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Review

Autophagy process is associated with anti-neoplastic function

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Autophagy is a highly conserved process of cellular degradation, which is present in yeast, plants, and mammals. Under normal physiological conditions, autophagy acts to maintain cellular homeostasis and regulate the turnover of organelles. In response to cellular stresses, autophagy prevents the accumulation of impaired proteins and organelles, which serves to inhibit carcinogenesis. On this basis, it is widely accepted that most tumor suppressors, such as beclin 1 associated proteins, forkhead box class O (FoxO) family proteins, multiple mammalian target of Rapamycin (mTOR) inactivators, and nuclear p53 play a role in inducing autophagy. Here, we focus on how the process of autophagy is associated with anti-neoplastic function.

Keywords autophagy; cancer; Beclin 1; FoxO; mTOR; p53

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Introduction

Autophagy is a highly conserved process of cellular degradation in eukaryotes by which damaged cytoplasmic proteins and organelles are delivered to the lysosome for degradation [1–6]. There are at least three types of autophagy including macro-autophagy, micro-autophagy, and chaperone-mediated autophagy (CMA), which differ in their physiological functions and modes of degradation. Macro-autophagy represents a process of engulfing cellular waste including misfolded or damaged proteins and organelles in a vesicle called autophagosome, and transferring for subsequent fusion with lysosome and degradation. Micro-autophagy, on the other hand, involves degradation by direct lysosome internalization of cellular waste products rather than fusion of a vesicle with a lysosome. CMA is a process of internalization of soluble proteins by the lysosome mediated by chaperones [7]. This review will focus on macro-autophagy, hereafter referred to as autophagy.

The process of autophagosome formation can be divided roughly into three steps, including initiation, elongation, and maturation. This is a highly complex process, the proximate regulation of which involves many proteins. First, the process is initiated with formation and expansion of free membranous structures called phagophores. The phagophore gradually grows into a double-membrane vesicle (called autophagosome) that sequesters cellular waste together with some cytoplasm. At present, there are more than 30 autophagy-related (Atg) genes that have been found to be involved in the process of autophagy [8]. Although the exact mechanism of phagophore formation is not clear, it has been determined that two ubiquitin-like complex, namely Atg12–Atg5–Atg16 complex and Atg8–PE localize on the phagophore, and this event is closely related to the formation of autophagosome [9]. There are at least three homologues of Atg8 in mammalian cells including microtubule-associated protein 1 light chain 3 (LC3), Gamma-aminobutyric acid receptor-associated protein (GABARAP), and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) [10]. LC3 is a protein that has been found to locate on the inner membrane of autophagosome and the amount of LC3-II is correlated with the extent of autophagosome formation [11]. As such, LC3 is one of the most important markers of autophagy. In addition, as p62 is the ligand of LC3, its degradation is also considered to be a positive marker for autophagy [12]. After fusion of the autophagosome and lysosome, the cargo with the inner membrane of the autophagosome enters the lysosome and is degraded by various lysosomal enzymes (**Fig. 1**).

The autophagic pathway is beneficial for cell survival under conditions of stress such as nutritional deprivation, as the ‘garbage’ comprised of proteins and organelles is degraded into ‘nutrients’ consisting of amino acids and nucleotides that may be recycled by the cell. Thus, in order to maintain cellular homeostasis, a basal level of autophagy is needed.

There is no complete agreement as to whether autophagy affects the process of carcinogenesis in a positive or a

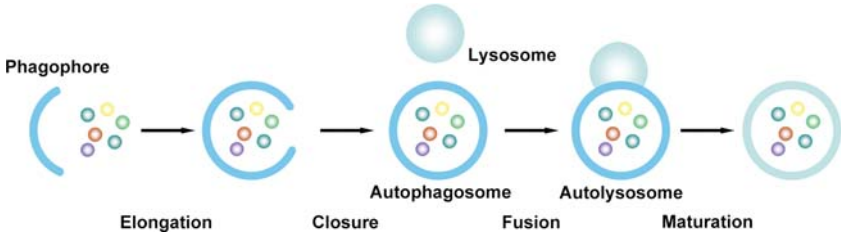

Figure 1 The cellular process of autophagy In macroautophagy, the cytoplasmic components to be degraded are first enclosed by a double-membrane structure called phagophore. With elongation, closure and fusion, the phagophore gradually grows into an autophagosome. The autophagosome with its contents is then transported by dynein motors to the lysosome that leads to the subsequent fusion and the formation of autolysosome. The autophagic body is then transferred into the autolysosome for degradation by lysosomal enzymes. The substrate material is degraded into small molecules that are then released into the cytoplasm and may be recycled.

