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Monitoring autophagy in cancer: from bench to bedside

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Abstract

Autophagy refers to an essential mechanism that evolved to sustain eukaryotic homeostasis and metabolism during instances of nutrient deprivation. During autophagy, intracellular cargo is encapsulated and delivered to the lysosome for elimination. Loss of basal autophagy *in vivo* negatively impacts cellular proteostasis, metabolism and tissue integrity. Accordingly, many drug development strategies are focused on modulating autophagic capacity in various pathophysiological states, from cancer to neurodegenerative disease. The role of autophagy in cancer is particularly complicated, as either augmenting or attenuating this process can have variable outcomes on cellular survival, proliferation and transformation. This complexity is compounded by the emergence of several selective autophagy pathways, which act to eliminate damaged or superfluous cellular components in a targeted fashion. The advent of sensitive tools to monitor autophagy pathways *in vivo* holds promise to clarify their importance in cancer pathophysiology. In this review, we provide an overview of autophagy in cancer biology and outline how the development of tools to study autophagy *in vivo* could enhance our understanding of its function for translational benefit.

Keywords

Autophagy, cancer, metabolism, tumour, organelles

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1. Introduction

Autophagy is an essential catabolic mechanism that evolved to sustain eukaryotic homeostasis during instances of nutrient deprivation. The term autophagy is translated from the Greek meaning "to eat oneself" and interestingly, was in usage before its incorporation into the scientific vernacular by Christian De Duve (Ktistakis, 2017). General or bulk autophagy is a non-selective process (herein referred to as macroautophagy) (Zachari and Ganley, 2017). Macroautophagy is a complex, multistep process involving (1) the sensing and identification of cellular cargo for destruction, (2) marking this cargo for recognition by the autophagy machinery, followed by (3) its encapsulation within a transient double membrane-bound organelle known as an autophagosome and (4) elimination of the sequestered contents *via* fusion of the autophagosome with an acidic compartment of the endolysosomal system, resulting in the formation of the autolysosome (Mizushima, 2018). This process results in the recycling of autophagic substrates, which is thought to fuel various metabolic pathways (White, 2015). Autophagy is an evolutionarily conserved process that is also essential for mammalian development. Impaired autophagic activity (termed "flux") is detrimental to cell and tissue function. Because of its links to organismal longevity, homeostasis and metabolism, autophagy has evolved to become an attractive therapeutic target for a variety of clinical indications (Galluzzi et al., 2017b). Indeed, dysregulated autophagic flux has emerged as a stand-alone hallmark of mammalian cancer (Hanahan and Weinberg, 2011). Recognising its vital biomedical importance, Professor Yoshinori Ohsumi received the 2016 Nobel Prize in Physiology or Medicine for pioneering the molecular basis of autophagy (Mizushima, 2017). Treatments that modulate autophagic capacity are widely thought to represent a therapeutically advantageous strategy to prevent cancer development (Mizushima, 2018). As we will see in this review, understanding autophagy presents a challenging conundrum in the context of cell transformation, cancer biology and chemoresistance. We summarise critical concepts related to our current knowledge of physiological autophagy pathways and their relationship to cancer biology. In particular, we discuss how developments that enable researchers to monitor autophagy pathways *in vivo* could clarify mechanistic questions of outstanding translational importance.

2. Classical and contemporary concepts in autophagy research

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The modern-day definition of autophagy broadly encompasses two sub-phenomena: macroautophagy and selective autophagy (Mizushima, 2018). The cargo to be degraded can vary from organelles, and proteinaceous aggregates to ferritin, lipids and carbohydrates. Macroautophagy is by far the best-studied autophagy pathway and refers to a non-selective or bulk digestion process, induced by many signals and stimuli (Zachari and Ganley, 2017). The most well-described induction stimulus in mammals is nutrient deprivation, resulting in the inhibition of the master nutrient sensor mTORC1 (Laplante and Sabatini, 2012). However, work by the laboratory of Terje Johansen has recently redefined the temporal dynamics of the autophagic response to amino acid deprivation (Mejlvang et al., 2018). Loss of GSK3-beta signalling is also associated with the initiation of macroautophagy, in addition to activation of AMPK and HIF signalling (Lin et al., 2012). The initiation and progression of autophagosome biogenesis involve the activation of autophagy-specific kinase complexes ULK1 (comprised of ULK1/2, FIP200, ATG101, ATG13), and VPS34 (including BECLN1) as well as the ATG16L1-ATG5-ATG12 conjugation machinery (Xie and Klionsky, 2007; Zachari and Ganley, 2017). The exact membrane origin or source that contributes to the nucleation of the transient autophagosome has been a controversial topic in the field for many years. However, a growing consensus suggests a multi-organellar contribution to autophagosome biogenesis, with subdomains of the endoplasmic reticulum (ER), ER and Golgi intermediate compartment (ERGIC), mitochondria and endolysosomal system playing key roles here (Bissa and Deretic, 2018; Ge et al., 2013; Hailey et al., 2010). ATG9A and ATG2 have both recently been reported to transport lipids to promote autophagosome formation (Valverde et al., 2019). Autophagosome closure and maturation constitutes a critical step that precedes autolysosome formation, yet we know less about its molecular regulation compared to upstream initiation events. It has been hypothesised that autophagosome sealing resembles a membrane scission event (Zhou et al., 2019). The budding yeast *Saccharomyces cerevisiae* has enabled the detailed study of these discrete steps at an unparalleled resolution, with recent work implicating the Rab5-dependent recruitment of ESCRT machinery in the closure of unsealed autophagosomes (Zhou et al., 2019). COPII-coated carriers ensure transport between the ER and Golgi complex and have also been shown to contribute to autophagosome membrane formation in yeast (Shima et al., 2019). Figure 1 provides a clear graphical overview of macroautophagy.

