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REVIEW

Autophagy and cancer: Modulation of cell death pathways and cancer cell adaptations

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Autophagy is intricately linked with many intracellular signaling pathways, particularly nutrient-sensing mechanisms and cell death signaling cascades. In cancer, the roles of autophagy are context dependent. Tumor cell–intrinsic effects of autophagy can be both tumor suppressive and tumor promotional. Autophagy can therefore not only activate and inhibit cell death, but also facilitate the switch between cell death mechanisms. Moreover, autophagy can play opposing roles in the tumor microenvironment via non–cell-autonomous mechanisms. Preclinical data support a tumor-promotional role of autophagy in established tumors and during cancer therapy; this has led to the launch of dozens of clinical trials targeting autophagy in multiple cancer types. However, many questions remain: which tumors and genetic backgrounds are the most sensitive to autophagy inhibition, and which therapies should be combined with autophagy inhibitors? Additionally, since cancer cells are under selective pressure and are prone to adaptation, particularly after treatment, it is unclear if and how cells adapt to autophagy inhibition. Here we review recent literature addressing these issues.

Introduction

Macro-autophagy is a complex multistep process that facilitates the degradation of damaged and excess proteins and organelles to generate macromolecular building blocks and fuel metabolic pathways (Dikic and Elazar, 2018). The autophagy pathway has critical roles in core biological processes such as mitochondrial function, cell death, immune surveillance, protein homeostasis, stress response, and metabolism. Accordingly, abnormalities in these processes and the disease-associated pathologies have been linked to aberrant autophagic degradation, most notably in aging, neurodegenerative diseases, and multiple forms of cancer. In this review, we focus on the protumorigenic role of autophagy in cancer, highlighting recent insights linking autophagy and apoptosis and other death pathways. With over 60 active clinical trials targeting autophagy in a variety of tumor types, it is critical to understand how the molecular mechanisms that connect these processes can be leveraged to enhance the benefit to patients and prevent relapse. The history of cancer therapy has proven that adaptation and acquired resistance to anticancer therapies represent perhaps the largest obstacle to overcome. Therefore, a critical, as yet incompletely understood, issue is whether autophagy inhibitors will be plagued by these same hurdles. Here we address this and other questions regarding autophagy inhibition as a cancer therapy.

Macro-autophagy

The evolutionarily conserved recycling processes that deliver surplus or damaged cytoplasmic material to lysosomes for degradation can be subdivided into three related processes: micro-autophagy, chaperone-mediated autophagy, and macro-autophagy. Micro-autophagy and chaperone-mediated autophagy involve direct delivery mechanisms to the lysosome, both of which can also be important in cancer; for a detailed discussion, readers are referred to an excellent recent review (Kaushik and Cuervo, 2018).

Macroautophagy (hereafter autophagy) is a multistep process involving >20 core autophagy proteins, called ATGs, that function to envelop cytoplasmic cargo within a double-membrane vesicle structure. These autophagosomes can subsequently fuse with acidic lysosomes, where pH-sensitive enzymes mediate the degradation of the cytoplasmic material (Dikic and Elazar, 2018; Fig. 1). The pathway is initiated by the Unc-51–like kinase (ULK) complex, which phosphorylates a phosphatidylinositol 3-kinase (VPS34), part of the Beclin1 complex necessary for initiation of the phagophore (Mizushima et al., 2011; Russell et al., 2013; He and Levine, 2010). Extension of the elongating phagophore membrane relies on two ubiquitin-like conjugation systems. The E1- and E2-like enzymes ATG7 and ATG10 conjugate ATG5 and ATG12. The resulting ATG5–12 conjugate binds to ATG16L1, and this complex acts as a E3-like enzyme in coordination with ATG7 as E1 and ATG3 as E2 to

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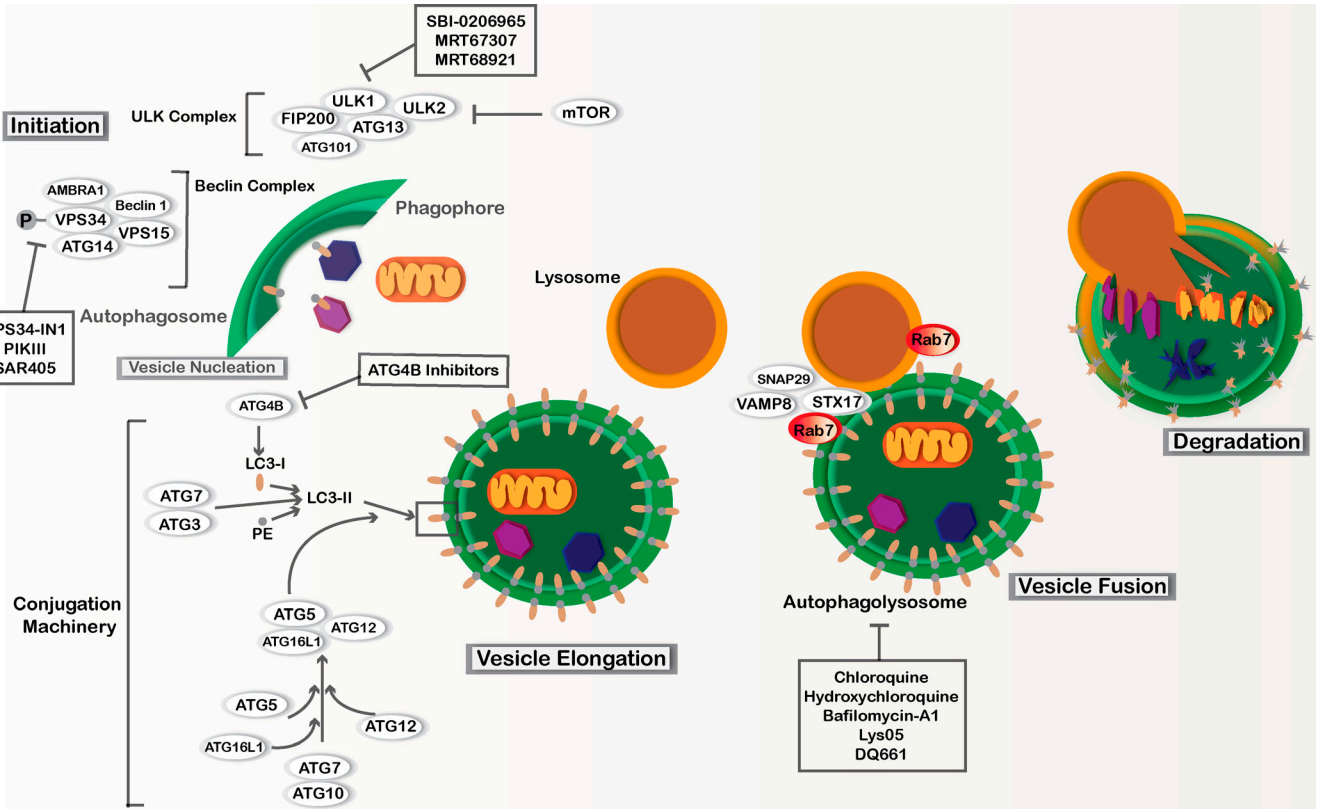

