Title

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Journal

The EMBO Journal, 41(13)

ISSN

0261-4189

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Publication Date

2022-07-04

DOI

10.15252/embj.2021110031

Peer reviewed

Review

THE EMBO JOURNAL

Cancer Review Series

The multifaceted role of autophagy in cancer

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Abstract

Autophagy is a cellular degradative pathway that plays diverse roles in maintaining cellular homeostasis. Cellular stress caused by starvation, organelle damage, or proteotoxic aggregates can increase autophagy, which uses the degradative capacity of lysosomal enzymes to mitigate intracellular stresses. Early studies have shown a role for autophagy in the suppression of tumorigenesis. However, work in genetically engineered mouse models and in vitro cell studies have now shown that autophagy can be either cancer-promoting or inhibiting. Here, we summarize the effects of autophagy on cancer initiation, progression, immune infiltration, and metabolism. We also discuss the efforts to pharmacologically target autophagy in the clinic and highlight future areas for exploration.

Keywords ATG; autophagy; cancer; chloroquine; metabolism
Subject Categories Autophagy & Cell Death; Cancer
DOI 10.15252/embj.2021110031 | Received 25 October 2021 | Revised 20 March 2022 | Accepted 8 April 2022 | Published online 10 May 2022
The EMBO Journal (2022) 41: e110031

Introduction

Macroautophagy (hereafter referred to as autophagy) is a lysosome-dependent catabolic process conserved from yeast to human. The basic function of autophagy is to maintain the integrity of proteins and organelles, and balance cellular energy and nutrient needs. The autophagy pathway is activated by a variety of intracellular stressors including damaged mitochondria, pathogen infection, protein aggregates, and nutrient limitation (Parzych & Klionsky, 2014). Short-term activation of autophagy is homeostatic and supports cell survival under stress by removing or counteracting the intracellular stressor. This activity is achieved through the formation of a double-membraned vesicle called an autophagosome that captures intracellular components for degradation (Zhao *et al*, 2021). Autophagic breakdown of encapsulated proteins, lipids, nucleic acids, or carbohydrates occurs after fusion with the lysosome, which provides the acid hydrolases to break down diverse macromolecules so that they can be transported back into the cytoplasm to support essential cellular processes such as translation and metabolism.

Mutational inactivation of the autophagy pathway is not common in cancer, suggesting that a complete loss of autophagy is not a major contributor to cancer development or is harmful to cell viability (Lebovitz *et al*, 2015). However, dysregulation of selective or bulk autophagy has been observed in several cancers and is linked to disease progression (Klionsky *et al*, 2021). The role of autophagy in cancer is complicated by several factors including stage of the disease, driver mutation, tissue types, and metabolic sensitivity. However, trends have emerged showing autophagy can play a tumor-suppressive role in cellular transformation or an oncogenic role in established disease. The tumor-suppressive role of autophagy has been linked to inhibiting stress and reactive oxygen species, while its oncogenic potential has been described to support cancer cell metabolism and inhibit immune infiltration (Amaravadi *et al*, 2016).

This review will focus on the role of autophagy in cancer, with particular emphasis on recent advances in the field. We will explore how cancer-driving pathways regulate autophagy activity and discuss the roles of autophagy in oncogenesis. Further, we will cover animal models of oncogenesis which perturb tumor autophagy or host autophagy. For those interested in general reviews of the autophagy pathway, please see the following reviews (Johansen & Lamark, 2020; King *et al*, 2021; Zhao *et al*, 2021).

ATG proteins and autophagy receptors

The formation and maturation of autophagosomes are supported by over 30 autophagy-related (ATG) proteins. ATG proteins can be categorized into 5 major functional groups (Fig 1). First is the Unc-51 Like kinase 1 (ULK1) protein kinase complex, which includes FAK family-interacting protein of 200 kDa (FIP200), ATG13, and ATG101. In cancer models, FIP200 is the most common component disrupted to inhibit autophagy. Second is the phosphatidylinositol-3-kinase catalytic subunit type 3 (PIK3C3; hereafter referred to as VPS34) complex, which includes the core subunits of phosphatidylinositide-3-kinase regulatory subunit 4 (PIK3R4) and Beclin 1 (BECN1). Additional regulatory binding partners of the VPS34 complex include ATG14, UV radiation resistance associated gene (UVRAG), SH3 domain-containing GRB2-like protein B1 (SH3GLB1), activating molecule in BECN1-regulated autophagy protein 1 (AMBRA 1) and Run domain Beclin 1-interacting and cysteine-rich domain-containing protein (RUBCN). Beclin 1

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This article is part of the Cancer Reviews series.

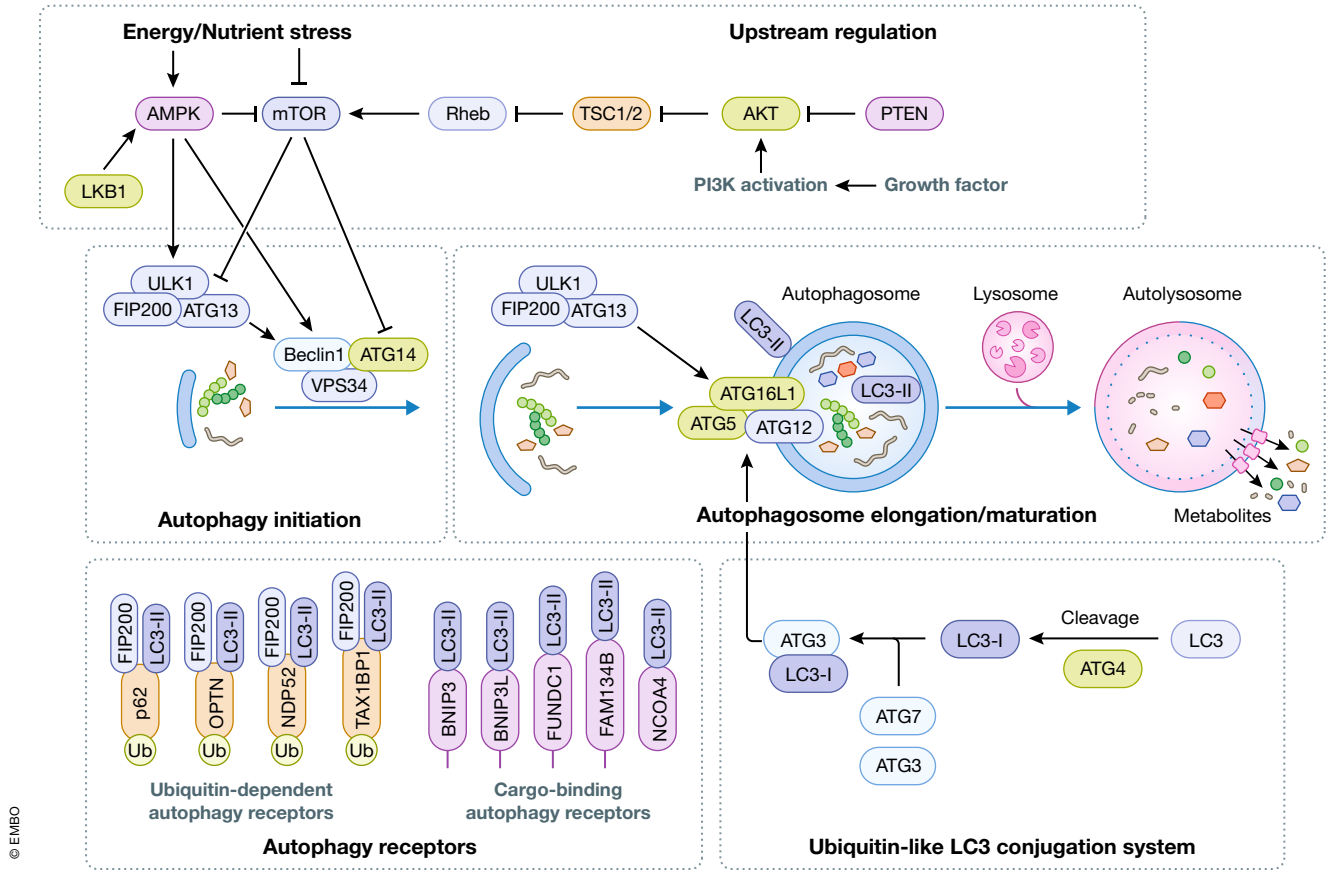

Figure 1. The autophagy pathway.

Autophagosome initiation is driven by protein and lipid kinase activity, which is sensitive to nutrient sensitive kinases mTORC1 and AMPK, and the PI3K-AKT pathway. Expansion of the autophagosome requires the activity of a ubiquitin-like conjugation system that lipidated LC3 to the autophagosomal membrane. Autophagy receptors bind LC3 to recruit cargo to the autophagosome. Recruitment of FIP200 by autophagy receptors can stimulate autophagosome biogenesis. Lysosomal fusion promotes degradation of autophagic cargo.

disruption is the most prevalent method used to inhibit this complex in cancer models. Third is the ubiquitin-like conjugation system, which contains two subgroupings of proteins: i. ATG3, ATG4, and ATG7 and ii. ATG7, ATG10, ATG5, ATG12, and ATG16L1. Together, these protein cascades are responsible for the conjugation of Atg8 family members to the autophagosomal membrane. ATG5 and ATG7 knockout mouse models are commonly used in autophagy studies, although an inducible dominant negative version of ATG4 has recently been used quite widely. Fourth is the trafficking of the transmembrane ATG9 protein. Fifth is the ATG2/WD repeat domain phosphoinositide-interacting protein (WIPI) lipid transfer system. In this review, we will pay particular attention to the first 3 groups of autophagy proteins as they are often targeted in transgenic mouse models of cancer.

ULK1 and VPS34 kinases

One of the earliest events in autophagosome biogenesis is the recruitment of ULK1 to the phagophore, a membrane structure that will expand to form the autophagosome. ULK1 and related ULK2 can both function as mammalian homologs to yeast Atg1. ULK1 and ULK2 are the most upstream components in the autophagy machinery and are the only protein kinases among the Atg proteins (Zhang *et al*, 2021). Inhibition or deletion of both ULK1 and ULK2 results in a profound defect in stress-induced autophagy. ULK1 promotes autophagy by phosphorylating several ATG protein targets including multiple subunits of VPS34 lipid kinase complexes (Fig 1; King *et al*, 2021). VPS34 is the catalytic component of these complexes phosphorylating phosphatidylinositol to form phosphatidylinositol 3-phosphate (PI3P). VPS34 is not exclusive to the autophagy pathway as it also plays a key role in cellular vesicle trafficking. The specific cellular function of VPS34 is driven by regulatory binding partners within complexes. VPS34 promotes autophagosome formation and maturation when complexed with Beclin 1 binding partners ATG14 and UVRAG, respectively (Kim *et al*, 2013). PI3P acts as an anchor to recruit downstream autophagy proteins and promotes autophagosome biogenesis and maturation (Zhang *et al*, 2021).