Textbook autophagosomes are classically depicted as single spheroids, yet advances in electron tomography have revealed the presence of multiple smaller vesicles of unknown function that are juxtaposed to mature autophagosomes (Biazik et al., 2015). The steps of cargo recognition, autophagosome closure and endolysosomal fusion rely upon ubiquitin-like conjugation systems of

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the ATG8 family of proteins (LC3A/B/C, and GABARAP/GABARAPL1/2) (Bissa and Deretic, 2018; Ganley, 2013). The end stage of all autophagy pathways depends on functional acidic compartments of the endolysosomal system to degrade the encapsulated cargo. A lysosome fuses with an autophagosome to form an "autolysosome", or an acidic Rab5/7-positive late endosome can fuse with an autophagosome to generate an "amphisome". An informative primer on autophagy nomenclature can be found in (Klionsky et al., 2014). The process of autophagosome-lysosome fusion is mediated by two independent autophagosomal SNARE protein complexes, syntaxin 17 and YKT6 (Matsui et al., 2018; Itakura et al., 2012).

Our understanding of the lysosome as a simple "suicide bag" has been dramatically revised by discoveries of its nutrient sensing properties in mammalian cells (Lim and Zoncu, 2016). Lysosomes orchestrate critical aspects of cellular metabolism, and also appear to be more phenotypically heterogeneous than previously thought. Recent findings demonstrate that lysosomes in distinct spatial cellular compartments are differentially acidic (Johnson et al., 2016), indicating that their function likely extends beyond mere digestion. This conceptional evolution in our understanding of the lysosome also necessitates a more in-depth evaluation of how nutrient status modulates crosstalk between autophagic compartments and the endolysosomal system.

The degradation of autophagic substrates acts to replenish amino acids necessary for protein synthesis and cellular homeostasis. This step of the pathway is associated with the reactivation of mTOR and a process known as autophagosome-lysosome reformation (ALR) (Chen and Yu, 2013). ALR is a terminal event that promotes lysosomal reformation from autolysosomes *via* membrane tubulation and scission. Ultimately, ALR sustains cellular lysosomal homeostasis during and after autophagy (Chen and Yu, 2013). A key regulator of this process is lysosome-associated PI(3)P, generated by the VPS34–UVRAG complex in an mTOR-dependent fashion (Munson et al., 2015).

Autophagy may also occur in the absence of canonical factors. A Rab9-pathway has been suggested to sustain autophagy in the absence of ATG5/ATG7/LC3-II (Nishida et al., 2016). Tripartite motif (TRIM31), an intestine-specific protein localised in mitochondria has also been implicated in Atg5/7-independent autophagy in gastrointestinal cells (Ra et al., 2016). In recent years, autophagy-independent functions of the ATG machinery have also manifested. Please consult (Galluzzi and Green, 2019) for an authoritative review of this emerging topic.

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In addition to non-selective macroautophagy, it is now well-established that autophagy can also be highly selective (Mizushima, 2018). Selective autophagy refers to the targeted destruction of damaged or superfluous cellular components. Cargo-specific autophagy receptors confer selectivity on various cellular constituents from organelles (organellophagy) to proteinaceous aggregates (aggrephagy) (Okamoto, 2014). Selective autophagy is a fast-moving domain of the field and involves an interplay between the post-translational modifications ubiquitylation and phosphorylation (Khaminets et al., 2016). The best-studied selective autophagy pathway to date is mitochondrial autophagy (mitophagy); however, pexophagy (Marcassa et al., 2018), ribophagy (Wyant et al., 2018) and ferritinophagy (Santana-Codina and Mancias, 2018) are also emerging as important determinants of cellular homeostasis. The following reviews provide a comprehensive overview of selective autophagy (Rogov et al., 2014; Galluzzi et al., 2017a). Reconciling the interplay between stimulus-induced and basal physiological autophagic turnover *in vivo* constitutes a significant challenge in the field (See section 4 for a discussion of selective autophagy).

Independently of the aforementioned pathways, chaperone-mediated autophagy (CMA) constitutes a distinct form of selective autophagy (Kaushik and Cuervo, 2018). The cytosolic chaperone HSC70 recognises protein substrates containing a distinct CMA-targeting motif (KFERQ) and targets these to the lysosomal surface, where they undergo active translocation *via* LAMP-2A (Kaushik and Cuervo, 2012, 2018). Levels of lysosomal LAMP-2A reflect the activity of CMA. Pioneering work by Ana Maria Cuervo and colleagues has demonstrated the critical importance of CMA in mammalian health and disease. Please consult (Kaushik and Cuervo, 2018) for a comprehensive overview of CMA.

3. Autophagy in cancer: a complex conundrum united by metabolic crosstalk

Autophagy is a disease-relevant process, yet it remains controversial whether dysregulated flux is a cause or consequence of any human disorder. From our perspective, the most accurate, evidence-based interpretation is that the role of autophagy in any physiological or pathological scenario appears to be highly context-dependent. This complexity becomes especially apparent when considering the astounding mechanistic heterogeneity of cancer. Accordingly, enhanced autophagy can either drive or diminish tumourigenesis depending on the cellular state and tissue type (Galluzzi et al., 2017b).

3.1 The protective influence of autophagy in cancer

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Insights from cell culture and pre-clinical animal studies have demonstrated that autophagy sustains tissue homeostasis and prevents pro-oncogenic environments through interplay with genome stability and anti-inflammatory signalling pathways (Amaravadi et al., 2016; Mathew et al., 2009).

Although core autophagy genes are not generally mutated in human cancers, polymorphisms and altered expression levels of autophagy-related proteins have been reported (Jiang and Mizushima, 2014). Additionally, autophagy genes have been implicated as both tumour enhancers and suppressors (White, 2015). Valuable insights into the role of autophagy in human oncology came from the discovery that the autophagy modulator Beclin-1 (*BECN1*) is lost in a substantial proportion of human breast, ovarian and prostate cancers (Liang et al., 1999; Yue et al., 2003; Qu et al., 2003).

