Chapter 12
Autophagy and Cancer Drug Discovery

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Abstract Autophagy is a highly conserved lysosomal degradation pathway that is important for maintaining cellular homeostasis by degrading bulk cytoplasm and superfluous or damaged organelles. Autophagy plays a dual role in cancer because it suppresses tumorigenesis and also promotes cancer cell survival for existing tumors. Therefore, targeting autophagy has become a promising therapeutic approach for preventing or treating cancers. With the rapid progression of autophagy research and our expanding knowledge on autophagy machinery and regulation pathways, many high-throughput screening assays have been established and conducted. Here, we summarize potential autophagy proteins and signaling pathways that could be drug targets for modulating autophagy. We also summarize novel compounds that have been discovered from high-throughput screening, which can either inhibit or promote autophagy.

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Abbreviations

3-MA	3-Methyladenine
4EBP1	4E-binding protein 1
AMP	Adenosine monophosphate
AMPK	5′ Adenosine monophosphate-activated protein kinase
Atg	Autophagy-related
ATM	Ataxia telangiectasia mutated
ATP	Adenosine-5′-triphosphate
BiFC–FRET	Bimolecular fluorescence complementation–fluorescence resonance energy transfer
CaMKKs	Calmodulin-dependent protein kinase kinases
DAPK1	Death-associated protein kinase 1
eIF4E	Eukaryotic translation initiation factor 4E
FACS	Fluorescence-activated cell sorter
FEZ1	Fasciculation and elongation protein zeta-1
FoxO	Forkhead box transcription factor class O
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GPCRs	G-protein-coupled receptors
HCQ	Hydroxychloroquine
IAV	Influenza A virus
IRS	Insulin receptor substrate
Jak3	Janus 3 kinase
LKB1	Liver kinase B1
MEF	Mouse embryonic fibroblasts
mLST8	Target of rapamycin complex subunit LST8
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NF-kappaB	Nuclear factor-kappaB
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
p19ARF	p19 Alternative reading frame
p70S6K1	p70 Ribosomal protein S6 kinase 1
PDK	Phosphoinositide-dependent protein kinase
PE	Phosphatidylethanolamine
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate

PKB	Protein kinase B
PKC	Protein kinase C
PLA2	Phospholipase A2
PPAR-γ	Peroxisome proliferator-activated receptor γ
PTEN	Phosphatase and tensin homolog
Raptor	Regulatory associated protein of mTOR
RFP	Red fluorescence protein
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RLuc	Renilla Reinformis luciferase
RTK	Receptor tyrosine kinase
SCOC	Short coiled-coil protein
SGK	Serum- and glucocorticoid-induced protein kinase
SREBP1/2	Sterol regulatory element-binding protein1/2
TAK1	Transforming growth factor-β-activated kinase-1
TFEB	Transcriptional factor EB
TSC	Tuberous sclerosis complex

1 Introduction

A fundamental goal in cancer research is to develop therapeutic drugs that selectively kill cancer cells, but have minimal cytotoxicity to the surrounding normal tissues. Over the past two decades, induction of apoptosis by therapeutically targeting the apoptotic pathway has become a major strategy to kill cancer cells because of its well-known genetically controlled pathways (Letai 2008). With the expanding understanding of molecular mechanisms of apoptosis, many chemotherapy drugs have been developed to treat cancer patients by inducing apoptosis. However, some tumors have developed resistance to traditional chemotherapeutic drugs that induce apoptosis. Therefore, an emerging and novel strategy for cancer therapy is to target not only the cell death pathway, but also the cell survival pathway.

Macroautophagy (referred to as autophagy hereafter) is a major intracellular degradation system that is mainly responsible for the degradation of long-lived proteins and other cellular contents (Levine and Klionsky 2004; Lum et al. 2005b). Autophagy is a bulk degradation system that is usually activated in response to an adverse environment, such as the deprivation of nutrients or growth factors (Kamada et al. 2004). Autophagy also plays a role in development (Levine and Klionsky 2004), aging, defense against microbial infections (Kirkegaard et al. 2004), and pathogenesis of many diseases, such as neurodegenerative diseases (Shintani and Klionsky 2004). As an important cellular homeostasis regulator, it is not surprising that autophagy also plays an important role in cancer. However, accumulating evidence suggests that autophagy plays differential roles in tumorigenesis and existing cancer.

