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REVIEW

Autophagy in cancer biology and therapy

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Abstract **The role of macroautophagy (hereafter autophagy) in cancer biology and response to clinical intervention is complex. It is clear that autophagy is dysregulated in a wide variety of tumor settings, both during tumor initiation and progression, and in response to therapy. However, the pleiotropic mechanistic roles of autophagy in controlling cell behavior make it difficult to predict in a given tumor setting what the role of autophagy, and, by extension, the therapeutic outcome of targeting autophagy, might be. In this review we summarize the evidence in the literature supporting pro- and anti-tumorigenic and -therapeutic roles of autophagy in cancer. This overview encompasses roles of autophagy in nutrient management, cell death, cell senescence, regulation of proteotoxic stress and cellular homeostasis, regulation of tumor-host interactions and participation in changes in metabolism. We also try to understand, where possible, the mechanistic bases of these roles for autophagy. We specifically expand on the emerging role of genetically-engineered mouse models of cancer in shedding light on these issues *in vivo*. We also consider how any or all of the above functions of autophagy proteins might be targetable by extant or future classes of pharmacologic agents. We conclude by briefly exploring non-canonical roles for subsets of the key autophagy proteins in cellular processes, and how these might impact upon cancer.**

Keywords autophagy, cancer, inflammation, metabolism, apoptosis, homeostasis

Introduction

Degradation of cellular contents is an important aspect of homeostasis and can occur via the proteasome or lysosome (Ciechanover, 2005). Autophagy is an evolutionary conserved pathway that facilitates the delivery of substrates, such as long lived proteins, damaged organelles and infectious pathogens, to the lysosome system. By doing so, autophagy can aid the clearance of toxic materials and increase nutrient availability in the cell thereby influencing a number of physiologic and pathological processes including development, pathogen infection, neurodegeneration and cancer. Various subtypes of autophagy exist including macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Several of these are involved in cancer biology. For example, CMA may have some protumorigenic roles (Kon et al., 2011). However, this review will focus on macroautophagy, in which a vesicular trafficking pathway operates to sequester and transport cytosolic content to the lysosomes. For the sake of simplicity, the term autophagy will be used to refer to macroautophagy hereafter.

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Autophagy at the molecular level

Core autophagy machinery

During canonical autophagy, a lipid bilayer (termed phagophore or pre-autophagosome) engulfs cytoplasmic material as it matures to form the autophagosome, a sealed bilayer vesicle, which eventually fuses with the lysosome system leading to the degradation of the autophagosome contents (Fig. 1). The origins of the autophagosome may involve various sources such as the endoplasmic reticulum (ER), plasma membrane and mitochondria (reviewed by Rubinsztein et al., 2012). However, whether the membrane source is determined by upstream signals that induce autophagy or whether the autophagosome content is affected by the origin of its membrane is yet unclear. A number of key players that are essential for autophagy have been initially identified in yeast and subsequently in higher eukaryotes. These include a family of ubiquitin-like proteins, such as LC3, which are involved in the phagophore maturation and cargo selection (Geng and Klionsky, 2008; Kraft et al., 2010). Membrane

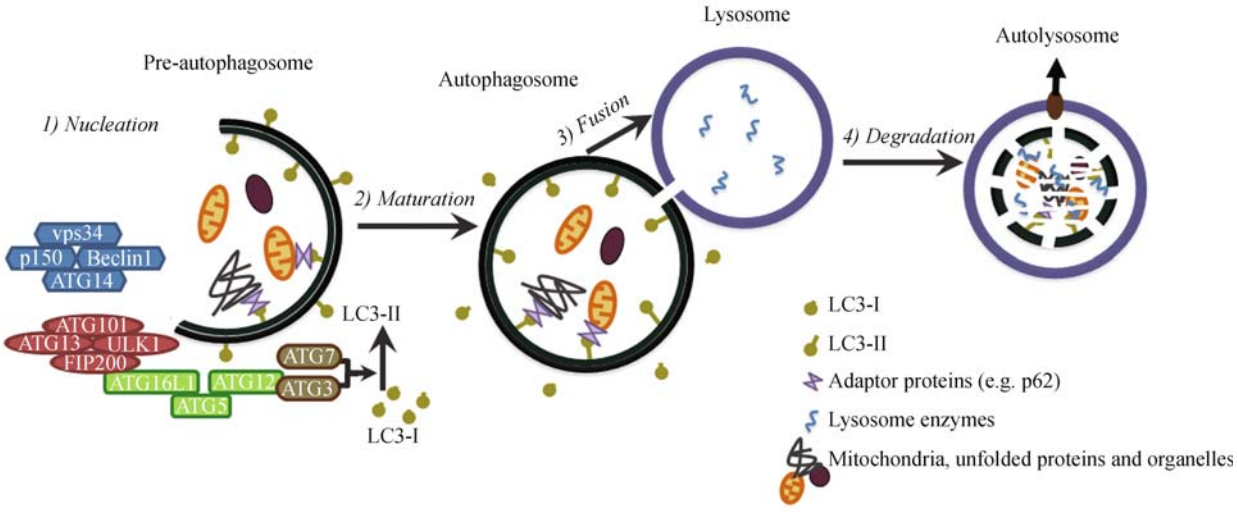

Figure 1 Molecular mechanism of autophagy. **1)** Nucleation of the pre-autophagosome structure requires the recruitment of the upstream ATG complexes including the Vps34, ULK and ATG5 complexes. The molecular mechanisms underlying the membrane recruitment of these complexes and their subsequent dissociation upon maturation of the autophagosome are unknown. **2)** Maturation of the pre-autophagosome requires the conjugation of cytosolic LC3-I to membrane bound LC3-II catalyzed by E1-, E2- and E3-like enzymes (ATG7, ATG3 and ATG5-ATG12, respectively). The maturing pre-autophagosome encapsulates cytosolic material (such as damaged mitochondria, unfolded proteins and organelles). Selective autophagy can be achieved through the ability of adaptor proteins (such as p62) to interact with LC3 and cellular cargoes. **3)** Autophagosome-lysosome fusion, resulting in autolysosome formation, is essential for autophagy flux. The molecular players facilitating this step are largely unknown. **4)** Degradation of the autophagosome content is catalyzed by lysosomal proteases resulting in the recycling of nutrients and energy back to the cytoplasm. The conclusion of this step denotes a complete autophagic flux.

targeting of LC3 is essential for autophagy and requires a series of ubiquitin-like conjugation events that lead to the conjugation of cytosolic LC3 (LC3–I) to membrane bound, phosphatidylethanolamine (PE)-conjugated form (LC3–II) (reviewed by Xie and Klionsky, 2007). These events are catalyzed by autophagy-related proteins (or ATG proteins) with E1-, E2- and E3-like enzymatic activities termed ATG7, ATG3 and ATG5–ATG12, respectively. The ATG5–ATG12 conjugate additionally forms a large protein complex with ATG16L1 which is essential for its function in autophagy but dispensable for its E3-like ligase activity. Depletion of any of ATG3, ATG5–ATG12, ATG16L1 or ATG7 completely abolishes autophagosome formation and leads to neonatal lethality in mouse models highlighting the role of autophagy during neonatal starvation period (Mizushima et al., 2001; Komatsu et al., 2005; Saitoh et al., 2008).