Consequently, human *BECN1* has been proposed as a haploinsufficient tumour suppressor gene. Consistent with this, heterozygote *Becn1* mice exhibited enhanced susceptibility to hepatic, mammary and lymphoid neoplasia (Qu et al., 2003). *BECN1* exerts critical cellular functions, although its role as a *bona fide* tumour suppressor appears somewhat contentious in the field (White et al., 2015). Recently, an interesting study focused on abolishing the interaction of endogenous Beclin-1 with the negative regulator of Beclin-1-dependent autophagy Bcl-2 (Pattingre et al., 2005) *in vivo*, *via* the generation of knock-in mice with a constitutively active variant of Beclin-1 (*Becn1*$^{F121A/F121A}$). Tissues from *Becn1* mutant animals exhibit enhanced levels of autophagic flux coupled to improvements in healthspan, longevity and a diminished incidence of age-related spontaneous cancer (Fernández et al., 2018). In another study, endogenous HER2 was reported to interact with Beclin-1 to inhibit autophagy and drive tumourigenesis. The enhanced levels of basal autophagy in *Becn1*^{F121A} mice also had an anti-tumourigenic influence on HER2-mediated cancer progression (Vega-Rubín-de-Celis et al., 2018). Treatment of mice harbouring HER2-breast cancer xenografts with an autophagy-inducing peptide (Tat-Beclin-1) diminished tumour growth *in vivo* and could represent an attractive therapeutic approach for HER2-positive breast cancer (Vega-Rubín-de-Celis et al., 2018). Interestingly, another autophagy regulator, UVRAG (described earlier for its role in ALR) is found to be mutated in colon cancer and is also proposed to be a haploinsufficient tumour suppressor (Liang et al., 2006).

Loss of autophagy is increasingly associated with the initiation of cancer. To this effect, autophagic cell death was recently shown to play a dominant role in restricting chromosomal instability during

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a process termed replicative crisis. During replicative crisis, pre-cancerous cells with dysregulated cell cycle checkpoints undergo continual division, leading to progressive telomeric DNA shortening and apoptosis. Cells that bypass this senescence-independent process harbour high levels of chromosomal instability and exhibit an increased propensity for oncogenic transformation, suggesting that replicative crisis is an essential anti-cancer mechanism (Nassour et al., 2019). Nassour et al. discovered that autophagy-deficient cells with deregulated cell cycle checkpoints could evade both crisis and cell death, and ultimately accrue chromosomal instability. Telomeric DNA damage generates cytosolic chromatin fragments that specifically activate cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes) signalling and the recruitment of the autophagy machinery (Nassour et al., 2019). These data suggest that cell death in replicative crisis is associated more with autophagy rather than canonical apoptosis. Overall, these findings highlight the protective nature of autophagy and raise potential caveats when considering its therapeutic inhibition in cancer.

Pre-clinical studies of cancer in mice have shown that loss of essential autophagy genes promotes tumourigenesis, and thus, the presence of functional autophagy in these contexts seems to be anti-oncogenic. Furthermore, the severity of tumourigenesis seems to be associated with oncogenic background and cellular context. To this effect, conditional hepatic ablation of *Atg5* leads to hepatotoxicity, inflammation and the development of benign liver tumours in mice (Takamura et al., 2011). Similar conditional experiments show that autophagy also exerts protective effects in the pancreas (Rosenfeldt et al., 2013). In pancreatic cancer characterised by mutant *Kras*, loss of pancreatic autophagy drives oncogenesis, yet malignancy appears to be restricted here (Yang et al., 2014). Furthermore, genetic ablation of essential autophagy genes induces the accumulation of the autophagy adapter protein p62 (SQSTM1), which is also thought to promote tumourigenesis (Wei et al., 2014).

Genetic inhibition of autophagy in a humanised mouse model of pancreatic ductal adenocarcinoma (PDAC; activated oncogenic *Kras, p53* KO double-mutant) is associated with an unfavourable prognosis. PDAC is a notoriously lethal cancer, characterised by high mortality and low survival rates (National Cancer Institute., 2018). PDAC mice with impaired autophagy (lacking *Atg5* or *Atg7*) exhibited accelerated tumour onset coupled with a metabolic state conducive to tumourigenesis (enhanced glucose uptake and anabolism) (Rosenfeldt et al., 2013). In another PDAC model driven by oncogenic KRAS (G12D) combined with ablation of the tumour suppressor Phosphatase and Tensin Homolog (PTEN), autophagy inhibition also promoted tumour

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development. These findings suggest that both p53 and PTEN can influence the contribution of autophagy to PDAC and reflects the context-dependent nature of this problem (Rosenfeldt et al., 2017). Treatment of *Kras p53* double KO mice with hydroxychloroquine (HCQ; an agent known to impair autophagy) was shown to enhance tumourigenesis. These data are pertinent due to the use of HCQ as an anticancer agent in clinical trials and highlight the need for studying drug treatments in cellular contexts that closely recapitulate the human condition (Rosenfeldt et al., 2013). Please consult Mainz and Rosenfeldt for an authoritative review on the study of autophagy in pre-clinical mouse models of cancer (Mainz and Rosenfeldt, 2018).

3.2 The pathological influence of autophagy in cancer

Conversely, cancer cells can also use autophagy to their pathological advantage. 95% of PDACs arise from activating *KRAS* mutations and have been linked with autophagy-dependent tumourigenic growth (Viale et al., 2014). Therefore anti-KRAS and anti-autophagy therapies, in general, have emerged as attractive strategies to treat PDAC (Viale et al., 2014). Chronic KRAS inhibition in PDAC mouse cell lines revealed a rare subpopulation of dormant tumour cells that can drive tumour relapse. These resistant cells survive oncogene ablation, become increasingly dependent on autophagy and exhibit a bioenergetic preference for mitochondrial respiration (oxidative phosphorylation) over glycolysis (Viale et al., 2014).

