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Impact of context-dependent autophagy states on tumour progression

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Abstract

Macroautophagy is a cellular quality control process that degrades proteins, protein aggregates and damaged organelles. Autophagy plays a fundamental role in cancer, where in the presence of stressors (e.g. nutrient starvation, hypoxia, mechanical pressure), tumour cells activate it to degrade intracellular substrates and provide energy. Cell-autonomous autophagy in tumour cells and cell-nonautonomous autophagy in the tumour microenvironment and in the host converge on mechanisms that modulate metabolic fitness, DNA integrity and immune escape and, consequently, support tumour growth. In this review we will discuss insights into the tumour-modulating roles of autophagy in different contexts and reflect on how future studies using physiological culture systems may help to understand the complexity and open new therapeutic avenues.

Introduction

Macroautophagy is a cellular process that captures and degrades intracellular proteins and organelles to maintain cellular metabolism and homeostasis. Under physiological conditions, basal autophagy is essential for the removal of protein aggregates and damaged organelles that could be toxic for the cells[1–4]. Organism-wide autophagy-deficient adult mice displayed disrupted glucose homeostasis, lipid accumulation, neurodegeneration, muscle atrophy and liver dysfunction, ultimately reducing lifespan[5]. In contrast, overexpression of the essential autophagy gene *Atg5* in mice induced moderate autophagy and prolonged survival[6]. Therefore, autophagy could be considered a crucial quality control system in mammalian cells.

Given its role in regulating key cellular processes, autophagy is involved in the pathophysiology of many diseases, including neurodegeneration and cancer. Its role in

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Competing Interest

A.C.K. has financial interests in Vescor Therapeutics and is an inventor on patents pertaining to KRAS- regulated metabolic pathways and redox control pathways in pancreatic cancer, targeting GOT1 as a therapeutic approach, targeting alanine transport, and the autophagic control of iron metabolism. A.C.K. is on the scientific advisory board of Rafael/Cornerstone Pharmaceuticals, and is advisor for OncoRev, and has been a consultant for Deciphera and Abbvie. The other authors declare no competing interests. M.A. is postdoctoral fellow at New York University Langone Health.

cancer, the focus of the present review, has been debated with studies showing either tumour suppressing or promoting functions[7]. For instance, autophagy inhibition enhanced the formation of benign neoplasms in the liver[8], suggesting on the one hand, that autophagy is important to suppress tumour initiation and on the other hand also implying it could promote the progression to malignant stages. Accordingly, other findings confirmed this dual role in genetically engineered mouse models (GEMM) of pancreatic ductal adenocarcinoma (PDAC)[9]. In established tumours, autophagy levels are higher in poorly vascularized areas where nutrients and oxygen are scarce[10]. The dependency on autophagy can vary between different tumour types; for example, pancreatic cancer cells are highly dependent on autophagy to grow and support metabolic demands[11]. Interestingly, the activation of autophagy is not limited to the tumour cell itself, but also occurs in stromal cells, immune cells and even in distant organs of the tumour-bearing host[12]. Emerging evidence indicates that autophagy in these other cell types and organs is similarly important to promote and maintain tumour growth, therefore, connecting autophagy in the tumour to autophagy in the microenvironment and, more globally, in the host. This article reviews the role of autophagy in the tumour and its macro- and micro-environment and aims to provide an overview of a complex and open-ended field of study.

The molecular underpinnings of autophagy

Autophagy can be divided into three main types. The first type is microautophagy in which the lysosomal membrane engulfs restricted parts of cellular organelles[13–15]. The second type is chaperone-mediated autophagy in which substrates destined for degradation are recognized by the chaperone protein HSC70 and trafficked to the lysosomal machinery[16–18]. The last and most commonly studied type is macroautophagy (hereafter referred as autophagy) in which parts of the cytoplasm and organelles are sequestrated into double-membrane vesicles called autophagosomes. After maturation, the autophagosome fuses with lysosomes to deliver cytoplasmic materials destined for degradation by lysosomal enzymes[19]. This bulk autophagy process, also called the canonical autophagy pathway, is believed to occur in response to nutrient deprivation where cells non-selectively degrade cytoplasmic entities to provide metabolites and maintain survival (the role of autophagy in supporting metabolic pathways has been reviewed in[20,21]). However, there are more selective forms of autophagy that target specific proteins or organelles to support precise homeostatic needs. Such selective autophagy pathways have been described for mitochondria (mitophagy), peroxisome (pexophagy), nucleus (nucleophagy), ribosome (ribophagy), endoplasmic reticulum (reticulophagy), protein aggregates (aggrephagy), lipid droplets (lipophagy), cytosolic iron storing complex ferritin (ferritinophagy) and pathogens (xenophagy). Molecular mechanisms underlying canonical and non-canonical autophagy as well as organelle-specific autophagy have been reviewed in more detail elsewhere[2,22–24].

Autophagy is initiated by the ULK1 complex (ULK1/ATG13/FIP200/ATG101) and class III PI3K (PI3KC3) complex I (VPS34/BECLIN 1/ATG14/AMBRA1/p115)[25,26]. Both complexes translocate to the endoplasmic reticulum (ER) to form a pre-autophagosomal structure (PAS). The ULK1 complex phosphorylates components of the PI3KC3 complex, which in turn initiates the production of local phosphatidylinositol-3-phosphate (PI3P). These PI3P-enriched ER subdomains are coined with the term omegasome and can be

identified by the presence of double-FYVE-containing protein 1 (DFCP1); the function of this factor is still unclear[27,28]. Additional sources of membranes from different organelles can be shuttled by ATG9 to the site of phagophore nucleation and seem to be important for effective autophagy initiation[29–32]. During phagophore expansion, most nascent ubiquitin-like ATG8s, such as LC3 and GABARAPs, are processed at their C-termini by the cysteine protease ATG4[33]. The resulting glycine-exposed C-terminus is activated by ATG7 and ATG3 and finally conjugated to a major membrane phospholipid, phosphatidylethanolamine (PE). ATG3-mediated LC3 lipidation is stimulated by the ATG5-ATG12-ATG16L1-WIPI2 complex[34–36]. The resulting lipidated membrane-bound form of LC3 (LC3-II) is not only important for the interaction with specific substrates destined for degradation, but also for sealing and maturation of autophagosomes (Figure 1).

The initiation of autophagy occurs in response to various stresses, the most important and well-characterized is starvation from amino acids, hormones and growth factors[37]. In this scenario, it has been proposed that the activation of autophagy is controlled by two major pathways, AMPK and mTOR. Upon a drop in nutrient levels, mTOR is inactivated and AMPK promotes autophagy by phosphorylating ULK1 on different serine residues, including ser777. In contrast, in the presence of sufficient nutrients, mTOR activity is high and prevents ULK1 activation by phosphorylation at ser757[38]. However, this “simple” model of duality between AMPK and mTOR does not apply to all circumstances. For example, pancreatic cancer cells, which are known to be metabolically plastic, induce autophagy in the presence of active mTOR signalling[39]; the activation of autophagy in this model is attributed to upregulated expression of core autophagy-lysosomal genes by the MiT/TFE family of transcription factors[39]. Interestingly, although mTOR is inactivated during autophagy initiation in various animal species, it is reactivated during periods of prolonged starvation to promote lysosome recycling and homeostasis[40]. Therefore, mTOR and AMPK may play various roles throughout the initiation, progression and termination steps of autophagy. Other pathways (e.g. Ca^{2+}-cAMP-PKA) may be involved depending on the type of stimulus and its duration [41]. Since most studies on signalling pathways governing autophagy were performed using starvation as a stimulus, it will be important for future studies to focus on other stimuli, such as hypoxia, matrix stiffness as well as mechanical and osmotic pressure, all of which are relevant to cancer pathophysiology.