2 Role of Autophagy in Cancer

2.1 Autophagy Is a Tumor Suppressor During Tumorigenesis

Accumulating evidence supports that autophagy functions as a tumor suppressor. The first evidence comes from the findings that there are increased spontaneous tumors in *Beclin 1* monoallelically deleted mice, a gene involved in the autophagy process in mammals (Huang and Tindall 2011; Tzivion and Hay 2011). Later, it was also found that knockout of *Bif-1*, another gene involved in autophagy, also leads to increased tumorigenesis in multiple organs (Huang and Tindall 2011; Takahashi et al. 2007). Because both Beclin 1 and Bif-1 also play a role in apoptosis by interacting with Bcl-2 family proteins, it is suspected that the tumor suppressor role of Beclin 1 and Bif-1 could be due to their multiple functions in addition to autophagy. However, the finding that liver-specific knockout of *Atg5* or *Atg7* leads to increased liver tumors clearly supports the notion that autophagy is a bona fide tumor suppressor (Takamura et al. 2011). More recently, it was found that liver-specific *TSC1* knockout mice, which have increased mTOR activation and decreased autophagy, also had increased liver tumors (Tzivion et al. 2011). Furthermore, various other tumor suppressors such as p53, DAPK, p19ARF, LKB, and PTEN all have shown to be able to induce autophagy, whereas oncogenes such as Bcl-2 and AKT suppress autophagy. Although the exact mechanisms by which autophagy suppress tumorigenesis are not completely understood, it is suggested that autophagy may help to remove damaged mitochondria, which in turn reduces mitochondrial mediated free radicals and, subsequently, genome instability (Mathew et al. 2009). In addition, increased inflammation has been consistently observed in the mouse liver with allelic loss of *Beclin 1* (Mathew et al. 2009), in autophagy-deficient tumor allografts (Degenhardt et al. 2006), and in Crohn's disease with the expression of a hypomorphic allele Atg16L1 (Cadwell et al. 2008). These results suggest that autophagy can limit inflammation. Furthermore, p62/SQSTM1, a multifunctional adapter protein, is usually accumulated in autophagy-deficient tissues (Komatsu et al. 2007; Ni et al. 2012). p62/SQSTM1 has been found to be a potential molecular link between autophagy inhibition and tumor development. Indeed, *p62/SQSTM1* knockout mice were protected from Ras-induced lung carcinomas compared to wild-type mice. *p62/SQSTM1* and *Atg7* double-knockout mice also have few liver tumors compared to *Atg7* liver-specific knockout mice (Takamura et al. 2011). p62/SQSTM1 may promote liver tumorigenesis through multiple mechanisms. First, p62/SQSTM1 interacts with Keap1, which is an inhibitor of the Nrf2 transcription factor. Nrf2 is important for regulation of cellular redox homeostasis. Interaction of p62/SQSTM1 with keap1 releases its inhibitory effect on Nrf2 and causes persistent Nrf2 activation (Komatsu et al. 2010). Activation of Nrf2 has also been found in liver tumor cells but not in adjacent normal tissues (Inami et al. 2011). Recent evidence indicates that expression of oncogenes such as Kras, Braf, and Myc can increase Nrf2 activation, which increases expression of antioxidant genes and leads to the detoxification of reactive oxygen species to promote cell survival and oncogene-driven

tumorigenesis (DeNicola et al. 2011). Moreover, Nrf2 activation can lead to up-regulation of Bcl-2, which inhibits apoptosis and increases cancer cell survival (Niture and Jaiswal 2012). Furthermore, Nrf2 activation can also redirect glucose and glutamine into anabolic pathways to promote cell proliferation (Mitsuishi et al. 2012). In addition to regulating Nrf2 activation, p62/SQSTM1 interacts with mTOR, raptor, and RAG to promote mTORC1 activation and cell proliferation (Duran et al. 2011). Finally, p62/SQSTM1 could also serve as a modulator of mitotic transit and genomic stability as well as the activation of nuclear factor-kappaB (NF-kappaB) (Duran et al. 2008; Mathew et al. 2009). Together, these findings indicate that autophagy suppresses tumorigenesis by multiple mechanisms, and autophagy inducers might be effective in preventing tumorigenesis.

2.2 Autophagy Acts as a Cell Survival Mechanism in Cancer Cells

While autophagy serves as a tumor suppressor mechanism during tumorigenesis, autophagy is activated in existing tumors to maintain tumor cell survival. Many conditions can activate autophagy in cancer cells such as starvation, growth factor deprivation, and hypoxia. Autophagy may also help recycle amino acids to maintain cellular biosynthesis and ATP levels for cell survival. It has been found that the number of autophagosomes is significantly increased in tumor cells that are located in hypoxic tumor regions, and cells with the deletion of autophagy genes in these regions are prone to undergo cell death. The induction of autophagy in hypoxic tumor regions can be hypoxia-inducing factor 1 α dependent or independent. Furthermore, pharmacological inhibition of autophagy or genetic knockdown of autophagy genes renders cancer cells as more sensitive to various chemotherapies both in vitro and in vivo, further supporting the notion that autophagy promotes cancer cell survival (Amaravadi et al. 2011; Ding et al. 2007a; Lum et al. 2005a). Oncogenic transformation by activation of RAS, which is important to promote tumor growth, also activates autophagy. Interestingly, we found that the combination of autophagy suppression and proteasome inhibition killed more RAS-transformed tumor cells than non-transformed normal cells (Ding et al. 2009). This indicates that RAS-transformed tumor cells seem to rely more on autophagy for survival than normal non-transformed cells. Thus, this finding supports the current idea for cancer treatment, which involves a combination of autophagy inhibition with traditional chemotherapy treatments to more selectively kill cancer cells with less cytotoxic effects on normal cells. Indeed, inhibition of autophagy using the antimalarial agent hydroxychloroquine (HCQ), a lysosomal inhibitor that inhibits autophagy by increasing lysosomal pH, together with other chemotherapy drugs is being actively assessed in the clinic (Amaravadi et al. 2011). Thus, identification of more novel autophagy inhibitors in addition to HCQ will help for development of more efficient cancer treatments that involve inhibition of autophagy in cancer cells.