Figure 1. **Macro-autophagy.** Macro-autophagy involves core autophagy proteins or ATGs and is subdivided into different stages, including phagophore initiation, vesicle nucleation, vesicle elongation, and autophagosome fusion with lysosomes. The ULK complex involves Unc-51–like autophagy activating kinases 1 and 2 (ULK1 and ULK2), ATG13, ATG101, and FAK family kinase interacting protein of 200 kD (FIP200). This complex can be regulated by nutrient availability via mTOR regulation as well as other signaling pathways to induce phagophore initiation. The Beclin complex is activated downstream of the ULK complex and is also necessary for phagophore initiation. The Beclin complex includes coiled-coil, moesin-like, BCL2 interacting protein (Beclin-1), activating molecule in Beclin-1 regulated autophagy (AMBRA-1), phosphatidylinositol 3-kinase catalytic subunit type 3 and regulatory subunit 4 (VPS34 and VPS15, respectively), and ATG14. Vesicle elongation depends on two ubiquitin-like conjugation systems. ATG5 is conjugated to ATG12 with the help of the E1-like enzyme, ATG7, and the E2-like enzyme, ATG10. The ATG5–ATG12 conjugate binds to ATG16L1, and together they act as a E3-like enzyme to facilitate the conjugation of microtubule-associated protein 1A/1B LC3 to PE. This second conjugation is also aided by ATG7 as well as the E1-like enzyme, ATG3. Prior to LC3-PE conjugation, LC3 is cleaved by the cysteine protease ATG4B. LC3-PE is incorporated into the autophagosome membrane. SNARE proteins including syntaxin-17 (STX17), synaptosome-associated protein 29 (SNAP29), and vesicle-associated membrane protein 8 (VAMP8) facilitate fusion between fully formed autophagosomes and lysosomes. The GTPase Rab7 is also important during fusion. After fusion occurs, the cytoplasmic material within the autolysosome as well as the intravesicular LC3-II is degraded by pH-sensitive enzymes found within the acidic compartments. Pharmacological agents that are currently used to inhibit autophagy in preclinical models are annotated.

conjugate phosphatidylethanolamine (PE) to the GABARAP/light chain 3 (LC3) family of proteins, the most well characterized being LC3B (Shpilka et al., 2011; Dikic and Elazar, 2018). The ATG4 family of cysteine proteases cleave the LC3 family members to create LC3-I, which is conjugated to PE to generate LC3-II (Li et al., 2011; Kirisako et al., 2000). Membrane-associated LC3-II associates with the autophagosome membrane and is critical as a target for recognition by adaptor proteins that bring specific substrates into the autophagosome for selective degradation. A handful of adaptor proteins have been identified, including the most well characterized, SQSTM1/p62, but also BNIP3, TAX1BP1, Optineurin, and NIX/BNIP3L, to name a few (Anding and Baehrecke, 2017). While LC3-II is dispensable for autophagosome formation, it is important for efficient autophagosome closure and fusion with lysosomes (Nguyen et al., 2016). Consequently, delayed closure and formation of inefficient autophagosomes can still occur in the absence of the conjugation machinery and LC3-II (Tsuboyama et al., 2016). Once closure is complete, the double-membrane autophagosome fuses with lysosomes using SNARE proteins, as well as the small GTPases, such as Rab7 (Yu et al., 2018; Hamasaki et al., 2013; Kirisako et al., 1999; Bento et al., 2013; Zhao and Zhang, 2019). Lysosomal enzymes then break down the cytoplasmic contents into amino acids and other macromolecular building blocks that are recycled into new macromolecules and fuel metabolic pathways.

Nutrient-sensing pathways tightly regulate autophagy induction, most notably by the mammalian targets of rapamycin complexes, mTORC1 and mTORC2 (Saxton and Sabatini, 2017). In the case of amino acid deprivation, the inhibitory mTORC complexes can no longer phosphorylate and inhibit the ULK complex, triggering autophagosome membrane nucleation (Hosokawa et al., 2009). Decreased energy availability and ATP:AMP ratios in the absence of sufficient glucose triggers the 5′ AMP-activated protein kinase, which can inhibit mTORC

signaling and also directly activate the ULK complex (Kim et al., 2011). Another major mechanism of autophagy regulation is transcriptional and mediated by master transcriptional regulators that activate expression of the core ATGs. These include the transcription factor EB (TFEB; Palmieri et al., 2011; Settembre et al., 2011), microphthalmia-associated transcription factor (Ploper et al., 2015; Perera et al., 2015), and the forkhead family of transcription factors (FOXO1 and FOXO3; Audesse et al., 2019; Zhao et al., 2007; Mammucari et al., 2007; Sengupta et al., 2009). Recently, the epigenetic regulator bromodomain-containing protein 4 was identified as a transcriptional repressor of autophagy and lysosomal genes (Sakamaki et al., 2017).

Transcriptional regulation of autophagy is thought to regulate the overall basal level of the process, and variations in transcriptional control may explain differences in basal autophagy between different cells/tissues (Perera et al., 2015). On the other hand, posttranslational regulation through kinases controlled by stress and nutrient-sensing pathways such as mTOR largely explain acute changes in autophagy. Additional cellular stressors, including hypoxia, metabolic stress, and ER stress, among others, can induce autophagy through mechanisms that usually converge on these major nutrient-sensing pathways. Moreover, these pathways interact with each other for more coordinated and intricate regulation mediated by feedback loops (Dikic and Elazar, 2018). For example, TFEB is negatively regulated by mTORC1 and the Ras-related GTP-binding protein D, which is an mTOR-activating Rag GTPase, a transcriptional TFEB target (Di Malta et al., 2017). Importantly, autophagy can participate in bulk degradation of cytoplasmic material, but there are also selective forms of autophagy that rely on intricate signaling cascades to target specific proteins or organelles including mitochondria, ribosomes, bacteria, ferritin, ER, and peroxisomes: mitophagy, ribophagy, xenophagy, ferritinophagy, ERphagy, and pexophagy, to name just a few. Details regarding selective autophagy processes have recently been reviewed (Anding and Baehrecke, 2017).

Autophagy and cancer

Tumor-cell intrinsic roles of autophagy in tumor growth

Autophagy plays a complex role in tumor development and progression. Autophagy was first suggested to function as a tumor-suppressive process, with the discovery of Beclin1 deletions across multiple tumor types and the ability of Beclin1 to function as a haplo-insufficient tumor suppressor in mice (Yue et al., 2003; Qu et al., 2003; Liang et al., 1999). Loss of other Beclin1 interacting proteins including endophilin B1 and UV radiation resistance-associated gene protein can also cause an increase in spontaneous tumors in mice and are mutated in human tumors, further supporting the Beclin complex as a tumor suppressor (Liang et al., 2006; Takahashi et al., 2007). It was reported, however, that in human tumors there is no significant loss of *BECN1* independent of the adjacent potent tumor suppressor *BRCA1* (Laddha et al., 2014). Additional tumor-suppressive functions of autophagy have emerged, including removal of damaged reactive oxygen species (ROS)–inducing mitochondria, maintenance of genomic stability, and a role in oncogene-induced senescence via degradation of the nuclear lamina (Levine and Kroemer, 2019; Wang et al., 2016; Belaid et al., 2013; Dou et al., 2015). Together, these studies suggest that autophagy prevents tumor initiation and early steps in tumor progression. Consistent with this, genetic inhibition of other core genes that are themselves only very rarely mutated in cancer, including *Atg5* and/or *Atg7*, confirmed that deletion of autophagy in tumor-prone mouse models following RAS pathway activation or TP53 deletion caused an increase in preneoplastic lesions and even tumor incidence (Rosenfeldt et al., 2013; Strohecker et al., 2013; Rao et al., 2014; Yang et al., 2014).