The complex cellular interactions governing autophagy

As autophagy in the tumour, the tumour microenvironment and in the host are important in promoting tumour progression, defining the potential physiological inputs that contribute to this process is a challenge that needs to be addressed in the cancer field. Some of these stressors have been already proposed and include nutrients and oxygen deprivation, anoikis, mechanical and osmotic pressure, ER stress and DNA damage[42,43] (Figure 2). Nutrient deprivation has been extensively studied in the field and reviewed elsewhere[2,44], hence we will focus briefly on the “less-studied” stressors that activate autophagy. ECM degradation (resulting in reduced ECM density) was shown to modulate glucose uptake in a large panel of cancer cell lines, however whether ECM detachment, nutrient uptake and autophagy constitute a continuous survival axis in cancer cells deserves further investigation[45]. Paradoxically, the mechanical pressure induced by cell interaction with stiff

matrix and cell-to-cell contact also activates autophagy via the YAP/TAZ pathway[46]. This contrasting example of ECM attachment versus detachment illustrates the complexity of cellular signalling governing autophagy activation.

The accumulation of unfolded proteins in the cytoplasm is a major inducer of ER stress and autophagy. Indeed, PERK-mediated phosphorylation of Eukaryotic Initiation Factor 2α (eIF2α), allows the specific translation of activating transcription factor 4 (ATF4), which in turn promotes the expression of a set of core autophagy genes[47–49]. ER stress promotes the release of calcium ions (Ca^{2+}) into the cytoplasm, leading to the activation of Ca^{2+}-dependent proteases, including Calpain. Calpain induces autophagy through mechanisms that are not yet fully elucidated and autophagy inducers, such as rapamycin, fail to induce autophagosome formation in cells lacking Calpain[50–52]. Ca^{2+} ions can also induce autophagy by inhibiting mTORC1 activity through the CAMKKβ/AMPK pathway[53].

Other interesting inducers of autophagy are osmotic pressure and fluctuations in pH and temperature. Applying osmotic pressure on cancer cells using gradients of sorbitol or mannitol induced autophagy through the mTOR pathway, which was dependent on the polycystin-2 membrane channel, a sensor for osmotic balance[54]. However, more physiologic models are needed to better understand the link between osmotic pressure and autophagy in cancer. Changes in pH are a well-known characteristic of the tumour microenvironment, where cancer cells survive in a hypoxic and acidic milieu[55,56]. In cancer cells, acute change in extracellular pH has been shown to modulate autophagy flux in two opposite directions, with an alkaline milieu promoting autophagy, contrary to an acidic milieu where autophagy is reduced[57]. However, once cancer cells have adapted to their acidic environment they activate autophagy flux and become even more resistant to autophagy inhibition[58]. It was found that only the non-acidic normoxic areas of tumours were sensitive to autophagy inhibition by chloroquine[58], providing a potential explanation for the limited benefit of chloroquine in the clinic as a single agent.

Autophagy in tumour cells

Cell-autonomous autophagy is an important process that tumour cells employ to preserve their metabolic fitness and reduce DNA damage, as demonstrated in various tumour types including melanoma, glioblastoma, breast, lung, pancreas, prostate and intestinal cancers[9,59–66] . A major focus was to determine the exact autophagy-derived substrates that cancer cells use to survive intense stress episodes and it was shown that autophagy maintains glutamate, α-ketoglutarate and nucleotide pools[67], which serve to fuel mitochondrial TCA cycle and preserve functional mitochondria[68]. Other studies have pointed to the importance of autophagy, and more specifically, mitophagy, in optimizing lipid catabolism and fatty acid oxidation[59,69,70]. Inhibition of autophagy in a model of lung adenocarcinoma, driven by $Kras^{G12D}$ mutation and lack of *Trp53* expression, resulted in the accumulation of defective mitochondria and the conversion of adenomas and adenocarcinomas to begin oncocytomas. In the absence of *Atg7*, mitochondria were unable to oxidize fatty acids (defective respiration) and ultimately accumulated in oncocytomas, suggesting a possible role of mitophagy in maintaining mitochondrial quality and lipid energy homeostasis in the tumour[69]. Recent evidence indicates that defective mitochondrial respiration following

autophagy inhibition may also be due to the alteration of iron metabolism, which is essential for the function of iron sulphur clusters involved in electron transfer within the mitochondrial respiratory chain[71,72]. Mechanistically, autophagy controls intracellular iron availability through NCOA4 that acts as a cargo receptor mediating ferritin degradation by autophagy to increase free iron in the cell[73]. Interestingly, mitophagy not only regulates mitochondrial quality, but also mitochondrial mass to optimize tumour progression[74]. In a model of KRAS-driven pancreatic cancer, authors showed that mutant KRAS induces the expression of BNIP3L, which in turn induces mitophagy in cancer cells, leading to reduced glucose consumption and increased redox buffering. BNIP3L deletion reduces mitophagy and induces the accumulation of mitochondria, which in turn enhance glucose consumption and reactive oxygen species production[74]. The increase in mitochondrial mass resulted in a decrease in cell proliferation under nutrient scarce conditions, leading to a delay in tumour progression *in vivo*. These studies and many others placed autophagy as a central node in modulating mitochondrial oxidative phosphorylation and, subsequently, buffering intracellular oxidative damages[67,75]. In a recent study, we provided direct evidence for the role of autophagy in regulating redox homeostasis in pancreatic cancer cells; namely, by demonstrating that autophagy maintains intracellular cysteine levels through recycling and targeting the cystine transporter SLC7A11 at the plasma membrane[76,77].

Through preserving the global metabolic and redox balance in the cell, autophagy protects DNA integrity. Indeed, autophagy inhibition increases DNA damage, micronuclei formation, chromosome instability and aneuploidy[78–81]. Interestingly, autophagy-competent cells were shown to use the error-free homologous DNA repair process, whereas autophagy-deficient cells rely on the error-prone non-homologous end joining repair[82]; however, the exact mechanisms underlying the switch in DNA repair processes are not completely understood (Figure 3). In response to genotoxic stress, chaperone-mediated autophagy is increased to degrade checkpoint kinase 1 (CHK1)[83], which in the nucleus compromises cell cycle progression and delays the DNA repair response[83]. Following DNA damage, histone2A is ubiquitinated by the E3 ubiquitin ligase RNF168, an essential step for the recruitment of DNA repair factors. In autophagy-deficient cells, the autophagic cargo p62 accumulates in the cell and binds to RNF168, thereby preventing its chromatin-ubiquitinating activity and the subsequent activation of the DNA repair response[84]. These two studies reveal distinct mechanisms linking autophagy and DNA integrity. Oncogene-induced replication stress activates the DNA damage response followed by autophagy activation[85]. The latter is needed for effective recovery from replication stress and to enhance the speed of the replication fork, possibly through maintaining intracellular nucleotide pools[85]. Indeed, knockout of *Atg5* and *Atg7* induces spontaneous replication stress and DNA damage[85]. Therefore, autophagy can promote tumour progression through multiple mechanisms, including by controlling mitochondrial function and mass, as well as ensuring DNA stability and replication.