Signaling pathways controlling autophagy and their link to cancer

A number of upstream signaling complexes can regulate autophagy including the uncoordinated-like kinase (ULK) and Vps34 complexes. The ULK complex is comprised of the protein kinase ULK1 and several regulatory components including ATG13, FIP200 and ATG101 that are required for optimal activity of the ULK1 kinase (Wong et al., 2013). A close homolog of ULK1, known as ULK2, exists and can also associate with ATG13, FAK-interacting protein 200 kDa (FIP200) and ATG101. Both ULK1 and ULK2 share high sequence similarities and are functionally redundant in regulating autophagy. Indeed, combined knockout of both ULK1 and ULK2 is required to completely disrupt amino acid starvation-induced autophagy (Lee and Tournier, 2011) and to recapitulate the neonatal lethality phenotype observed in mouse knockout models of other autophagy essential genes (Cheong et al., 2011; Cheong et al., 2014). The kinase activity of ULK1 is essential for its function in autophagy, however the precise mechanism by which the ULK complex contributes to autophagosome formation remains largely unknown. Recent studies have started to shed light on potential substrates of the ULK1 kinase (Di Bartolomeo et al., 2010; Russell et al., 2013). The ULK complex is essential for certain subtypes of autophagy such as during amino acid starvation and cytotoxic response. However, membrane targeting of LC3 can take place in the absence of the ULK complex such as during autophagy induced by glucose starvation and an autophagy related process known as LC3-associated phagocytosis (Cheong et al., 2011; Florey et al., 2011).

A number of upstream signaling molecules that influence the ULK complex activity have been identified of which the most studied is the mammalian target of rapamycin (mTOR) complex 1 (mTORC1). During nutrient rich conditions and in the presence of growth factors, mTORC1 has been shown to inhibit autophagy by phosphorylating ULK1 and ATG13 (Ganley et al., 2009; Jung et al., 2009). When mTORC1 is

inactivated, for example by amino acid deprivation or cytotoxic response, the ULK complex becomes hypophosphorylated and thereby activated leading to autophagy stimulation. The control of autophagy by mTORC1 indicates that autophagy is regulated in cancers with deregulated mTORC1 activity and during anti-cancer therapy targeting the mTOR kinase.

The ULK complex can also be regulated by the cellular ATP sensor, AMP-activated protein kinase (AMPK). AMPK can regulate the ULK1 kinase activity either by directly phosphorylating ULK1 or, indirectly, by supressing the mTORC1 pathway. The consequences of ULK1 phosphorylation by AMPK are complex and may be dependent on the upstream stimulus or phosphorylation sites on ULK1. During nutrient availability, AMPK can phosphorylate ULK1 on sites that inhibit ULK1 activity and are subsequently dephosphorylated in the absence of amino acids (Shang and Wang, 2011). On the other hand, glucose starvation can induce the phosphorylation of ULK1 by AMPK on alternative sites resulting in enhanced ULK1 activity and thereby autophagy (Kim et al., 2011a). However, glucose starvation-induced autophagy can occur in cells lacking the expression of ULK1 (as well as ULK2) (Cheong et al., 2011; Gammoh et al., 2013) but is defective in cells lacking AMPK expression (Kim et al., 2011a). Here, the phosphorylation of alternative autophagy components by AMPK may be more relevant (Kim et al., 2013a).

The Vps34 complex is comprised of a number of proteins, including the Vps34 phosphatidylinositide 3-kinase, p150, Beclin 1 and ATG14, and is essential for phagophore formation and proper recruitment of ATG proteins during autophagy. The activity of the Vps34 is essential for autophagy and pharmacological inhibition of its activity, for example using 3-methyladenine (3-MA), disrupts autophagosome formation. Recent findings suggest that Beclin 1 can be subjected to phosphorylation by major oncogenic signaling pathways. In one study, activated Akt has been shown to phosphorylate Beclin 1 leading to its enhanced ability to bind Vimentin intermediate filaments and 14-3-3 thereby inhibiting its function in autophagy (Wang et al., 2012a). Expression of a Beclin 1 mutant that lacks Akt phosphorylation sites leads to enhanced autophagy and reduced tumorigenesis in a xenograft model. In addition, Beclin 1 was also shown to be a direct substrate for phosphorylation by activated epidermal growth factor receptor (EGFR) leading to inhibition of its function in autophagy (Wei et al., 2013). In this case, expression of a phosphomimetic mutant of Beclin in a non-small-cell lung carcinoma (NSCLC) cell line supresses autophagy and correlates with enhanced tumorigenesis and resistance to tyrosine kinase therapy. These studies suggest that Beclin 1 may act as a key molecule to relay upstream signals that sense cellular transformation and response to anti-cancer therapy and are supportive of a tumor suppressive function of Beclin 1, and possibly autophagy. Beclin 1 however has multiple non-autophagy-related functions. Addressing the effects of these modifications on such functions of Beclin 1 may be essential to determine the contribution of autophagy to the tumor suppressive role of Beclin 1.

Transcriptionally, autophagy is controlled by several different pathways. However, of particular note in cancer biology is the p53 transcriptional program. p53 is a major tumor suppressor in a wide variety of malignancies and a large number of p53 target genes regulate autophagy in cancer cells. The first of these to be discovered was the Damage Regulated Autophagy Modulator (DRAM), which may participate in relatively direct regulation of the autophagy pathway, the DRAM protein being resident in the lysosome at steady-state (Crighton et al., 2006). Since then a number of other p53 target genes including p53 upregulated modulator of apoptosis, PUMA (Yee et al., 2009); tp53-inducible glycolysis and apoptosis regulator, TIGAR (Bensaad et al., 2009); ULK1 and others (Kenzelmann Broz et al., 2013) have been discovered to regulate autophagy. The mechanism of action of these varies. For example, TIGAR is an enzyme that participates in the pentose phosphate metabolic pathway. Reductions in autophagy engagement occur on induction of TIGAR gene expression. However, these changes in autophagic rates occur secondarily downstream of metabolism-linked reductions in reactive oxygen species (ROS) generation (Bensaad et al., 2009). Taken together, the existence of multiple p53-linked changes in autophagy suggests that autophagy regulation may be intimately linked to the tumor suppressive effects of p53.

The degradative function of autophagy

Autophagic degradation of cellular contents can be of either general or selective nature. It is thought that deviation from optimal growth conditions, such as during nutrient deprivation, hypoxia and cell detachment, induces non-selective degradation of cytoplasmic material. However, selective autophagic degradation of cellular contents, such as damaged mitochondria (mitophagy), ER membranes (reticulophagy), unfolded protein aggregates (aggrephagy) and intracellular pathogens (xenophagy), can also take place during basal conditions or under certain stimuli (reviewed by Reggiori et al., 2012). Specific recognition of the autophagosome cargo is mediated through the ability of LC3 and its homologs to bind to receptors which act as adaptor proteins between membrane-bound LC3 and the cytoplasmic cargo. The first identified and perhaps most studied receptor is the ubiquitin binding protein p62 (alternatively known as sequestosome-1) which can bind LC3 and ubiquitinated cellular targets through its LC3 interaction region (LIR) motif and ubiquitin associated (UBA) domain, respectively. Disruption of autophagy and not the proteasome system results in the upregulation of p62 levels, suggesting that autophagy is also a major negative regulator of p62 levels and thereby activity (Bjørkøy et al., 2005; Gao et al., 2010b). Other well-

established cargo receptors involved in selective forms of autophagy include neighbor of BRCA1 gene 1 protein, NBR1; nuclear dot protein 52, NDP52; NIX (Johansen and Lamark, 2011) and optineurin, OPTN (Wild et al., 2011). It is also important to note that LC3B, which is often studied as a marker of autophagosome formation and the activity of the lipid conjugation machinery, serves as a prototypical member of the LC3 family in humans, which contains at least six members (LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2). Elegant recent work has shown that these LC3 family members might serve different functions in the core autophagy process (Weidberg et al., 2011). This may be related to the demonstration from proteomic studies of substantially different ranges of interacting proteins unique to each LC3 subfamily (Behrends et al., 2010). When LC3 family members are used to track the autophagy process in experiments designed to probe the role of autophagy in cancer, these observations must be borne in mind.