However, these studies also showed that after a tumor is fully established, autophagy inhibition often results in less aggressive cancers. Successful tumor cells have to cope with harsh environments characterized by nutrient depletion, hypoxia, and other stresses. Autophagy is able to help cells cope with many of these stressors; thus although autophagy may serve to prevent tumor initiation, it often promotes tumor cell survival in more advanced cancers. Many studies have confirmed that genetic deletion of autophagy regulators (often Atg7 or Atg5) in established tumors from a variety of tissue types causes a dramatic reduction in tumor growth and a corresponding increase in survival of the animal. These effects have been attributed to blocked tumor growth and increased tumor cell death mediated by an accumulation of dysfunctional mitochondria, reduced fatty acid oxidation, reduced glycolytic capacity, elevated DNA damage, and impaired tumor cell metabolism (Guo et al., 2011, 2013, 2016; Karsli-Uzunbas et al., 2014; Yang et al., 2011, 2018; Poillet-Perez et al., 2018; Degenhardt et al., 2006; Strohecker et al., 2013; Xie et al., 2015).

Cancer cells vary in their dependence on autophagy. Some cancer cell lines are acutely sensitive to genetic inhibition of autophagy, while others show very little change in viability in the absence of autophagy. While little is known about what drives autophagy dependence in cancer, tumors with RAS-RAF-MEK-ERK pathway activation are often more sensitive to autophagy inhibition. Pancreatic ductal carcinomas (PDACs) for example, where RAS mutations are very common, are exquisitely sensitive to autophagy inhibition (Yang et al., 2011, 2014; Perera et al., 2015). TP53 status may also affect autophagy dependence, and loss of p53 in a humanized PDAC mouse model with KRAS activation eliminated the antitumor effects of autophagy inhibition (Rosenfeldt et al., 2013). Additional studies showed that these effects may apply only to germline deletion of TP53, as somatic loss of heterozygosity of TP53, i.e., the more common form of p53 deletion in human tumors, did not have the same effect (Yang et al., 2014). Pediatric brain cancer cells with a constitutively active BRAF(V600E) mutation are more sensitive to both genetic and pharmacological autophagy inhibition (Levy et al., 2014). Additionally, a variety of cancer cells without RAS pathway mutations have also been identified as extremely autophagy dependent in vitro (Maycotte et al., 2014; Towers et al., 2019).

Many general cytotoxic chemotherapy drugs as well as multiple targeted therapies such as kinase inhibitors have been proposed to induce autophagy as a cytoprotective measure in cancer cells. For example, inhibitors of the MAP kinase pathway

can induce autophagy in pancreas cancer, and combinations of these kinase inhibitors with autophagy inhibition can enhance tumor cell killing (Bryant et al., 2019; Kinsey et al., 2019). These effects can even extend to overcoming acquired resistance to specific drugs. For example, patients with BRAF(V600E)-driven brain tumors that had become resistant to the BRAF inhibitor vemurafenib could be resensitized to the kinase inhibitor by addition of an autophagy inhibitor (Mulcahy Levy et al., 2017; Levy et al., 2014).

Autophagy also promotes a stemness phenotype, not only in normal stem cells (Warr et al., 2013; Vázquez et al., 2012; García-Prat et al., 2016), but also in "cancer stem cells" that can maintain a quiescent or dormant state, self-renew, and regenerate an entire tumor at limiting dilutions (Meacham and Morrison, 2013; Smith and Macleod, 2019). In breast cancer models, genetic manipulation of core autophagy proteins can diminish the tumor-initiating properties of specific cancer stem cell populations in mice (Yeo et al., 2016). Similarly, in chronic myeloid leukemia (CML), the addition of autophagy inhibition to kinase inhibitors led to the complete elimination of functionally defined CML stem cells (Bellodi et al., 2009). These effects may be due to autophagy-regulated metabolic changes in the stem cells (Kuntz et al., 2017). Together, these basic cell biology, preclinical, and clinical studies support a cell-autonomous, tumor-suppressive role for autophagy at early stages in oncogenesis and a tumor-promotional and therapy-resistance role in more established tumors.

Non–cell-autonomous roles of autophagy in tumor growth

The tumor-promotional roles of autophagy are both cell autonomous and nonautonomous (Yang et al., 2018) and are becoming increasingly recognized as important in controlling how the tumor microenvironment (TME) affects tumor growth (Fig. 2). However, as with the tumor cell-autonomous functions, non–cell-autonomous effects can be both pro- and antitumor. For example, autophagy in the tumor cells can affect whether immune cells in the TME recognize the tumor to either enhance or inhibit the antitumor immune response. Autophagy in the tumor cells was shown to be necessary for immunogenic cell killing following chemotherapy treatment to allow efficient tumor infiltration by antitumor dendritic cells and T-lymphocytes (Michaud et al., 2011). Moreover, autophagy induction by caloric restriction mimetics before treatment with anticancer drugs could improve anticancer immunosurveillance and decrease tumor burden (Pietrocola et al., 2016). These studies have been interpreted as meaning that tumor cell autophagy should be maintained or even enhanced to elicit a productive antitumor immune response.

Several other studies, however, have demonstrated the opposite effect, i.e., tumor cell autophagy may reduce antitumor immune responses, for example, by showing that inhibition of autophagy in the tumor cells can enhance natural killer (NK) cell infiltration and reduce tumor growth (Baginska et al., 2013; Mgrditchian et al., 2017). Genetic or pharmacological autophagy inhibition caused an increase in the transcript levels of the cytokine CCL5, mediated by decreased PP2A phosphatase activity and subsequent activation of JNK- and c-Jun–mediated transcription of CCL5 to attract NK cells into the TME (Mgrditchian et al., 2017).

It has also become clear that autophagy in the immune cells themselves is important. Pharmacological inhibition of autophagy with chloroquine (CQ) in tumor-associated macrophages causes a switch from the tumor-promotional M2 phenotype to an M1, tumor-killing, phenotype. CQ-reset macrophages decreased the immunosuppressive T cell populations and enhanced antitumor T cell immunity in mouse models (Chen et al., 2018a). Inhibition of autophagy in $CD8^+$ T cells can also promote tumor rejection in mice by shifting T cell biology to a more effector memory phenotype and producing more cytokines, including IFNγ and TNFα. These effects were linked to enhanced glucose metabolism and altered epigenetic regulation in the T cells (Devorkin et al., 2019).

Processes that use components of the autophagy machinery but are independent of the degradative process of autophagy itself also affect tumor cell growth. Core autophagy proteins have been implicated in nonconventional secretory pathways (Dupont et al., 2011), host–pathogen interactions during infections, immune signaling, and inflammation (Cadwell and Debnath, 2018; Galluzzi and Green, 2019). LC3-associated phagocytosis (LAP) utilizes a subset of core ATGs, in addition to the LAP-specific protein Rubicon, which enhances LAP but inhibits autophagy, to facilitate the conjugation of LC3 to phagosomal membranes. Inhibition of LAP, but not canonical autophagy, in tumor-associated macrophages causes an M1 (antitumor) phenotype that induces a STING-dependent type 1 IFN and antitumor T cell response (Cunha et al., 2018). Interestingly, the core LC3 conjugation machinery, including ATG5 and ATG7, is shared between autophagy and LAP; therefore the majority of studies conducted in mice to date using genetic knockout (KO) of these proteins cannot differentiate between LAP and canonical autophagy (Heckmann and Green, 2019).