The use of autophagy by tumour cells goes beyond the above-mentioned pathways and includes aspects related to immune surveillance. In a recent study, pancreatic cancer cells were found to target the MHC-I molecule through the cargo receptor NBR-1, for degradation by the autophagosome/lysosomal system[86,87]. This degradation process was dependent on canonical autophagy, but not on LC3-associated phagocytosis (LAP). Indeed,

genetic and pharmacologic autophagy inhibition enhanced anti-tumour immune response and sensitized tumours to immunotherapy[87]. In addition, breast tumour cells escape immune killing by activating autophagy to degrade granzyme B produced by natural killer (NK) cells[88]. Another study found that hypoxia-induced autophagy in tumour cells protects them against the cytolytic activity of T-cells[89]. Lung tumours with a high mutational burden were shown to have increased autophagy to compromise immunoproteasome activity and antigen presentation and the inhibition of autophagy using ULK1 inhibitors was found to synergize with PD-1 blockade to reduce tumour growth[90]. In a mammary tumour model, the ULK1 complex member FIP200 has been shown to interact with 5-Azacytidine induced 2 (AZI2) to inhibit Tank binding kinase 1 (TBK1)-derived interferon response[91]. Accordingly, FIP200 deletion resulted in enhanced interferon signalling and increased CD8+ T-cell infiltration[91]. Functional genetic screens, *in vitro* and *in vivo*, revealed the involvement of core autophagy genes in a conserved pathway allowing cancer cell evasion from cytotoxic T lymphocytes and mediating resistance to TNF and IFN-γ in multiple syngeneic tumour models[92]. Another set of CRISPR screens revealed that autophagy in pancreatic tumour cells serves as a determinant factor to escape CD8+ T cell-mediated killing; specifically, autophagy rendered tumour cells more resistant to TNF-α-induced cell death[93]. Taken together, these studies emphasize the importance of cell-autonomous autophagy for tumour survival; however, emerging evidence indicate that cell-nonautonomous autophagy in the tumour microenvironment is also determinant for tumour cell fate (Figure 3).

Autophagy in the tumour microenvironment

The tumour microenvironment is composed of stromal, immune, nerve and endothelial cells. Stromal cells, such as fibroblasts, are the most prevalent type and are responsible for excessive intra-tumoral collagen and extracellular matrix (ECM) deposition, a characteristic shared by many aggressive cancers. This fibrotic stromal reaction, also called desmoplasia, is dependent on autophagy in stromal cells[94]; it stiffens the tumour microenvironment, thereby limiting the accessibility of cancer cells to nutrients and oxygen. In this austere milieu, the crosstalk between the stromal compartment and tumour cells becomes crucial for survival. Interestingly, using co-cultures of human pancreatic cancer and stellate cells tumour-derived factors were found to stimulate autophagy flux in stellate cells, which in turn released the non-essential amino acid (NEAA) alanine at high concentrations. This study showed that when essential nutrients like glucose and glutamine are scarce in the microenvironment, tumour cells shift their dependency on NEAA to fuel TCA cycle and lipid biosynthesis[95]. In 2020, a follow-up study identified SLC38A2 as the main transporter expressed by pancreatic cancer cells to mediate the import of stellate cells-derived alanine[96]; however, whether the expression of SLC38A2 in pancreatic cancer cells is by itself regulated by autophagy warrants further investigation. Autophagy in pancreatic stellate cells has been shown to be associated with reduced overall survival in patients and inhibition of autophagy in stellate cells reduced primary tumour growth and liver metastasis in mice[97]. In addition to direct cell-cell communication, the stiffness of the ECM has been shown to be an important driver of autophagy within fibroblasts and ECM stiffness was shown to activate AMPK by stabilizing its protein half-life via surface Integrin-αV[98]. Accordingly, stiffness-induced autophagy in stellate cells conferred a growth advantage for pancreatic tumour cells when co-injected into mouse pancreata[98].

Endothelial cells constitute an important component of the tumour microenvironment, and execute autophagy to modulate intra-tumour vasculature. Indeed, ablation of *Atg5* specifically in the murine endothelial compartment provoked the formation of immature and tortuous blood vessels within melanoma tumours, leading to a reduction in tumour growth[99]. However, autophagy inhibition in endothelial cells did not affect the metastatic potential of cancer cells[99], suggesting that autophagy in different cell types may preferentially promote either tumour growth and/or metastasis. Different studies highlighted the responsiveness of tumour endothelial cells to activators of the stimulator of interferon genes (STING) pathway. Intra-tumour injection of STING agonists cGAMP or RR-CDA increased the expression of genes related to type-I interferon and angiogenesis in endothelial cells, which led to enhanced CD8+ immune cell infiltration[100,101]. Accordingly, combination of STING activation with immune checkpoint blockade induced a complete regression of melanoma, colon and breast tumours[100,101]. Since pro-autophagy proteins and cargoes including ATG12, ATG5 and p62 can bind elements in the STING pathway and target them for degradation[102–104], it is tempting to speculate that autophagy in endothelial cells can modulate immune cell activation at least partly through inhibiting the STING pathway. This possibility deserves further investigations.

Autophagy is an important physiological process for immune cell differentiation, survival and fitness[105–107] but in the context of cancer has been shown to mediate immunosuppressive actions to facilitate tumour progression. For example, autophagy is essential for the survival and function of the immunosuppressive regulatory T-cells (Tregs)[108]. Other groups found that ablation of essential core autophagy genes in T-cells provoked a significant increase in their tumour killing capacities[109]. In addition, autophagy inhibition elicits antitumour immune response by favouring the polarization of the anti-inflammatory M2 macrophage to the proinflammatory M1 phenotype[110,111]; cytokines released by M1 macrophages promote a more effective chemotaxis, leading to enhanced T-cell intra-tumour infiltration. Interestingly, tumour cells undergoing chemotherapy produce factors that induce the expression of the surface glycoprotein TIM-4 on tumour-associated macrophages[112]. TIM-4 interacts with AMPK to activate autophagy in macrophages upon uptake of dying tumour cells, therefore leading to reduced antigen presentation and increased tumour resistance to conventional chemotherapies[112]. These studies clearly show that autophagy in stromal and immune cells is highly connected to cell-autonomous autophagy in tumour cells and autophagy at both levels seems to be essential for successful tumour progression (Figure 3).