It is also important to note that encapsulation of cellular contents by the autophagosome per se is not sufficient for the degradative function of autophagy. The eventual fusion between autophagosomes and lysosomes is an essential step for a complete "autophagic flux" which results in the degradation of the autophagosome content and the recycling of nutrients back into the cytoplasm. Pharmacological inhibition of the lysosome activity, such as by using chloroquine (CQ) or the V-ATPase inhibitor bafilomycin A1, can often have similar effects to the inhibition of ATG protein expression, but this is not a given in any particular scenario. The specificity of such inhibitors and their use during autophagy research and anti-cancer treatment will be further discussed below.

Autophagy in cancer biology

Autophagy is commonly referred to as a 'double-edged' sword in cancer biology. Genetic experiments in cancer cell lines or in mouse models have been performed where autophagy function has been removed via ablation of expression of ATG proteins. This has revealed both pro-tumorigenic and anti-tumorigenic phenotypes associated with autophagy ablation, including changes in cell proliferation, programmed cell death, senescence, metabolism and cell-cell interactions, as discussed in the following section. The findings of this section are categorised and grouped in Table 1.

Protumorigenic roles of autophagy

Tumour cell survival and dormancy under nutrient deprivation

Perhaps the best understood role of autophagy is in the response of cells to nutrient starvation. Relatively non-selective, or bulk, autophagy is thought to be a response of cancer cells to shortages in amino acid supply, glucose supply or to other energy stress conditions. Under these conditions, cell mass is expended for the gain of a self-limited survival advantage. For example, cytokine-withdrawn, apoptosis-resistant cells that cannot utilize extracellular nutrients can survive in this state for several weeks *in vitro*, dependent upon the function of ATG5 or ATG7 (Lum et al., 2005). Hypoxic or nutrient-starved cancer cell lines are also sensitive to RNAi against autophagy genes such as ATG5 and Beclin 1 (Boya et al., 2005; Degenhardt et al., 2006; Wilkinson et al., 2009). Generally, autophagy represses the onset of metabolic catastrophe under these conditions. However, in some tumor cell lines, the direct engagement of programmed cell death may occur in the absence of autophagy (Boya et al., 2005).

In vitro, nutrient starvation responses are accompanied by cessation of cell proliferation (Lum et al., 2005). In cancer

Table 1 Summary of pro- and anti-cancer processes which autophagy promotes. Each of these may be a composite of multiple mechanistic roles of autophagy, determined by the work of several groups. Please see the text for details.

Pro-cancer	Anti-cancer
Tumour cell dormancy and maintenance of metabolism under nutrient stress	Clearance of toxic organelles and damaged proteins, ameliorating genotoxic insults
Survival and avoidance of senescence by cancer cells in no chemotherapy setting	Contribution to programmed cell death mechanisms
Survival by varied mechanisms of cancer cells upon chemotherapuetic treatment	Repression of NF-κB-driven, or other, inflammatory gene expression programmes
Tolerance of the aneuploid state	Facilitation of oncogene-induced death and senescence in certain settings
Cell survival by clearance of toxic organelles and damaged proteins, and reduction in associated ROS production	Elimination of oncogenic drivers of proliferation and aberrant cell survival
Degradation of signaling molecules or scaffolds such as p62, Src and TAX1BP1	Possibly- although currently unlikely- direct function as a programmed cell death mechanism
Activation of canonical NF-κB signaling	Maintenance of genomic stability
Required for mitochondrial metabolism in KRAS mutant lung cancers	Anti-tumor immune responses
Anti-senescence in p53 wild-type, KRAS mutant pancreatic cancers	Suppression of angiogenesis
	Increased glucose metabolism upon autophagy loss in KRAS-driven pancreatic cancer may be tumor promoting when p53 is also absent

biology, this state of cellular quiescence, which goes in hand with survival under unfavorable conditions, is referred to as tumor cell dormancy. However, the *in vivo* relevance of autophagy in tumor cell dormancy is less clear. In xenograft models, using *in vitro* transformed cells, there is significant cell death associated with the hypoxic and nutrient-starved core regions of tumors, which is exacerbated by loss of autophagy function (Degenhardt et al., 2006). However, in addition to the nutrient buffering effects of autophagy, this could potentially also involve selective autophagy pathways involving degradation of p62 or mitochondria (Mathew et al., 2009). Furthermore, *in vivo* dormancy of cells is difficult to monitor experimentally. One interesting study demonstrates the potential of inducible systems for manipulating levels of autophagy regulators in tumor cell xenografts (Lu et al., 2008). Inducible RNAi against a proposed autophagy promoter, aplasia Ras homolog member 1 (ARHI), in ovarian cancer cell xenografts, was used to demonstrate a temporal correlation between inhibition of autophagy and regrowth of dormant cell masses. However, it will be important to apply such temporally controllable approaches to core ATG genes in order to interpret these results fully. It is also possible that autophagy may be involved in maintaining cell survival in dormant micro-metastases, thus contributing to long-term tumor spread. However, no specific experimental analysis has been performed of this yet. Due to the bulk autophagy-repressing effect of many tumor-promoting pro-growth signals it is also possible that certain tumor cells have deficiencies in autophagy engagement in response to nutrient limitation. For example it has been shown that leucine withdrawal from human melanoma cells, both *in vitro* and in xenograft models, synergises with autophagy inhibition in inducing apoptosis (Sheen et al., 2011). This raises the possibility of a synthetic lethal approach to selectively target tumor cell viability, via combined autophagy inhibition and dietary restriction.

Autophagy in survival responses to proteotoxic and mitochondrial stress

Autophagy may also function in proteotoxic stress responses to clear damaged or unfolded protein molecules. For example, reticulophagy may be involved in clearing unfolded proteins from the ER under stress conditions. Ablation of autophagy in transformed cells may lead to elevated proteotoxic stress, impaired mitochondrial turnover by mitophagy and subsequent ROS production, consequent from both of these events (Mathew et al., 2009). In the case of aneuploid cells–common in most tumor types due to the prevalence of chromosomal instability–it is possible autophagy acts to restore the stoichiometry of the proteome (Stingele et al., 2012). Activation of autophagy is indeed observed in cells that have an unbalanced chromosome complement and may be associated with cellular sensitivity to agents such as CQ (Tang et al., 2011). Taken together, the above roles for autophagy will result in avoidance of cellular stress and subsequent clearance of cancer cells via engagement of programmed cell death mechanisms. In the aneuploidy setting, it can also be envisaged how the proteotoxic stress response in particular might further promote the toleration of genetic diversity created by genomic instability.

Autophagy as pro-survival mechanism to other stresses

Autophagy mediates survival responses to chemotherapy in certain settings. There are numerous examples of these and the reader is referred to recent reviews (Sui et al., 2013). This raises the possibility that pharmacological targeting of autophagy might sensitize to therapeutic regimens in the clinic. For example, treatment of chronic myeloid leukemia (CML) cells with clinical agents, such as the tyrosine kinase inhibitor imatinib, which targets the enzymatic activity of the Bcr-Abl oncoprotein, is only partially effective. In CML samples there is an imatinib-resistant cancer stem cell population that is only destroyed by imatinib treatment and combined inhibition of autophagy for example via RNAi against ATG5 or ATG7 or CQ treatment (Bellodi et al., 2009). Loss of autophagy function here is associated with elevated proteotoxic stress. More broadly, in such examples of autophagy-mediated resistance to chemotherapy, it is likely a wide range of mechanistic functions of autophagy participate in cell survival, including but not limited to proteostatic and mitophagic roles. However, more detailed mechanistic investigations are still required in the majority of such settings.