negative manner. However, most studies have considered autophagy to be a mechanism that acts against carcinogenesis because most inducers of autophagy act as tumor suppressors. Moreover, studies have shown that autophagy-related genes are often down-regulated or even absent in multiple types of cancer cells [13,14]. That autophagy acts against carcinogenesis are mainly based on two mechanisms. For one, the stimulation of autophagy functions to limit the local necrosis, which reduces the level of inflammatory response, and thus might contribute to the inhibition of carcinogenesis [15]. Second, in addition to eliminating damaged proteins and organelles, autophagy serves to reinforce proper function of the cell cycle check point, which reduces the rate of carcinogenesis [16].

As autophagy plays crucial roles in both normal physiological conditions and inhibition of carcinogenesis, understanding the molecular mechanisms of the induction is essential. Here we discuss pathways or mechanisms to understand the process of autophagy in cancer development. These include two positive regulatory pathways that are associated with Beclin 1 and forkhead box class O (FoxO), and a negative regulatory pathway that is associated with the mammalian target of Rapamycin (mTOR). In addition p53 acts to both positive and negative regulatory influence on autophagy in a manner associated with subcellular localization, which is in turn dependent on multiple post-translational modifications.

Beclin 1 and associated proteins

Beclin 1 is the homologue of yeast Atg6/Vps30, which is the most important up-regulator of autophagy, and as such also has a significant association with tumorigenesis. The autophagosome formation is facilitated by a specific protein complex vacuolar protein sorting 34 (Vps34) Phosphatidylinositol 3-kinase (PI3-kinase) [17], the core of which contains Beclin 1 [18], VPS15 [19], and the proteins such as ultraviolet radiation resistance-associated gene (UVRAG) [20], Bax-interacting factor 1 (Bif-1) [21], activating molecule in Activating molecule in BECN1-regulated autophagy protein 1 (Ambra 1) [22] and Beclin 1-associated autophagy-related key regulator (Barkor) [23] [**Fig. 2(A)**]. The roles of Beclin 1, UVRAG, and Bif-1 in the interplay of autophagy and tumorigenesis have been well described, and we therefore pay attention to these three proteins in this review.

Beclin 1 regulates autophagy via Beclin 1/PI3K3C complex. The process of autophagy is down-regulated when Beclin 1 binds anti-apoptosis factor Bcl-2. Beclin 1 together with Bcl-2 constitutes a sensor that responds to nutritional level, and accelerates autophagy by diminishing the interaction between these two proteins under conditions of starvation [24]. As for the function of Beclin 1, it has gradually been found to be haploinsufficient in its function as a tumor suppressor [25]. For example, monoallelic loss of Beclin 1 has been observed in sporadic breast, prostate, and ovarian cancers [26,27]. Similarly, *in vivo* experiments have shown that Beclin $1^{+/-}$ mutant causes increased morbidity in lymphoma, heptocellular carcinoma, and adenocarcinoma of the lung [28,29].

As a tumor suppressor, UVRAG is often found to be mutated in colon cancer [20]. Through binding with Beclin 1, UVRAG promotes autophagy by increasing the interaction between Beclin 1 and PI3K3C as well as enhancing the activity of PI3K3C [20]. Bif-1 is another molecule which interacts with Beclin 1, and activates PI3K3C through its interaction with Beclin 1 via UVRAG, resulting in stimulation of autophagy under conditions of nutrition deprivation [21]. Bif-1 has also been identified as a tumor suppressor [30–33] as many spontaneous tumors have been identified in Bif-1 knockout mice.

On this basis, the presumption has been established that Beclin 1, UVRAG, and Bif-1 exert tumor-suppressive function through up-regulation of autophagy. However, all these proteins have been shown to play important roles in apoptosis as well as in numerous other regulatory processes [18,20,21]. Therefore, whether their anti-neoplastic functions are dependent on autophagy needs more advanced study.