2.3 Paradoxical Role of Autophagy in Cell Death

As discussed above, cancer cells use autophagy as a cell survival pathway when facing a variety of stresses including hypoxia, growth factor deprivation, starvation, endoplasmic reticulum (ER) stress, or damaging stimuli as well as proteasome inhibition (Ding et al. 2007a, b; Hu et al. 2012; Lum et al. 2005a). By doing so, autophagy helps to maintain mitochondrial quality control and genome stability and also provides more fuel for supporting mitochondrial metabolism and energy homeostasis. In addition to cancer cells, genetic deletion of *Atg5* or *Atg7* from mouse livers leads to increased cell death and liver injury, suggesting that even basal autophagy is an important cell survival pathway for normal liver cells (Komatsu et al. 2007; Ni et al. 2012). Together, this evidence strongly supports the notion that autophagy is a cellular protective mechanism. Indeed, accumulating evidence has demonstrated that inhibition of autophagy can lead to more cell death in cancer cells or make cancer cells more susceptible to chemotherapy (Degenhardt et al. 2006; Ding et al. 2007b; White 2012). These findings are very significant for cancer therapy because many cancer cells eventually become resistant to chemotherapeutic drugs, and inhibition of autophagy can make these drug-resistant cancer cells more susceptible for drug-induced cell death. In line with this, discovery of novel autophagy inhibitors by biomedical research would be beneficial for cancer therapy. While most evidence supports that autophagy is a cell survival mechanism for cancer cells, it has also been hotly debated whether autophagy could be a cell death mechanism, which is referred to as "autophagic cell death." Many early studies only employed morphological approaches when defining autophagic cell death. Therefore, criteria used in these studies for autophagic cell death may not be appropriate for definition of autophagic cell death because coexistence of autophagy with cell death does not guarantee that autophagy contributes to cell death. In this case, autophagy could be detrimental, protective, or just a bystander. However, with better understanding of autophagy machinery and molecular pathways, it is now relatively easier to address this issue. It seems that under certain circumstances or in some particular cell types, autophagy may contribute to cell death. During the developmental stage of Drosophila, programmed cell death is required for the degradation of the salivary glands. Interestingly, both autophagy and apoptosis are induced during this process. Using Atg8 and Atg18 mutants, autophagy was found to contribute to the cell death (Berry and Baehrecke 2007). In mammalian cells, autophagy has also been found to contribute to cell death when cells are exposed to certain chemotherapeutic drugs (Kanzawa et al. 2005; Shimizu et al. 2004), radiation(Moretti et al. 2007), hypoxia (Azad et al. 2008), and cytokines such as INF-γ (Pyo et al. 2005). In all these cases, siRNA knockdown or genetic deletion of key autophagy genes suppresses cell death, while overexpression of these genes promotes cell death. Exactly how autophagy induces cell death is not clear, although it is generally thought that excessive autophagy may non-selectively degrade essential cell components. Moreover, whether there is a real "autophagic cell death" is also debatable, because the presence of autophagy may actually just

promote either apoptosis or necrosis (Shen et al. 2012). Nevertheless, in certain cancer cells such as murine fibrosarcoma L929 cells (Yu et al. 2004), breast cancer MCF7 cells (Akar et al. 2008), or glioblastoma cells (Kanzawa et al. 2005; Takeuchi et al. 2005), induction of autophagy seems to promote cell death. In this case, autophagy inducers would be beneficial for the treatment of these cancers. Therefore, usefulness of autophagy inhibitors or inducers for cancer treatment may depend on the specific cancer type. More work is definitely needed to further clarify why certain cancers behave differently in response to the modulation of autophagy. However, it should be noted that in most cases, inhibition of autophagy can further promote cell death. In line with this notion, the approach to use autophagy inhibitors alone or in combination with a chemotherapy drug for clinical trials is currently under investigation.

3 Targeting Different Autophagy Pathways for Cancer Therapy

More than 30 autophagy-related (Atg) genes have been identified in mammalian cells, and many of them play a critical role in regulating the formation of double-membrane autophagosomes, which is described in Chap. 2 in detail. Many signal transduction pathways that regulate autophagy have also been revealed and described in detail in Chap. 4. Here we focus on these pathways and autophagy molecular machinery that have been shown to be potential drug targets for modulating autophagy.

3.1 Drug Development for Targeting Signaling Pathways Regulating Mammalian Autophagy

3.1.1 Targeting mTOR

Among the signaling pathways that regulate autophagy, the inhibition of the mammalian target of rapamycin (mTOR) has been placed as a central key signaling pathway for regulation of autophagy induction (Jung et al. 2010; Yang and Klionsky 2010). mTOR exists in two heteromeric complexes, mTORC1 and mTORC2. However, it seems that rapamycin-sensitive mTORC1 plays a major role in the regulation of autophagy and cell growth, although there is evidence that rapamycin-insensitive mTORC2 may also regulate autophagy in some specific tissues.

The first generation of mTOR inhibitors is rapamycin and its analogs. Rapamycin (also known as Sirolimus) was first discovered as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample and has an antifungal function (Sehgal et al. 1975; Vezina et al. 1975). Later it was found that it also has potent immunosuppressive and anti-proliferative effects. In mammalian cells, rapamycin binds

with an intracellular protein FKBP12, and the rapamycin–FKBP12 complex binds to mTOR in a region adjacent to the mTOR kinase domain to inhibit mTORC1 but not mTORC2. However, chronic treatment with rapamycin can also block mTORC2 function in some cells. Many rapamycin analogs have also been discovered, and they all inhibit mTOR activity by binding with FKBP12. All of these rapamycin analogs contain only minor structure modifications to increase their solubility and stability. Currently, rapamycin and its analogs CCI-779, RAD-001, and AP23573 are in clinical trials for treating various cancer patients. As we discussed above, although rapamycin and its analogs have showed inhibitory effects on cell growth, they also simultaneously induce the cell survival autophagy process in cancer cells. This may help to explain why the therapeutic effects of rapamycin for cancer are far more satisfactory. However, in various cell culture and animal models, the combination of autophagy inhibitors, such as chloroquine with rapamycin or other mTOR inhibitors, has shown increased tumor cell death and tumor regression (Huang et al. 2011; Loehberg et al. 2012), although the beneficial effects of this approach on human cancer therapy are currently not clear.

In addition to rapamycin and its analogs, the second generation of mTOR inhibitors has been developed, and these are small-molecule mTOR kinase inhibitors that inhibit mTOR by targeting its ATP-binding site pocket. By binding the catalytic site of mTOR, these mTOR kinase inhibitors can inhibit both mTORC1 and mTORC2, and therefore have broad applications. These inhibitors include Torin1, PP242, and PP30. It has been shown that both Torin1 and PP242 could fully suppress 4EBP-1 phosphorylation, whereas rapamycin has only mild inhibitory effects on 4EBP-1 phosphorylation. Thus, these mTOR kinase inhibitors have been shown to have greater efficacy for inhibiting cell proliferation and inducing autophagy than rapamycin (Guertin and Sabatini 2009). The mTOR inhibitors that have been shown to induce autophagy in cancer cells are listed in Table 12.1 including rapamycin and its analogs, ATP-binding competitive inhibitors, and inhibitors with unknown mechanisms.