Autophagy, metastasis and cancer stem cells

Autophagy in tumour cells and their microenvironment impacts the ability of tumour cells to disseminate into secondary organs, for example by promoting the release of pro-migratory factors, including interleukine-6, matrix metalloproteinase 2 (MMP2) and WNT5A[113]. It is also involved in the biogenesis and release of exosomes, which may facilitate cancer cell metastasis[114]. In addition, autophagy has been shown to either promote or supress epithelial-to-mesenchymal transition (EMT)[115], a process preceding cancer cell extravasation from the primary tumour to distant organs. Two studies in breast cancer and glioblastoma have shown that the master EMT regulators TWIST and SNAIL can be degraded by autophagy through

interaction with BECLIN 1[116,117]. Another study in squamous cell carcinoma demonstrated that autophagy inhibition resulted in accumulation of p62, which in turn bound to TWIST to stabilize its expression and promote metastasis[118]. On the other hand, knockout studies of different core autophagy genes found the reversed EMT phenotype, also known as, mesenchymal-to-epithelial transition (MET)[113,119].

Following extravasation from the primary tumour site, cancer cells need to survive matrix detachment in the bloodstream, a process termed anoikis, which was shown to be dependent on autophagy[120]. Upon detachment, cells upregulate protein kinase R (PKR)–like endoplasmic reticulum kinase (PERK), which in turn activates AMPK and inhibits mTOR signalling, leading to autophagy induction. Interestingly, the dynamics of PERK signalling to AMPK and mTOR were only observed during anoikis and could not be recapitulated using ER stress inducers[120]. Other mechanisms of resistance to anoikis via autophagy have been reported such as the activation of the NF-κB (nuclear factor-κB) pathway [121] and the autophagic degradation of RHOA, which regulates anoikis by modulating cytoskeleton dynamics[122]. Indeed, the role of autophagy in anoikis resistance and metastasis has been validated in different cancer types, including hepatocellular carcinoma and ovarian cancer[123–125]. In addition, a link between autophagy and cancer stemness was established. Given their ability to self-renew, cancer stem cells are thought to be important for tumour aggressiveness and metastasis initiation[126] (reviewed in [127]). Accordingly, autophagy promotes cancer stem cell-like phenotypes by increasing the number of CD44-positive over CD24-negative cells and supporting their ability to form mammospheres in breast cancer models[128,129]. In addition to the role of autophagy in solid tumours, selective autophagy forms such as AMPK/FIS1-mediated mitophagy have been shown to promote leukaemia stem cell self-renewal ability, which is essential to drive the genesis of acute myeloid leukaemia[130]. Mechanistically, it was found in a model of hepatic cancer that mitophagy degrades p53 through its association with PINK1, thereby reducing p53 phosphorylation by PINK1 and its translocation into the nucleus, where p53 binds to NANOG promoter to prevent the expression of *OCT4* and *SOX2*[131]. Therefore, bulk or selective autophagy positively regulate stemness and self-renewal ability of cancer stem cells. While there is convincing evidence to support a role for autophagy in driving cancer cell migration and invasion, the role of autophagy as a metastasis-promoter or suppressor seems to be dependent on tumour stage. Using a mammary tumour model, where autophagy was inhibited at different stages of tumour progression, autophagy was found to reduce primary tumour growth, but enhance spontaneous metastasis[132]. This phenotype was dependent on the accumulation of NBR1 cargo, as genetic ablation of the latter in autophagy-deficient cells reversed the spontaneous increase in metastasis[132]. Similarly, ablation of RUBICON induced autophagy, which in turn decreased the metastatic index in mice. Interestingly, inhibition of autophagy, at the lysosomal level, with chloroquine in this study had no impact on metastasis as compared to the genetic knockout of *Atg5* and *Atg12*[132]. In contrast, another study showed that both chloroquine and tumour-specific *Atg5* deletion reduce lung metastasis in a melanoma model, although the effects of both interventions on the tumour microenvironment were different[99]. The discrepancy in the results of these two studies could be due to the difference in tumour types (breast cancer and melanoma) and/or the genetic background of animals. They also suggest that different mechanisms may operate following

genetic autophagy targeting *versus* pharmacological inhibition of lysosome function in the context of metastasis; an important detail that should be considered in the design of translational studies.

Autophagy in organs distant from the primary tumour

In addition to autophagy in tumour cells and their microenvironment, it is remarkable to note that host autophagy (in other tissues/organs) has also a critical impact on tumour progression and growth. Early studies using organism-wide autophagy deficient animals, showed that neonates lacking essential autophagy genes *Atg5* and *Atg7* only survive for few hours after birth[133]. In adults, autophagy-deficient mice survive for two to three months, but experience excessive muscle wasting, liver damage and inflammation, neurodegeneration and adipose tissue lipolysis, leading to premature death[5]. Fasting was lethal in host autophagy-deficient adult mice due to hypoglycaemia, emphasizing a global role for autophagy in regulating systemic metabolism and tissue metabolic homeostasis[5]. Using a complementary approach, some groups engineered mice overexpressing moderate levels of Atg5[6]. In these animals, the increase in autophagy levels was associated with resistance to oxidative stress, improved lean mass and extended overall survival[6]. Although the induction of autophagy through Atg5 overexpression is clear, it is possible that Atg5 may exert some autophagy-independent functions that could have contributed to the observed phenotype[134]. All these prominent effects of autophagy on organism physiology raised the question on whether or not host autophagy has an impact on tumour development. Interestingly, whole-organism deletion of autophagy (Atg7 knockout model), reduced autochthonous $Kras^{G12D}$- and $Trp53^{-/-}$-driven lung cancers, even more potently than tumour-specific autophagy inhibition[5]. These findings in the same transgenic background were recently corroborated by showing that transient systemic *Atg5* deletion restricts tumour growth by reducing glucose and lactate uptake[135]. Consequently, the reduction in carbon sources slowed down the activity of essential metabolic pathways required for tumour growth, such as TCA cycle and serine biosynthesis[135]. To specifically show the contribution of host autophagy to tumour growth, autophagy-competent cancer cells were subcutaneously implanted into mice with competent or deficient host autophagy[136]. The study showed a clear host-autophagy dependent decrease of tumour growth in different cancer types, including melanoma, urothelial carcinoma and lung cancer[136]. This was accompanied with systemic metabolic alterations, where a drop in arginine levels was most remarkable[136]. Dysfunction or alteration in *de novo* arginine synthesis makes tumour cells dependent on exogenous arginine uptake and occurs in many human cancers that are known as arginine auxotrophs[137] and indeed, the subset of tumour allografts that regressed in autophagy-deficient hosts were arginine auxotrophs too[136]. The low levels of circulating arginine were due to increased circulating levels of the hepatic enzyme arginase-1 (ARG1), which degrades arginine. Indeed, the inhibition of autophagy in the host or specifically in the liver, both induced hepatic stress leading to the release of ARG1 from hepatocytes into the bloodstream and the resulting degradation of arginine[136]. As expected, an arginine-rich diet partially rescued circulating arginine levels and tumour growth in autophagy-deficient mice. These findings indicate that host autophagy is critical to supply arginine-auxotrophic tumours with arginine[136]. In another study, the same group showed that host autophagy has an immunomodulatory function on the anti-tumour immune response[138]. The authors describe the effect to be specific for tumours