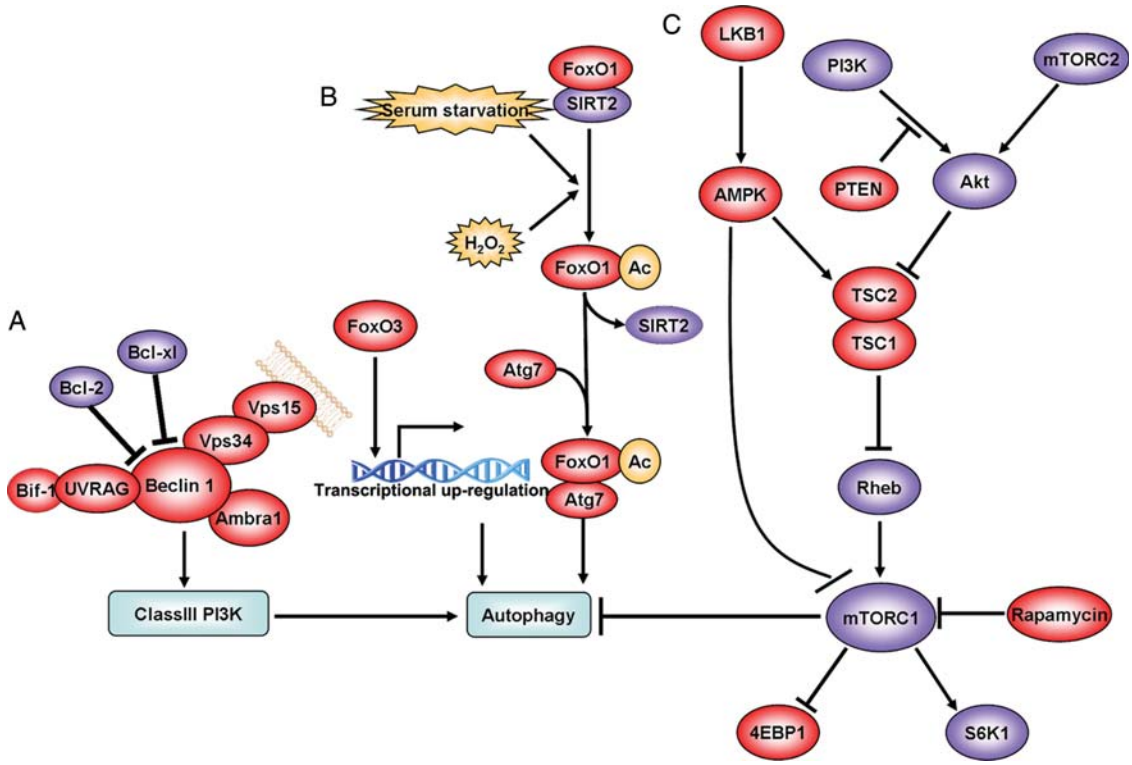

Figure 2 The molecular pathways for regulation of autophagy (A) The PI3K3C complex contains VPS15, VPS34, Beclin 1, UVRAG, Bif-1, and Ambra 1. Under amino acid deprivation, PI3K3C activation triggers the process of autophagy. The process of autophagy is down-regulated when Beclin 1 binds to Bcl-2. UVRAG is one of the beclin 1 interactors and promotes autophagy by increasing the interaction between Beclin 1 and PI3K3C, as well as enhancing the activity of PI3K3C itself. Bif-1 is also able to activate PI3K3C through its interaction with Beclin 1 via UVRAG, resulting in the stimulation of autophagy in nutrition deprivation. (B) In muscle cells, FoxO3 induces autophagy by enhancing expression of both autophagy-related genes and autophagy regulation genes. Moreover, cytosolic FoxO1 is required for autophagy in response to oxidative stress or serum starvation. In response to these stimuli, FoxO1 is acetylated by dissociation from SIRT2, which is a NAD^+-dependent histone deacetylase, and then binds to Atg7, an E1-liked protein, to up-regulated the autophagic process. (C) In cancer cells, the mTOR pathway is frequently activated which inhibits autophagy. In the PI3K/Akt pathway, activated Akt stimulates Rheb by inhibiting the TSC1/TSC2 complex, followed by activation of mTOR. In this pathway, PTEN acts as the antagonist of PI3K, which triggers autophagy by inhibiting the function of Akt. Moreover, activated LKB1 stimulates TSC2 through AMPK phosphorylation, resulting in the down-regulation of mTOR by the subsequently activation of Rheb. In that manner, the autophagy inducers in mTOR pathway includes LKB1, AMPK, TSC1/TSC2 complex, and PTEN.