3.1.2 Targeting PI3K and AKT

Many PI3K and AKT inhibitors have been, and are being, developed. For the PI3K inhibitors, there are adenosine triphosphate (ATP)-competitive inhibitors of PI3K, which can target to all class I PI3K such as XL147 (Exelixis), BKM120 (Novartis), and GDC0941 (Genetech). The isoform-specific PI3K inhibitors include BYL719 (Novartis) and CAL-101 (Calistoga) (Zhang et al. 2011). The advantage of the isoform-specific inhibitors of PI3K is that they have increased potency, fewer off-target effects, and decreased side effects. For the AKT inhibitors, both ATP-competitive and allosteric AKT inhibitors are being developed. AZD5363, GDC-0068, GSK2141795, and GSK690693 are ATP-competitive inhibitors which target to three different isoforms of AKT. Allosteric AKT inhibitors, such as MK-2206, bind to the AKT PH domain to promote an inactive conformation of the AKT protein, which is unable to bind to the plasma membrane (Zhang et al. 2011). All of these PI3K and AKT inhibitors have either been in

Table 12.1 mTOR inhibitors

Compound	Mechanism and target	Effect	Cancer type/disease	Reference
Rapamycin (Sirolimus)	Bind to FKBP12	Autophagy inducer	Malignant glioma cell	Sarbassov et al. (2005); Takeuchi et al. (2005)
Everolimus (RAD-001)	Rapamycin analogue	Autophagy inducer	Lung cancer/Huntington's disease	Kim et al. (2008)
Temsirolimus (CCI-779, Torisel)	Rapamycin analogue	Autophagy inducer	Mantle cell lymphoma	Yazbeck et al. (2008)
Torin1	ATP-competitive inhibitor	Autophagy inducer	MEF cell	Thoreen et al. (2009)
AZD8055	ATP-competitive inhibitor	Autophagy inducer	Human embryonic kidney293	Huang et al. (2011)
PP242	ATP-competitive inhibitor	Autophagy inducer	Leukemia	Janes et al. (2010)
MG-2477	Akt/mTOR	Autophagy inducer	Non-small-cell lung carcinoma cell line (A549)	Viola et al. (2012)
E Platinum	mTOR	Autophagy inducer	Gastric carcinoma BGC-823 cells	Hu et al. (2012)
Ku-0063794	mTOR	Autophagy inducer	H4 cell	Nyfeler et al. (2011)
OSI-027	mTOR	Autophagy inducer	Chronic myeloid leukemia	Carayol et al. (2010)
WYE-354	mTOR	Autophagy inducer	H4 Cell	Nyfeler et al. (2011)
LD9-4	mTOR	Autophagy inducer	Lung cancer A549 cells	Hao et al. (2011)

phase I or II clinical trials and have shown some antitumor activities. Because all these inhibitors inhibit AKT, which ultimately leads to the suppression of mTOR, it is suggested that these inhibitors will activate autophagy, which will limit their antitumor effects. Indeed, the allosteric AKT inhibitor MK-2206 has been shown to increase autophagy in leukemia cells (Simioni et al. 2012). However, because of the structure similarity among class I, II, and III PI3Ks, the specificities of these PI3Ks will affect their effects on autophagy. For example, both LY294002 and wortmannin are ATP-competitive PI3K inhibitors which have similar inhibition potency on class I, II, and III PI3K in vitro (Vanhaesebroeck et al. 2001). Indeed both the inhibition and induction of autophagy by LY294002 have been reported in many cell culture models (Blommaart et al. 1997; Takeuchi et al. 2005). 3-Methyladenine (3-MA), another widely used autophagy inhibitor, which was thought to inhibit class III PI3K to suppress autophagosome formation (Seglen and Gordon 1982), actually shows a dual-effect on autophagy depending on the duration of its treatment. At a shorter time point (less than 9 h), 3-MA

Table 12.2 PI3K–AKT inhibitors

Compound	Mechanism and target	Effect	Cancer type/disease	Reference
3-Methyladenine (3-MA)	Class III PI3K inhibitor	Autophagy inhibitor	Fibrosarcoma cell	Ito et al. (2007)
Wortmannin	Class III PI3K inhibitor	Autophagy inhibitor	Pancreatic cancer cell	Blommaart et al. (1997)
PX-866	PI3K inhibitor	Autophagy inducer	Human glioblastoma	Koul et al. (2010)
LY294002	PI3K inhibitor	Autophagy inhibitor	L929 cells (murine aneuploid fibrosarcoma cell line)	Blommaart et al. (1997); Wu et al. (2009)
XL147	PI3K inhibitor	Autophagy inducer	Pancreatic cancer cell	Mirzoeva et al. (2011)
CAL-101	AKT inhibitor	Autophagy inducer	Chronic lymphocytic leukemia cell	Mahoney et al. (2012)
MK-2206	AKT inhibitor	Autophagy inducer	Glioma/leukemia cell	Cheng et al. (2012); Simioni et al. (2012)
AZ7328	AKT inhibitor	Autophagy inducer	Human bladder cancer	Dickstein et al. (2012)
Perifosine	AKT inhibitor	Autophagy inducer	Human lung cancer cell	Fu et al. (2009)

inhibits autophagic flux but it increases autophagic flux after prolonged treatment (more than 9 h) in cultured cells. The dual-effect of 3-MA on autophagy regulation is due to its differential temporal effects on class I and III PI3K in which 3-MA blocks class I PI3K persistently, but only suppresses class III PI3K transiently (Wu et al. 2010). It is possible that the effects of PI3K inhibitors on autophagy would depend on different cell types (the basal expression level and activity of class I, II, and III PI3K), dose of PI3K inhibitors, and duration of treatment. Nevertheless, it does not matter if PI3K and AKT inhibitors induce or inhibit autophagy because, when combined with other autophagy inhibitors that target downstream lysosomes, such as chloroquine or bafilomycin A1, they should further suppress tumor cell proliferation and increase cell death. Not all known PI3K and AKT inhibitors [such as CH5132799, AS-252424, CAY10505, A66, and BKM120 (NVP-BKM120)] have been tested for their effects on autophagy (Maira et al. 2012; Pomel et al. 2006; Tanaka et al. 2011; Tyagi et al. 2012). The PI3K and AKT inhibitors that have been shown to inhibit or activate autophagy in cancer cells are listed in Table 12.2.