Ubiquitin-like conjugation systems

Autophagosome formation is dependent on two ubiquitin-like conjugation cascades that promote the coupling of Atg8 homologs to phosphatidylethanolamine (PE) of the autophagosomal membrane. Mammals have 7 isoforms of Atg8 including the microtubule-

associated protein light chain 3 (LC3) family (LC3A, LC3B, LC3B2, and LC3C) and the GABA type A receptor-associated protein (GABARAP) family (GABARAP, GABARAP1, and GABARAPL2/GATE16), which are all conjugated to PE on the autophagosomal membrane (Kumar *et al*, 2020; Fig 1). The first ubiquitin-like system involves the activity of ATG7 (acting as E1) and ATG10 (acting as E2), which facilitates an isopeptide bond formation between ATG12 to ATG5 (resembling that of ubiquitin to its substrate). ATG5-ATG12 then associates with ATG16L1 to form the core of the enzymatic complex (acting as E3) that conjugates LC3 to PE. The processing of LC3 and other Atg8 family members depends on the second ubiquitin-like pathway required for lipidation (Zhang *et al*, 2021). LC3 is first processed by C-terminal cleavage by ATG4 (acting as a protease) and is then activated by ATG7 (E1). Activation by ATG7 promotes transfer of LC3 to ATG3 (E2) to form an ATG3-LC3 conjugate. ATG3-LC3 is then recruited to the autophagosomal membrane by the ATG12-ATG5-ATG16L1 complex (acting as an E3), where LC3 is finally lipidated with PE (Fig 1; Kabeya *et al*, 2000). The ATG12-ATG5-ATG16L1 enzyme is phosphorylated directly by ULK1 in response to most autophagy-inducing stressors (Tian *et al*, 2020). Ablation of lipidation of Atg8 isoforms in mammals inhibits nearly all autophagy. As a result, deletion of proteins required for lipidation are very common targets for inhibiting the autophagy pathway *in vitro* and *in vivo*.

Autophagy receptors

Autophagosome cargo is often enriched for substrates that have been ubiquitinated. Selective capture of ubiquitinated substrates is facilitated by a class of proteins called autophagy receptors (Fig 1). Autophagy receptors bind ubiquitinated proteins through ubiquitin binding domains and recruit them to the autophagosome by associating with LC3 family members through a LC3-interacting region (LIR) (Johansen & Lamark, 2020). Some membrane-embedded autophagy receptors that promote the degradation of membranous organelles lack a ubiquitin binding domain. These transmembrane adaptors can be activated by phosphorylation or increased protein levels to trigger the uptake of damaged organelles into autophagosomes (Palikaras *et al*, 2018). More recently, autophagy receptors have been shown to bind LC3 outside the LIR domain, adding another layer of selectivity and complexity to selective autophagy (Marshall *et al*, 2019). Since autophagy receptors are digested by lysosomal proteases along with other autophagy cargos, their levels tend to be inversely correlated with autophagic flux, making them useful markers for studying autophagy.

The best characterized autophagy receptor is p62 (also called sequestosome 1). p62 is implicated in several forms of selective autophagy, including, but not limited to, aggrephagy (degradation of protein aggregates), xenophagy (degradation of pathogens), lipophagy (degradation of lipids), and mitophagy (degradation of mitochondria; Johansen & Lamark, 2020). In addition to binding LC3 and ubiquitin p62 can bind other signaling molecules such as raptor (a subunit of the mTORC1 complex) and Kelch-like ECH-associated protein 1 (KEAP1), which is a regulator of nuclear factor erythroid 2-related factor 2 (NRF2; Katsuragi *et al*, 2015). Notably, NRF2 and KEAP1 are frequently mutated in cancer (Rojo de la Vega *et al*, 2018). As a result of these additional interactions, p62 functions as a regulator in amino acid sensing and oxidative stress pathways (Katsuragi *et al*, 2015). Some functions of p62, such as the positive regulation of mTORC1 through raptor, are autophagy-independent. Chromosomal amplification of 5q in renal cancer leads to increased p62 levels, which fortifies the cell against redox stress and enhances oncogenesis (Li *et al*, 2013). Like members of the Atg8 family there is a significant degree of functional overlap possible among the autophagy receptors, which typically contain ubiquitin binding and LIR domains to promote the targeting of autophagy cargo (Johansen & Lamark, 2020).

Autophagy receptors have more recently been shown to promote the formation of autophagosomes by recruiting FIP200 to autophagic cargo. When p62 is recruited to ubiquitinated autophagic cargo, it promotes degradation by binding the claw domain of FIP200 to stimulate the recruitment of autophagy-initiating enzymes (Turco *et al*, 2019). The ability to recruit FIP200 is shared by many autophagy receptors, including NBR1, NDP52, TAX1BP1, CCPG1, and OPTN (Fig 1; Ravenhill *et al*, 2019; Vargas *et al*, 2019; Turco *et al*, 2021; Zhou *et al*, 2021). Importantly, the recruitment of FIP200 by these receptors has been shown to be important, and in some cases sufficient, to promote the degradation of specific cargo including mitochondria, ER, protein aggregates, and pathogens (Ravenhill *et al*, 2019; Vargas *et al*, 2019; Zhou *et al*, 2021). Not all autophagy receptors bind ubiquitin to identify cargo for autophagosomal capture. For example, some autophagy receptors are localized to the lipid bilayer of targeted organelles such as mitochondria or ER (Fig 1). In addition, NCOA4 is stabilized to transfer ferritin to autophagosomes when iron is low, but ferritin does not require ubiquitination for NCOA4 binding (Gubas & Dikic, 2022). The selective degradation of cargo such as mitochondria or antigen presenting receptors without a concomitant activation of bulk autophagy has significant effects on tumor biology, which will be discussed in further detail in subsequent sections.

Upstream signals and autophagy regulators

The formation of autophagosomes and therefore the rate of cellular degradation is tightly controlled by the activity of ULK1, VPS34, and ATG16L1-containing enzyme complexes described above. Accordingly, several signal transduction pathways converge on these three enzymes to control the initiation and rate of autophagy (King *et al*, 2021). Autophagy is stress responsive and as such many of the upstream pathways regulating autophagy-promoting enzymes are controlled by well characterized stress-sensitive kinases. For example, nutrient deprivation is one of the earliest and best characterized stresses capable of activating autophagy. One of the first nutrients showed to regulate autophagy levels was branched chain amino acids (Mortimore & Schworer, 1977). Withdrawal of amino acids inhibits the activity of mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), which is prominently linked to autophagy regulation (Ravikumar *et al*, 2003). In addition to amino acids, mTORC1 activity is enhanced by growth factors through the class I phosphatidylinositol 3-kinase (PI3K)-Akt pathway. mTORC1 is a potent repressor of the autophagy pathway. Under nutrient and growth factor sufficiency, mTORC1 blocks autophagy through inhibitory phosphorylation of ULK1 and the ATG14 subunit of VPS34 complexes (Fig 1; King *et al*, 2021). However, upon starvation mTORC1 activity is rapidly inhibited and the repressive phosphorylation on ULK1 or VPS34 complexes is removed, thereby activating these enzymes and autophagy. Dephosphorylation of ULK1 and

VPS34 complexes under nutrient withdrawal is facilitated by the serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform (PP2A)-B55α phosphatase complex, which is activated upon starvation (Wong *et al*, 2015; Fujiwara *et al*, 2016).

In cancer, mTORC1 activity is often elevated, promoting cellular growth through control of translation and other biosynthetic processes. Pathological mTORC1 activation is promoted through activation of PI3K signaling, which is achieved through activation of receptor tyrosine kinase signaling or inactivation of the lipid phosphatase phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN; Jewell & Guan, 2013). PI3K signaling promotes AKT-mediated phosphorylation and inactivation of the tuberous sclerosis complex (TSC), thus relieving the inhibitory effect of TSC on mTORC1. Analysis of precancerous lesions from patients harboring TSC mutations revealed a repression of autophagy, which was reversible upon pharmacological mTORC1 inhibition (Reis *et al*, 2021).

Another important stress responsive kinase upstream of the autophagy pathway is AMP activated protein kinase (AMPK). AMPK is a serine/threonine kinase, whose activity is sensitive to intracellular ratios of AMP/ADP to ATP. In times of energetic stress, cellular AMP and ADP levels rise. The binding of AMP and ADP to a regulatory subunit of the AMPK kinase complex increases phosphorylation on the activation loop of the AMPK catalytic subunit, thereby promoting kinase activity of the complex (Xiao *et al*, 2007; Dunlop & Tee, 2013). Notably, serine/threonine–protein kinase STK11 (LKB1) is a tumor suppressor kinase that directly phosphorylates and activates AMPK. In addition to the regulatory effects of AMP and ADP on AMPK, intracellular levels of an intermediate metabolite in glycolysis, fructose-1-6-bisphosphate can affect AMPK activity (Zhang *et al*, 2017). Specifically, lower levels of fructose-1-6-bisphosphate increases the activity of LKB1 on AMPK localized to the lysosome, thereby providing another stimulatory input to AMPK upon glucose scarcity (Zhang *et al*, 2017). Under energetic stress, AMPK regulates many metabolic processes, including inhibiting lipid synthesis, glycolysis, and protein synthesis. AMPK also promotes autophagy by targeting many of the same autophagy components as mTORC1. However, AMPK-mediated phosphorylation of autophagy components is activating rather than inhibitory (Kim *et al*, 2011). Specifically, AMPK can activate ULK1 through direct phosphorylation, or VPS34 lipid kinase through phosphorylation of the Beclin 1 subunit (Kim *et al*, 2013). AMPK is also capable of indirectly activating autophagy through inhibition of mTORC1. AMPK inhibits mTORC1 through direct phosphorylation of the complex on the raptor subunit, or through activation of the inhibitory TSC complex (Inoki *et al*, 2003; Gwinn *et al*, 2008). Taken together, AMPK can act as a powerful autophagy inducer by relieving the repressive effects of mTORC1 signaling and simultaneous activation of key autophagy enzymes.

Together, the regulation of autophagy machinery by the coordinated activity of mTORC1 and AMPK ensures that during acute starvation autophagy is upregulated to generate sufficient nutrients to support essential cellular processes. In solid tumors, the ability of autophagy to compensate for nutrient limitation promotes survival and cancer progression. Autophagy-deficient mice are born phenotypically normal, but die within 24 h after birth (Kuma *et al*, 2004). Postnatal lethality poses difficulties in studying the role of autophagy in disease, and as a result, most studies of the role of autophagy in cancer utilize a tissue-specific knockout of autophagy genes.

Selective autophagy

Selective autophagy relies on the binding of autophagy receptors to membrane-associated LC3/GABARAP family members and ubiquitin. Localized activation of autophagy enzymes coupled with the ubiquitination of damaged organelles or proteins drives many types of selective autophagy (Johansen & Lamark, 2020). Unlike starvation-induced autophagy, activation of selective autophagy is not always accompanied by a reduction in total protein levels for the autophagy receptors involved. For example, degradation of a single invading bacteria or a few mitochondria does not require enough p62 to affect protein levels, which also complicates the analysis of selective autophagy in disease (Losier *et al*, 2019). However, the lack of alteration in levels of proteins associated with autophagic flux does not indicate that defects in selective autophagy are devoid of biological impact. For example, inhibition of mitophagy can affect cellular metabolism and viability. Similarly, defects in clearance of protein aggregates because of aggrephagy inhibition can stimulate cancer cell survival and exacerbate neurodegeneration. Here, we will limit our discussion of selective autophagy to mitophagy and aggrephagy. However, there are several in-depth reviews of selective autophagy in normal and disease states (Mancias & Kimmelman, 2016; Lamark *et al*, 2017; Kirkin & Rogov, 2019; Liu *et al*, 2020).