Furthermore, Bryant and colleagues demonstrated that KRAS inhibition enhances autophagic flux in human and mouse PDAC cell lines (Bryant et al., 2019). Acute ablation of mutant KRAS or selective ERK inhibition in PDAC cells both resulted in the same metabolic effect by increasing autophagic activity at multiple levels (enhanced autophagosome flux, phosphorylation and activation of AMPK and Beclin-1, downregulation of mTOR signalling, and increased transcription of autophagy-related genes). In addition to enhanced autophagic flux, both ERK inhibition and KRAS silencing also influenced metabolic state, evidenced by diminished glycolysis and altered mitochondrial function. Bryant et al. demonstrated that combinational therapy targeting the increased autophagy and increased ERK activity is an effective treatment against KRAS driven PDAC in mice (Bryant et al., 2019; Kinsey et al., 2019). These data highlight the complex interplay between autophagy, metabolism and cancer progression. For an excellent overview of autophagy and metabolic crosstalk in cancer, please consult (Kimmelman and White, 2017).

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Cancer cells also have increased metabolic requirements to support their accelerated growth (Rabinowitz and White, 2010). The microphthalmia/transcription factor E (MIT/TFE) family of transcription factors regulate energy metabolism by controlling the expression of genes that regulate both autophagy and lysosomal biogenesis. These factors also have established roles in promoting tumourigenesis (Perera et al., 2015; Kauffman et al., 2014) and have been reviewed recently in (Perera et al., 2018).

3.3 Autophagy as a therapeutic target in cancer

The clinical benefit of autophagy inhibition in human cancer remains controversial (Mainz and Rosenfeldt, 2018). Nonetheless, clinical trials aiming to modulate levels of autophagy in cancer are underway (Levy et al., 2017a). To date, chloroquine (CQ) and its derivative HCQ are the only repurposed drugs used in the clinic to inhibit autophagic flux and are already approved to treat malaria. CQ and HCQ have been associated with favourable clinical outcomes for cancer therapy (Levy et al., 2017a). CQ was first tested on 18 glioblastoma patients and showed positive results, increasing survival from 11 to 33 months when combined with radiation therapy and the alkylating agent, temozolomide (Briceño et al., 2003). Follow up studies verified the benefits of CQ in improving survival of glioblastoma patients (Briceño et al., 2007; Sotelo et al., 2006). HCQ has been used in various clinical trials involving different malignancies, with therapeutic dosage determined by the cancer subtype and pre-existing treatment regimen (Rangwala et al., 2014b; a). Results from these trials have been varied, with HCQ treatment exerting little effect on patient health compared to controls (Rosenfeld et al., 2014; Goldberg et al., 2012; Mahalingam et al., 2014). Furthermore, CQ was also shown to sensitise cancer cells to chemotherapy through autophagy-independent mechanisms. Thus, it is necessary to consider that the effects of CQ/HCQ may occur independently of direct flux inhibition (Maycotte et al., 2012).

Accordingly, although lysosomotropic drugs such as HCQ attenuate autophagic flux, these compounds have been reported to activate a parallel noncanonical autophagy pathway that drives LC3 lipidation on endosomal membranes (Jacquin et al., 2017). Nonetheless, CQ and HCQ treatment undoubtedly compromise autophagic flux *in vitro*. However, readers should be aware that recent mechanistic insights have emerged comparing CQ to other lysosomal inhibitors such as Bafilomycin A1. CQ and HCQ inhibited autophagy by impairing autophagosome-lysosomal fusion, rather than by affecting the acidity and degradative activity of the autolysosome. CQ/HCQ administration has also been reported to cause defects in Golgi complex organisation in mouse

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intestinal and kidney cells (Mauthe et al., 2018). These findings merit vital mechanistic consideration when interpreting and evaluating autophagy-associated compounds as precision therapeutics for cancer. As outlined in section 2, autophagy is a multistep pathway with several nodes representing attractive targets for drug discovery (e.g. inhibitors of ULK1/2, ATG4B, p62). At present, vital tool compounds exist, but their utility in disease models remains to be determined. Please consult (Limpert et al., 2018) for an up-to-date overview of small molecule modulation of autophagy in cancer.

A large number of studies have shown that cancer cell subtypes respond differentially to autophagic inhibition. To this effect, RNAi-based studies in various breast cancer cell lines revealed a differential dependency on autophagy for proliferation and survival (Maycotte et al., 2014). In cancer subtypes where autophagy is dispensable for survival, autophagy inhibition may have only a modest effect. Accordingly, the degree of autophagy inhibition required for therapeutic efficacy may vary depending on its combination with a given chemotherapeutic strategy. In order to integrate autophagy therapeutics as part of a meaningful precision medicine approach, it will be necessary to devise standardised clinical protocols that incorporate the autophagy-dependence of cancer subtypes for predicted patient benefit. For a detailed overview of autophagy therapeutics and clinical trials in cancer, please refer to the excellent review by Mulcahy Levy and colleagues (Levy et al., 2017a).

In BRAF-mutant melanoma, therapeutic inhibition of BRAF acts to increase autophagy (Ma et al., 2014), and thus combination therapies targeting the increased autophagy as well as BRAF inhibition are proposed to combat tumourigenesis (Levy et al., 2017b; Wang et al., 2017).

