this thesis.