Mitophagy

The autophagic degradation of mitochondria (hereafter referred to as mitophagy) is selective for damaged mitochondria. Specifically, depolarization of the outer mitochondrial membrane (OMM) or hypoxia-induced accumulation of mitophagy receptors can block mitochondrial fusion and trigger isolation from the rest of the mitochondrial compartment, thereby promoting its engulfment into an autophagosome (Liu *et al*, 2012; Palikaras *et al*, 2018). Efficient removal of damaged mitochondria is essential to limit the release of reactive oxygen species (ROS), mitochondrial DNA, and activators of intrinsic apoptosis from damaged mitochondria into the cytosol.

Ubiquitin dependent mitophagy

The best characterized mechanism for the clearance of damaged mitochondria involves the PTEN-induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin. This pathway is stimulated by the depolarization of the mitochondrial membrane, which results in stabilization and accumulation of PINK1 on the OMM (Jin *et al*, 2010; Shi & McQuibban, 2017). On the OMM, PINK1 phosphorylates and activates Parkin and ubiquitin (Vives-Bauza *et al*, 2010; Kane *et al*, 2014; Kazlauskaite *et al*, 2014; Koyano *et al*, 2014). Activated Parkin targets mitochondrial proteins for ubiquitination, which promotes the recruitment of ubiquitin binding domain-containing autophagy receptors (Fig 1; Geisler *et al*, 2010; Chen & Dorn, 2013; Sarraf *et al*, 2013). Despite being well characterized *in vitro*, the requirement for PINK1/Parkin in physiological mitophagy remains more elusive. For example, knocking out PINK1 in mice does not result in a profound change in the number of mitochondria (Gautier *et al*, 2008; McWilliams *et al*, 2018). This may be a result of PINK1/Parkin-mediated mitophagy being activated in rare instances or compensation by other mitophagy-inducing pathways, which are discussed below.

Ubiquitin-independent mitophagy

Ubiquitin-independent mitophagy uses mitochondria-localized adaptors that have transmembrane domains and LIRs. These adaptors are regulated by protein stability and post-translational modification. BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L; also known as NIX; Mellor & Harris, 2007) are two such mitophagy adaptors. BNIP and BNIP3L are BH3-only containing members of the Bcl2 family that also contain LIR domains (Bellot *et al*, 2009). Transcription of BNIP3 and BNIP3L is sensitive to hypoxia via stabilization of the hypoxia-inducible factor transcriptional coactivator. BNIP3 is also transcriptionally regulated by forkhead box O3a (FOXO3a) and nuclear factor kappa B (NF-kappaB) signaling (Mellor & Harris, 2007; Dhingra *et al*, 2013; Chaanine *et al*, 2016). Phosphorylation BNIP3 and BNIP3L near the LIR motif can increase their affinity to LC3 and upregulate mitophagy (Zhu *et al*, 2013; Rogov *et al*, 2017; Poole *et al*, 2021).

Like BNIP3, FUN14 domain-containing 11 (FUNDC1) is an OMM protein that binds LC3 to promote mitophagy under hypoxia (Liu *et al*, 2012; Lampert *et al*, 2019). FUNDC1 is subject to inhibitory phosphorylation by the Src kinase and activating phosphorylation by ULK1. In addition to the pathways involving adaptor proteins like FUNDC1, many more proteins have been implicated in mitophagy regulation that have been described in depth (Palikaras *et al*, 2018; Vara-Perez *et al*, 2019).

Mitophagy defects in cancer

Alterations in mitophagy may play an important role in supporting the metabolic requirements of a cancer cell. For example, deletion of the mitophagy adaptor BNIP3 is found in triple-negative breast cancer and was shown to have tumor-suppressive functions in the MMTV-PyMT murine breast cancer model (Chourasia *et al*, 2015). In this model, BNIP3 deletion resulted in a reduction in the rate of mitophagy and an increase in ROS-induced HIF1α expression (Chourasia *et al*, 2015). Concordantly, siRNA-mediated knockdown of BNIP3 in breast cancer cell lines injected into the fat pad of syngeneic mice resulted in a reduction of primary tumor growth and metastases (Manka *et al*, 2005). Reduction of BNIP3 has been linked to poor mitochondrial health and an increased reliance on glycolysis (Fig 2) (Chourasia *et al*, 2015; Lyons *et al*, 2017). While aerobic glycolysis had been linked to metastasis, the release of reactive oxygen species by the accumulation of defective mitochondria has also been shown to promote metastatic potential (Ishikawa *et al*, 2008;

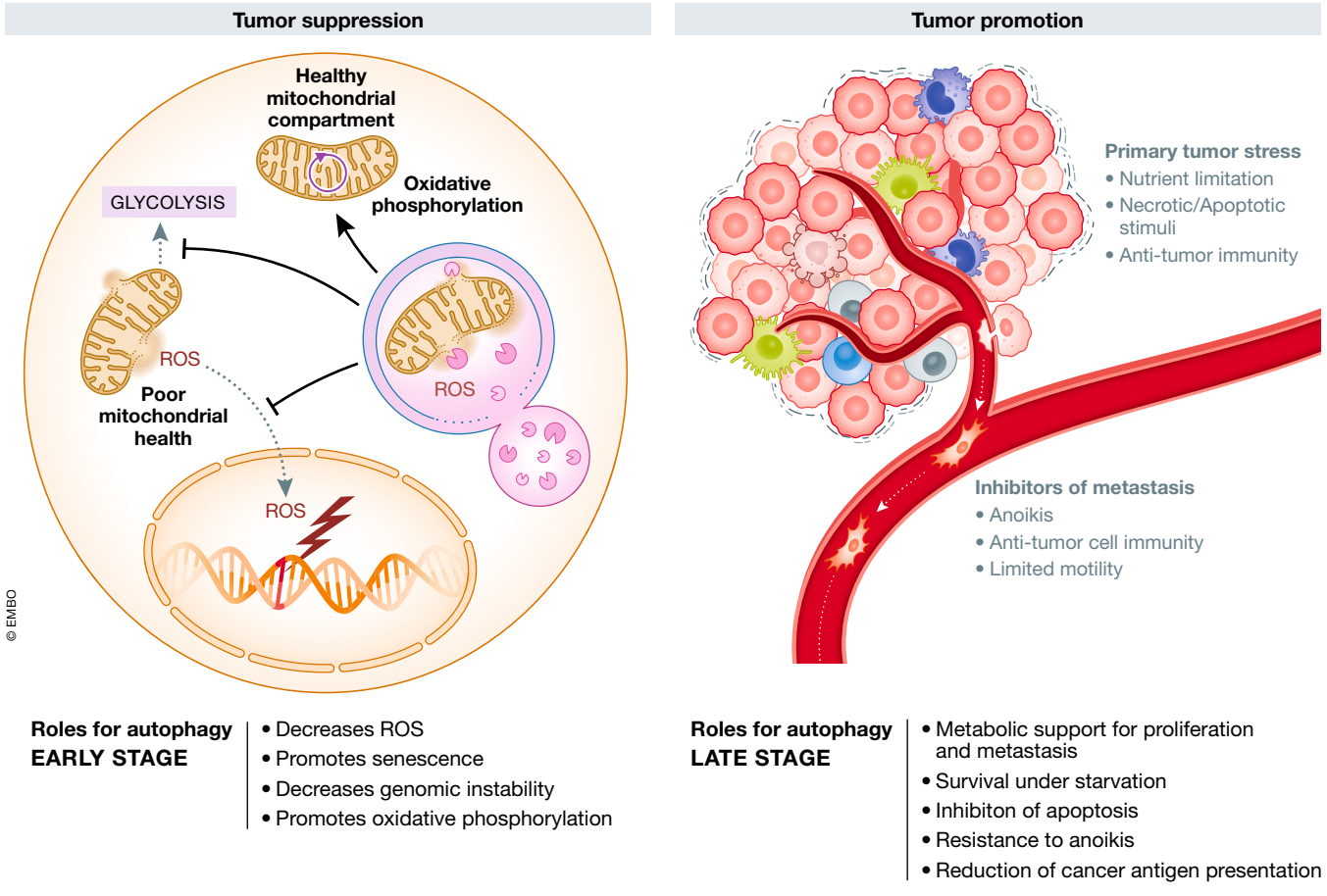

Figure 2. The dynamic role of autophagy in oncogenesis.

Autophagy can inhibit tumor initiation through efficient removal of damaged mitochondria, which reduces glycolysis, ROS levels, and DNA damage. Autophagy supports later stage tumor growth by promoting metabolic remodeling, inhibiting apoptosis and anoikis, and suppressing anti-tumor immunity (see text for details).

Porporato *et al*, 2014). In triple-negative breast cancer, BNIP3 repression is predictive of poor metastasis free survival (Chourasia *et al*, 2015).

Conversely, in several cancers expression of BNIP3 and family member BNIP3L are positively correlated with poor prognosis, which may suggest an oncogenic role for these mitophagy receptors (Petrova *et al*, 2015; Chen *et al*, 2017; Macher-Goeppinger *et al*, 2017). However, this correlation may be in part due to the hypoxic signature of advanced malignancies and the role of the HIFα transcription factor in driving expression of BNIP3 and BNIP3L. The best evidence for an oncogenic role for BNIP3L comes from a study of KRAS proto-oncogene (KRAS) and tumor suppressor protein p53 (hereafter referred to as p53)-driven pancreatic cancer. In this model, KRAS activation drives BNIP3L expression and enhances redox capacity (Humpton *et al*, 2019). Deletion of BNIP3L resulted in an increase in mitochondrial number and improved survival. The expression of BNIP3 and BNIP3L is tissue specific and, in PDAC, BNIP3 expression was not observed except in BNIP3L knockout tumors, indicating a partial compensatory mechanism in these cells. Therefore, it will be important in the future to tease apart the role of these two mitophagy receptors in the promotion or inhibition of malignancy while also mediating the oncogenic effects of tumor hypoxia.