3.1.3 Targeting PI3K and mTOR

Activation of mTOR is the most downstream target of the PI3K signaling pathway, and thus inhibition of mTOR has been a critical strategy for cancer therapy.

Table 12.3 Dual PI3K–mTOR inhibitors

Compound	Mechanism and target	Effect	Cancer type/disease	Reference
NVP-BEZ235 (BEZ235)	PI3K and mTOR	Autophagy inducer	Glioma	Fan et al. (2010)
PI-103	PI3K and mTOR	Autophagy inducer	Glioma	Fan et al. (2010)
XL765	PI3K and mTOR	Autophagy inducer	Pancreatic cancer cell	Mirzoeva et al. (2011)
BGT-226	PI3K and mTOR	Autophagy inducer	Breast cancer	Sanchez et al. (2011)
NVP-BGT226	PI3K and mTOR	Autophagy inducer	Head and neck cancer cell	Chang et al. (2011)

As discussed above, because of the negative-feedback loop inhibition on AKT by mTOR, inhibition of mTOR leads to activation of PI3K and AKT. To circumvent this problem, dual inhibitors that target both PI3K and mTOR have been developed. Dual PI3K–mTOR inhibitors such as NVP-BEZ235, NVP-BGT226, XL765, and SF-1126 have been extensively studied in preclinical animal models for cancer treatment (Amaravadi et al. 2011; Zhang et al. 2011). However, results from these mouse models reveal that the dual PI3K–mTOR inhibitors induce tumor stasis rather than tumor regression. It was further revealed that the dual PI3K–mTOR inhibitors activate autophagy, and in combination with the autophagy inhibitor (chloroquine) can promote tumor cell death and cause tumor regression in a preclinical glioblastoma model. More importantly, this combination approach also increased cell death in glioma cells mutant for PTEN. Interestingly, combination of inhibitors for mTORC1 only with autophagy inhibitors did not promote cell death (Fan et al. 2010). These results suggest that blockade of three targets, PI3K, mTOR, and autophagy, may be required for inducing efficient cell death in PTEN mutant glioma. More studies are needed to determine whether simultaneous inhibition of PI3K–mTOR and autophagy would be a general effective approach for treating cancers with activated PI3K/AKT/mTOR signaling. The dual PI3K–mTOR inhibitors that have been shown to induce autophagy in cancers are listed in Table 12.3.

3.1.4 Targeting AMPK

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase which acts as a sensor of cellular energy levels (Hardie et al. 2012). AMPK is a heterotrimeric complex which is highly conserved through evolution. The AMPK complex contains a catalytic subunit (AMPK-α) and two regulatory subunits (AMPK-β and AMPK-γ). A high AMP/ATP ratio reflects low-energy status, which can directly activate AMPK through AMP binding. AMP binds to the CBS domain in the γ

subunit resulting in conformational changes in the heterotrimeric complex. This conformational change then promotes the phosphorylation of Thr^{172} in the α subunit by several upstream kinases which include the tumor-suppressor liver kinase B1 (LKB1), calmodulin-dependent protein kinase kinases (CaMKKs), and transforming growth factor-β-activated kinase-1 (TAK1) (Alexander and Walker 2011; Hurley et al. 2005; Xie et al. 2006). AMPK is a master regulator of glucose, cholesterol, and lipid metabolism in various organs such as liver, skeletal muscle, and adipose tissue. AMPK stimulates glucose transport and fatty acid oxidation in skeletal muscle while it increases fatty acid oxidation and decreases cholesterol and triglyceride synthesis as well as glucose output in the liver. Thus, AMPK plays a critical role in lowering blood glucose levels in hyperglycemic individuals and is a key therapeutic target for diabetes. In addition to the regulation of glucose and lipid metabolism, AMPK can also regulate mTOR activity. Once activated, AMPK phosphorylates TSC2 and stimulates TSC2 GAP activity toward Rheb, which results in the inhibition of mTOR. AMPK-induced inhibition of mTOR leads to the inhibition of protein translation and activation of autophagy (Inoki et al. 2003). In addition, AMPK can also directly phosphorylate UNC-51-like kinase 1 (ULK1), the mammalian homologue of yeast Atg1, leading to ULK1 activation and induction of autophagy (Egan et al. 2011; Kim et al. 2011; Zhao and Klionsky 2011). Thus, AMPK agonists seem to behave similarly to mTOR inhibitors that can simultaneously inhibit cancer cell growth and induce autophagy.

Indeed, epidemiology studies indicate that there is strong evidence that AMPK agonists have suppressive inhibitory effects on tumorigenesis and cancer growth. In an observational cohort study, it was found that people with type 2 diabetes who used metformin had a reduced risk of cancer (Libby et al. 2009). Several other epidemiology studies have also found that oral short-term low-dose metformin treatment suppressed colorectal cancer (Higurashi et al. 2012), liver cancer (Zhang et al. 2012), pancreatic cancer (Bodmer et al. 2012), breast cancer (Col et al. 2012), and lung cancer (Lai et al. 2012). Metformin was also associated with a survival benefit for patients with various solid tumors (Currie et al. 2012; Romero et al. 2012). Experimentally, administration of metformin alone reduced human gastric cancer cell proliferation in vitro and in vivo (Kato et al. 2012). However, because AMPK agonists can induce autophagy, it remains to be studied whether the combination of AMPK agonists (such as metformin) with autophagy inhibitors (such as chloroquine) would further enhance the anticancer effects beyond each agent alone.