Beyond immune cell–mediated effects, autophagy in other nontumor cells within the TME can increase growth-promoting stimuli. For example, inhibition of autophagy in stroma-associated pancreatic stellate cells causes a decrease in secreted alanine, which is critical for PDAC cell growth. This mechanism involves a significant amount of cross-talk between the different cell types within the TME. The PDAC cells induce autophagy in the stellate cells to increase secretion of nonessential amino acids, which are then used by the PDAC cells to fuel the tricarboxylic acid cycle (Sousa et al., 2016). Recently, it was also shown that deletion of host autophagy causes a decrease in circulating arginine, and since many tumor cells are arginine auxotrophs, this can cause a corresponding decrease in tumor growth (Poillet-Perez et al., 2018). Another study showed that transplantation of autophagy-deficient, dormant tumors into autophagy-proficient hosts caused a reactivation of tumor growth mediated by TNF and IL-6–like signaling in a *Drosophila melanogaster* model (Katheder et al., 2017). Importantly, the dormant tumors remained small when they were transplanted into an autophagy-deficient host. These studies suggest that multiple mechanistically distinct mechanisms contribute to autophagy-dependent promotion of tumor growth from the microenvironment (Fig. 2).

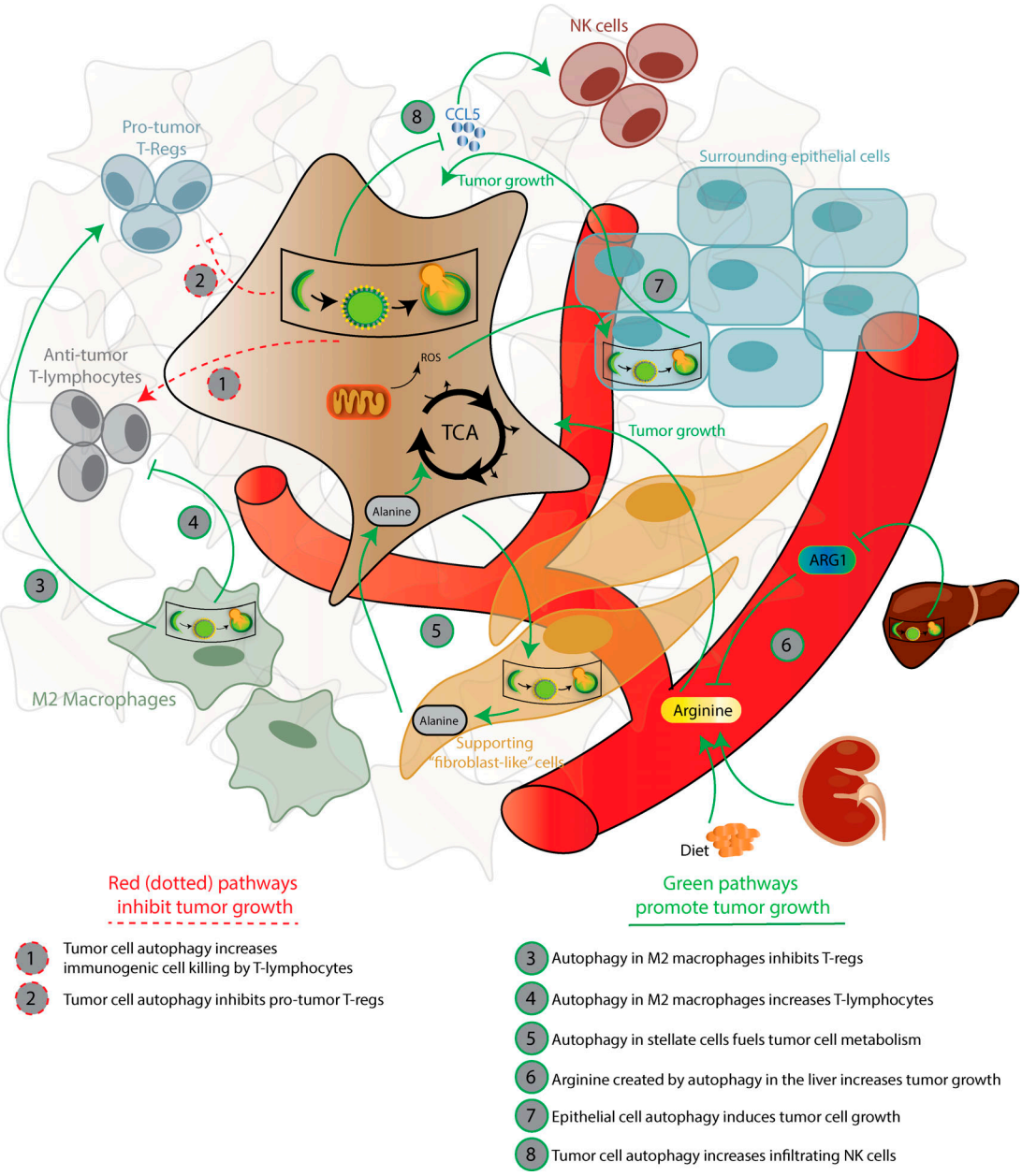

Figure 2. **Non–tumor cell-autonomous roles of autophagy in cancer.** The red dotted lines indicate pathways that have been implicated in the tumor-suppressive roles of autophagy in cancer. These mechanisms include how autophagy in the tumor cells can increase the presence of infiltrating antitumor T-lymphocytes and decrease protumor regulatory T cells in the TME. Green lines indicate pathways that have been implicated in the tumor-promotional roles of autophagy in cancer. Autophagy in the tumor cells can inhibit the antitumorigenic NK cells via inhibition of the cytokine, CCL5. Autophagy in nontumor cells within the TME can also affect the tumor growth. Autophagy in the supporting fibroblast-like cells and surrounding epithelial cells can support metabolism and tumor cell proliferation. Autophagy in macrophages can increase protumor regulatory T cells and decrease infiltrating tumor-suppressive T cells. In $CD8^+$ T cells, loss of autophagy increases the memory effector phenotype and also increases the antitumor cytokine IFNγ. Circulating arginine supplied from the kidneys and diet can also support tumor cell proliferation. Autophagy in hepatocytes is important for regulating the arginine-degrading enzyme arginase-1 (ARG1).