In another recent study it was shown that upon treatment of transplanted mouse lymphomas driven by an overexpressed MYC proto-oncogene, where additional genetic aberrations have disabled the apoptotic machinery, chemotherapeutic agents do not clear cancer cells but instead induce senescence (Dörr et al., 2013). These senescent cells display an extreme proteotoxic stress response and thus co-opt autophagy in order to maintain their viable, albeit non-proliferative, status. Here, targeting of autophagy, by CQ treatment, is required to further remove senescent cells from the animal and remove these traces of the lymphoma. Again, this example argues that complete eradication of cancerous material may require multiple strategies, one of which is targeting of autophagy.

Selective autophagy pathways that result in degradation of individual toxic protein molecules may also be involved in maintaining tumor cell viability. These include those targeting activated Src oncoprotein (Sandilands et al., 2012a), or other tyrosine kinases such as Ret (Sandilands et al., 2012b), in non-adherent or focal adhesion kinase deleted tumor cells. In the former instance this is mediated by c-Cbl, a dual E3 ubiquitin ligase and cargo receptor protein, although ubiquitin ligase activity is not required for cargo receptor function. Other examples include the autophagic sequestration of putative signaling protein substrates for cell survival, such as those associated with the NDP52 and TAX1BP1 cargo

receptor molecules in KRAS-mutated lung cancer cells (Newman et al., 2012). However, the *in vivo* relevance of these observations is yet unclear and needs to be tested.

Antitumorigenic roles of autophagy

Homeostatic roles of autophagy may suppress tumorigenesis

Despite the above roles for autophagy in survival of cancer cells, it is also possible that selective elimination of toxic material, which might otherwise be carcinogenic in healthy cells, may constitute an anti-tumorigenic function of autophagy. Indeed, experimental studies show that the genetic disruption of autophagy can lead to spontaneous tumor formation and accelerated tumor growth in various mouse models. Genetic manipulations that disrupt autophagy in healthy mice, by monoallelic deletion of Beclin 1, mosaic deletion of ATG5 or tissue specific knockout of ATG7, resulted in the development of spontaneous tumors particularly those of the liver (Qu et al., 2003; Yue et al., 2003; Takamura et al., 2011). It is possible that the liver is highly exposed to multiple insults rendering it susceptible to compromised autophagy. However, it is also possible that autophagy may be required during later stages of tumor development and its disruption may be lethal once the tumor is formed. More tissue-specific and temporal studies are required to address these issues. This will be discussed later (see *Humanised GEM models to integrate autophagy research in cancer*). How does autophagy contribute to tumor suppression? Studies have shown that the disruption of autophagy in healthy cells can lead to the accumulation of protein aggregates, dysfunctional organelles, ER structures and lipid droplets (Komatsu et al., 2005; Mizushima and Komatsu, 2011) subsequently resulting in ROS production, oxidative stress, DNA damage response and genomic instability (Karantza-Wadsworth et al., 2007; Mathew et al., 2007; Mortensen et al., 2011). Autophagy can thereby selectively maintain homeostasis by acting as a cellular waste disposal during normal cellular functions or in the presence of tumorigenic insults. Importantly, these mechanisms are true 'double-edged swords'; they have also been shown in various settings, as described above, to maintain tumor cell survival and thus promote tumorigenesis.

Cross talk of autophagy with programmed cell death and senescence

Dying cells frequently exhibit an increase in their autophagosome content. Although autophagy is more likely to play a pro-survival role, as described above, certain cell death triggers appear to require autophagy for efficient killing in the presence or absence of caspase activation. For an example, inhibiting autophagy by RNAi against various ATG genes was shown to suppress apoptotic cell death induced by PUMA and Bax overexpression (Yee et al., 2009). PUMA and Bax proapoptotic activity was associated with the selective removal of mitochondria by autophagy. Similarly, overexpression of HRAS in ovarian epithelial cells leads to non-apoptotic cell death that can be supressed by autophagy inhibition (Elgendy et al., 2011). This form of death is dependent on the expression of the BH3-only protein, Noxa, and is associated with an elevation in cellular autophagosome content. Furthermore, overexpression of the Forkhead Box Protein O1 (FOXO1) in a NSCLC cell line can induce autophagy-dependent cell death that is supressed by RNAi against ATG5 (Zhao et al., 2010). FOXO1 is thought to induce autophagy by binding to and possibly activating ATG7. In this system knockdown of ATG7 expression reverts FOXO1-mediated suppression of tumorigenesis in a xenograft.

On the other hand, autophagy may inhibit tumorigenesis by facilitating oncogene-induced senescence during early stages of tumor formation. Suppression of autophagy by RNAi against ATG7 or ATG5 delayed the onset of senescence induced by HRAS overexpression in fibroblasts (Young et al., 2009). However, in this model the effect of autophagy knockdown was unapparent upon prolonged HRAS overexpression suggesting that autophagy inhibition only delays the senescence phenotype. Similarly, knockdown of ATG5 also suppressed HRAS and BRAF-induced senescence in melanocytes (Liu et al., 2013). Interestingly, ATG5 expression, and thereby autophagy, was decreased in melanoma tissues compared to benign melanocytic nevi and lower ATG5 levels in melanoma patients correlated with poorer prognosis of the disease. Unexpectedly, the reintroduction of ATG5 in melanoma cells restored autophagy and inhibited cell growth by inducing senescence, suggesting that in this case restoring autophagy in already established tumors can have a growth inhibitory effect. Further understanding of the factors that control expression of ATG genes and the cellular substrates engulfed by autophagosomes in this model may be of therapeutic relevance.

Autophagy is also relevant to relay the killing and growth inhibitory effects of therapeutic agents. One of the first evidence supporting this comes from a study where autophagy inhibition suppressed non-apoptotic cell death induced by etoposide and staurosporine in Bax/Bak double knockout MEFs (Shimizu et al., 2004). In addition, non-apoptotic cell death induced by zVAD treatment (a general caspase inhibitor) requires autophagy for efficient killing (Yu et al., 2004; Yu et al., 2006). Similarly, the anti-leukemic effects of arsenic trioxide can be compromised when autophagy is disrupted using RNAi against a panel of ATG sequences as well as pharmacological inhibition of lysosomes (Goussetis et al., 2012). Here autophagy appears to be dispensable for the growth of leukemic cells *in vitro* but may enhance the anti-tumor effects of arsenic trioxide through the degradation of the Bcr-Abl oncogene. On the other hand, treatment of various cell lines (including HEK293T, iBMK and NIH3T3) with arsenic appear to increase autophagosome

content by inhibiting autophagic flux (Lau et al., 2013). The variation of these results, which may be dependent on the difference in cell lines tested and inhibitor doses used, should be further addressed to carefully dissect the anti-tumor effects of arsenic and its clinical cytotoxicity.