with high mutational burden. Organism-wide deletion of *Atg7* reduced tumour growth by enhancing T-cell infiltration. Single-cell transcriptomics and histology analysis showed increased tumour infiltration of macrophages, dendritic cells and T-cell populations[138]. This response was dependent on STING and the resulting activation of interferon-γ signalling. It has been shown that mitophagy mitigates STING signalling through clearing mitochondrial DNA[139]. Therefore, it is possible that host autophagy inhibition impairs the clearance of mitochondrial or genomic DNA, which leads to STING activation. There may also be a possible role of tumour endothelial cells in the observed phenotype. As mentioned above, the endothelial compartment in the tumour is known to be responsive to STING agonists and is capable to effectively activate the interferon response to promote T-cell infiltration. Interestingly, liver-specific autophagy inhibition recapitulated the different phenotypes observed in organism-wide autophagy inhibition[138], highlighting the prominent role of hepatic autophagy in remodelling metabolic and immune aspects in the primary tumour. In another example, using a doxycycline-inducible Atg4B dominant negative mouse model (refer to Figure 1 regarding the role of ATG4), our laboratory has previously shown that inhibition of host autophagy delays tumour take[140]. This effect is likely, in part, due to a reshaping of the tumour microenvironment, which hampered metabolic crosstalk between tumour and stellate cells[140]. Given the widespread expression of the Atg4b dominant negative, it is highly probable that autophagy inhibition in distant tissues is also contributing to this phenotype. In line with the Atg4b dominant negative model, removal of *Atg13* in eye tumour cells or in their surrounding environment (epithelial cells) in *Drosophila Melanogaster* impaired autophagy and reduced tumour cell growth and similar data were obtained with *Atg14* deletion[141]. Interestingly, supplementation with *Atg13* DNA in the host or specifically in the tumour-bearing organ rescued autophagy and tumour growth, suggesting that both cell-autonomous and host autophagy are required for tumour growth and invasiveness. Mechanistically, tumour cells increased interleukin 6 (IL6) production, which possibly acted in an autocrine manner to increase reactive oxygen species generation within tumour cells, which in turn induced autophagy in the microenvironment. In a series of allograft experiments, the authors transplanted autophagy-competent or -deficient tumour cells into hosts with functional or inactive autophagy and found that systemic autophagy had a great impact on the growth capability of tumours[141]. In the same model it was shown that autophagy in host organs increases as tumour progresses and muscle wasting occurs[142]. The full-body removal of either *Atg13* or *Atg14*, decreased tumour growth and reduced muscle atrophy, weight and motility loss, and body fat alterations. Specific expression of Atg13 in the tumour and the tumour microenvironment rescued tumour growth, but failed to induce muscle wasting, indicating that systemic body wasting was only dependent on systemic autophagy, while tumour growth depended on both cell-autonomous and host autophagy. This global wasting state was associated with an increase in circulating amino acids, including arginine and glutamine, which was reversed in autophagy-deficient flies. Early in the wasting process, autophagy-competent hosts showed depletion of glycogen stores and an increase in haemolymph sugar (trehalose), which was normalized in autophagy-deficient counterparts[142]. Therefore, similarly to what has been described in mouse models, host autophagy can control the systemic metabolism and mobilization of nutrients in tumour-bearing hosts. It is worth mentioning that the mobilization of nutrients into the circulation preceded the exponential growth of tumour, suggesting that it may contribute to

accelerate tumour progression[142]. Using two food regimens with different carbon isotopes, the authors show that amino acids, lipids and sugars can be mobilized from host tissues to the tumour in an autophagy-dependent fashion. The findings paint a picture where the progressive loss of body biomass, orchestrated by autophagy, serves to fuel tumour growth and progression. Together, the data support a clear role for systemic or host autophagy in promoting tumour progression and cancer cachexia (Figure 4). In an attempt to provide a translational context, another team inhibited systemic autophagy intermittently to mimic what would happen in a clinical setting of cancer therapy[135] and was found to extend the survival of mice and allow recovery of normal organs, while maintaining its inhibitory effects on lung tumour growth[135]. An analogous intermittent autophagy inhibition approach using the aforementioned Atg4B dominant negative system in pancreatic cancer, showed similar effects in mouse models[9]. Whether or not these findings could be recapitulated by hydroxychloroquine treatment requires further investigation, given the known potency issues of hydroxychloroquine (as recently reviewed recently in [43]) and the fact that it targets the lysosome and is not selective for autophagy.

Modulation of autophagy in cancer treatment

In addition to driving the progression of many human cancers, autophagy has been shown to be elevated in response to cancer therapies, suggesting that it may also serve as a resistance mechanism. The inhibition of autophagy using hydroxychloroquine in combination with chemotherapeutic agents (e.g. gemcitabine, nab-paclitaxel, carboplatin, paclitaxel) showed an improvement in progression-free survival in lung cancer patients and improved the response to chemotherapy in lung and pancreatic cancers[143,144]; the addition of hydroxychloroquine in the neoadjuvant setting may also facilitate tumour resection due to local tumour shrinkage[143,145]. Conversely, in some circumstances, induction of autophagy in combination with chemotherapy may also be beneficial to enhance the recruitment of immune cells into the tumour bed and promote tumour killing[146]. The inhibition of the ERK pathway has been proposed as a treatment for RAS-mutant cancers where the RAS-RAF-MEK-ERK pathway is hyperactivated and drives cancer initiation and progression[147,148]. Three independent studies found that inhibition of the ERK pathway leads to autophagy activation[149–151]. Concurrent inhibition of the ERK pathway with trametinib and autophagy with hydroxychloroquine led to a drastic regression of tumour growth in patient-derived xenograft models of pancreatic cancer, melanoma and colorectal cancer[149,150]. The combination was also successful to reduce pancreatic and metastatic liver lesions in a patient, along with normalization of the circulating levels of cancer antigen 19–9[149]. Multiple ongoing clinical trials are assessing this approach in patients. In addition, the combination of dabrafenib (BRAF inhibitor), trametinib and hydroxychloroquine was safe and produced a high response rate in BRAF V600-mutant melanoma patients[152]. A recent study revealed that autophagy may also be a resistance mechanism in response to the CDK4/6 inhibitor-based therapy, suggesting that adding hydroxychloroquine may provide a greater therapeutic benefit in this context[153]. Using a genetic CRISPR loss-of-function screen, insulin-like growth factor 1 receptor (IGF1R) was identified as a sensitizer to hydroxychloroquine treatment[154]and dual inhibition of IGF1R and ERK pathways increased dependency on autophagy[154]. Accordingly, the inhibition of autophagy, IGFR1 and ERK pathways led to a reduction in survival in cancer cell lines and organoids[154].

Pancreatic tumours are known to be particularly refractory to immunotherapy. We have recently found that hydroxychloroquine treatment combined with dual immune checkpoint blockade (anti-PD1 and anti-CTLA4) induced a drastic regression of pancreatic tumours[87]. Combining chemotherapy, hydroxychloroquine, and anti-CTLA4 is currently being assessed in pancreatic cancer patients. Similar data were obtained in melanoma models, where inhibition of palmitoyl-protein thioesterase 1 by hydroxychloroquine synergized with anti-PD1 therapy to impair tumour growth and improve survival in mice[155]. Therefore, modulation of autophagy levels in combination with existing clinically-approved drugs has therapeutic benefit in preclinical and clinical settings. Optimizing these combination approaches, developing robust biomarkers of responsiveness, and bringing forward more potent and selective autophagy inhibitors will be critical for future success.