Autophagy also influences the survival of dormant breast cancer cells and metastatic tumour reoccurrence (Vera-Ramirez et al., 2018). Pharmacogenetic experiments demonstrated an essential role for ATG7 in this context, with selective inhibition of autophagic flux in dormant breast cancer cells showing promise as a potential anti-cancer strategy (Vera-Ramirez et al., 2018). The same approach also appears encouraging for epithelial ovarian cancer, which has a high mortality rate following relapse (Pagotto et al., 2017). Ovarian cancer stem cells (CSCs) are a rare cellular population that exhibit high levels of basal macroautophagy and chemoresistance and are predicted to be a significant contributor to malignancy. Genetic ablation of *ATG5* or pharmacological inhibition of autophagic flux significantly diminished the tumourigenic potential of CSCs *in vitro* and *in vivo*, indicating a potentially effective strategy to combat chemoresistance and neoplastic

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relapse in this scenario (Pagotto et al., 2017). Relevant links between autophagy and metastatic colon cancer have also emerged. Ragusa and colleagues identified a subset of cancer stem/progenitor cells, capable of PROX1-mediated metabolic adaptation in an autophagy-dependant manner. These metabolic and autophagic changes in cancer cells appear to confer resistance to metabolic stress and promote their metastatic growth. Thus, inhibition of autophagy as part of combination therapy has been proposed as a strategy to attenuate the growth advantage of PROX1+ metastatic colon cancer cells (Ragusa et al., 2014). CMA and human cancer are also interconnected, with loss of LAMP-2A associated with attenuated tumourigenic potential (Kon et al., 2011; Thorburn and Debnath, 2011).

A study by Karsli-Uzunbas et al. highlighted the challenges of broadly inhibiting autophagy in the clinic using a mouse model of KRAS driven-lung cancer, in which tumours developed before the systemic genetic ablation of *Atg7* (Karsli-Uzunbas et al., 2014). In this context, autophagy inhibition by *Atg7* whole body KO markedly decreased tumourigenesis in adult mice. Despite this elegant demonstration, these animals had reduced overall survival (two to three months) with an increased predisposition to bacterial infection and the onset of neurodegeneration (Karsli-Uzunbas et al., 2014). These data demonstrate that acute autophagy inhibition may be therapeutically beneficial in cancer, although several parameters (therapeutic window, drug safety, length of usage and drug site of action) will need to be carefully established to prevent secondary complications (Towers and Thorburn, 2016). This example also underscores the importance of developing targeted therapeutic strategies, which is particularly pertinent to post-mitotic tissues such as neurons, where autophagy is essential to sustain neural integrity.

In healthy cells, autophagy is predicted to protect against malignant transformation by degrading toxic or superfluous cellular components (White, 2015). Nutrient deprivation is one of the best-characterised signalling events known to induce macroautophagy (Laplante and Sabatini, 2012). In recent years, fasting and calorie restriction mimetics (e.g. hydroxycitrate) (Madeo et al., 2014) have garnered increased attention due to their reported influence on restricting cancer growth *in vitro* and *in vivo* (Pietrocola et al., 2016; Lee et al., 2012). In athymic mice with subcutaneous fibrosarcoma, a 48h starvation period or the use of calorie-restricted mimetics triggered an autophagy-dependent anti-cancer immune response. This beneficial effect was abolished when cancer cells were rendered autophagy-deficient upon *Atg5* knockdown (Pietrocola et al., 2016). Autophagy-activating agents have also been demonstrated to improve the therapeutic response to multiple malignancies evolving in immunocompetent hosts (Galluzzi et al., 2017). Although the implementation of prolonged

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fasting may be infeasible or unsuitable for many patients, alternate nutrient restriction approaches with similar benefits may represent a more sustainable intervention (Nencioni et al., 2018). For example, selective restriction of non-essential dietary amino acids (serine and glycine) in combination with biguanide inhibition of mitochondrial respiration has been associated with enhanced survival in cancer-prone mice (Maddocks et al., 2017). Neoadjuvant metformin treatment in breast cancer patients undergoing chemotherapy has also been shown to induce a systemic metabolic profile akin to that observed in fasting (Cuyàs et al., 2019). Interestingly, metformin is also known to inhibit mitochondrial complex I (Wheaton et al., 2014). These distinct studies underscore a significant convergence upon mitochondrial metabolism in the context of cancer (Maddocks et al., 2017; Cuyàs et al., 2019).

Autophagy is required for adult mice to survive nutrient deprivation, and its loss creates a catabolic dependence on nutrient stores, leading to metabolic imbalance (Karsli-Uzunbas et al., 2014). As this imbalance depletes essential nutrients from fat, muscle and liver, it has been postulated to reflect the cachexia commonly associated with cancer progression (Poillet-Perez and White, 2019). Interestingly, autophagy-deficient mice have recently been identified to have reduced circulating levels of the semi-essential amino acid arginine (Poillet-Perez et al., 2018). Many human tumours are arginine auxotrophs and derive arginine from the host to sustain metabolism, growth and function. Diminished levels of circulating arginine in autophagy-deficient mice impaired tumour growth, and could be partially reversed by dietary supplementation of arginine (Poillet-Perez et al., 2018). Thus, host autophagic activity may sustain tumour growth *via* supplementation with necessary amino acids. Accordingly, in some instances, autophagy inhibition could represent a potential therapeutic strategy to restrict the bioavailability of certain nutrients and thus, tumourigenesis (Poillet-Perez and White, 2019).

The precise mechanistic relationship between autophagy, fasting, FMDs and the potentiation of chemotherapeutic efficacy in human subjects is unclear (Caccialanza et al., 2019). Extensive large-scale clinical studies are ultimately required to establish the efficacy of fasting and FMDs for cancer therapy (Caccialanza et al., 2019; Nencioni et al., 2019). It will also be essential to determine the parameters required to obtain the maximum benefit from FMDs and related interventions. Are these interventions beneficial for a majority of patients, or should they be selectively employed in a patient-specific fashion, i.e. depending on the patient profile, cancer type and stage? Furthermore, at what stage would autophagy inhibition prove most therapeutically efficacious? Answering these

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questions will be essential in harnessing the therapeutic potential of any autophagy-based intervention.

A key challenge in the field is the development of clinical-grade diagnostics to monitor autophagy signalling in human patients. The capacity to rapidly assess autophagic status in clinical biospecimens will ultimately impact our physiological and mechanistic understanding of targeted treatments in this area and will be vital for shaping their evolution. Until these tools emerge, phenotypic profiling of patient-specific cancer subtypes may be a prerequisite to determine the value of autophagy modulation alongside ongoing treatment. Clinical trials aimed at uncovering cancer-specific autophagy biomarkers are underway and reviewed in (Levy et al., 2017a).