Parkin is encoded by the *PARK2* gene, a large gene that is in the genomically unstable region *FRAG6E,* which is a fragile region and mutational hotspot in cancer (Toma *et al*, 2013). In kidney cancer driven by HIF stabilization, loss of Parkin results in a reduced overall survival (Toma *et al*, 2013). Deletion of *Park2* in a ras-driven model of pancreatic cancer accelerated oncogenesis, further underscoring the potential tumor-suppressive functions of Parkin (Li *et al*, 2018). Ablation of Parkin in this model resulted in an increase of mitochondrial iron and HIF stabilization, which in turn promoted glycolysis (Li *et al*, 2018). Due to the location of *PARK2* in a genomically fragile site Parkin loss has also been found in several cancers, although in many instances the role of mitophagy disruption has not been explored (Vara-Perez *et al*, 2019). It is important to determine the biological consequences of Parkin loss because Parkin has functions that extend beyond promoting the recycling of damaged mitochondria. For example, Parkin forms multiple E3–ligase complexes to ubiquitinate cell cycle proteins including polo-like kinase (PLK1), Aurora A&B, and cyclins E1, D, B1 (Gong *et al*, 2014; Lee *et al*, 2015). The discovery that Parkin can directly ubiquitinate cell cycle proteins explained the observations that Parkin deletion can increase cell division and lead to errors in chromosomal segregation (Gong *et al*, 2014; Lee *et al*, 2015). In addition to a role in regulating cell cycle through chromosomal segregation and control of cyclin stability, Parkin may also control damage-sensitive impediment of cell cycle. This role was identified through a genetic interaction with ataxia telangiectasia mutated (ATM), which is a kinase involved in DNA damage response (Sarraf *et al*, 2019). The deleterious effects of deleting both ATM and Parkin were described through a mechanism involving a Parkin-dependent sequestration of activated TANK-binding kinase 1 (TBK1) to damaged mitochondria, thereby inhibiting TBK1 function at the centrosome (Sarraf *et al*, 2019). Interestingly, this mechanism serves to blur the lines between the classical mitophagy activity of Parkin and its role in regulating chromosomal segregation, further muddling the role(s) Parkin may play in oncogenesis.

Aggrephagy

Cancer cells are routinely under oxidative stress and have activated unfolded protein response pathways. To prevent the deleterious effects of these pathways, cancer cells use autophagy and the ubiquitin proteasome pathway to control the levels of protein aggregates. Protein aggregates form from accumulation of misfolded proteins, which protects the cell from the adverse effects of soluble cytosolic misfolded proteins. Protein aggregates are often ubiquitinated, which links these aggregates to autophagy receptors that promote their clearance (Ravikumar *et al*, 2002; Bjørkøy *et al*, 2005). The E3-ubiquitn ligase TRIM16 has been shown to play an important role in both the biogenesis and clearance of proteotoxic aggregates. TRIM16 promotes both the ubiquitination and autophagic clearance of proteotoxic aggregates in conjunction with p62 (Jena *et al*, 2018). In a xenograft mouse model, tumors engineered with a deletion of TRIM16 regressed upon induction of oxidative stress when compared to the parental line (Jena *et al*, 2018).

The complex role of autophagy in cancer

Cancer cells grow despite a constant exposure to intrinsic and extrinsic stressors, which would kill or halt growth in normal cells. The ability of autophagy to counteract stress can have diverse effects. For example, the ability of autophagy to clear-damaged mitochondria reduces ROS and inhibits glycolysis, contributing to tumor suppression. Conversely, autophagy inhibits apoptosis and promotes survival in the nutrient poor tumor microenvironment, contributing to oncogenesis. These conflicting effects of stress-reduction by the autophagy pathway have led to the theory that autophagy may initially play a role in tumor suppression by maintaining cellular homeostasis, which switches to a protooncogenic role as the tumor develops (Fig 2). Beyond the intrinsic effects of autophagy in the cancer cell is a more recent focus on the effects of autophagy in the tumor stroma, including its role in supporting the metabolic requirements of the tumor and immune surveillance. Lastly, autophagy may contribute to the metastatic potential of the tumor by either suppressing detachment induced death (referred to as anoikis) or by promoting cancer stem cells (Fig 2). These complexities will be discussed in further detail below.

Tumor-suppressive functions of autophagy

The mutation of autophagy genes in cancer is rare and as a result much of what we know about autophagy and cancer is gleaned from genetically engineered mouse models (GEMM). The earliest hint of a role for autophagy in cancer came from the characterization of Beclin 1 as a candidate tumor suppressor in breast cancer (Liang *et al*, 1999). However, the proximity of *BECN* to *BRCA1* on 17q21 meant that its monoallelic loss in cancer may be partly driven by co-deletion of *BRCA1*. Supporting this notion, *BECN1* deletion is not observed independently of *BRCA1* in breast cancer, while deletions of *BRCA1* without *BECN1* inactivation are observed (Laddha *et al*, 2014). Conditional deletion of *Fip200*, which encodes a binding protein required for both ULK1 and ULK2 activity, resulted in a repression of tumor growth in a MMTV-PyMT model of breast cancer, indicating that the tumor-suppressive effects of Beclin 1 may be

partially autophagy-independent (Wei *et al*, 2011). Similarly to *Becn1*, deletion of *Atg5* or *Atg12* leads to an increase in metastases in PyMT-driven mouse model of breast cancer that was driven by the accumulation of the autophagy receptor neighbor of BRCA1 gene 1 (NBR1; Marsh *et al*, 2020). Notably, this increase in metastasis upon *Atg5* or *Atg12* deletion underscores the diverse roles that autophagy plays in metastasis. When the effects of autophagy inhibition on primary tumor growth are controlled for, an increase in metastatic outgrowth could be observed upon inducible ATG5 or ATG12 disruption, uncovering a specific role for autophagy in the suppression of metastatic outgrowth (Marsh *et al*, 2020). Several additional studies have implicated *BECN1* in the development of ovarian and breast cancer (Qu *et al*, 2003; Yue *et al*, 2003; Cicchini *et al*, 2014). *BECN1* deletion results in genomic instability, thereby contributing to cancer development (Fig 2; Karantza-Wadsworth *et al*, 2007; Delaney *et al*, 2020).

While additional clarity is needed on the mechanisms underlying Beclin 1 tumor suppression, it seems that in some genetically driven mouse models of breast cancer that wnt family member 1 (WNT1) is involved (Cicchini *et al*, 2014). A recent unbiased screen of the proteome identified the involvement of e-cadherin and alpha catenin in Beclin 1 tumor-suppressive function (Wijshake *et al*, 2021). Study of other organs in *Becn1*$^{+/-}$ mice has uncovered an increased susceptibility to tumor development in the lung and liver (Qu *et al*, 2003; Yue *et al*, 2003), in addition to some type of breast tumors (Cicchini *et al*, 2014). Interestingly, the lung and livers of *Becn1*$^{+/-}$ mice exhibit reduced levels of p53. The link between the tumor suppressors p53 and Beclin 1 is through regulation of p53 deubiquitination. Mechanistically, this is achieved by Beclin 1-dependent regulation of ubiquitin-specific protease USP10, which stabilizes p53 through cleavage of p53 ubiquitin chains (Yuan *et al*, 2010; Liu *et al*, 2011). Collectively, work on Beclin 1 has yielded some clues into the tumor-suppressive functions of the autophagy pathway, while also identifying some autophagy-independent functions of Beclin 1 that may contribute to its tumor-suppressive function.

Since Beclin 1 has well-established autophagy-independent functions, a concerted effort has been made to explore the tumor-suppressive functions of the autophagy pathway by knocking out genes that are both essential and autophagy specific. These studies have provided evidence that autophagy can play an oncogenic or tumor-suppressive function. Additional models that show tumor suppression by autophagy include the conditional deletion of Atg5 and Atg7 in the liver, which resulted in limited tumor development consisting of age-dependent benign adenomas rather than the well-developed hepatocellular carcinoma in the *Becn*1$^{+/-}$ background (Qu *et al*, 2003; Takamura *et al*, 2011). Reduction of *ATG5* by monoallelic deletion or promoter methylation have both been described to reduce autophagy in melanoma and are associated with poor survival (Liu *et al*, 2013; García-Fernández *et al*, 2016). Stable knockdown of *ATG5* in melanoma cells reduced senescence in BRAF-induced oncogenesis, indicating a role for *ATG5* suppression in the tumor initiation (Young *et al*, 2009; Liu *et al*, 2013). In BRAF-driven melanoma, autophagy is required to overcome the senescence triggered by BRAF inhibitors. Deletion of *Atg7 in Braf*^{V600E} -driven melanoma resulted in a reduction in tumor initiation, further strengthening the link between autophagy and tumor initiation in melanoma (Fig 2; Young *et al*, 2009; Xie *et al*, 2015). Notably, the tumor suppressive effects of *Atg7* disruption are lost if the *Braf*^{V600E}-driven melanoma also has disruption of *Pten*, underscoring some of the underlying complexities in trying to characterize the role of autophagy in oncogenesis (Xie *et al*, 2015).

Autophagy-mediated promotion of oncogenesis

Autophagy is a stress-responsive pathway and in most cases promotes survival and a return to cellular homeostasis. Perhaps the most intuitive example of this is the recycling of cytosolic components to produce compounds to support metabolism and metabolic adaptation when nutrients become limiting. A solid tumor expanding from a preneoplastic lesion into a malignant or invasive tumor will experience limitations in oxygen, glucose, and amino acids as it grows beyond the ability of nutrients to freely diffuse from the normal vasculature. The ability to recycle nutrients gives tumor cells that upregulate autophagy a competitive advantage under starvation pressure. More information on cell competition in the tumor microenvironment is reviewed here (Parker *et al*, 2020). Malignant solid tumors often have areas that are anoxic or hypoxic because tumor angiogenesis is haphazardly organized compared to the vasculature of normal tissue. In addition to nutrient stress, DNA damage, activation of the unfolded protein response (UPR), and organelle damage are all capable of activating autophagy in tumors. To compensate for the growth-suppressing effects of these cancer-related stresses autophagy is frequently elevated in cancer. Autophagy also plays tumor cell non-autonomous roles in promoting cancer growth. For example, in *Kras*-driven lung cancer a conditional whole-body deletion of ATG7 was more potent in promoting tumor regression than inhibiting autophagy in the cancer cells (Karsli-Uzunbas *et al*, 2014). Tumor regression was linked to metabolic deficiencies in the tumor cells that were reliant on host nutrient availability, which was altered due to autophagy inhibition in the liver and tumor microenvironment (Fig 2; Karsli-Uzunbas *et al*, 2014). This trend of autophagy supporting the metabolic requirements of an aggressive tumor, either intrinsically or non-autonomously, have been reproduced in several models and expose a pattern by which autophagy supports cancer progression (Amaravadi *et al*, 2016, 2019).

Autophagy can also promote certain aspects of tumor cell biology that enhances metastasis, including metabolic remodeling, inhibiting anoikis, evading immune cells, and improving motility (Fig 2; Peng *et al*, 2013; Chourasia *et al*, 2015; Sharifi *et al*, 2016). However, it is important to note that the effects of autophagy on metastasis are also dependent on tumor stage and genetic drivers (Chen *et al*, 2013; Lock *et al*, 2014; Marsh *et al*, 2020). Below we discuss in more detail the tumor-promoting activities of autophagy, which are broken down by target organ.