Autophagy and metastasis

Metastasis is a multistep process in which cancer cells acquire invasive phenotypes such as motility, the ability to survive under detached conditions, and stem cell–like properties including a more mesenchymal morphology by way of epithelial-to-mesenchymal transition (EMT; Massagué and Obenauf, 2016). Pathology studies in breast cancer and melanoma patients identified a correlation between elevated LC3B puncta staining and poorer outcomes and detected especially high LC3 expression in the metastatic lesions (Lazova et al., 2010, 2012; Zhao et al., 2013; Han et al., 2011). In addition to these correlative studies, autophagy has been shown to play a causative role in metastasis. Both genetic and pharmacological inhibition of autophagy eliminated lung metastasis in a syngeneic breast

cancer mouse model after orthotopic injection of the mouse mammary carcinoma 4T1 cell line (Sharifi et al., 2016). Autophagy has also been associated with EMT, a particular hallmark of metastatic dissemination that is linked to increased invasiveness as well as cancer stem cell phenotypes (Li et al., 2013; Kim et al., 2016). Inhibition of autophagy-mediated degradation of RNA processing bodies, or p-bodies, mitigates a mesenchymal-to-epithelial transition, ultimately reducing metastatic outgrowth (Shinde et al., 2019). An additional mechanism by which autophagy can regulate cell migration and metastasis is dependent on autophagy-mediated regulation of focal adhesion dynamics. Autophagosomes interact with and turn over focal adhesion complexes to destabilize the cell-matrix contact sites, allowing for increased cell motility. This involves the autophagy cargo receptor NBR1 as well as direct interactions between LC3B and the focal adhesion protein, paxillin (Sharifi et al., 2016; Kenific et al., 2016).

In line with the competing pro- and antitumorigenic roles of autophagy at the primary tumor site, there have also been studies showing that autophagy can both promote and inhibit metastatic outgrowth at secondary sites. Metastatic cancer cells can be maintained in a dormant state for long periods of time at a secondary site only to initiate growth at a later time, resulting in appearance of metastatic tumors (Sosa et al., 2014). Transcriptomic analysis of surviving pancreatic cancer cells after oncogene ablation identified elevated expression of autophagy and lysosome-regulating genes, suggesting a role for these processes in the survival of subpopulations of dormant cells (Viale et al., 2014). Similarly, in breast and ovarian cancer models, autophagy has been shown to promote the survival and outgrowth of dormant cell populations (Lu et al., 2008; Vera-Ramirez et al., 2018). Specifically, in D2.0 mouse mammary carcinoma cells (derived from murine mammary hyperplastic alveolar nodules) that can remain dormant in the lung for months before outgrowth, pharmacological autophagy inhibition caused a drastic reduction in metastatic outgrowth in the lung, suggesting that autophagy is critical for survival of the dormant cells (Vera-Ramirez et al., 2018). However, using the same D2.0 model, a recent study confirmed elevated autophagic flux in the dormant cells, but in contrast concluded that inhibition of autophagy did not eliminate the dormant cells but instead caused them to emerge from the dormant state to reinitiate proliferation, causing an increase in metastatic outgrowth (La Belle Flynn et al., 2019). The discrepancy between these two studies could depend on differential host immunity (i.e., athymic mice vs. intact mice), different experimental procedures (i.e., time frame for quantification and imaging), or different means used to inhibit autophagy (i.e., pharmacological or genetic). Other mechanisms can also provide competing pro- and antimetastatic signals, even though the same autophagy-related proteins were manipulated. For example, in melanoma, the autophagy cargo receptor SQSTM1/p62, which is reduced in cells where autophagy is active, can interact with select RNA-binding proteins to stabilize specific prometastatic mRNAs (Karras et al., 2019). After autophagy inhibition, the resulting accumulation of p62 can interact with and stabilize the pro-EMT and prometastatic protein, Twist1 (Qiang et al., 2014; Bertrand et al., 2015). Conversely, p62 can also target prometastatic proteins for autophagic degradation, leading to a decrease in metastatic potential (Tan et al., 2018).

Targeting autophagy in cancer patients

While additional studies are necessary to fully understand the mechanisms of the tumor-suppressive and tumor-promotional roles of autophagy, both cell autonomously and nonautonomously, pharmacological and therefore systemic inhibition of autophagy is already moving forward in the clinic. A variety of pharmacological autophagy inhibitors and inducers are in development, and it is known that many approved drugs can affect autophagy (Rubinsztein et al., 2012; Kaizuka et al., 2016). However, the lysosomal inhibitor CQ and its derivative hydroxychloroquine (HCQ) are the only drugs that are currently used in patients with the deliberate goal of targeting autophagy (Towers and Thorburn, 2016). The first wave of clinical trials with CQ/HCQ in combination with other therapies showed promising results, indicating that target doses of the autophagy-inhibitor drugs could be reached with minimal toxicity (Levy et al., 2017). Some antitumor effects were also observed. Glioblastoma patients treated with CQ combined with radiation and the alkylating agent, temozolomide (TZD), tripled their median survival over control patients (33 mo compared with 11 mo; Briceño et al., 2003). In melanoma, 41% of patients treated with CQ and TZD showed a partial response or stable disease, and 84% of patients with brain metastasis derived from a variety of solid tumors treated with CQ and radiation reported a 1-yr survival rate compared with 55% of patients treated with radiation alone (Rangwala et al., 2014; Rojas-Puentes et al., 2013). BRAF(V600E) mutant brain cancers are particularly dependent on autophagy for survival, and combined therapies with the BRAF-targeted therapy vemurafenib and CQ synergistically reduces cancer cell viability (Levy et al., 2014). These results led to the clinical use of CQ in vemurafenib-resistant brain cancer patients who showed favorable results when treated with a CQ/vemurafenib combination (Mulcahy Levy et al., 2017). Despite these initially promising studies, recent clinical trials have provided lackluster results. This is likely due to a variety of factors, including almost no biomarkers to identify the correct patients ideal for treatment, a need for more specific and potent autophagy inhibitors, and a lack in understanding of potential mechanisms of resistance.

Novel autophagy inhibitors

While only CQ and HCQ have been approved for use in patients, other potent autophagy inhibitors are working their way through the pipeline. These include upstream inhibitors that target VPS34, including VPS34-IN1 (Bago et al., 2014), PIK-III (Dowdle et al., 2014), and SAR405 (Ronan et al., 2014). Other agents are in development that target the ULK1 kinase such as SBI-0206965 (Egan et al., 2015), MRT67307, and MRT68921 (Petherick et al., 2015). Pharmacological inhibitors of the cysteine protease ATG4B are also being optimized to block LC3 conjugation (Akin et al., 2014). The targets of these agents are annotated in Fig. 1, but the exact mechanism of action is described in detail elsewhere (Chude and Amaravadi, 2017).

The pharmacological reagents with arguably the most clinical potential are CQ and quinacrine derivatives such as Lys05 and DQ661. Lys05, similar to CQ/HCQ, de-acidifies the lysosome and is thought to have increased antitumor potential over CQ or HCQ (Chude and Amaravadi, 2017). Up until recently, the exact target of CQ, HCQ, and Lys05 was unknown; however, due to the increased lysosomal specificity and potency of DQ661, an in situ photoaffinity pull-down assay could be used. While palmitoyl-protein thioesterase 1 was identified as the direct target of all the CQ and quinacrine derivatives, DQ661 appears to be the most specific and have the most potent antitumor activity in preclinical models (Rebecca et al., 2017, 2019).