3.1.5 Targeting Bcl-2 Family Proteins

Bcl-2 family proteins play an important role in regulating cell death. However, recent evidence suggests that Bcl-2 can also regulate autophagy. By directly binding to Beclin 1, anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1 can suppress autophagy (Liang et al. 1999; Pattingre et al. 2005). The binding of Bcl-2 to Beclin 1 dissociates Beclin 1 from Vps34, resulting in decreased Vps34 kinase activity and inhibition of autophagy. In contrast to anti-apoptotic Bcl-2 family proteins,

pro-apoptotic BH3-only proteins such as Bnip3, Bnip3L/Nix, Bad, Bik, Noxa, Puma, and Bim induce autophagy by displacing the inhibitory Bcl-2 from the Beclin 1 complex. In general, it seems that anti-apoptotic Bcl-2 family proteins inhibit, but pro-apoptotic Bcl-2 family proteins promote, autophagy. Thus, the small-molecule BH3 mimetics, which are pharmacological ligands of the BH3-binding domain of Bcl-2, would induce autophagy by disrupting Bcl-2–Beclin 1 interaction. Indeed, several BH3 mimetics such as ABT737, HA14-1, and (−)-gossypol can induce autophagy in various types of cancer cells (Lian et al. 2011; Maiuri et al. 2007).

BH3-only proteins (or BH3 mimetics) have been shown to induce mitochondria-mediated apoptosis by direct or indirect activation of pro-apoptotic multidomain proteins from the Bcl-2 family such as Bax and Bak (Labi et al. 2008). Thus, similar to mTOR inhibitors and AMPK agonists, BH3 mimetics also have dual roles on cell death and autophagy. Because of their abilities to induce apoptosis alone or augment the anticancer effects of other conventional chemotherapy drugs, some of the BH3 mimetics are currently being evaluated in clinical trials. Although it is promising to use the combination of BH3 mimetics and autophagy inhibitors strategy in treating cancers, the results are not yet clear.

3.2 Targeting Transcription Factors

Autophagy involves the formation of double-membrane autophagosomes, which is a dynamic process requiring multiple protein complexes. Although it has been suggested that some of the existing membranes such as ER and mitochondrial membranes could be used, it is not surprising that transcriptional regulation of many autophagy genes can play an important role in autophagy. Three decades ago, it was shown that administration of cycloheximide, a general inhibitor of protein synthesis, could block glucagon-induced autophagy as demonstrated in rat hepatocytes (Papadopoulos and Pfeifer 1986). In yeast, it was also found that in the presence of cycloheximide, autophagosomes are significantly smaller than normal, indicating that de novo protein synthesis could play a role in the regulation of autophagosome expansion (Abeliovich et al. 2000). Indeed, recent evidence has supported that autophagy genes are regulated at a transcriptional level in response to stress. Below, we discuss several key transcription factors that regulate gene expression for autophagy and lysosome biogenesis.

3.2.1 FoxO3

FoxO (forkhead box transcription factor class O) transcription factors regulate diverse cellular functions including metabolism, oxidative stress, differentiation, cell cycle, and cell death. It has been shown that FoxO is the first transcription factor that is necessary and sufficient to induce autophagy in the *Drosophila* larval fat body (Dobson et al. 2011). Over-expression of FoxO3 induces the

transcription of multiple autophagy genes including *LC3B*, *Gabarapl1*, *atg12*, *atg4B*, *vps34*, *ulk2*, *beclin 1*, *Bnip3*, and *Bnip3l* in mouse skeletal muscle (Zhao et al. 2007). It has been demonstrated that FoxO3 directly binds to the promoters of *LC3B*, *Gabarapl1*, *atg12*, *Bnip3l*, and *Bnip3* to activate their gene transcription (Mammucari et al. 2007). As a result, constitutively active FoxO3 promotes lysosomal proteolysis and leads to muscle wasting by activating autophagy in mouse skeletal muscle.

The activity of FoxO is inhibited by growth factors and PI3K/AKT pathways. AKT phosphorylates FoxO and negatively regulates its transcriptional activity by promoting its nuclear extrusion into the cytosol. In addition to AKT, FoxO can also be phosphorylated on different sites by other kinases including AMPK and JNK, which promote its transcription activity, and ERK and IKKb, which inhibit its transcription activity (Tzivion et al. 2011). FoxO protein levels are also regulated by ubiquitin-dependent protein degradation. The E3 ligase SKP2 binds to AKT-phosphorylated FoxO1 at Ser 256 whereas the E3 ligase MDM2 binds to ERK-phosphorylated FoxOs. Interestingly, MDM2 can induce both FoxO mono-ubiquitination and poly-ubiquitination. Mono-ubiquitination promotes FoxO translocation to the nucleus and increased transcriptional activity whereas poly-ubiquitination targets FoxO for degradation (Huang and Tindall 2011). Sirt1, a mammalian NAD^+-dependent protein deacetylase, deacetylates FoxO to increase a subset of its target genes such as antioxidant genes. In addition to regulating expression of autophagy genes, it was recently found that activation of FoxO increased levels of glutamine production (van der Vos et al. 2012). This resulted in mTOR inhibition by preventing the translocation of mTOR to lysosomal membranes, which in turn activated autophagy (Fu and Tindall 2008).

Although the small molecules that directly activate FoxO remain to be discovered, the activity of FoxO could be modulated by indirectly targeting its posttranslational modifications. For example, the PI3K/AKT, Sirt1, and JNK inhibitors can all modulate FoxO activity and thus modulate the expression of autophagy genes and autophagy. Because these agents are not specific for FoxO, future work is definitely needed to identify direct specific FoxO agonists.

3.2.2 TFEB

Completion of the autophagic process relies on the fusion of autophagosomes with lysosomes to form autolysosomes. Within autolysosomes, the autophagic contents are then broken down by lysosomal enzymes. Thus, it is important that lysosomal activities are coordinated to respond to cellular stress and needs. Recently, it was found that the transcriptional factor EB (TFEB), a basic helix-loop-helix leucine zipper transcription factor of the Myc family, is a master regulator for controlling the expression of lysosomal genes (Settembre et al. 2011, 2012). Over-expression of TFEB in cultured cells results in the biogenesis of lysosome and enhanced lysosomal degradation capacity (Settembre et al. 2012). Indeed, over-expression of TFEB can reduce mutant huntingtin levels in cultured cells (Settembre et al. 2012).