Breast cancer

Ablation of the tumor suppressor *Palb2* in the mouse mammary gland impedes repair of DNA damage and redox regulation, leading to tumor development (Bowman-Colin *et al*, 2013). In this model, impairment of autophagy by monoallelic deletion of *Becn1* reduced *Palb2*-assocaited tumorigenesis (Huo *et al*, 2013). Notably, this oncogenic effect of *Becn1* deletion was not observed in *p53* null mice, which indicates context-dependent function of Beclin 1 in tumorigenesis (Huo *et al*, 2013). *Becn1* deletion results in only a

partial inhibition of autophagy and likely has impacts outside the autophagy pathway, which weakens the link between autophagy and oncogenicity in this model. Further evidence for a role of autophagy in breast cancer came from crossing the conditional knockout of *Fip200* in a PyMT-driven mouse model of breast cancer. Mammary tumors in the *Fip200* null mice were significantly smaller and occurred with less frequency than in autophagy proficient mice (Wei *et al*, 2011). Mechanistically, *Fip200* ablation is proposed to suppress oncogenesis through accumulation of the autophagy receptor p62 and regulation of NF-kappaB signaling (Wei *et al*, 2014). Various syngeneic breast cancer xenograft models that have disrupted autophagy through deletion of *Becn1*, Atg5, or Atg7 have also highlighted a role for autophagy in supporting tumor growth (Baginska *et al*, 2013; DeVorkin *et al*, 2019; Li *et al*, 2020). Xenograft studies with breast cancer cells have also uncovered a role for autophagy in the regulation of metastasis. In non-metastatic cells, the DNA-binding protein (DEDD) promotes VPS34 stability and autophagic degradation of twist family bHLH transcription factor 1 (TWIST1) and snail family transcriptional repressor 1 (Snail), which are two important regulators of epithelial-mesenchymal transition (EMT) and metastasis (Lv *et al*, 2012). The autophagic degradation of EMT-related transcription factors has also been described in other cancer types (Qiang *et al*, 2014; Catalano *et al*, 2015; Qin *et al*, 2015).

Lung cancer

Non-small-cell lung cancer driven by the activation of the oncogenic allele $Kras^{G12D}$ gives rise to the development of adenocarcinomas in the context of a functional immune system (Jackson *et al*, 2001; Kwon & Berns, 2013). Elevated autophagy in response to KRAS activation is required for mitochondrial homeostasis, metabolic adaptation, and stress adaptation (Guo *et al*, 2011, 2013; Lock *et al*, 2011; Yang *et al*, 2011; Karsli-Uzunbas *et al*, 2014). Deletion of Atg7 or Atg5 in *Kras*-driven lung tumors reduces tumor size and results in the development of predominantly benign oncocytomas, which exhibited accumulation of defective mitochondria (Guo *et al*, 2013; Rao *et al*, 2014). The effects of autophagy have also been explored in the $Kras^{G12D}$; $p53^{-/-}$ model, which shows more aggressive adenocarcinoma than those in the *p53* wild-type mice. Dual ablation of autophagy and *p53* resulted in an increase in tumor volume, which indicates that, similarly to work in breast cancer, p53 plays a role in oncogenesis following inhibition of autophagy (Guo *et al*, 2013; Rao *et al*, 2014). In addition to *KRAS*, mutations in the kinase domain of Braf are found in ~3% of lung cancer (Davies *et al*, 2002). Combinational mutation of ATG7 and $Braf^{V600E}$ resulted in an increase on early-stage tumor formation, but ultimately resulted in a reduced tumor burden and metabolic dysfunction (Strohecker *et al*, 2013), highlighting the tumor-suppressive and oncogenic roles of autophagy (Fig 2). Fasting in conditional whole body *Atg7* knockout mice was lethal and lead to muscle wasting and hypoglycemia. However, anti-tumor activity of autophagy inhibition was achieved prior to systemic metabolic dysfunction in lung cancer models indicating the existence of a therapeutic window (Karsli-Uzunbas *et al*, 2014).

Pancreas

Autophagy is elevated in pancreatic cancer and inhibition of autophagy by shRNA knockdown of ATG5 was first used to link autophagy to tumor development (Yang *et al*, 2011). Similarly to breast and lung cancer, the mutational landscape seems to be a critical factor in determining if autophagy plays a role in pancreatic cancer. Oncogenic activation of *KRAS* and inactivation of *p53* are common in pancreatic cancer (Ying *et al*, 2016). In the humanized genetically modified mouse model of pancreatic cancer driven by $Kras^{G12D}$, autophagy inhibition by deletion of Atg5 or Atg7 results in an inhibition of high-grade pancreatic lesions and pancreatic ductal carcinoma (PDAC) (Rosenfeldt *et al*, 2013). Importantly, tumors in mice with ablation of both copies of *p53* and *Kras* activation are no longer sensitive to autophagy (Rosenfeldt *et al*, 2013). However, a subsequent study using a pancreas specific $p53^{+/-}$, which is subject to loss of heterozygosity during disease progression, showed that autophagy deficiency was still able to block cancer progression in this model that more closely mimics the tumor development in humans (Yang *et al*, 2014). Collectively, these studies show that autophagy plays an oncogenic role in the progression of pancreatic cancer, while also underscoring the importance of considering the timing and makeup of accompanying genetic lesions in recapitulating human disease. Additionally, a study in the $Kras^{G12D}$ model showed that reductions in autophagy, as opposed to complete inhibition, exhibit very different results than complete loss of autophagy (Görgülü *et al*, 2019). To modulate autophagy in the *Kras*-driven cancer, Atg5 was either reduced to one allele or completely deleted. While $Atg5^{-/-}$ co-deletion reduced tumor formation when compared to *Kras* controls, a single allele deletion of *Atg5* resulted in an increase in tumor formation and metastases (Görgülü *et al*, 2019). The increase in metastasis and tumor burden with partial autophagy inhibition may raise concerns about unintended results from drug-induced autophagy inhibition. However, in a genetic model using an inducible and reversible dominant negative point mutant of $ATG4B^{C74A}$ meant to mimic the reversible inhibition of an autophagy-targeting drug, autophagy inhibition resulted in tumor regression without significant side effects (Yang *et al*, 2018). While additional details still need to be resolved regarding the role of autophagy in pancreatic cancer, including the effects of autophagy in heterogeneous cell types found in pancreatic cancer (Milan *et al*, 2021), the preponderance of data suggests that inhibition of autophagy may have a therapeutic role. The potential of inhibiting autophagy has spurred on multiple clinical trials using combination therapy with hydroxychloroquine (NCT04145297, NCT03825289, NCT04132505).

Melanoma

Early work in melanoma showed an elevation of autophagy, which correlated with poor therapeutic response (Ma *et al*, 2011). But the exact contribution of autophagy in the development of melanoma remains unclear. As described in the tumor-suppressive section above, *Atg7* deletion with wildtype Pten worsens overall survival (Rosenfeldt *et al*, 2021). *BRAF* and *NRAS* mutations are common in cutaneous melanoma (Schadendorf *et al*, 2015). However, using a model that inhibits autophagy by *Atg7* deletion in melanoma driven by *Braf* activation and allelic loss of *Pten*, an inhibition of tumor growth was observed as well as significantly extended overall survival (Xie *et al*, 2015). One possible explanation for this discrepancy is that downregulation of autophagy and *Pten* deletion have both been implicated in avoidance of oncogene-induced senescence and the timing or exclusivity of these mechanisms to escape senescence may warrant further study to explain this

genetic interaction. While the role of autophagy in breast, lung, pancreatic, and melanoma cancers has been studied in some detail, autophagy has also been examined in additional genetic mouse models and allografts, which were summarized in a recent review (Klionsky *et al*, 2021).

Chaperone-mediated autophagy in cancer

Chaperone-mediated autophagy (CMA) is a type of selective autophagy that uses different proteins to promote cellular degradation and does not involve formation of an autophagosome. Unlike conventional autophagy which targets ubiquitinated and damaged organelles, CMA is known to target fully functional proteins to exert its effects on important cellular processes such as cell cycle (Kon *et al*, 2011; Hubbi *et al*, 2014; Zhou *et al*, 2016) and metabolism (Kon *et al*, 2011; Lv *et al*, 2011; Xia *et al*, 2015; Arias & Cuervo, 2020). In CMA, individual cargo are delivered to the lysosome via a heat shock-cognate chaperone of 70 kDa (hsc70), which recognizes a pentapeptide motif on the cargo protein (Kirchner *et al*, 2019; Arias & Cuervo, 2020). The translocation of substrate into the lysosome requires lysosome-associated membrane protein type 2A (LAMP2A), which acts in concert with hsc70. In this pathway, protein levels of LAMP2A are limiting and are used as a proxy for measurement of CMA capacity (Cuervo & Dice, 2000). Chaperone-mediated autophagy is upregulated in several cancers and contributes to tumor growth (Kon *et al*, 2011). Inhibition of CMA by LAMP2A knock-down suppresses tumor growth (Ding *et al*, 2016; Han *et al*, 2017). However, not all roles of CMA support tumor growth and, like autophagy, CMA has been reported to have tumor-suppressive attributes in early stages of tumor formation (Arias & Cuervo, 2020). Additionally, CMA in cancer cells can promote tumor growth through regulation of immune cells in tumor microenvironment. In glioblastoma, the inactivation of CMA in the tumor suppresses the proinflammatory activity of pericytes, resulting in an increase of anti-tumor T cells and reduction of tumor growth (Valdor *et al*, 2019).

In the future, it will be interesting to determine if the next generation of autophagy inhibitors have an effect on CMA, or whether the development of specific inhibitors of CMA are feasible and beneficial in cancer treatment.

Autophagy in cancer stem cells

Cancer stem cells (CSCs, also known as tumor-initiating cells) are found in many types of cancer and contribute to tumor heterogeneity, metastases, and chemotherapeutic resistance (Kaur *et al*, 2014; Smith & Macleod, 2019). The first CSC was identified in acute myeloid leukemia (Lapidot *et al*, 1994), but have since been identified in several cancer types including solid tumors. In normal stem cells, autophagy has been reported to promote stemness through regulation of metabolic stress, hypoxia, mitochondrial turnover, and oxidative stress (Liu *et al*, 2010; Tra *et al*, 2011; Oliver *et al*, 2012; Vázquez *et al*, 2012; Warr *et al*, 2013; García-Prat *et al*, 2016; Naik *et al*, 2019; He *et al*, 2021). It is through some of these same mechanisms that autophagy supports CSCs survival. However, it was the ability of autophagy to preserve the CSC population in chronic myelogenous leukemia (CML) that raised a significant amount of interest in studying the therapeutic potential of targeting autophagy to selectively kill CSCs. The ability of CSCs to upregulate autophagy to survive chemotherapeutics was first clearly illustrated in CML treated with imatinib, a tyrosine kinase inhibitor of the BCR-ABL fusion protein. Treatment with imatinib in combination with either pharmacological or genetic inhibition of the autophagy pathway resulted in much better killing of CSCs than imatinib alone (Bellodi *et al*, 2009). This study spurred on several clinical trials targeting autophagy in combination with front-line therapeutics. However, most of these studies used hydroxychloroquine, a lysosomotropic agent that only indirectly block autophagy, and have met with limited success (Horne *et al*, 2020). The use of more targeted drugs against autophagy is underway and their functionality in combinational therapy is of intense interest to the field. It has since been shown that upregulation of autophagy is a common mechanism by which CSCs survive pharmacological stressors and the nutrient limitation of the stem cell niche (Nazio *et al*, 2019).