An appraisal of the vast literature associated with modulating autophagy in cancer reveals a clear and unifying theme: it is a context-dependent process. Thus, the rigorous *in vivo* profiling of autophagy in relevant pre-clinical cancer models represents a distinct challenge for the field.

4. Monitoring autophagy pathways *in vivo*: from bench to bedside

Ultimately, implicating autophagy in any physiological system depends upon sensitive assays that can probe this process in a precise and cell-specific fashion. Our present-day understanding of these pathways in health and disease is ultimately due to continual advances in the development and refinement of tools to monitor autophagy using microscopy and biochemistry. We next outline critical developments in laboratory-based autophagy detection, with a view to the development of clinical diagnostics.

4.1 Monitoring autophagy in the laboratory

4.1.1 Macroautophagy

Autophagy has been classically studied in cell culture using either metabolic or chemical stimuli. The most reliable readouts of autophagy are those based on converging data, e.g. monitoring autophagy flux of LC3 and p62 (and other autophagy associated factors) in the presence of the lysosomal vATPase inhibitor (e.g. Bafilomycin A1), and supported by fluorescence-based reporter assays or electron microscopy (the latter in the case of extreme phenotypes) (McWilliams and Ganley, 2019a). All of these assays have provided powerful insights into the mechanistic regulation of stimulus-induced macroautophagy in cultured cells. Tracking LC3 has provided powerful

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insights into autophagosome formation (Kuma et al., 2017). Immunodetection or GFP-labelling of N-terminal LC3 has enabled the visualisation of phagophores and autophagosomes in cells as puncta or ring-like structures (Kuma et al., 2017). Pioneering work from Noboru Mizushima and colleagues using GFP-LC3 transgenic reporter mice revealed that macroautophagy also proceeds at basal levels to varying degrees across all mammalian tissues (Mizushima et al., 2004). Numerous studies in "macroautophagy-deficient" mice support the physiological significance of these findings, where loss of basal macroautophagy *via* conditional tissue-specific genetic ablation of *Atg5/7* has profound effects in mammals depending on the cell subtype affected, e.g. selective loss of autophagy in the nervous system results in severe neurodegeneration, whilst the selective loss of macroautophagy in liver cells results in systemic glucose dysregulation and the onset of tumourigenesis (Takamura et al., 2011; Komatsu et al., 2006).

Such fluorescence-based reporter systems have enabled researchers across many fields to interrogate the physiological significance of macroautophagy *in vivo*. Observations using the GFP-LC3 approach require validation with lysosomal inhibitors to reliably interpret and infer changes in autophagic flux. This control is necessary due to the acid labile properties of GFP, whose fluorescence becomes quenched in the acidic microenvironment of the lysosome. Such a validation approach is particularly challenging with rodent models, as the effects of lysosomal inhibitors *in vivo* are far more variable than under controlled *in vitro* conditions. Since the initial development of the GFP-LC3 reporter mice, other probe systems have emerged with different properties that enable the facile detection of autophagy *in vivo* without the use of inhibitors. These approaches exploit the acid-resistance properties of other fluorescent proteins, e.g. those in the red spectrum. To this effect, tandem reporter systems have emerged as powerful tools to monitor autophagy pathways in cells and tissues. Tandem reporters involve the use of red-green fusion proteins (e.g. mCherry-GFP) that can demarcate both autophagosomes and autolysosomes. In merged images using this approach, yellow structures represent autophagosomes, whereas mCherry-only puncta represent autolysosomes (due to the quenching of GFP). Consequently, when autophagosome-lysosome fusion is blocked, only the number of yellow puncta increases. This method has been successfully deployed in mice and includes the tandem fluorescent-tagged (TfLC3) reporter mouse model (mRFP-EGFP-LC3) and mCherry-GFP-LC3B reporter mice (McWilliams et al., 2018a; Kimura et al., 2007).

Another complementary approach to track autophagic flux *in vivo* involves the GFP-LC3-RFP-LC3ΔG probe. This approach exploits ATG4-mediated cleavage to generate two products: GFP-

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LC3 and RFP-LC3ΔG. Ultimately, GFP-LC3 becomes degraded by autophagy and is quenched, whereas RFP-LC3ΔG remains stable (Kaizuka et al., 2016). Measurements of cellular GFP-fluorescence intensity against total RFP-LC3ΔG fluorescence intensity provide a ratiometric readout of autophagic flux in cells and tissues. RFP-LC3ΔG cannot be lipidated due to the absence of c-terminal glycine, thus enhanced autophagy results in a decreased GFP:RFP ratio. Many of these tandem tag approaches have also been validated in *Atg*-null backgrounds (Kuma et al., 2017). As these reporter systems can measure basal autophagy *in vivo* without the need for validation by inhibitors, they provide a powerful way to clarify the contribution and importance of basal macroautophagy in healthy as well as neoplastic cells *in vivo*. For an excellent review of recent developments in this area, please consult (Kuma et al., 2017).

4.1.2 Selective autophagy

Damaged or defective cellular components can have deleterious effects on cellular function and viability. The sensing, identification and targeted destruction of such components can occur through selective autophagy. In this instance, damaged/defective cellular components become decorated with an "eat-me" signal (e.g. ubiquitin), and selectivity is conferred by cargo-specific receptors that engage the autophagy machinery (Rogov et al., 2014). Several receptors are associated with the selective autophagic degradation of a range of organelles, including mitochondria (mitophagy), peroxisomes (pexophagy), ferritin (ferritinophagy), endoplasmic reticulum (reticulophagy), nuclei (nucleophagy) and ribosomes (ribophagy). An excellent overview of these processes can be found in (Galluzzi et al., 2017a).

Many of these selective autophagy pathways require detailed characterisation *in vivo*, and we have much to learn regarding their role in promoting or preventing tumourigenesis. We will focus on mitophagy due to recent advances in our molecular and physiological understanding of this process in tissues.