Cooperation between autophagy, apoptosis, and necroptosis

Mechanisms of cell death

Cell death is a highly regulated and programmed process and has recently been subdivided into 12 interconnected subclasses based on distinct signaling pathways and has been extensively reviewed elsewhere (Galluzzi et al., 2018). The 12 subroutines have been historically grouped into three major categories based on cell morphology, including apoptosis, necrosis, and autophagic cell death (Green and Llambi, 2015; Kerr et al., 1972; Galluzzi et al., 2018). Apoptosis can be initiated either by external stimuli reliant on membrane-bound death receptor signaling or via the intrinsic pathway that is dependent on mitochondrial signaling and is stimulated by internal cues such as DNA damage or ER stress. Both forms of apoptosis share a dependence on caspase cascades converging on cleavage of the executioner caspases 3 and 7, resulting in an orderly cell death process characterized by plasma membrane blebbing, chromatin condensation, cell shrinkage, and nuclear fragmentation. In contrast, necrosis is morphologically distinct and is accompanied by cell swelling and lysis without nuclear condensation. While necrosis can occur after an overwhelming insult lacking controllable signaling cascades, it has become clear in recent years that it can also be programmed and driven by distinct signaling pathways. For example, necroptosis involves activation of receptor-activating protein kinase 3 (RIPK3), which then activates mixed-lineage kinase domain-like pseudokinase (MLKL) that serves as the mediator of cell lysis and death (Galluzzi et al., 2018).

The third major form of cell death, autophagic cell death, is the most controversial; it involves the appearance of large vacuoles and is reliant on the core autophagy machinery (Green and Llambi, 2015). There are distinct contexts where autophagy appears to be necessary for physiological cell death, particularly during *Drosophila* development (Berry and Baehrecke, 2007; Denton et al., 2009). Autophagic cell death has been reported in cancer cells as a potential safeguard to prevent RAS-induced oncogenic transformation (Elgendy et al., 2011). ATG5 was reported to be necessary for apoptosis-independent death observed in epithelial cell lines transformed with either HRAS or KRAS (Byun et al., 2009). ROS-inducing agents also stimulate autophagic cell death that cannot be attenuated with caspase inhibitors in different cancer cell lines (Chen et al., 2008). While these initial studies may have reported autophagic cell death, all other forms of cell death were often not properly ruled out. Generally, autophagy is a prosurvival and cytoprotective mechanism, and failed attempts at survival mediated by autophagy often accompany cell death morphologies (Shen et al., 2012). Thus, it is often difficult to exclude the possibility that activation of autophagy in a dying cell was not in fact contributing to the death but was instead an effort by the cell to avoid dying. The current recommendation (Galluzzi et al., 2018) is that the term "autophagic cell death" should refer only to a death process that requires the autophagy machinery but does not involve the other major death mechanisms such as apoptosis or necroptosis.

Recently, autophagic cell death was shown to be important in epithelial cells during crisis after telomere dysfunction, implying that activation of an autophagy-dependent form of death acts as a principal tumor-suppressive barrier (Nassour et al., 2019). The debate of prosurvival versus prodeath autophagy in cancer cells is, of course, relevant to the tumor-suppressive versus tumor-promotional roles of the process, as well as how cancer cells respond to therapy. Defining stimuli that induce autophagic cell death while identifying strategies that inhibit the cytoprotective functions of autophagy will be critical to optimize the therapeutic use of autophagy inhibitors in the clinic. Additional complications may arise when a single stimulus can induce autophagic cell death but only under some contexts. For example, Azad et al. (2008) showed that the DNA damaging agent etoposide can induce apoptotic cell death in normal oxygen conditions; however, the mechanism of cell death switched to autophagic cell death in hypoxia. Interestingly, while it is clear that some cancer cells are prone to die after autophagy inhibition, either genetic or pharmacological, it is unclear exactly how these cells are dying. Caspase activation has been noted in these instances, but any nonapoptotic contributions to the death such as different types of programmed necrosis have not been ruled out.

Autophagy promotion of other forms of cell death

Beyond autophagic cell death, it is clear that the autophagic machinery can be intertwined with the apoptotic pathways, and the two can either cooperate or inhibit each other depending on the context and inducing stimuli (Doherty and Baehrecke, 2018). While the above experiments suggest that autophagy plays a direct role in cell death, there are a number of experiments indicating a more indirect role where autophagy affects apoptosis. Different anti-apoptotic proteins have been identified as autophagy substrates. For example, some cells with elevated autophagic flux are more sensitive to FAS-induced apoptosis due to selective autophagic degradation of the tyrosine phosphatase, FAP-1/PTPN13. Interestingly, these results were stimulus specific, as FAP-1 specifically modulates FAS-induced apoptosis; accordingly, elevated autophagy in the exact same cells does not sensitize cancer cells to a very similar apoptotic stimulus, such as TNF-related apoptosis-inducing ligand (TRAIL)–induced apoptosis (Gump et al., 2014). In hippocampal astrocytes, autophagic degradation of the anti-apoptotic protein, Cav-1, was shown to be important for autophagy-induced apoptosis in the presence of palmitic acid (Chen et al., 2018b). Additionally, in mouse embryonic fibroblasts, caspase-8 can complex with Atg5

and interact with LC3 and p62 on the autophagosome membrane. The intracellular death-inducing signaling complex, including Fas-associated death domain (FADD), a necessary adaptor protein for caspase-8 activation, is also recruited to the complex and interacts with ATG5 on ATG16L- and LC3-positive autophagosomes. These interactions promote caspase-8 self-processing to propagate the caspase signaling cascade and cause cell death (Young et al., 2012; Tang et al., 2017).

Autophagy inhibition of cell death

Although the above examples show that under certain circumstances autophagy can promote apoptosis, autophagy more commonly affects cell death by reducing apoptosis. Genetic inhibition of core ATGs causes an increase in the pro-apoptotic protein, PUMA (p53 up-regulated modulator of apoptosis) and a corresponding increase in complete mitochondrial outer membrane permeabilization, usually the point of no return in apoptosis (Thorburn et al., 2014). Interestingly, PUMA is not a direct autophagic substrate but instead is transcriptionally regulated by the pro-apoptotic transcription factor, FOXO3a (Warr et al., 2013). The autophagic turnover of FOXO3a mediates changes in PUMA mRNA levels, and deletion of a single intronic FOXO3 binding forkhead response element (FHRE) in the endogenous PUMA gene eliminated both pharmacological and genetic autophagy inhibition-induced PUMA expression (Fitzwalter et al., 2018). Consequently, the PUMA FHRE was shown to be critical for synergistic induction of apoptosis when pharmacological autophagy inhibition with CQ was combined with other cytotoxic agents including etoposide, doxorubicin, and the p53-targed therapy, Nutlin-3a. Interestingly, these experiments were conducted in a cell model characterized as autophagy independent, where treatment with CQ alone did not cause cell death. Instead, autophagy inhibition pushed the cells closer to their apoptotic threshold, allowing addition of the other drug to more easily induce apoptosis. Other molecularly distinct mechanisms by which autophagic turnover of a protein can reduce apoptosis sensitivity have also been identified. For example, the initiator caspase and pro-apoptotic protein caspase-8 has also been identified as an autophagic substrate to coordinate crosstalk mechanisms between autophagy and apoptosis after treatment with TRAIL (Hou et al., 2010).

Autophagic degradation can also inhibit necroptosis. Proteins critical for necroptosis, including RIPK1, RIPK3, TBP1, and TRIF, all of which contain RIP homotypic interaction motifs, are autophagy substrates. Inhibition of autophagy leads to accumulation of these proteins and subsequent activation of TNF- and Toll-like receptor–mediated necroptosis (Lim et al., 2019).