From genetic models, we know that autophagy plays a role in supporting the CSC population in non-myelogenous cancers. For example, in breast cancer inhibition of autophagy by deletion of *Becn1* or *Fip200* results in reduced CSC number and inhibition of tumor growth (Gong *et al*, 2013; Yeo *et al*, 2016). While these studies indicate that inhibition of autophagy may be a viable approach to inhibit CSCs, it may take the use of new and more specific autophagy inhibitors in order to successfully target CSC population in susceptible tumors.

Autophagy in the regulation of anti-tumor immunity

Therapeutic promotion of anti-tumor immunity has had a profound impact on the treatment of a variety of cancers in numerous organs, including kidney, skin, lung, head and neck, and bladder cancers (Egen *et al*, 2020). An analysis of genetic circuits used by diverse cancer cells to evade detection and killing by immune cells has identified a conserved role for autophagy genes (Lawson *et al*, 2020). The strength of an anti-tumor immune response is governed by a variety of factors, including presentation of cancer antigens to prime T cells, secretion of pro-tumor and anti-tumor chemokines, T cell infiltration, T cell activation and killing, and clearance of tumor cell debris. Existing therapies focus heavily on inhibitory checkpoint proteins. Immune checkpoints are ligand–receptor pairs, which elicit a suppression of the immune cell activity (Egen *et al*, 2020). A well-studied example is programmed cell death protein 1 (PD-1) that is expressed on T cells and its ligand PD-L1 that is expressed on cancer cells. Activation of this immune checkpoint greatly dampens the tumor-killing activity of T cells. Drugs that exploit this pathway block the checkpoint, often with monoclonal antibodies, and activate the anti-tumor immune response (Braun *et al*, 2021). Another checkpoint protein, Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) has also been targeted in immunotherapy with good success. However, even in tumors that have seen markedly improved outcomes using checkpoint inhibitors, such as in kidney cancer, a large proportion of patients remain resistant leading to the search for new inhibitory receptors (Andrews *et al*, 2019; Braun *et al*, 2021). While the use of checkpoint inhibitors has been a game changer for some diseases, there remains a large fraction of patients

who do not respond. Some factors that contribute to checkpoint inhibitor resistance are changes in immune effectors or antigen presentation (Sucker *et al*, 2017). Inhibition of autophagy by deletion of *Fip200* or *Becn1* in PyMT-driven breast cancer and B16-F10 melanoma leads to an increase in cytokines that recruit anti-tumor immune cells, indicating that inhibition of autophagy may be a viable approach to sensitizing some cancers to immunotherapy (Wei *et al*, 2011; Mgrditchian *et al*, 2017). Additionally, autophagy-dependent control of extracellular ATP has been shown to play an important role in the control of immune surveillance (Kepp *et al*, 2021). Recent work in the autophagy field has uncovered links between the autophagy pathway and key steps in the anti-tumor immune response.

One common cause of immune evasion is mutations that can lead to the inhibition of major histocompatibility complex class 1 (MHC-I; Rooney *et al*, 2015). However, in absence of genetic alterations to MHC-I related proteins, cancer cells can also downregulate MHC-I through an autophagy-dependent process. In pancreatic cancer, MHC-I was specifically targeted for autophagic degradation through its interaction with the autophagy receptor NBR1 (Fig 3; Yamamoto *et al*, 2020). In this model inhibition of autophagy restores MHC-I and increases antigen presentation, thus enhancing T-cell function and reducing tumor growth in a syngeneic model (Yamamoto *et al*, 2020). Autophagy inhibition by knockdown of ATG3 or ATG7, or pharmacologically using chloroquine (CQ) or ULK1 inhibitors also resulted in a sensitization of pancreatic and lung cancer to checkpoint inhibitors (Yamamoto *et al*, 2020; Deng *et al*, 2021). Autophagy has also been linked to the increased production of $CD8^+$ memory T cells and reduction in tumor growth in autophagy-deficient syngeneic mouse cancer models (Fig 3; DeVorkin *et al*, 2019). Mechanistically, it was shown that ablation of autophagy by deletion of ATG5 resulted in elevated production of interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF-α; DeVorkin *et al*, 2019). Interestingly, a high-throughput CRISPR screen in multiple cancers identified autophagy as a key pathway contributing to evasion of T-cell killing and resistance to TNF-induced cytotoxicity (Lawson *et al*, 2020). Pharmacological inhibition of autophagy using VPS34 inhibitors has been shown to increase inflammation and T-cell infiltration (Noman *et al*, 2020).

Autophagy also plays a role in macrophage-dependent anti-tumor immunity. Autophagy has been described to promote M2 macrophage polarization, which is generally considered to be anti-inflammatory and promote tumorigenesis (Fig 3; Sanjurjo *et al*, 2018; Wu *et al*, 2020). Inhibition of autophagy in pancreatic cancer using transient induction of a dominant-negative ATG4B mutant resulted in an increase in macrophage tumor infiltration. Depletion of macrophages resulted in an impairment of tumor regression upon autophagy induction in this model (Yang *et al*, 2018). While acute

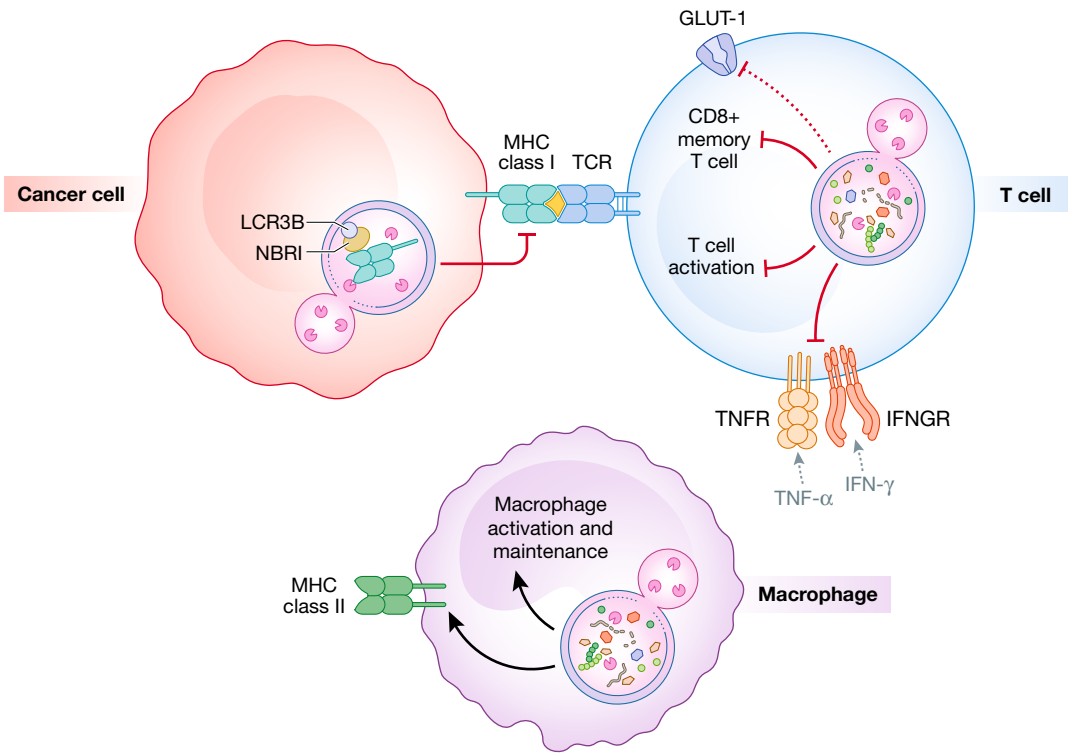

Figure 3. Roles for autophagy in anti-tumor immunity.

Autophagy inhibition promotes anti-tumor immunity through a variety of factors. Tumor cells can degrade MHC Class I through the autophagy receptor NBRI reducing T-cell activation. Autophagy inhibition in T cells also promotes activation of T cells. Autophagy in macrophages creates substrates for MHC Class II and promotes differentiation and maintenance. Systemic inhibition of autophagy in macrophages inhibits tumor-associated macrophage differentiation and increases macrophage infiltration in the tumor.

inhibition of autophagy does not seem to damage adaptive immunity, autophagy plays an important role in the differentiation and survival of stimulated monocytes (Zhang *et al*, 2012; Starobinets *et al*, 2016). Autophagy has been implicated in the antigen delivery to MHC Class II on macrophages, thereby stimulating T cells (Dengjel *et al*, 2005). Autophagy and LC3-associated phagocytosis (LAP) have been linked to the polarization of M2 macrophages, which are generally considered to promote oncogenesis (Liu *et al*, 2015; Cunha *et al*, 2018). The contribution of autophagy to the immune response to cancer can also be cell non-autonomous (Yang *et al*, 2018; Poillet-Perez *et al*, 2020). In a model of hepatic cancer with high mutational burden, the conditional deletion of *Atg7* in the host resulted in an increase in inflammatory cytokine production and greater $CD4^+$ T-cell tumor infiltration (Poillet-Perez *et al*, 2020). This indicates that host autophagy plays a role in enhancing the growth of hepatic tumors with high mutational burden by inhibiting T cell-mediated anti-tumor immunity (Poillet-Perez *et al*, 2020).

Similarly to how autophagy can play dual roles in tumor suppression and oncogenesis, the role of autophagy in immune surveillance is nuanced. As described above, autophagy can play a role in silencing T-cell activation (Fig 3). However, it can also increase immune surveillance induced by chemotherapeutics. Fasting, a physiological way to induce autophagy in many organs of the body, can have a beneficial effect when combined with chemotherapeutics that target DNA repair and replication (Mizushima *et al*, 2004; Lee *et al*, 2012). Notably, the beneficial effects of starvation were lost in mice that lacked T cells, indicating that starvation can enhance chemotherapy-induced immunosurveillance (Pietrocola *et al*, 2016). Like T cell-deficient mice, knockdown of ATG5 also resulted in an attenuation of starvation-induced immunosurveillance (Pietrocola *et al*, 2016). The studies above demonstrate some of the potential effects of autophagy inhibition in the promotion of anti-tumor immunity. However, autophagy has also been shown to play a role in T-cell priming by dendritic cells and development of anti-viral $CD8^+$ T cells. Therefore, careful analysis of the immune response needs to be characterized when testing autophagy inhibition in future pre-clinical models.