FoxO family proteins

The mammalian FoxO transcriptional factor family includes four members: FoxO1 [34], FoxO3 [35], FoxO4 [36], and FoxO6 [37], which have been reported to serve as potential tumor suppressors by inducing cell cycle arrest, DNA repair and apoptosis [38–40]. Recently, it has been demonstrated that some FoxO family members is able to induce autophagy. For instance, fasting in Drosophila [41] or overexpression of FoxO3 [42,43] in muscle cells induces autophagy by enhancing the expression of autophagy-related genes. Moreover, cytosolic FoxO1 has also been found to be one of the up-regulators of autophagy, and the expression of FoxO1 paralleled with the induction of autophagy has been detected in human colon cancer [44]. This in turn has raised the possibility that FoxO family proteins may operate through an autophagy mechanism to suppress tumorigenesis.

Experiments have shown that FoxO3 is dephosphorylated and localizes in the nucleus of starved cardiomyocytes, resulting in activation of autophagic pathway genes including Atg12, LC3, and Gabarapl 1. *In vivo* experiments have also shown that the level of autophagy is increased in mice with increased activity of FoxO3 induced by ischemia or reperfusion in heart [42] [**Fig. 2(B)**].

However, the exact manner in which FoxO3 regulates autophagy is not clear yet. It has been demonstrated that FoxO3 up-regulates autophagy through up-regulating several autophagy-related genes as well as some autophagy regulation genes including PI3K3C, BCL2/adenovirus E1B interacting protein 3 (Bnip3), and BCL2/adenovirus E1B interacting protein 3-like (Bnip3l) [43]. In addition, the binding activity of FoxO3 with the promoters of Bnip3 and Bnip3l is increased in starved skeletal muscle, which results in increased level of autophagosome formation. In further support of this mechanism, the level of autophagy

induced by constitutively active FoxO3 (ca-FoxO3) in Bnip3 knockdown skeletal muscle is notably decreased. However, this phenomenon has not been evaluated in cancer cells.

Recently, our group has found that in response to oxidative stress or serum starvation, cytosolic FoxO1 is essential for the induction of autophagy in a transcription-independent manner. This process depends on acetylation of FoxO1 resulting from its dissociation with the histone deacetylase sirtuin-2 (SIRT2) [44,45]. Moreover, acetylated FoxO1 specifically interacts with Atg7, which causes up-regulation of autophagy [44]. In addition, *in vivo* experiments have shown that ectopic expression of FoxO1 decreases the size of tumor in nude mice, while this inhibition of tumorigenesis has not been found in cells with FoxO1-RNAi. Furthermore, FoxO1 expression and p62 degradation simultaneously takes place in normal human tissue while significantly decreases in human colon cancer, which indicates that FoxO1-mediated suppression of tumor growth might also depend on an autophagic mechanism [44] [**Fig. 2(B)**]. Thus, it is possible that cytoplasmic FoxO1 plays a critical role in linking autophagy and tumor suppression. However, it is important to point out why some FoxO family members, such as FoxO1 and FoxO3, induce autophagy by different pathways. Whether they are co-incident to play a role in inducing autophagy needs more advanced study in the future.

Autophagy up-regulators in signaling pathways through mTOR

Much of the information about the relationship of autophagy and oncogenesis comes from the study of pathways associated with mTOR that is a central regulatory protein in numerous processes of cell metabolism including cell growth, proliferation, and survival under both physiological and pathological conditions, including tumorigenesis [46]. This molecule is one of the main constituents of two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [46]. mTOR is inhibited under nutrient starvation, which is a crucial step for induction of autophagy [47,48]. Although the exact mechanism how mTOR regulates autophagy remains elusive, it has been reported that mTOR contributes to the hyperphosphorylation of Atg13 and thus reducing the binding affinity of Atg13 with Atg1 [49]. Since Atg13–Atg1–Atg17 complex is essential for the control of initiation of phagophore, mTOR plays a role in down-regulation of the process of autophagy. In cancer cells, mTOR is frequently activated, which tends to inhibit autophagy and enhance cell growth. In addition, multiple crucial factors in the mTOR pathway are closely related to cancer. As such, mTOR may be exploited to investigate the relation between cancer and autophagy.