Conclusive remarks and future perspectives

Here, we have reviewed early and recent literature on the role of autophagy in cancer, highlighting a role of autophagy on three main levels: 1) in the tumour, 2) in the tumour microenvironment and 3) in the host. Autophagy and/or mitophagy in tumour cells is important to maintain functional mitochondrial metabolism, redox balance and DNA integrity. In the tumour microenvironment, autophagy in stromal cells is crucial for tumour supply with nutrients. Autophagy in both immune and tumour cells converge into mechanisms allowing tumour immune tolerance and, therefore, to the establishment of immune evasion. Even autophagy in organs distant from the primary tumour has significant impact on systemic metabolism and inflammation, which can shape the microenvironment of the tumour itself.

The physiological stressors that modulate tumour autophagy *in vivo* are various and complex. Depending on the location in the tumour, tumour cells are exposed to distinct stressors, resulting insubstantial heterogeneity in autophagy levels, directly impacting cell metabolism and growth. This heterogeneity may also explain why many conventional therapies are not very successful in treating patients with aggressive cancers. Previous studies have helped to understand the role of autophagy particularly in the context of nutrient starvation, while the impact of other environmental factors on autophagy has not been well characterized. Although culture models of nutrient starvation helped to elucidate the mechanistic underpinning of the autophagic machinery[156,157], addressing its role in the cancer setting requires a more physiological environment. For example, 3D culture platforms may better mimic the behaviour of tumour cells and help to define which environmental factors modulate and induce the autophagic activity in tumour cells. Importantly, 3D organoids may be cultured in media with more physiological metabolite concentrations compared to the supraphysiological levels of glucose and metabolites in cell culture [158]. The successful use of such models to identify key physiological autophagy regulators may hold the promise of optimally modulating autophagy levels in the tumour. Such discoveries can be aided by sophisticated tools, such as functional genomics in 3D conditions, and may pave the way for more targeted, autophagy-focused anticancer therapies.

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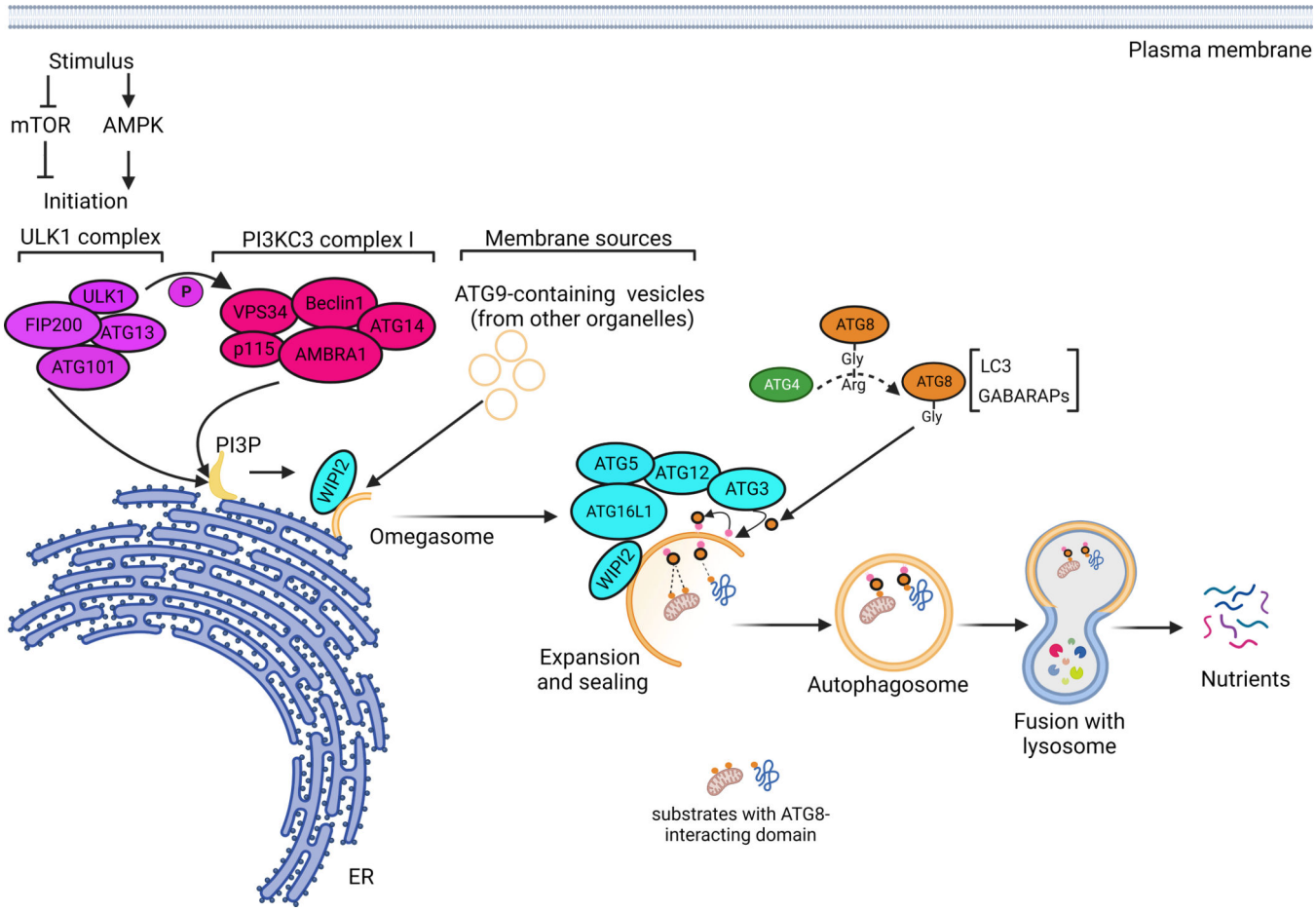

Figure 1. Molecular mechanisms of autophagy.

In response to environmental stresses, autophagy initiation is thought to take place at the endoplasmic reticulum membranes, where the ULK1 complex activates the PI3KC3 complex by phosphorylation. The PI3KC3 complex initiates the production of phosphatidylinositol-3-phosphate (PI3P) on ER subdomains forming a structure called the omegasome. In addition to PI3P provided by PI3KC3 complex, ATG9-containing small vesicles, originating from the membranes of other organelles, are involved in autophagy initiation. From the omegasome structure, a series of reactions involving a large panel of ATGs will take place to allow autophagosome elongation, sealing, maturation and fusion with lysosomes. ATG4, including ATG4B, cleaves Pro-ATG8 forms, such as LC3 and GABARAPL, to allow their conjugation into major phospholipids on the forming autophagosome. This lipidation reaction is catalysed by a complex involving WIPI2, ATG16L1, ATG5, ATG12, ATG7 and ATG3. The lipidation of ATG8s, such as LC3, is important for autophagosome elongation and maturation, but also for the interaction with cargos destined for autophagic degradation; it is therefore a major and critical step in the autophagic process.