Autophagy and tumor cell metabolism

The recycling of nutrients by autophagy is necessary to meet the altered metabolic demands of some cancers (Nyfeler & Eng, 2016). There are several properties that can contribute to a cancers autophagy dependency including intrinsic factors like switching to anaerobic metabolism or an increased need for the biosynthetic building blocks required for continuous cell division (Strohecker *et al*, 2013). Extrinsically, autophagy-dependent cancer cells have to contend with the stressors of hypoxia, immune response, and chemotherapeutics (Kimmelman & White, 2017). In RAS-driven tumors autophagy was shown to support glycolysis and maintain nucleotide levels by supplying amino acids under starvation (Fig 4; Guo *et al*, 2011). In KRAS-driven lung cancer, autophagy supports AMPK signaling (Eichner *et al*, 2019), which was recently tied to lung cancer growth through promotion of lysosomal gene expression (Eichner *et al*, 2019). In pancreatic cancer, melanocyte inducing transcription factor (MITF), transcription factor binding to IGHM enhancer 3 (TFE3), and transcription factor EB (TFEB) drive autophagy and are necessary for maintaining intracellular amino acid pools (Perera *et al*, 2015). As discussed in the section on mitophagy, mutations or deletions of mitophagy adaptors are also linked to metabolic reprogramming of cancer cells. Collectively, we have learned from these studies that autophagy plays an important role in promoting the metabolic alterations in multiple cancer models.

Host autophagy promotes tumor cell metabolic deficiencies

Cancer cells have altered metabolic requirements that can be supported by metabolites produced by autophagy in distant organs (Fig 4). For example, several cancer cells increase their production of nucleotides by inhibiting expression of argininosuccinate synthase 1 or argininosuccinate lyase, which are necessary for arginine biosynthesis (Fig 5; Soria *et al*, 2021). As a result, these cells are reliant on circulating arginine that is provided by autophagy in the liver (Dillon *et al*, 2004; Rabinovich *et al*, 2015). If Atg7 is conditionally knocked out in the liver or whole body, tumor growth is reduced (Poillet-Perez *et al*, 2018). The exact mechanisms by which arginine promotes cancer cell survival remains unclear, but might involve activation of mTOR, polyamine synthesis, or production of nitric oxide (Morris, 2016).

In addition to distally produced nutrients, the tumor can also stimulate nutrient production from the tumor microenvironment. In pancreatic cancer, the involvement of the stroma in supporting cancer cell metabolism is well established (Feig *et al*, 2012; Sousa & Kimmelman, 2014). Pancreatic cancer is nutrient poor leading the tumor cells to scavenge protein from its surroundings. The amino acid alanine, rather than glucose or arginine, is the major carbon source for pancreatic cancer (Fig 5; Kamphorst *et al*, 2015). Pancreatic stromal cell autophagy is required for the release of alanine into the tumor microenvironment, where it is scavenged by the tumor cells (Sousa *et al*, 2016). Consequently, stable knockdown of ATG5 or ATG7 in pancreatic stellate cells resulted in a reduction in alanine in the tumor microenvironment, inhibition of tumor growth, and an increase in disease-free survival (Sousa *et al*, 2016). The discoveries linking systemic autophagy to impairments in tumor metabolism and growth are an exciting advance in the field. Future work to discover the link between an acquired addiction to circulating nutrients and cancer sensitivity to autophagy inhibitors will help in the identification of tumors that should be prioritized inclusion in future clinical trials targeting autophagy.

The therapeutic potential of targeting autophagy

The importance of autophagy in maintaining cellular and organismal homeostasis is well known. For example, conditional knockout of *Atg7* in adult mice leads to increased rates of infection, neurodegeneration, and metabolic deficiencies (Karsli-Uzunbas *et al*, 2014). These disorders limited mouse survival to 2–3 months due to neurodegeneration and starvation-induced hypoglycemia and cachexia (Karsli-Uzunbas *et al*, 2014). However, this study as well as others identified a therapeutic window where the anti-cancer effects precede systemic dysfunction (Yang *et al*, 2011). Moreover, the preponderance of neurological disorders also indicated that an autophagy inhibitor that could not cross the blood brain barrier would be better tolerated (Amaravadi *et al*, 2019). Another model system, which used a whole-body inducible dominant negative mutant of the ATG4B enzyme, also found that anti-cancer effects could be achieved by inhibiting host autophagy, without lethality or

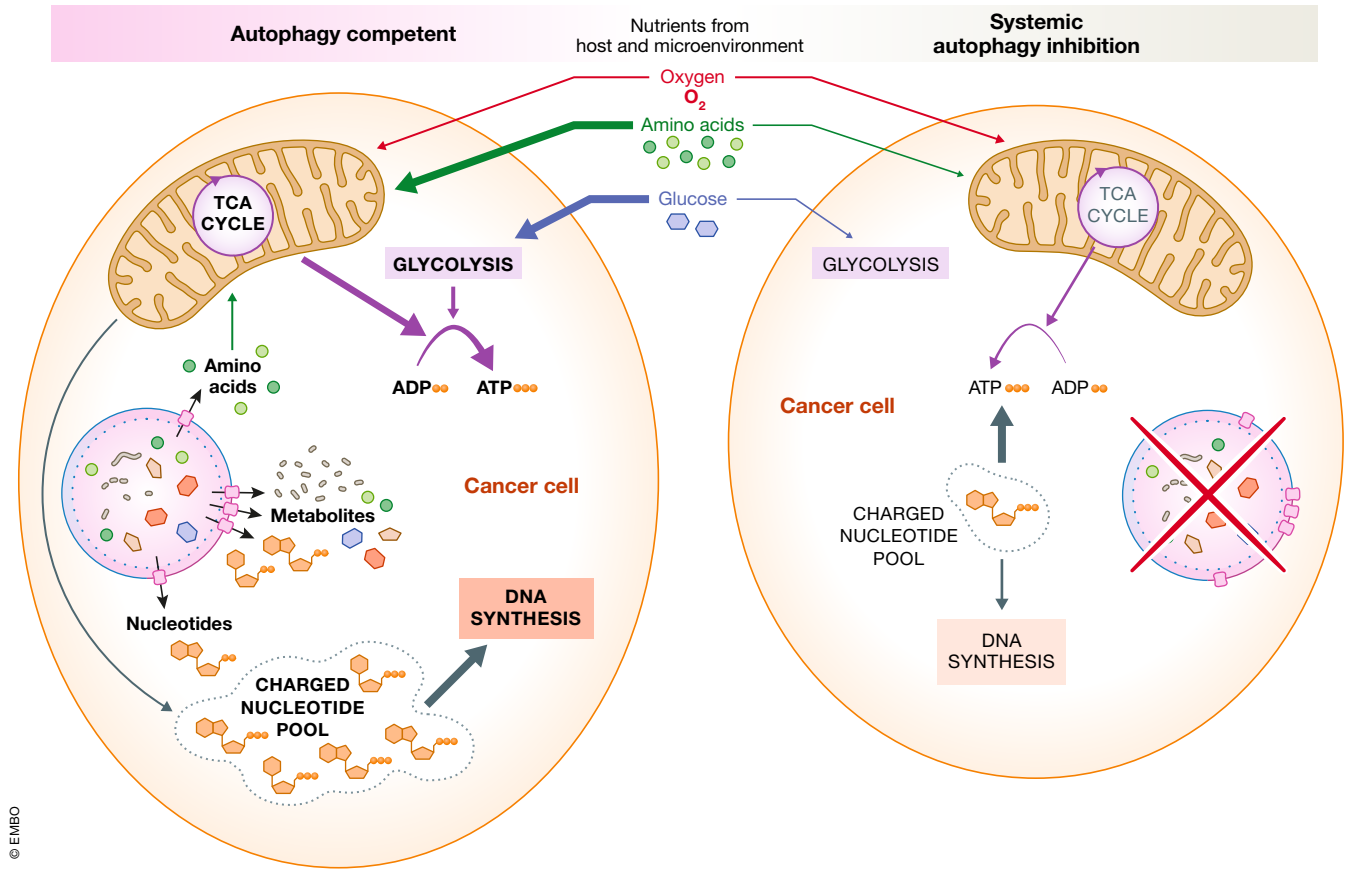

Figure 4. Autophagy supports tumor metabolism.

Left: Autophagy supports maintenance of charged nucleotide and adenosine pools within the cancer cell. Host autophagy maintains circulating amino acids and glucose that can be used as metabolic inputs for oxidative phosphorylation and glycolysis for the cancer cell. Right: Systemic autophagy inhibition reduces mitochondrial amino acid oxidative phosphorylation, resulting in a decrease in ATP. Energy stored in charged nucleotide pools is used to support ATP production leading to a depletion in nucleotides that can be used to support cancer cell DNA synthesis.

increased oncogenesis arising from transient systemic inhibition of autophagy (Yang *et al*, 2018).

While there is a possible therapeutic window for autophagy inhibitors, evidence from pre-clinical models indicates that only some tumors will be autophagy sensitive. For example, factors that have been linked "autophagy addiction" in cancer include RAS and BRAF proto-oncogene activation, MHC I downregulation, and arginine auxotrophy (Yang *et al*, 2011; Levy *et al*, 2014; Mulcahy Levy *et al*, 2014, 2017; Yang & Kimmelman, 2014; Xie *et al*, 2015; Poillet-Perez *et al*, 2018). Because of the involvement of host metabolism and immunity in the sensitivity of cancer to autophagy inhibition, it is important wherever possible that future studies use immune competent animal models to address these variables.

To date, clinical trials in cancer that target autophagy, alone or in combination, have relied on chloroquine derivatives. The best studied of these derivatives is hydroxychloroquine (HCQ), which has been used to treat malaria and rheumatoid arthritis for several decades. These drugs block autophagy by deacidifying the lysosome and prevent autophagosome fusion (Mauthe *et al*, 2018). However, HCQ is not an autophagy-specific inhibitor and its effect on cancer cells can be autophagy-independent (Maycotte *et al*, 2012; Maes *et al*, 2014; Eng *et al*, 2016). One of the first trials was a small trial in glioblastoma multiforme, which added chloroquine to surgery, radiotherapy, and chemotherapy (Briceño *et al*, 2003). Although autophagy was not considered in the execution of this study, they observed that chloroquine improved the response rate to the anti-neoplastic treatments. Subsequent studies identified the potential anti-cancer effects of autophagy inhibition, and several clinical trials were initiated with HCQ. The outcomes of these clinical trials are summarized in Table 1 if results were reported (see Table 1).