The mTOR signaling pathway is the convergent point of multiple signal transduction pathways. Two upstream pathways are responsible for the activation of mTOR, including the phosphoinositide-3-kinase (PI3K/Akt) cell survival pathway and the adenosine monophosphate-activated protein kinase (AMPK)/extracellular signal-regulated kinase (ERK) pathway [50,51]. Although the specific mechanism of PI3K/Akt regulation of mTOR has not been clearly elaborated, PI3K/Akt has been confirmed to bring about stimulation of mTOR function through two pathways. In the first pathway, activated PI3K/Akt stimulates Ras homolog enriched in brain (Rheb) by inhibiting the tuberous sclerosis complex 1/2 (TSC1/TSC2), followed by the activation of mTOR [52]. Here phosphatase and tensin homolog deleted on chromosome TEN (PTEN) acts as a phosphatase functioned as the antagonist of PI3K [53]. In the second pathway, the mTORC1 binding protein, proline-rich AKT1 substrate 49 (PRAS49), is phosphorylated by Akt, then binds to 14-3-3 protein and dissociates from mTORC1, which directly activates the mTOR pathway [54]. There is another negative regulatory pathway of mTOR, which fulfills its function by serine/threonine kinase 11 (STK11/LKB1). Activated LKB1 stimulates TSC2 through AMPK phosphorylation, resulting in the down-regulation of mTOR by the subsequently activation of Rheb [**Fig. 2(C)**].

As mTOR is a down-regulator of autophagy, the activator of mTOR always inactivates autophagy through mTOR pathway, and vice versa. In that manner, the inducers of autophagy in mTOR signaling pathways includes LKB1, AMPK, TSC1/TSC2 complex, and PTEN, while the suppressors of autophagy in the same pathways are Akt and Rheb. In the upstream of mTOR, multiple mTOR activators are oncogenes, which suppress autophagy through the activation of mTOR. For example, Akt is a proto-oncogene that is observed to be activated in most human cancers. Similarly, it has been discovered that the level of Rheb mRNA is appreciably elevated in breast, lung, prostate, colon, pancreatic, ovarian, and melanoma cancer cells, which demonstrates that Rheb is an oncogene. On the other hand, most proteins involved in pathways down-regulating mTOR are cancer suppressors. For instance, LKB1 as an inactivator of mTOR is a tumor suppressor closely related to Peutz–Jeghers syndrome (also known as Intestinal Multiple hamartoma) [46]. Moreover, AMPK integrates diverse intracellular signals and balances intracellular energy, which facilitates tumor suppressors exerting their functions via the activation of AMPK [48]. In addition, TSC1 and TSC2 are both mTOR inhibitors and tumor suppressors. Mutation of either of TSC1 and TSC2 can induce tuberous sclerosis syndrome, which is

characterized by multiple benign tumors in various organs. Various tumors develop in the human body resulting from PTEN mutation, which causes Cowden syndrome, Bannayan－Riley－Ruvalcaba syndrome, Lhermitte－Duclos disease, and Proteus syndrome [46]. In addition, tumor angiogenesis increases when PTEN is deleted [55], further indicating the mechanism by which PTEN acts as a tumor suppressor.

Based on these findings, almost all activators of mTOR are oncogenes while almost all of its inhibitors are tumor suppressors. At the same time, mTOR acts as an inhibitor of autophagy. Therefore, it is reasonable to reach the conclusion that oncogenes in mTOR signaling pathways inhibit autophagy while tumor suppressors enhance autophagy.

Application of p53 in regulating the autophagic process

Although it is accepted that most tumor suppressors act as up-regulators of autophagy while most oncogenes down-regulate autophagy, this view has recently been challenged by experimental results with p53.

It is well known that p53 is a tumor suppressor that is mutated in 30－50% of breast cancer, 50% of lung cancer and various other human cancer tissues [56]. In different sub-cellular locations, p53 exerts tumor-suppressive function by different mechanisms. In the nucleus, p53 acts as a transcriptional factor and transactivates a variety of crucial regulatory proteins associated with cell cycle, apoptosis and metabolism, and thus suppresses carcinogenesis [57]. On the other hand, in the cytoplasm, in response to a variety of stimuli which induce cell death, p53 rapidly moves into the mitochondria, triggering the process of mitochondrial outer membrane permeabilization and bringing about release of pro-apoptosis factor from the mitochondria, leading the cell to apoptosis. Thus, p53 exerts a prominent oncosuppressive function [58].