In addition to regulating lysosome biogenesis, TFEB can also directly regulate autophagy. Over-expression of TFEB increases autophagy flux in HeLa cells by increasing the expression of autophagy genes. Under starvation conditions in both cultured cells and in mice in vivo, it was found that TFEB translocated from cytosol into the nuclei, and this was associated with increased transcription of TFEB target genes both for autophagy and lysosomal biogenesis (Settembre et al. 2011). Mechanistically, it was found that TFEB can be phosphorylated by the extracellular signal-regulated kinase (ERK) and mTOR, which retains TFEB in the cytoplasm under normal nutrient conditions (Martina et al. 2012; Settembre et al. 2011, 2012). Moreover, TFEB colocalizes with mTORC1 on the lysosomal membrane and serves as the sensor for cellular nutrients. When levels of cellular nutrients are high, mTORC1 phosphorylates TFEB and inhibits its activity. Conversely, starvation as well as pharmacological inhibition of mTORC1 activate TFEB by promoting its nuclear translocation and in turn promoting autophagy (Martina et al. 2012; Settembre et al. 2012). Therefore, inhibition of mTOR can activate autophagy not only through the modulation of the ULK1 complex but also through the increased expression of autophagy and lysosomal genes by activating TFEB.

However, it should be noted that TFEB has been suggested to function as an oncogene in the kidney. In a subset of renal tumors, both mRNA and protein levels of TFEB are dramatically increased in tumor cells compared to normal tissues (Davis et al. 2003; Kuiper et al. 2003). Translocation carcinoma, which has aberrant expression of melanocytic markers, is thought to also be driven by enhanced TFEB nuclear translocation and activation (Srigley and Delahunt 2009). Moreover, genetic deletion of the tumor-suppressor gene *tsc2* leads to persistent activation of mTORC1 and in turn phosphorylates TFEB (Pena-Llopis et al. 2011). Contradictory to other studies, this results in TFEB nuclear translocation and activates expression of a subset of genes including *V-ATPase*, an important component of the late endosomes/lysosome which regulates their acidification. The reasons behind these contradictory data are not clear, but it could be possible that the amino acid-mediated mTOR activation (through the regulator) and growth factor-mediated mTOR activation (through PI3K-tsc1/tsc2) are different. More studies are needed to clarify these conflicting findings in the future. Nevertheless, although reagents that directly activate TFEB have not been reported, modulation of mTOR may serve as an indirect approach for TFEB activation.

3.2.3 C/EBPβ

Cellular metabolic states are dynamically changed with circadian oscillation, which is a well-regulated process in response to limited nutrients for maintaining energy homeostasis in mammals. As an important catabolic pathway, it was recently found that autophagy activation fluctuates to coordinate circadian rhythm in mouse liver (Ma et al. 2011). There is a cyclic induction of autophagy gene expression to meet the needs for autophagy induction during circadian rhythm changes. C/EBPβ was identified as a potent activator of autophagy in the mouse liver during circadian

changes through functional analysis of various transcription factors and cofactors in the liver (Ma et al. 2011). C/EBPβ is a basic leucine zipper transcription factor transcribed from an intronless gene. There are three protein isoforms from this single mRNA due to alternative translation initiation at three in-frame methionine initiator codons. C/EBPβ is an important regulator for a variety of physiological processes including metabolism, cellular differentiation, and stress response. In the liver, C/EBPβ is rhythmically expressed in response to both circadian and nutritional signals. In primary cultured hepatocytes, starvation-increased expression of C/EBPβ stimulates the expression of autophagy genes and is sufficient to trigger autophagy induction. siRNA knockdown of C/EBPβ in mouse liver in vivo abolishes diurnal hepatic autophagy rhythm (Ma et al. 2011). All these results support that C/EBPβ is a key integrator of nutritional and circadian signals that regulates autophagy status in the liver.

Interestingly, over-expression of C/EBPβ leads to increased autophagy resulting in non-apoptotic cell death in various human breast cancer cell lines (Abreu and Sealy 2010). Although the expression of autophagy genes was not determined in the C/EBPβ over-expressed breast cancer cells, over-expression of C/EBPβ resulted in an increased number of acidic vesicles (Abreu and Sealy 2010). Moreover, cells with over-expressed C/EBPβ can engulf neighboring cells in culture. However, whether autophagy is involved in this process is not clear (Abreu and Sealy 2012). Interestingly, C/EBPβ over-expression stimulates the engulfment of live cells but not dead apoptotic cells. It is suggested that C/EBPβ-mediated engulfment may serve as a tumor suppressor by removing unwanted cells that may have DNA mutations. However, more studies are needed to further expand our knowledge on how C/EBPβ affects autophagy in cancer cells, and more importantly, to identify pharmacological approaches to modulate C/EBPβ.

3.3 Protein Posttranslational Modifications

Autophagy is regulated by various posttranslational modification processes. These modifications include ubiquitination, phosphorylation, lipidation, and acetylation. The autophagy core machinery proteins such as Atg5, Atg7, Atg12, and LC3 undergo ubiquitin-like conjugation to form complexes that regulate the formation of double-membrane autophagosomes. Autophagy cargos and damaged mitochondria are often ubiquitinated, which seems to be important for their selective removal by autophagy (Ding et al. 2010; Johansen and Lamark 2011; Karbowski and Youle 2011). Phosphorylation of ULK1 and dephosphorylation of Atg13 are required for autophagy induction (Egan et al. 2011; Kim et al. 2011). LC3 needs to be lipidated by conjugation with phosphatidylethanolamine (PE) to ensure the formation of autophagosomes. Accumulating evidence now suggests that protein acetylation is an evolutionarily conserved mechanism regulating autophagy. In yeast, Atg3 is acetylated during starvation by Esa1p, a histone acetylase. The acetylation of Atg3 is necessary for Atg8 lipidation and autophagy activation (Yi et al. 2012).