The molecular mechanism by which the degradative function of autophagy facilitates cell death or senescence and thereby suppresses tumorigenesis remains largely unknown. Regulation of cellular ROS levels by autophagy may contribute to cell death particularly in cases where ROS accumulation is essential for the cell death trigger. Autophagy can trigger ROS production by inhibiting the activities of antioxidant defense machinery such as catalase (Yu et al., 2006). However, in some cases it is possible that autophagy may be advantageous to a subpopulation of cells that can tolerate the death insult, possibly by accumulating DNA mutations as a result of ROS accumulation, and may thereby grow into more aggressive tumorigenic clones. Alternatively, it is also possible that high rates of autophagy may directly contribute to cell death in the absence of caspase activation in a process termed "autophagic cell death." Whether excessive "self-eating" is a mechanism by which cells can die or is a non-causal association with the cell death phenotype is not fully understood. Furthermore, direct association between components of the autophagy and cell death machinery have been described. In these cases however, it is likely that the autophagy machinery acts in influencing cell death independently of their role in autophagy. For an example, Beclin 1, ATG5 and ATG12 have been shown to interact with the apoptotic machinery and promote cell death in a mechanism independent of their autophagic function (Yousefi et al., 2006; Rubinstein et al., 2011; Wirawan et al., 2012).

Specific elimination of oncoproteins

Targeted protein degradation by selective autophagy can also have important anti-tumorigenic roles. Perhaps the most studied autophagy substrate is p62. Studies indicate that accumulation of p62 in the absence of autophagy is a major contributor of tumorigenesis (Mathew et al., 2009; Takamura et al., 2011). Deregulation of p62 levels may alter antioxidant response through binding to and activating the nuclear factor-erythroid 2-related factor-2 (NRF2) transcription factor (Komatsu et al., 2010). This transcription factor promotes a gene expression program that results in detoxification of the cytosol, and the consequence of this may be to-paradoxically-both protect cells from DNA damage and tumor initiation via oncogenic mutation, but also to protect tumor cells and keep them viable under stress conditions. The accumulation of NRF2 is thought to occur via the accumulation of p62 downstream of autophagy inhibition and consequent ablation of the function of an ubiquitin ligase for NRF2, the Keap1-Cullin3-Rbx1 complex. This occurs via accumulated p62 binding directly to and sequestering Keap1. Furthermore, p62 accumulation may contribute to tumor progression by interacting with TRAF6 and promoting NF-κB activation as well as associating with mTORC1 and stimulating its activity leading to enhanced tumor growth (Duran et al., 2011). Additionally, autophagy can regulate proliferation by mediating the degradation of the Wnt signaling components DVL2 and β-catenin (Gao et al., 2010a; Petherick et al., 2013). The relevance of elevated Wnt signaling during tumor progression in the absence of autophagy is yet to be determined and may be differentially required in different tumor models. Furthermore, anti-cancer agents induce the autophagic degradation of certain oncoproteins such as PML/PARA and Bcr-Abl (Isakson et al., 2010; Goussetis et al., 2012) suggesting that in these settings autophagy augments the anti-tumor effects of drug treatment by the selective degradation of oncoproteins.

DNA damage response and mitotic instability

Several lines of evidence suggest that autophagy acts to preserve genomic integrity by limiting metabolic stress, DNA damage response, genome amplification and aneuploidy (Karantza-Wadsworth et al., 2007; Mathew et al., 2007). How does autophagy disruption, for example by monoallelic loss of Beclin 1 or gene knockout of ATG5, induce genomic instability? It is possible that autophagy may directly implicate DNA replication by maintaining ATP levels required for efficient DNA replication and damage response. Alternatively, specific degradation of damaged mitochondria during mitophagy is an important mechanism to control ROS levels and oxidative stress which might otherwise induce DNA damage. During mitophagy, PINK1 is activated by dysfunctional mitochondria leading to the subsequent activation of the E3 ubiquitin ligase PARK2 which ubiquitinates the mitochondrial outer membrane proteins (reviewed by Jin and Youle, 2012). The autophagy machinery in turn recognizes the damaged mitochondria by the aid of various adaptor proteins and targets their degradation. Interestingly, PARK2 is a tumor suppressor suggesting that clearance of damaged mitochondria through mitophagy is a major tumor suppression mechanism (Cesari et al., 2003). Furthermore, autophagy may regulate tumorigenesis by regulating midbody degradation (Pohl and Jentsch, 2009; Kuo et al., 2011). Midbodies appear to be inherited asymmetrically in postmitotic cells and accumulate in cells with defective autophagy. The inability of cells to clear midbody structures correlates with increased tumorigenicity and colony formation *in vitro*. How the accumulation of midbody derivatives associates with tumorigenicity and whether their presence affects chromosome segregation in subsequent cell division events are still to be explored.

Inflammatory signaling pathways and stromal interactions

The dynamic association and co-evolution between the tumor

stroma, consisting of the tumor associated fibroblasts, endothelial cells and immune cells, and the tumor cell compartment can significantly determine tumor progression and therapy response. The tumor microenvironment is characterized by hypoxic conditions, lack of growth factors and nutrients, all of which can stimulate autophagy both in the tumor stroma and the tumor itself. The stimulation of autophagy can, in addition to its intracellular functions, influence secretion of various pro-inflammatory signals to the extracellular surrounding (Dupont et al., 2011; Michaud et al., 2011; Narita et al., 2011). Such signals may be important determinants of angiogenesis and immunosurveillance (reviewed by Maes et al., 2013) as well as the interaction between tumor cells and the surrounding fibroblasts (Capparelli et al., 2012). It is plausible that autophagy may impact the relationship between the tumor and its surrounding stroma, however more detailed studies on the nature of such relationship are not yet explored.

Inflammatory response is key determinant during various stages of tumorigenesis and during anti-cancer treatment (Grivennikov et al., 2010). Autophagy can influence the tumor associated immune response by directly affecting the function of the immune system (for recent reviews Levine et al., 2011; Kuballa et al., 2012; Deretic et al., 2013) or by influencing secretory pathways and "eat me" signals in tumor cells. A number of studies have shown that autophagy can suppress immunoevasion in the context of the tumor. Inhibiting autophagy, by for an example FIP200 deletion or RNAi against ATG5 or Beclin 1, increased chemokine production and infiltration of effector T cells to the tumor site (Noman et al., 2011; Wei et al., 2011;Garg et al., 2013). These results were supported by a more recent finding where the deletion of ATG7 in a KRAS-driven NSCLC model in mice resulted in elevated cytokine production accompanied by macrophage infiltration in the region surrounding the lesion compared to tumors with intact autophagy (Guo et al., 2013). Importantly, no overall survival advantage was observed in mice harbouring ATG7 deletion despite the benign nature of such tumors. The mortality in this case is due to pneumonia as a result of increased inflammation rather than the tumor burden. Furthermore, in xenograft models of cancer using cells that are *in vitro* transformed with Bcl2, autophagy deficiency leads to enhanced inflammation (Mathew et al., 2009). However, the direct consequences, or otherwise, of increased inflammatory and immune responses remains to be determined in these *in vivo* examples.