Autophagy regulation of switching between forms of cell death

The autophagosome can also act as a scaffolding complex to mediate switching between apoptosis and necroptosis. In mouse prostate cells with loss of the tumor suppressor *MAP3K7*, RIPK1 and the necrosome are recruited to the autophagosome membrane in a p62-dependent manner to facilitate TRAIL-induced necroptosis. Interference with RIPK1, RIPK3, or MLKL or recruitment of the complex by knocking down p62 caused the mode of death to switch from necroptosis to apoptosis after treatment with TRAIL (Goodall et al., 2016). A similar model of drug-induced necroptosis was described in rhabdomyosarcoma cells, in which treatment with the small-molecule BCL-2 inhibitor obatoclax induced formation of the necrosome, including FADD, RIPK1, and RIPK3, on the outer membrane of autophagosomes via interactions with ATG5 (Basit et al., 2013). Higher ratios of necroptosis compared with apoptosis in dying cancer cells can elicit a greater antitumor immune response and activation of $CD8^+$ T cells (Yatim et al., 2015; Snyder et al., 2019), suggesting that the autophagy machinery through this scaffolding function may play an integral role in immunogenic cell death. These results show that the formation of the autophagosome membrane is a critical event in mediating cell death, suggesting that autophagy inhibition at earlier stages that block autophagosome formation may have opposing effects compared with later-stage autophagy inhibitors that affect autophagic turnover. More work is needed to fully understand the stimuli, cellular contexts, and necessary interacting proteins to fully elucidate the interplay between autophagy, apoptosis, and necroptosis.

Autophagy as a mechanism to evade apoptosis in cancer and its treatment

One of the key hallmarks of cancer is "evading cell death," particularly evading programmed cell death pathways such as apoptosis. There have been a number of mechanisms to describe how cancer cells can evade apoptosis: loss of the classic death receptors, inactivation of different caspases, and aberrant expression of the BH3 proteins that regulate mitochondrial-mediated intrinsic death pathways. Many of these mechanisms have been reviewed elsewhere (Fulda, 2009). Up-regulation of autophagy as a prosurvival mechanism has also been implicated as a mechanism for cancer cells to evade apoptosis. Autophagy deficiency increases attachment-induced apoptosis (anoikis) classified by elevated cleaved caspase-3 expression in HRAS(V12)-transformed mouse embryonic fibroblasts (Lock et al., 2011). In addition, immunohistochemistry staining in tumors derived from HRAS(V12)-transformed cells grown in nude mice showed a dramatic increase in active caspase-3 staining when core autophagy genes *Atg5* or *Atg7* were also knocked out (Guo et al., 2011). In both cell lines and primary cells isolated from glioblastoma patients harboring a BRAF(V600E) mutation, pharmacological autophagy inhibition with CQ induced apoptosis (Levy et al., 2014; Mulcahy Levy et al., 2017). Cells without RAS pathway mutations are also particularly dependent on autophagy to evade apoptosis, and shRNA and CRISPR-mediated screens have shown that some human cancer cell lines undergo programmed cell death when core autophagy genes are knocked down/knocked out or after pharmacological autophagy inhibition with CQ (Maycotte et al., 2014; Towers et al., 2019).

A large body of literature indicates that cancer cells can up-regulate autophagy to evade chemotherapy-induced apoptosis, resulting in chemoresistance (Mohammad et al., 2015). Preclinical cell culture and animal studies showed that pharmacologically targeting autophagy increases cell killing when combined with other chemotherapeutic agents such as cisplatin in esophageal squamous cell carcinoma, the histone

deacetylase inhibitor suberoylanilide hydroxamic acid in CML (Carew et al., 2007; Liu et al., 2011), and BRAF inhibitors in brain and pancreatic cancer (Mulcahy Levy et al., 2017; Bryant et al., 2019; Kinsey et al., 2019). These studies and others have provided the rationale to launch dozens of clinical trials combining autophagy inhibition with a variety of different chemotherapies (Levy et al., 2017).

While there is little doubt that autophagy inhibition can increase cell killing with other agents, the hypothesized mechanism for why this works is based on the idea that the accompanying chemotherapy induces a protective prosurvival form of autophagy that, upon inhibition, leads to cell death. This hypothesis implies that the increased protective autophagy is downstream of the chemotherapy target, whether that be general DNA damage or specific tumor-driving kinases. An alternate hypothesis based on the mechanistic understanding of how autophagy inhibition enhances drug-induced apoptosis via the FOXO3/PUMA mechanism described above proposes that autophagy may be the upstream mediator and may instead affect how "primed" a cell is to die (Fitzwalter and Thorburn, 2018). Importantly, these two hypotheses (autophagy inhibition works to enhance cancer therapy because it is blocking cancer drug-induced autophagy that counteracts the death signal; and autophagy inhibition works because it pushes cancer cells closer to their apoptotic threshold) are likely not mutually exclusive. It is important to tease apart the exact mechanisms that link these processes to better leverage autophagy inhibition as a cancer therapy.

Adaptation to autophagy inhibition

Can cancer cells evade autophagy inhibition? To date there have been few, if any, studies to directly address this question. There have yet to be clinical trials conducted that empirically test for resistance to pharmacological autophagy inhibition in patients. However, previously published trials already show hints of acquired resistance. Most of the trials with published results have been phase I or II, focused on safety and pharmacokinetics-pharmacodynamics, and were not designed for this type of analysis. Nonetheless, in the phase I clinical trial with CQ and TZD, Rangwala et al. (2014) described a patient who initially responded to the therapy but eventually succumbed to a brain metastasis with persistent growth after 4 mo on therapy. Two canine patients in a phase I trial with HCQ and doxorubicin in dogs showed complete initial responses but eventually were removed from the study due to subsequent progressive disease (Barnard et al., 2014). In the phase I trial combining the proteasome inhibitor bortezomib with HCQ, 45% of the patients achieved stable disease that eventually progressed after 9–14 wk (Vogl et al., 2014).

Such results suggest it is plausible that tumors can adapt to autophagy inhibition (Fig. 3). Purely pharmacological mechanisms of resistance to autophagy-inhibiting drugs such as CQ have been identified. The extracellular pH of the TME can prevent drug uptake (Collins et al., 2018), and cancer cell lines grown in acidic media are resistant to CQ-induced toxicity. Moreover, in tumors grown in mice, normoxic regions with physiological pH showed an expected increase in LC3 expression indicative of a CQ-induced block of autophagy, whereas the hypoxic regions with a more acidic pH were significantly less responsive to CQ (Pellegrini et al., 2014).