In the process of autophagy, p53 acts in very different ways in the nucleus and the cytoplasm. Nuclear p53 activates autophagy through inactivation of the mTOR pathway. To be more specific, p53 activates TSC2 and AMPK activators Sestrins 1 and 2, while the activated TSC2 and AMPK subunits subsequently down-regulates the mTOR pathway [59,60]. There are several molecules that are associated with regulation of p53 function which affects the regulation of autophagy by p53. Although p53–specific E3 ubiquitin ligase MDM2 contributes to p53 degradation, the existence of $p14^{ARF}$ blocks the function of MDM2 by rapid binding with MDM2 [61]. Moreover, a p53 target gene *ISG20L1* has been recently identified, which is located in the nucleolar and perinucleolar regions of the nucleus [62]. After knockdown of ISG20L1, levels of autophagy in response to genotoxic stress are dramatically decreased, proposing another mechanism as to how nuclear p53 induces autophagy. In addition, recent studies have found that p73, as a member of p53 family, also functions as autophagy inducer through transactivation of Atg genes [63] (**Fig. 3**).

On the other hand, the manner in which p53 in the cytoplasm impacts autophagy is completely different [64]. However, the specific mechanism of cytosolic p53-mediated autophagy suppression has not been determined.

The inhibition of autophagy by p53 is thought to be correlated with its shuttling between nucleus and cytoplasm [64]. When the p53 nuclear export signal is abolished, p53 accumulates in the nucleus, which increases the autophagy dramatically [64]. These results suggest that p53 inhibits autophagy in a transcription-independent manner. Evidence indicates that p53 nucleus－cytoplasm shuttling is

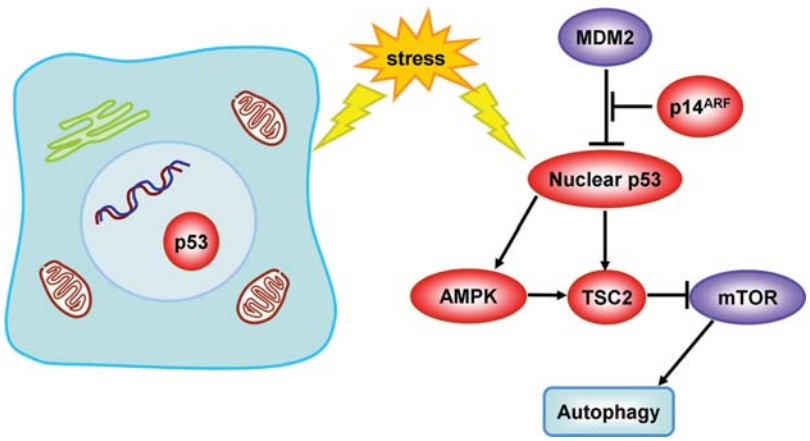

Figure 3 The regulatory mechanism of nuclear p53 on activating autophagy In the nucleus, p53 activates autophagy through inactivation of the mTOR pathway. p53 transactivates TSC2 and AMPK, which are down-regulators of the mTOR pathway, causing the subsequent activation of autophagy. In addition, p53-specific E3 ubiquitin ligase MDM2 may contribute to p53 degradation. $p14^{ARF}$, which binds with MDM2, blocks this function of MDM2.

facilitated by multiple post-translational modifications including Poly(ADP)ribosylation of p53 [65], mono-ubiquitination by MDM2 [66], and phosphorylation of serine on the C terminus [67]. Moreover, the transcription factor FoxO3 is able to promote p53 cytoplasmic accumulation by increasing its nuclear export [68]. However, the signaling network which regulates p53 is complex, thereby leaving an open question about how p53 functions in autophagy.

Conclusion and future prospects

Autophagy is a crucial cellular metabolic process that functions both in physiological and pathological conditions. In this review, we discussed the relationship between autophagy and carcinogenesis. Based on multiple studies, it has been concluded that autophagy plays an anti-neoplastic role in cells. This conclusion has been confirmed by findings regarding Beclin 1 and its association with its interacting proteins, FoxO family proteins, multiple proteins in the signaling pathways of mTOR and the nuclear p53. However, some recent studies have raised the question that the anti-neoplastic function of UVRAG and several other genes may not depend on the induction of autophagy. Several autophagy regulation proteins are involved in multiple cellular processes, suggesting that their tumor suppressive functions may be achieved by mechanisms other than autophagy. Moreover, the tumor suppressor p53 has been reported to act in a distinctly different manner. It is a breakthrough in view of the fact that the differences in effects result from differences in sub-cellular location. Further studies focused on the exact mechanisms how autophagy affects the process of tumorigenesis should be conducted and the relationship between autophagy and tumorigenesis should be further elaborated. Any novel points of view will likely facilitate investigation into research on the relationship between autophagy and carcinogenesis, and the research focused on the interplay of autophagy and carcinogenesis will further contribute to our overall understanding of cancer.

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