Mechanistically, one potential intersection of autophagy with inflammation is in the regulation of NF-κB signaling. NF-κB signaling is a relatively well-understood transcriptional signaling pathway that results in the nuclear localization of heterodimeric transcription factors, canonical and non-canonical NF-κB. Particularly in the case of canonical NF-κB, many of the target genes that are transactivated are cytokines that participate in driving inflammation in the surrounding tissue. A number of links between autophagy and the regulatory machinery for NF-κB signaling have been proposed. Generally these suggest that autophagy would inhibit the function of canonical NF-κB. For example, autophagic degradation of NEMO (Fliss et al., 2012) or Bcl10 (Paul et al., 2012) may repress canonical NF-κB. Alternatively, it has been suggested that autophagic degradation of IκBα in response to either bortezomib treatment or TNFα treatment mediates a propagation of canonical NF-κB signaling by autophagy (Colleran et al., 2011; Jia et al., 2012), although this is less easy to reconcile with *in vivo* models demonstrating upregulation of canonical NF-κB targets in autophagy-deficient KRAS-driven lung tumors (Guo et al., 2013). Perhaps different aspects of canonical NF-κB regulation are predominant in different tumor settings. Interestingly, autophagy also engages non-canonical NF-κB signaling by degradation of the TAX1BP1 and NDP52 cargo receptor proteins in KRAS-mutated lung cancer cells, although this is more likely to impact on gene targets involved in cell proliferation than in inflammation (Newman et al., 2012). Furthermore, there is no evidence that sequestration of these particular cargo receptors affects canonical NF-κB signaling in this setting (Newman et al., 2012). Finally, it is also likely that NF-κB transcriptional activity itself feeds back on the autophagy process, as demonstrated by several studies (Djavaheri-Mergny et al., 2006; Barré and Perkins, 2010).

In other cases, autophagy activation may be important to signal for the recruitment of immune cells and for the suppression of angiogenesis. Autophagy machinery has been shown to cooperate with mTOR for efficient production of interleukin 6/8 during RAS-induced senescence (Narita et al., 2011). In this system, it is proposed that autophagy is required to generate amino acids for optimal activity and subcellular localization of mTOR. In addition, autophagy may be important for dendritic cells and T lymphocytes to recognize the tumor site by enhancing ATP levels in dying cells. The inability of tumor cells to produce ATP in the absence of autophagy impeded their response to therapy by disrupting the activation of immune cells (Michaud et al., 2011). Furthermore, autophagy suppression in various mouse models resulted in enhanced angiogenesis associated with the tumor region. Defects in autophagy were associated with an increase in circulating factors such as erythropoietin and Gastrin Releasing Peptide (GRP) which are stimulants of vascularization (Lee et al., 2011; Kim et al., 2013b). The relevance of these findings and the adverse effects of inhibiting autophagy should be carefully considered during anti-cancer treatment.

Humanised GEM models to integrate autophagy research in cancer

The above examples highlight a plethora of potentially pro- and anti-tumorigenic functions for autophagy in cancer. As described, it is plausible that individual mechanisms, for example the role of mitophagy in mediating both cell survival

and reducing DNA damage, have effects in both directions, dependent upon context. Indeed, in true cancer contexts *in vivo*, it is likely the dominant effect of either net autophagy, or individual autophagic mechanisms, will be determined by stage and mutational spectrum of the cancer. However, true extrapolation from human cancer cell lines, or *in vitro* transformed cells, to the *in vivo* state is difficult. In this regard, one of the most promising developments for unpicking the role of autophagy in cancer comes from the application of humanised, genetically engineered mouse models (GEM models). These sophisticated model systems use conditional or sporadic genetic drivers of tumorigenesis and progression in various tissue types. These drivers mimic mutations seen in human cancers in order to produce disease that mirrors the human condition. Combined with companion *in vitro* experiments, using human cancer cell lines and transformed primary cell lines, these GEM models will assist in the unpicking of the context specific function of autophagy genes in cancer.

An excellent paradigm for the study of autophagy in cancer, and powerful combination of *in vitro* and GEM model studies, comes from the investigation of the role of autophagy in cancers where the KRAS proto-oncogene is mutated. This will be explored in depth here. A few additional examples will also be highlighted.

KRAS-driven cancer and autophagy

The KRAS proto-oncogene is mutated in a high proportion of pancreatic, lung and colorectal cancers, and several other cancer types at lower frequency. Cells transformed *in vitro* with activated mutants of the KRAS proto-oncogene become sensitized to the loss of ATG protein function, implying a role for autophagy in mediating proliferation and/or cell survival on this genetic background. Indeed, loss of ATG protein function diminishes tumorigenicity (Guo et al., 2011) and induces senescence (Wang et al., 2012b) in such *in vitro* transformed cells. Furthermore, KRAS mutant cancer cell lines derived from human cancers have significantly impaired *in vitro* proliferation upon autophagy disruption. For example, standard retroviral RNAi against key autophagy genes renders such cells incapable of proliferating under anchorage-deficient conditions (Lock et al., 2011). Lentiviral RNAi against ATG proteins leads to cell death even under conventional culture conditions (Guo et al., 2011; Newman et al., 2012). CQ-mediated targeting of lysosomal function may also produce similar effects although it is not clear that all aspects of the cellular response stem from inhibition of autophagic flux. The metabolic profiles of KRAS mutant cells that are compromised for autophagy function are significantly altered. For example dysregulated balance between aerobic glycolytic activity and oxidative phosphorylation is observed (Guo et al., 2011; Lock et al., 2011; Yang et al., 2011). It is possible such metabolic alterations reflects problems caused by the inability of autophagy to participate in mitochondrial homeostasis (mitophagy) in these cells (Guo et al., 2011; Kim et al., 2011b; Yang et al., 2011) or by other yet to be determined mechanisms, either involving lysosomal degradative activity or otherwise.

Overall these observations suggest that targeting of autophagy in KRAS mutant cancers might be beneficial, at least in some types and stages of cancer. This has begun to be tested using GEM models of KRAS-driven lung adenocarcinomas (with or without p53 loss). Tumour formation in this model is sensitive to ablation of autophagy function by homozygous deletion of the ATG7 gene at the inception of tumorigenesis (Guo et al., 2013). In this context cells are driven down an alternative tumor pathway ultimately forming a more benign form of lung tumor, called an oncocytoma. One possibility is that this is related to metabolic demands of the default tumor pathway that cannot be met without a functioning autophagy apparatus, reflecting some of the *in vitro* evidence discussed above. Autophagy-deficient KRAS-driven lung tumors are abundant in undegraded mitochondria and have defective lipid homeostasis, possibly reflected by the inability of cell lines from these tumors to participate in mitochondrial oxidative metabolism of fatty acids (Guo et al., 2013). Again, it remains to be determined precisely how the function of the autophagy pathway is reprogramming metabolism and whether alterations in mitophagy are the only component of this phenotype. There is also an inflammatory response of unestablished origin in these ATG7-deficient, KRAS-driven lung tumors, at least when p53 is absent (Guo et al., 2013). The inflammatory infiltrate might either result in immunosuppression of the cancer or, if this were to be overcome, create a suitable microenvironment for tumor progression. The role of inflammation in this model remains to be directly explored. Interestingly, KRAS driven pancreatic tumors in a GEM model of pancreatic ductal adenocarcinoma are sensitive to treatment with CQ, although it remains to be determined that this is entirely via effects on autophagy (Yang et al., 2011). However, recent studies demonstrate that the effect of ATG7 loss is switched from tumor suppressive to tumor promoting in the absence of p53 in KRAS-driven pancreatic ductal adenocarcinoma (Rosenfeldt et al., 2013). An upregulation of glucose metabolism is seen upon ATG7 loss. Such observations argue that ATG7, and by extension autophagy, is not critically required for tumorigenesis per se in KRAS-driven cancer, but remodels cellular function via metabolism and other routes such that, dependent upon the genetic background, this may either contribute to or impede tumor growth and progression.