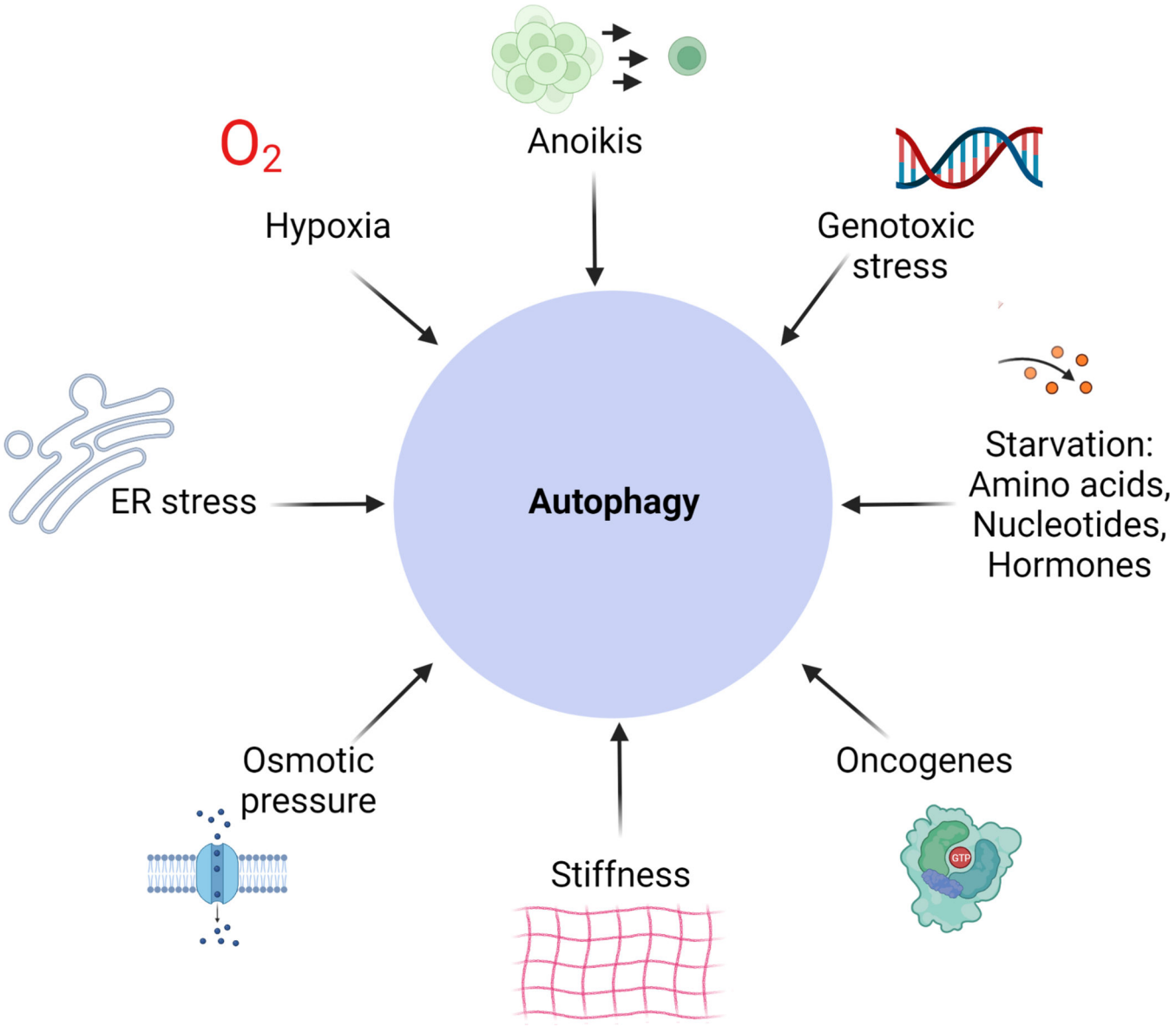

Figure 2. Cellular responses modulating autophagy.

A distinct set of stressors can activate autophagy in cancer cells. They do not act isolated from each other and may co-exist in the cell to activate autophagy, resulting in a highly complex signalling network.

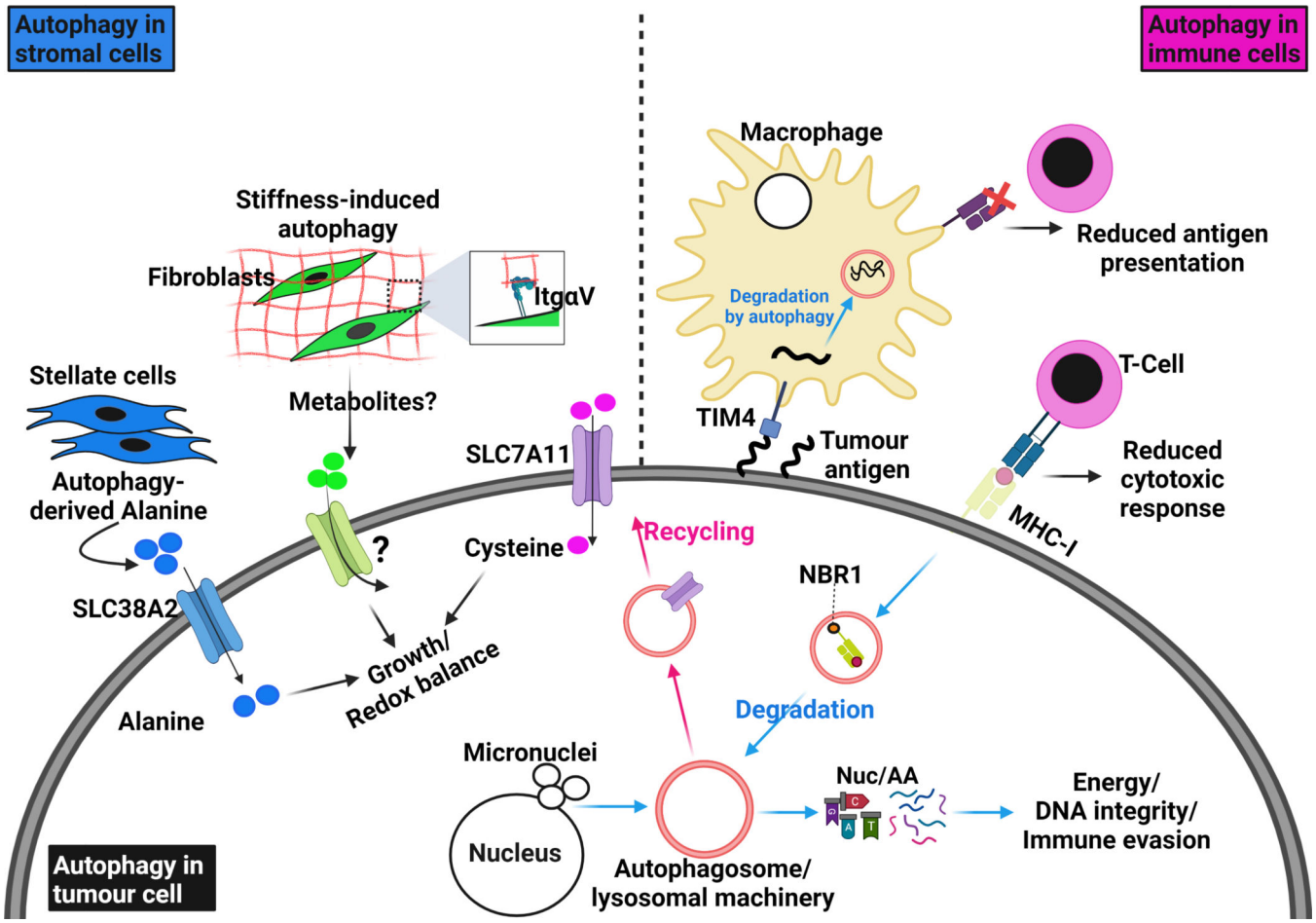

Figure 3. Autophagy in the tumour and surrounding microenvironment.