While some of the HCQ trials have shown promise, the efficiency of autophagy inhibition and autophagy-independent effects have led to a search for more targeted and potent inhibitors. ULK1 inhibitors SBI-0206965, ULK101 and MRT403, which should also inhibit ULK2, should be much more specific than HCQ in targeting the autophagy pathway. Inactivation of ULK1&2 will abrogate most bulk autophagy. However, it will spare LAP, which may be more beneficial for preserving immune function. In terms of specificity, SBI-0206965 may also target FAK and aurora kinases, while ULK101 inhibits members of the CAMK family (Egan *et al*, 2015; Martin *et al*, 2018). MRT403 was recently shown to sensitize CSCs from patient-derived CML to imatinib (Ianniciello *et al*, 2021). This is an

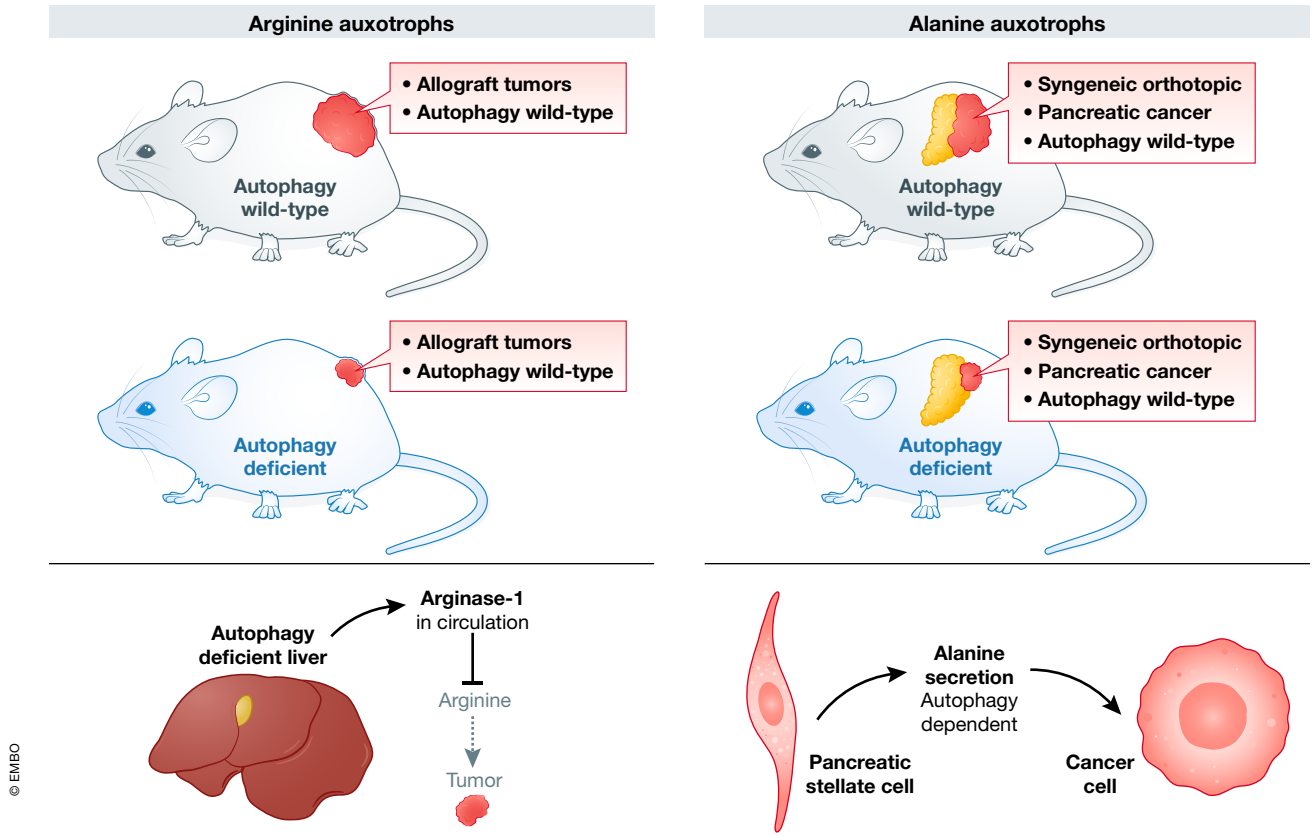

Figure 5. Tumor growth is supported by host autophagy.
Left: Autophagy inhibition in the liver increases plasma arginase-1, depleting circulating arginine. Ablation of host autophagy decreases the growth of autophagy competent cancer cells. Right: Autophagy inhibition in pancreatic stellate cells reduces alanine delivery to autophagy competent pancreatic cancer cells.

Table 1. Clinical trials targeting autophagy.

Condition	Intervention	Results	References
Refractory metastatic pancreatic Cancer	HCQ	No response to HCQ treatment	NCT01273805 (Wolpin *et al*, 2014)
Previously untreated Pancreatic Cancer	HCQ, Gemcitabine	No overall survival improvement or progression-free survival with HCQ. Statistically significant response rate was increased in HCQ arm	NCT01506973 (Karasic *et al*, 2019)
Pancreatic Cancer	HCQ, gemcitabine, abraxane	Autophagy inhibition correlates with increased disease-free survival. 61% of patients exhibited decrease in biomarker CA 19-9. *p53* mutational status did not affect response	NCT01978184 (Boone *et al*, 2015)
Solid Tumor	HCQ prior to surgical resection	Increase in apoptosis in resected tumors, no clinical outcomes reported	NCT02232243 (Wang *et al*, 2018)
Renal Cell Carcinoma	HCQ, everolimus	Increase in progression free survival with HCQ was observed.	NCT01510119 (Haas *et al*, 2019)
Brain and Central Nervous System Tumors	HCQ, temozolomide, Radiation	Results posted, no associated publications	NCT00486603
Metastatic Renal Cell Carcinoma	HCQ, IL-2	Results posted, no associated publications	NCT01550367
Glioblastoma	HCQ, temozolomide, radiation	No improvement in overall survival. Dose limiting toxicity prevented escalation of HCQ. Autophagy inhibition was not consistently achieved	NCT00486603 (Rosenfeld *et al*, 2014)

exciting development and the sensitivity of CSCs to autophagy inhibition was one of the early drivers for the application of autophagy inhibitors in the clinic (Bellodi *et al*, 2009).

VPS34 is the catalytic component of multiple lipid kinase complexes, which are necessary for autophagy and endocytosis. Specific inhibitors of VPS34, like SAR405, therefore effects both autophagosome and late endosome compartments (Ronan *et al*, 2014). Inhibition of VPS34 with SAR405 inhibited the growth of multiple cancer types in a xenograft model and promoted immune infiltration in the B16-F10 melanoma model (Noman *et al*, 2020). As a result, VPS34 inhibitors sensitized tumors to anti-PD-L1 checkpoint inhibitors. Despite their recent success in pre-clinical models, VSP34 inhibitors have not yet made it into clinical trials.

ATG4B is a protease that can activate LC3 for conjugation of LC3 to phosphatidylethanolamine and also plays a role in its deconjugation in the final steps of autophagosome closure. Overexpression of mutant ATG4B results in an inhibition of autophagy and accumulation of defective autophagosomes. Inhibitors of ATG4B have been developed and tested *in vitro* and mouse models (Akin *et al*, 2014; Chu *et al*, 2018; Fu *et al*, 2019). ULK1, VPS34, and ATG4B inhibitors are of interest, but have not yet made it to clinical trials.

As mentioned above, the use of general autophagy inhibitors that cannot cross the blood-brain barrier is a strategy to limit neurotoxicity. However, this would not address the more uncommon, but potentially serious damage to the liver, retina, and other tissues that can occur with high dose and prolonged use (Geamănu (Pancă) *et al*, 2014; Madhavan *et al*, 2016). Another approach is to inhibit selective forms of autophagy that are relevant to the disease in order to retain the majority of autophagy-dependent activity in normal cells. Autophagy receptors are attractive targets as they recruit specific ubiquitinated cargo to the autophagosome and their individual deletion does not eliminate all forms of autophagy. For example, the p62-NRF2-KEAP1 signaling pathway in liver cancer regulates metabolic reprogramming in response to oxidative stress (Saito *et al*, 2016). In pancreatic cancer, which has high basal autophagy, targeting of specific autophagy receptors has uncovered distinct in tumor-suppressive effects. Knockdown of NBR1 inhibited autophagic MHC-I turnover and immune evasion in pancreatic cancer (Yamamoto *et al*, 2020), while knockdown of optineurin caused an inhibition in colony formation and activation of apoptosis (Ali *et al*, 2019).

Targeting of mitophagy receptors in cancer has also been the focus of significant study (see preceding section "Mitophagy in cancer" for additional details). These studies have yielded contrasting results with BNIP3 knockdown in triple-negative breast cancer reducing tumor growth (Chourasia *et al*, 2015), while *Park2* or *Bnip3L* deletion caused an increase in pancreatic cancer growth (Li *et al*, 2018; Humpton *et al*, 2019). In spite of the promising results from inhibition of autophagy receptors by knockout or knockdown, the therapeutic potential of targeting selective autophagy remains to be realized due to a lack of drugs to target these receptors selectively.

Synergy of dual inhibition of autophagy and Ras pathways in cancer

In response to chemotherapeutics, cancer cells can upregulate autophagy to promote survival (Sui *et al*, 2013). The ability of autophagy inhibition to sensitize cancer cells to targeted therapeutics has been exemplified by, but is not limited to, recent work in RAS-driven pancreatic cancer. Activation of KRAS signaling is common in pancreatic cancer and drives autophagy induction. In KRAS-driven cancers pharmacologic or genetic inhibition of RAS-ERK signaling activates autophagy (Bryant *et al*, 2019; Kinsey *et al*, 2019; Lee *et al*, 2019). While inhibition of KRAS-signaling alone is not effective in pancreatic cancer, the inhibition of autophagy in conjunction with targeting MAPK or ERK1/2 downstream of RAS results in a decrease in tumor formation (Infante *et al*, 2014; Bryant *et al*, 2019; Kinsey *et al*, 2019; Lee *et al*, 2019). RAS also activates BRAF, which is frequently mutated in melanoma, central nervous system tumors, and colorectal cancers. Cancers treated with the BRAF inhibitor vemurafenib often develop resistance to the drug and display a concomitant increase in autophagy levels (Ma *et al*, 2014). Inhibition of autophagy with CQ has been described to re-sensitize tumors to vemurafenib or other inhibitors targeting kinases downstream of BRAF (Mulcahy Levy *et al*, 2017). Collectively, these studies show that in "autophagy addicted" cancers the combination of autophagy inhibitors with frontline treatments holds significant therapeutic promise.

Challenges and future directions

Despite intense interest in targeting autophagy in cancer clinical trials, no definitive therapeutic edge has been provided by combination of HCQ to approved therapeutics. It is obvious that autophagy inhibitors are unlikely to be effective as a single agent therapy for cancer. Encouragingly, there seems to be a therapeutic window for autophagy inhibition and use of these antimalarials have largely been devoid of serious metabolic dysfunction, neurological problems, or liver injury. The use of newer and more specific autophagy inhibitors will hopefully be able to exploit autophagy addicted cancers in a more efficacious manner. Work in GEMM has also shown that certain mutations result in sensitivity to autophagy inhibition, while others do not (Karsli-Uzunbas *et al*, 2014). Therefore, expanded experimentation in GEMMs may uncover which cancer types may benefit from autophagy inhibitors. Additionally, in the last decade we have seen a rapid advance in our understanding of the regulatory mechanisms controlling selective autophagy. However, the inhibition of selective autophagy in GEMM or with drugs is significantly more challenging than the study of blocking all autophagy. Despite these challenges, targeting selective autophagy, in particular mitophagy, represents an attractive target for future study and therapeutic development.

Acknowledgments

The Russell laboratory acknowledges support from the CIHR grants #462274 and #153034; and NIH grant GM51586 (K.L.G.). The authors thank Damian Gatica and Karyn King for their input. Figures created with Biorender.com.

Disclosure and competing interests statement

K.L.G. is a cofounder of and has equity interest in Vivace Therapeutics. R.C.R. has equity interest in Benchmark Bioscience.

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