Regulation of Nrf2 by autophagy and its role in cancer

Some insight into the signaling pathways downstream of KRAS in the lung come from the observation that lung tumors driven by active BRAF V600E, which in turn drives the Raf-MEK-MAPK pathway arm of the KRAS signaling network, display a similar reliance on ATG7 for determining

tumor cell fate in the GEM model setting (Strohecker et al., 2013). However, in this system there is an interesting initial acceleration of formation of low-grade tumors, before a subsequent failure to progress, the latter observation mirroring the KRAS situation (above). Interestingly, the loss of ATG7 results in hyperelevation of level of the oxidative stress response transcription factor, NRF2. However, loss of NRF2 phenocopies ATG7 loss and is epistatic with ATG7 loss (Strohecker et al., 2013). So the accumulation of NRF2 upon ATG7 loss on this genetic background is unlikely to be causing the tumor proliferation defect, despite some reported anti-tumor roles for the NRF2 signaling axis. It is also noteworthy that several cancer cell lines where autophagy loss has been shown to be synthetic lethal with activation of the KRAS pathway, such as A549 and NCI-H23, also bear mutant alleles of KEAP1 that, in any case, result in constitutive activation of NRF2 signaling, regardless of autophagy status (Shibata et al., 2008). In other instances, the loss of autophagy proteins such as ATG7 and ATG5 may predispose to tumorigenesis without additional oncogenes, for example in the liver, and in this scenario failure to degrade p62 is implicated (Inami et al., 2011; Takamura et al., 2011). Deregulated NRF2 signaling is seen here and it is possible that, in this context, elevated NRF2 signaling might promote the cancer.

Current limitations of GEM models for autophagy research

Despite the power of GEM models, the nature of these experiments dictates that they are currently best suited to studying the role of autophagy proteins in the early stages of tumorigenesis. They generally involve conditional mutation of an ATG gene at the same moment in time as initiation of tumorigenesis (by recombination of conditional alleles of tumor driving oncogenes and tumor suppressors). Thus, given the pleiotropic role of autophagy in cancer biology, which leads to early onset changes in tumorigenesis and changes in the fate of early tumor cells, it is somewhat difficult to extrapolate the findings of these models to the clinical situations where high-grade tumors have been free to evolve without any intervention in the autophagy pathway. Findings from these models can be compared with results from dosing fully-formed, frankly malignant tumors with agents such as CQ or rapamycin analogs, which are tolerated by mice at the doses required to alter autophagic flux. However, the widely non-selective nature of these agents means that observed effects on tumor physiology, while likely relevant to a clinical scenario where tumors are treated with these particular agents, are far from definitive in defining the role of the autophagy pathway per se. Future improvements in modeling autophagy in GEM models will doubtless involve use of dual recombinase (i.e. temporally regulable forms of Cre and Flp recombinase) to temporally separate conditional induction of tumorigenesis from genetic manipulation of autophagy, or inducible RNAi systems-again to target the function of autophagy proteins in fully formed tumors.

Therapeutic manipulation of autophagy

Findings from GEM models and *in vitro* experiments, where effects of autophagy manipulation are phenotypically evident, strongly highlight the significance of targeting autophagy during anti-cancer treatment. Small molecule modulators, including both inhibitors and activators of autophagy, may be of clinical relevance not only during cancer treatment, but also during treatment of other diseases such as neurodegeneration, diabetes and atherosclerosis. Our current understanding of the pharmacological manipulation of autophagy comes from the utilization of non-specific inhibitors such as CQ and 3-MA. The use of these inhibitors in combination with anti-cancer therapeutics, such as DNA damaging agents and inhibitors of the PI3K-AKT and mTOR pathways, is supportive of a pro-survival role of autophagy in cancer. Thereby, in these cases the use of autophagy inhibitors would enhance the efficacy of conventional therapy that counteractively upregulates autophagy. However, it is also possible that autophagy activation is beneficial in cases where autophagy acts as a tumor suppressive pathway (as discussed above). In addition to conventional therapy, autophagy activation has been achieved through the use of peptides that act by binding to negative regulators of the pathway (Shoji-Kawata et al., 2013) or by dietary deprivation of certain amino acids, such as leucine (Sheen et al., 2011).

Although efforts to develop specific autophagy modulators are still ongoing, a number of clinical trials have already been initiated to tackle aggressive cancers such as glioblastoma multiforme and metastatic solid tumors using CQ in combinational therapy (reviewed by Maes et al., 2013). In these studies, CQ treatment appeared to increase survival median and reduce tumor burden suggesting that pharmacological inhibition of autophagy may be effective in the clinic. However, it remains to be verified whether the anti-cancer effects of CQ are due, at least in part, to autophagy inhibition as autophagy-independent effects of CQ have been described (Maycotte et al., 2012; Musiwaro et al., 2013). Furthermore, the advantage of using specific autophagy inhibitors over CQ would also need to be addressed and whether these may have reduced undesirable effects.

What are the potential molecules that can be targeted to provide specific manipulation of autophagy? The targetable autophagy players and their specificity are highlighted in Fig. 2. Although autophagy-independent activities have been ascribed to almost all ATG gene products (see below), these non-canonical roles may have reduced relevance when targeting autophagy during disease. This remains to be determined. Because of its kinase activity, ULK1 is of increasing interest as a target to inhibit autophagy. However, under certain conditions autophagy can still take place in the

absence of ULK1 expression and cancers with high mTOR activity might stimulate autophagy without ULK1 activation. On the other hand, targeting the enzymatic activities of the ubiquitin-like conjugation system, which appear to be dispensable for their autophagy-independent functions, may be a potential alternative. Furthermore, inhibiting lysosome-autophagosome fusion would also provide some specificity although the molecular players regulating this step and whether they are specific to autophagy are still unknown. It is important to consider, however, that inhibiting later stages of autophagy, where autophagosome formation and thereby sequestration of cytoplasmic materials is unaffected, may produce different clinical outcomes compared to inhibiting upstream processes required for phagophore maturation. Also, prolonged disruption of downstream events in certain cases may indirectly compromise autophagosome formation and therefore impose dual effects on autophagy.

Apart from adverse complications that may rise because of the lack of specificity of pharmacological inhibitors, additional side effects may result from autophagy inhibition in healthy tissues and in cancer-associated stromal cells. The fact that prolonged disruption of autophagy in various tissues, particularly in the liver, can result in spontaneous tumors would require careful, long-term assessment of the use of autophagy inhibitors. Furthermore, the role of autophagy in the function of the immune system during cancer and whether autophagy disruption in the whole organism may compromise the individual's immunity has not yet been addressed. Autophagy inhibition in tumor cells may adversely affect the recruitment of the immune cells to the tumor region. It would be hoped that tumor cells with high levels of autophagy will pose increased sensitivity to lower doses of autophagy inhibitors. This, along with dietary manipulation, may help overcome adverse effects of these inhibitors.