Autophagy is involved in complex crosstalk between tumour cells and their surrounding microenvironment. Cell-autonomous autophagy in tumour cells is important to relieve replication stress and maintain DNA stability by removing damaged chromosomes and micronuclei, which are the result of aberrant proliferation. It also degrades and recycles essential components for redox homeostasis like the cystine transporter SLC7A11. As energy demands in tumour cells are progressively increasing, they produce factors that activate the stromal component in their microenvironment. Tumour-stimulated stromal cells activate autophagy to release amino acids, such as alanine, which in turn are up-taken by tumour cells through specific transporters, like SLC38A2, to fuel their metabolic networks. Integrin-mediated interaction with stiff extracellular matrix (ECM) directly activates autophagy in stromal cells, such as fibroblasts and stellate cells, and provides tumour cells with growth advantages. More studies are still needed to understand whether ECM stiffness modulates nutrients release from stromal cells. Therefore, autophagy in tumour cells and stromal cells provides the former with sufficient amino acids and nucleotide pools to survive intense episodes of stress. The degradation of surface proteins such as MHC-I by autophagy allows tumour cells to escape anti-tumor immunity; MHC-I degradation and delivery to autophagosomes is dependent on the cargo NBR1. Interestingly, autophagy in immune cells can also sense and degrade tumour antigens through TIM4 and reduce their presentation to

cytotoxic T-cells. All these examples illustrate the autophagy-dependent crosstalk that exists between tumour cells and their surrounding microenvironment.

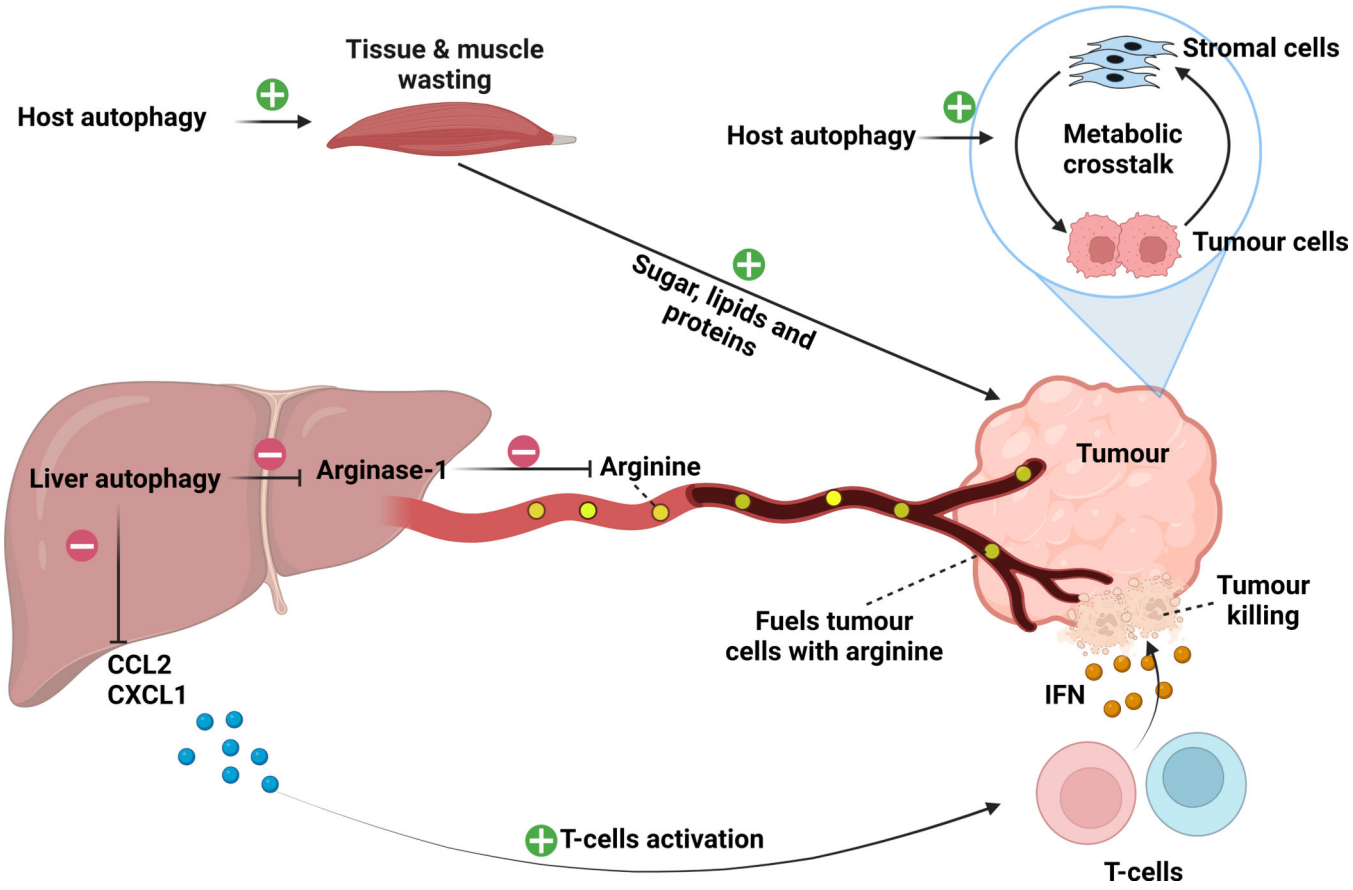

Figure 4. Autophagy in the tumour-bearing host.

Inhibition of host autophagy by expressing a dominant negative version of Atg4B impairs tumour growth by disrupting the metabolic crosstalk between tumour cells and stromal cells and may have other systemic effects in various tissues. In *drosophila melanogaster*, host autophagy has been shown to induce tissue and muscle wasting to fuel tumour cells with sugars, lipids and proteins. In another model, liver-specific inhibition of autophagy, through genetic *Atg7* ablation, induced a stress response in the liver, leading to the release of Arginase-1 into the circulation. High circulating Arginase-1 promoted the degradation of the amino acid arginine that is necessary for the growth of a subset of arginine auxotroph tumours. In parallel, liver-specific autophagy inhibition induced the production and release of pro-inflammatory cytokines from the liver into the circulation. This pro-inflammatory state activated the CD4+ and CD8+ T-cell immune response, resulting in increased tumour killing through the IFN-γ pathway. Green positive sign indicates activation. Red negative sign indicates inhibition.