4.1.3 Focus on mitophagy

Mitophagy is by far the most studied mode of selective autophagy in mammalian cells. For recent reviews on this topic, please consult (Palikaras et al., 2018; Rodger et al., 2018). At present, it seems that the term "mitophagy" was first used in a review by Scott and Klionsky in 1998 (Scott and Klionsky, 1998), and subsequently promoted by Lemasters in 2005 (Lemasters, 2005). The earliest description resembling our present-day understanding of mitophagy is the sequence of events leading to "mitochondrial degeneration", first visualised over 100 years ago using Janus

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green dye in tissue cultures, in the landmark cell biology studies of Margaret Reed Lewis and Warren Lewis (Lewis and Lewis, 1915). It is essential to recognise that the idea of monitoring mitochondrial turnover has captivated investigators over the past several decades. This is evidenced by extensive work from the 1950s-1970s using electron microscopy, which identified the presence of mitochondria inside lysosomes within a variety of mammalian tissues under steady-state conditions (Clark, 1957; Novikoff and Essner, 1962). Radiolabelling of mitochondrial proteins was also used as a surrogate assay to measure mitochondrial turnover (Fletcher and Sanadi, 1961).
A revival of this research area occurred in 2008, with the demonstration that overexpression of the RBR E3-ubiquitin ligase Parkin in HeLa cells could drive dramatic levels of mitochondrial turnover (mitophagy) in response to chemical agents that dissipate mitochondrial membrane potential (Narendra et al., 2008). This critical proof-of-principle study established a new framework to investigate the selective turnover of mitochondria in tissue culture. It was subsequently elaborated by many laboratories that mitochondrial depolarisation stabilises and activates the mitochondrial-associated ubiquitin kinase PINK1 (McWilliams and Muqit, 2017). Ultimately, the substrates of PINK1 are Parkin and ubiquitin, which are both phosphorylated at their respective Serine 65 residues. Through a coordinated series of events, depolarised mitochondria become decorated in a coat of ubiquitin *via* feed-forward amplification signalling. "Mitochondrial ubiquitylation" serves as a potent signal for the recruitment of the autophagy machinery and the elimination of the damaged organelle. This stress-induced pathway has dominated the attention of researchers in the field of mitophagy, yet its role in mitochondrial elimination *in vivo* remained obscure until the advent of mitophagy reporter mice (Jang et al., 2018; Palikaras et al., 2018).

Like fluorescent macroautophagy reporter mice that revealed a landscape of tissue-specific bulk degradation, the recent advent of mitophagy reporter systems and their corresponding mouse models have also redefined our understanding of mammalian mitochondrial destruction *in vivo* (McWilliams et al., 2016; Sun et al., 2015). The development of the *mito*-QC and mitochondrial Keima (mt-Keima) mouse models signified an important advance in the field of mitochondrial biology (McWilliams et al., 2016; Sun et al., 2015). Keima is a coral-derived pH-sensitive fluorescent protein, which can fluoresce in different wavelengths, depending on acidification state. In the mt-Keima mouse model, Keima is present in the mitochondrial matrix due to a COX8 targeting sequence and emits green-fluorescence. A shift to red-fluorescence is observed upon mitophagic delivery to the lysosome (Sun et al., 2015). In the *mito*-QC mouse model, cytosolic mitochondrial networks are visible in yellow due to a tandem mCherry-GFP tag on the outer mitochondrial membrane *via* the mitochondrial targeting sequence of FIS1 (McWilliams et al.,

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2018a, 2016, 2018b). Upon delivery of mitochondria to the lysosome as in mitophagy, GFP fluorescence is quenched yet the mCherry signal remains stable. The aforementioned reporter systems provide a simple approach to measure mitophagy, and a related reporter system known as mito-Timer enables a temporal assessment of mitochondrial age and biogenesis *in vivo* (Wilson et al., 2019). The latter system exploits the fluorescence shift of DsRed1-E5 from green to red over time.

As with any model, all systems have different strengths and limitations, but collectively these mice have facilitated a converging conceptual advance in our understanding of mitophagy as a steady state process during mammalian development and disease (McWilliams and Ganley, 2019b; Jang et al., 2018; Palikaras et al., 2018; Kuma et al., 2017). Under steady state conditions, mitophagy appears to be a highly pervasive process with striking heterogeneity, even between cells of the same organ. These observations at steady state contrast dramatically with the notion of mitophagy as an induced stress response (Rodger et al., 2018; Palikaras et al., 2018; Jang et al., 2018). Furthermore, the demonstration that basal mitophagy is evolutionarily conserved from Drosophila to mammals using both mt-Keima and *mito*-QC is further evidence that suggests that the modulation of basal mitochondrial turnover is likely to be critical to tissue-specific homeostasis and metabolism (Lee et al., 2018; Sun et al., 2015; McWilliams et al., 2016). The idea that distinct basal and stress-evoked pathways orchestrate mitophagy *in vivo* is exemplified by the many demonstrations that basal mitophagy is unaffected in cells and tissues that lack a functional PINK1-Parkin signalling pathway (either PINK1 KO or Parkin Ser65 KI mutant mice) (McWilliams et al., 2018b; a). Consistent with this, subsequent data demonstrated that p62 (SQSTM1) is likely to play a crucial role in modulating basal mitophagy *in vivo* (Yamada et al., 2018).

Much more work will be required to decipher the molecular determinants of basal mitophagy in vivo. Nonetheless, it is clear that many pathways likely operate to coordinate the elimination of defective or damaged mitochondria in a context-specific fashion (Rodger et al., 2018; Jang et al., 2018; Palikaras et al., 2018). A significant challenge in the field will be to reconcile in vitro observations to in vivo pathophysiology. A step in this direction has emerged recently by Soutar and colleagues, who identified that the concentration of serum and more specifically albumin in tissue culture media had a potent influence on mitochondrial membrane potential and activation of the PINK1-Parkin pathway (Soutar et al., 2019). Higher albumin levels in FBS/FCS required greater concentrations of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) to depolarise mitochondria and activate PINK1-Parkin-dependent mitophagy in cultured cells.