Despite the increasing evidence highlighting autophagy as an attractive target during anti-cancer treatment, a number of unanswered questions remain. Can complete ablation of autophagy by gene knockout systems in mouse models be recapitulated by partial inhibition of autophagy using pharmacological inhibitors? Would targeting different stages of autophagy give different outcomes? Can cells adapt to autophagy inhibition by upregulating alternative degradation pathways such as the proteasome, chaperone-mediated autophagy or lysosomal degradation? Does specifically targeting autophagy have a clinical advantage over non-specific inhibitors that target for an example the endocytic/

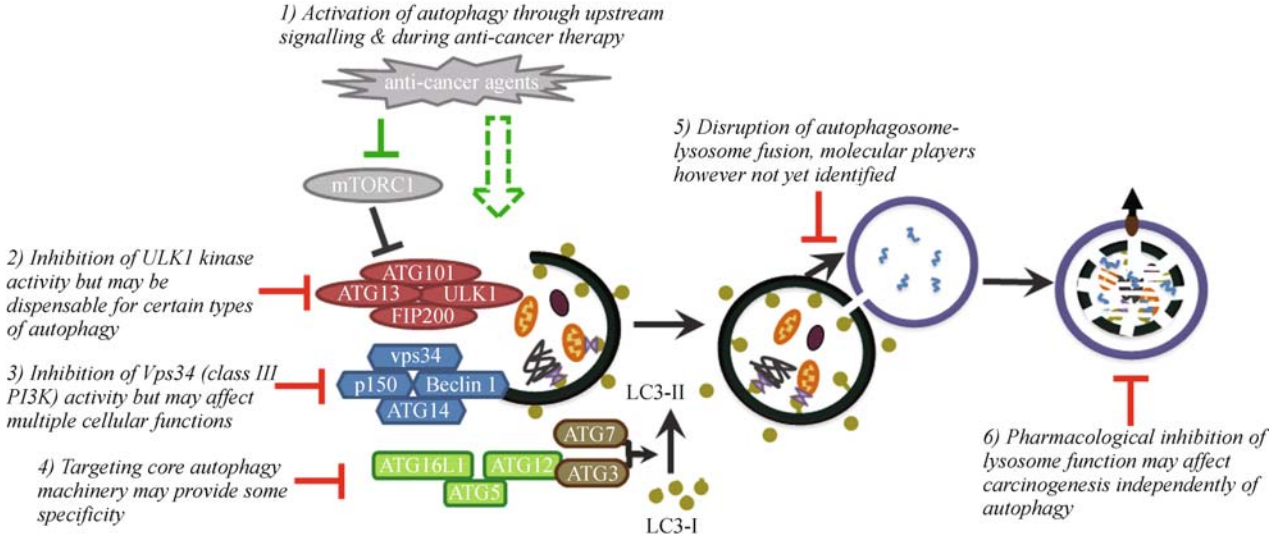

Figure 2 Potential manipulation of autophagy during anti-cancer therapy. Highlighted using red lines are potential molecules that can be targeted to inhibit autophagy meanwhile in green line are potential ways to activate autophagy. **1)** Autophagy can be activated using anti-cancer agents (e.g. etoposide & cisplatin), mTOR inhibitors (e.g. rapamycin & CCI-779), small peptides and leucine starvation. **2)** Inhibiting ULK1 kinase activity is of interest to disrupt autophagy. However, ULK complex proteins have non-autophagic functions and autophagy can take place in their absence. **3)** The class III PI3K, Vps34, is currently being targeted to inhibit autophagy using inhibitors such as 3-MA and wortmannin. Vps34 is also involved in endosomal trafficking and its inhibition can disrupt autophagy as well as multiple cellular functions. **4)** Targeting core autophagy machinery may provide some specificity. Individual members of the ATG5 complex have autophagy-independent activities involving DNA damage response, apoptosis, immune response and mitochondrial function. Disrupting the complex formation (e.g. by inhibiting ATG5-ATG12 conjugation) may specifically abrogate autophagy. E1- and E2-like enzymes have non-autophagy functions; however targeting their enzymatic activities may lead to specific inhibition of autophagy. **5)** Potential disruption of autophagosome-lysosome fusion may be specific to autophagy. The molecular players involved are not yet identified. **6)** Pharmacological inhibition of lysosome function (e.g. using CQ or Bafilomycin A1) may affect carcinogenesis independently of autophagy. Furthermore, the disruption of autophagy at either early stages involving autophagosome formation or later events that abrogate autophagic degradation can lead to differential consequences during anti-cancer therapy as discussed in the text.

lysosome pathway? Finding the appropriate autophagy inhibitor will shed light on such questions and accelerate our understanding of the impact of autophagy during the pathogenesis of cancer.

Non-canonical roles for autophagy proteins

A major assumption in many of the above studies discussed this far is that ablation of function of ATG proteins results in specific ablation of the canonical autophagy pathway, i.e. non-selective and selective degradation of cytosolic components via autophagic vesicles. Relatively non-specific drugs such as CQ may mimic some, but certainly not all, effects of ATG protein depletion. This may be a boon for clinical applications, but is unsatisfactory in many instances for firmly implicating 'classic' autophagy, mechanistically, in a given cellular phenomenon. This is particularly important because a number of observations underscore non-canonical roles of ATG proteins in processes that are independent of autophagy. A long-standing example is the Beclin 1- and the more recently characterized Beclin 2- gene(s), which have roles in controlling other membrane trafficking processes that potentially impact upon cancer cell signaling (He et al., 2013). More recent observations include a role of ATG5 expressed downstream of DNA damage responses in causing mitotic errors – these might readily impact on genomic instability in cancer (Maskey et al., 2013). Also, ATG7 affects the transcriptional output of the p53 pathway, independent of its E1-like enzymatic activity required for autophagy (Lee et al., 2012). As p53 is a major tumor suppressor that acts via predominantly via gene transcription, this may have substantial impact upon tumor progression. In another example, ATG12-ATG3 conjugates control mitochondrial function independent of mitophagy (Radoshevich et al., 2010).

It is reasonable to expect that other non-canonical roles for ATG proteins will be determined in the near future. Incorporation of these into a more nuanced picture of the various different roles of ATG proteins will give greater clarity for clinical application. This is particularly important given that a drug against a given ATG protein complex may not thus affect all of the roles ascribed to autophagy or may have ATG protein specific effects. This picture is further complicated by the recent observations of forms of *bona fide* macroautophagy, in lower eukaryotes at least, that only require a subset of the ATG proteins for completion (Chang et al., 2013).

Are there novel roles to be discovered for autophagy in cancer?

The evidence discussed above suggests multiple, mechanistically distinct roles for autophagy in growth and evasion of cellular death or senescence of tumor cells. However, relatively unexplored areas of the function of autophagy proteins in cancer lie within the spread of cancer *in vivo*. Such spread involves multiple distinct stages that may be affected by autophagy; for example, the ability of autophagy to maintain metabolism in dormant tumor cells, such as upon colonisation of initially hostile niches during the metastatic process. Furthermore, it is not apparent, even in the GEM models of cancer, whether effects of autophagy are particular to the cancer stem cell compartment or the main bulk of the epithelium in the tumor. This is an important distinction with ramifications for mechanisms of tumorigenesis, as well as tumor therapy. Another interesting area of potential clinical significance is the possible involvement of autophagic degradation of cellular mass in the wasting of muscular tissues during the cancer cachexia response (Penna et al., 2013). Future exploration of these areas is likely to further implicate autophagy proteins in different stages and aspects of cancer.

Additionally, with the advent of modern techniques that are giving us unparalleled insight into the cancer genome and proteome, such as next-generation sequencing, functional screens and cutting edge mass spectrometry techniques, it is likely that numerous new protein cargoes and components of the autophagy pathway will be identified, along with perturbations at a genetic or functional level in autophagy-related molecules in various cancers. Detailed investigation of the transcriptome of cells and tumors under various stress conditions will also identify cohorts of genes that are co-regulated by transcriptional programmes that reflect dynamic regulation of the autophagy process. These approaches will give us yet more insight into the roles of this important process in cancer.

Conclusions

In summary, it is clear that autophagy, and more broadly ATG proteins, participate in many different mechanisms that promote or repress cancer in different tumor scenarios. To leverage our knowledge of the autophagy pathway appropriately to treat cancer, we need to understand in detail the different roles of ATG proteins. This will enable us to consider if and how we might wish to treat autophagy in different tumor types, and to be able to design therapeutic agents accordingly.

Compliance with ethics guidelines

Noor Gammoh and Simon Wilkinson declare that they have no conflict of interest.

This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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