In addition to pharmacological mechanisms of resistance, there are also likely to be genetic mechanisms of resistance; however, very little work has been done to address this question. A recent study used a rapid CRISPR/Cas9 assay to analyze how KO of 12 different core autophagy genes affects cancer cell viability and growth during the first 7 d after gene loss (Towers et al., 2019). By comparing each gene to loss of known essential and nonessential genes, cell lines where multiple autophagy genes behaved like essential genes could be identified. But, even in autophagy-dependent cell lines where CRISPR-mediated KO of *ATG7* caused the majority of the cells to die within 48 h after editing, at much later time points *ATG7*-deficient clones could be isolated. Intriguingly, the selected $ATG7^{-/-}$ clones that were derived from autophagy-dependent cells grew at equal rates compared to the WT cells from which they were derived, even in autophagy-inducing conditions such as nutrient starvation or hypoxia (Towers et al., 2019). These adapted cells were also resistant to pharmacological inhibitors of autophagy such as CQ. These results indicate that under enough selective pressure, autophagy-dependent cancer cells can adapt to circumvent autophagy inhibition. $ATG7^{-/-}$ clones derived from originally autophagy-dependent cells acquired an increased dependence on the master antioxidant transcriptional regulator, nuclear factor erythroid 2-related factor 2 (NRF2), and this was critical to maintain protein homeostasis (Fig. 3). NRF2 has previously been linked to autophagy, and p62 sequesters and inhibits the NRF2-negative regulator, KEAP1 (Komatsu et al., 2010). Moreover, a recent genome-wide CRISPR screen showed that KO of a large subset of autophagy genes results in up-regulation of the NRF2 signaling pathway (Kerins et al., 2019). As a consequence of NRF2 up-regulation, the autophagy-deficient cells developed an increased sensitivity to pharmacological proteasome inhibitors, a phenotype that was exacerbated with NRF2 knockdown (Towers et al., 2019). These results suggest that tumors that originally start off sensitive to pharmacological autophagy inhibition may be able to adapt and acquire mechanisms of resistance to these therapies. More studies investigating this phenomenon are needed to understand the underlying molecular mechanisms that might provide insight into the correct combinatory therapies to prevent resistance to autophagy inhibition. It will be important to incorporate analysis of these mechanisms into clinical trials, e.g., to investigate if NRF2 expression correlates with clinical response to autophagy inhibition.

Conclusions

Together, the preclinical and clinical studies suggest that autophagy inhibition may be a viable cancer therapy (Levy et al., 2017). However, these initial studies also indicate that both inherent and acquired mechanisms of resistance may be a significant hurdle to overcome before autophagy inhibition can be a truly effective therapy in cancer patients. Additional studies are needed to better identify which patient populations are best suited for autophagy inhibition, and the initial studies in RAS

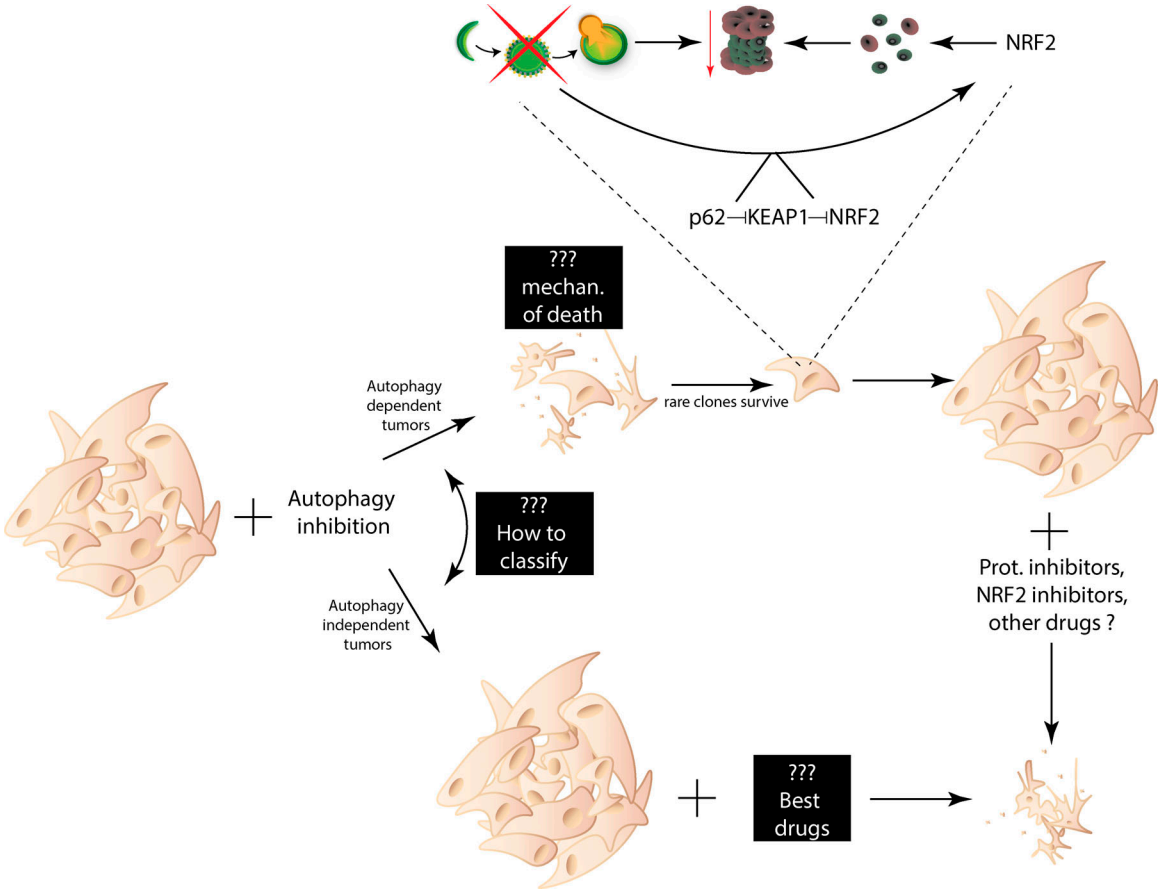

Figure 3. **Cancer cells can adapt to autophagy inhibition.** There are still a number of "black boxes" when it comes to targeting autophagy as a cancer therapeutic. While we know that some cancers are particularly sensitive to autophagy inhibition, the exact biomarkers that dictate autophagy dependence remain at large. It is also unclear if autophagy-independent cells may be exquisitely sensitive to other targeted agents. Recently, it was shown that in autophagy-dependent cancer cell lines that die after acute autophagy inhibition, rare clones can survive by up-regulating NRF2 to maintain protein homeostasis. Consequently, the cells with acquired autophagy independence gained new targetable susceptibilities, i.e., proteasome inhibitors. There are likely additional mechanisms cells can use to circumvent autophagy inhibition and corresponding novel susceptibilities that have yet to be discovered.

pathway mutated tumors may provide a framework for such studies. It is also unlikely that autophagy is consistently inhibited in cancer patients treated with HCQ (Rosenfeld et al., 2014; Wolpin et al., 2014; Boone et al., 2015). Better pharmacological autophagy inhibitors are needed, such as the dimeric quinacrines, which are more potent and selective autophagy inhibitors (Rebecca et al., 2017).

Additional basic cell biology studies are needed to better understand autophagy's various functions in cancer cell biology and to understand the compensatory mechanisms that are up-regulated when autophagy is inhibited. For example, it is unclear how cells that survive loss of autophagy can maintain mitochondrial homeostasis and turn over ER or ribosomes, all of which use LC3-conjugated autophagosomes. But even larger questions still remain, such as what expression and/or mutational landscape dictates autophagy dependence in the first place? When autophagy is inhibited in autophagy-dependent cancer cells, what is the mechanism of subsequent death? Are there specific therapies that work better in autophagy-independent tumors? Lastly, based on the changes in cell biology that occur in the context of autophagy inhibition and the complex interactions with the TME, what combination therapies will lead to the most robust patient responses? Ultimately, the goal is to have a rational mechanistic basis with which to target autophagy manipulations in tumors to maximize beneficial effects, by, for example, increasing the tumor cell killing capacity of other agents while at the same time circumventing the mechanisms by which cancer cells will adapt to the autophagy manipulation. Achieving this goal may allow long-lasting benefits from autophagy-targeted therapies in patients but will require much deeper understanding of how autophagy controls the cell biological processes that drive cancer cell behavior.

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