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However, this does not alter the depolarising effect of other agents known to activate this stress-associated pathway (e.g. combined oligomycin and antimycin-A treatment) (Soutar et al., 2019). Such studies are particularly vital in the context of cancer biology, where recapitulating metabolic state is crucial for biological relevance (Voorde et al., 2019). It will be of considerable interest to determine the contribution of basal mitophagy to metabolic plasticity in cancer.

Interestingly, aside from their role in modulating depolarisation-induced mitophagy *in vitro*, PINK1-Parkin signalling has long been linked with cancer biology. Indeed, Parkin has been proposed as a potential tumour suppressor (Bernardini et al., 2017; Veeriah et al., 2010). Links have also emerged between Parkin and glioblastoma *via* regulation of G1/S cyclins (Gong et al., 2014). In terms of tumour metabolism, loss of Parkin has been reported to drive the Warburg effect, a hallmark of many tumours (Zhang et al., 2011). For an excellent review of Parkin signalling in oncology, please consult (Bernardini et al., 2017). Recently, a cullin RBR E3 ligase known as ARIH1 has been implicated in Parkin-independent mitophagy associated with protecting cancer cells from chemotherapy-induced cell death (Villa et al., 2017). Furthermore, prolonged mitotic arrest in cancer cells is associated with the induction of mitophagy, with AMPK and PFKFB3 modulating the metabolic adaption and survival of cancer cells in this context (Doménech et al., 2015).

Mutations in PINK1 and Parkin are most known for their role in the neurodegenerative movement disorder, Parkinson's disease (PD) (Rodger et al., 2018; McWilliams and Muqit, 2017), yet the contribution of dysregulated mitophagy to PD pathology remains unclear. In terms of cancer biology, the inverse co-morbidity relationship between neurodegeneration and carcinogenesis is well known (Bajaj et al., 2010). However, an intriguing and somewhat overlooked point of convergence stems from epidemiological data demonstrating that PD patients have an increased risk of developing melanoma, but not other cancers (Bose et al., 2018; Ascherio and Schwarzschild, 2016). Because defective macroautophagy is also associated with neuropathology (Menzies et al., 2017), it will be interesting to investigate if this constitutes a pathogenic convergence point between these distinct disease states.

In terms of modulating mitophagy in humans, Urolithin A (UA) has recently been reported to have metabolically-beneficial effects on mitochondrial function in humans (Andreux et al., 2019). Although UA modulates mitophagy in *C. elegans* and influences mitochondrial function in rodent

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models (Ryu et al., 2016), it remains to be determined how UA administration influences mitophagy signalling in human tissues.

4.2 Monitoring autophagy pathways in the clinic

Despite significant advances in the experimental monitoring of macroautophagy and selective autophagy, there is still a long road to travel before reliable diagnostics can infer meaningful information about these cellular processes in the clinic. Autophagy is a context-specific process with different effects dependent upon cell and cancer type. Accordingly, the diagnostic value of measuring autophagy pathways in clinical specimens is advantageous for discovery science, but of limited use to clinical diagnostics at present (Galluzzi et al., 2017b). Many caveats exist with the current methods to monitor autophagy pathways, including the need to monitor flux as opposed to the level of LC3 lipidation. Furthermore, methods of measuring these can vary dramatically from laboratory to laboratory, and LC3 levels can also differ across samples. The emerging landscape of selective autophagy pathways indicates a growing need to identify pathway-specific receptors. Even more sensitive reporter systems will also be required to discriminate between these processes both *in vitro* and *in vivo*. Such developments will be essential to resolve the contribution of selective autophagy or macroautophagy pathways to a given cancer subtype.

In order to effectively develop autophagy-specific therapies for the clinic, it will be essential to standardise longitudinal measurements of autophagy across many laboratories. Significant efforts in the global autophagy community are already underway to do this (Klionsky et al., 2016; Galluzzi et al., 2017a). Given the vast mechanistic heterogeneity in cancer cell biology, distinct autophagy-related biomarkers will likely emerge that are suitable for subsets of particular cancers. It will be exciting to determine the predictive and prognostic value of such autophagic signatures in the context of cancer predisposition, progression and chemoresistance.

5. Summary and future outlook

It is difficult to generalise a single role for autophagy in cancer, as this multistep process can confer survival advantages to both tumour and host. More than any other disease field, cancer biologists have embraced complexity and recognised the context-dependent nature of autophagy and its emerging crosstalk with other pathways. This integrative approach surely signals a bright future

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where the development of precision therapeutics that drive or diminish autophagy signalling in defined contexts could provide a clinically meaningful benefit for cancer patients. The rigorous and sensitive profiling of autophagy in well-controlled pre-clinical models will undoubtedly contribute towards achieving this goal.

Conflict of interest

None

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Figure Legend

Figure 1: Generalised overview of mammalian macroautophagy

Macroautophagy is a conserved process that degrades a variety of different types of cellular cargo in a non-selective fashion, ranging from protein aggregates to defective organelles and even intracellular pathogens. Autophagosome biogenesis involves crosstalk between various organelles. Many organellar compartments (ER, Golgi complex, endosomes, and mitochondria) have been shown to contribute to the phagophore, and the resultant autophagosome. Mature autophagosomes are often juxtaposed to multiple smaller vesicles as depicted here. Following the encapsulation of cargo, autophagosomes undergo fusion with acidic compartments of the cellular endolysosomal system. The fusion of autophagosomes with lysosomes generates the terminal compartment of the autophagy pathway: the autolysosome, required for the completion of autophagy. However, when macroautophagy and endocytosis converge, late endosomes can fuse with autophagosomes to generate an amphisome. Created with BioRender.