In mammals, TIP60, a yeast Esa1p homologue, mediates the acetylation of ULK1 to promote autophagy induction. In response to starvation, glycogen synthase kinase-3 (GSK3) phosphorylates TIP60, which enhances its affinity to bind with ULK1 resulting in ULK1 acetylation. Acetylation increases ULK1 kinase activity and thus promotes autophagy induction in response to starvation (Lin et al. 2012). Moreover, other autophagy core machinery proteins have also been shown to be regulated by acetylation. Under nutrient-rich conditions, the acetyltransferase p300 directly acetylates Atg5, Atg7, LC3, and Atg12 to inhibit autophagy (Lee et al. 2008; Lee and Finkel 2009). In contrast, Sirt1, an NAD-dependent deacetylase, deacetylates Atg5, Atg7, LC3, and Atg12 to promote autophagy when p300 is dissociated from these Atg proteins under starvation conditions (Lee et al. 2008). Sirt1 knockout mice die during the neonatal stage, which resembles the phenotype of Atg5 or Atg7 knockout mice (Lee et al. 2008). Activation of Sirt1 by resveratrol or inhibition of histone acetylases by spermidine induces autophagy and increases longevity in yeast, nematodes, and flies (Eisenberg et al. 2009; Marino et al. 2011; Morselli et al. 2011). Although it seems that acetylation of different autophagy proteins may differentially regulate autophagic response, targeting acetylation modification may be a useful approach for modulating autophagy.

3.4 mTOR-Independent Autophagy

Autophagy can also be activated independent of mTOR. Drugs such as lithium, carbamazepine, and valproic acid are used to treat a range of neurological and psychiatric diseases. These drugs lower intracellular inositol and inositol 1,4,5-trisphosphate (IP_3) levels and induce autophagy independent of mTOR activity (Sarkar et al. 2005). Mechanistically, it was found that these drugs can affect the intracellular levels of Ca^{2+} and cyclic AMP (cAMP). Indeed, calpain inhibitors, L-type Ca^{2+} channel agonists, and chemicals which decrease intracellular cAMP can all induce autophagy independent of mTOR (Ravikumar et al. 2010). In a GFP-LC3 image-based high-throughput screen, eight compounds were identified that induce autophagy in an mTOR-independent manner (see below Sect. 4.1 and Table 12.4), although the exact mechanisms by which these compounds induce autophagy are not well understood (Zhang et al. 2007).

4 Methods to Discover Drugs That Modulate Autophagy

With the rapidly expanding understanding of autophagic machinery and the molecular signaling pathways that regulate autophagy, many high-throughput autophagy screening assays have been developed, and have led to identification of various small molecules that either activate or inhibit autophagy. Here, we discuss several reported assays for autophagy screening.

Table 12.4 mTOR-independent and mTOR-dependent autophagy inducers

Compound	Mechanism and target	Effect	Cancer type/disease	Reference
Nigericin	mTOR-independent	Autophagy inducer	Human glioblastoma H4 cell	Zhang et al. (2007)
Wiskostatin	mTOR-independent	Autophagy inducer	Human glioblastoma H4 cell	Zhang et al. (2007)
Fluspirilene	mTOR-independent	Autophagy inducer	Human glioblastoma H4 cell	Zhang et al. (2007)
Niguldipine	mTOR-independent	Autophagy inducer	Human glioblastoma H4 cell	Zhang et al. (2007)
Trifluoperazine	mTOR-independent	Autophagy inducer	Human glioblastoma H4 cell	Zhang et al. (2007)
Nicardipine	mTOR-independent	Autophagy inducer	Human glioblastoma H4 cell	Zhang et al. (2007)
Penitrem A	mTOR-independent	Autophagy inducer	Human glioblastoma H4 cell	Zhang et al. (2007)
Perhexiline	mTOR-dependent	Autophagy inducer	MCF-7 (breast cancer cell)	Balgi et al. (2009)
Niclosamide	mTOR-dependent	Autophagy inducer	MCF-7 (breast cancer cell)	Balgi et al. (2009)
Amiodarone	mTOR-dependent	Autophagy inducer	MCF-7 (breast cancer cell)	Balgi et al. (2009)
Rottlerin	mTOR-dependent	Autophagy inducer	MCF-7 (breast cancer cell)	Balgi et al. (2009)

4.1 GFP-LC3 High-Content and High-Throughput Assay

One high-throughput image-based assay to screen small molecules that modulate autophagy is to use a cell line that stably expresses GFP-LC3. This assay takes advantage of the distinctive pattern of cellular GFP-LC3 upon autophagy induction. Under nutrient-rich conditions, the level of autophagy is low and GFP-LC3 displays a diffuse pattern. However, during autophagy induction, GFP-LC3 translocates to the autophagosomal membrane and displays a punctate pattern. The number of GFP-LC3 puncta per cell can be quantified and represent the number of autophagosomes in each cell.

We recently developed an image-based assay using mouse embryonic fibroblasts (MEF) stably expressing GFP-LC3. Briefly, MEF-GFP-LC3 cells were dispensed at 2,000 cells/well/25 μl in a 384-well black wall/clear bottom, collagen-coated plate (BD Bioscience, San Jose, CA) for 5 h to allow cell attachment to the plate followed by addition of various concentrations of chloroquine (0.31–20 μM) or DMSO as a vehicle control. After 16-h incubation, the cells were fixed with 8 % paraformaldehyde and nuclei were stained with Hoechst 33342. After washing twice with PBS, the assay plate was subjected to quantitative image analysis by an ArrayScan VTI

HCS Reader (Thermo Scientific, Pittsburgh, PA) with a 20× objective and an Omega XF100 dual-bandpass filter set at excitation/emission wavelengths of 385 nm/461 nm (Hoechst) and 485 nm/515 nm (GFP), respectively. Typically >200 cells per well were examined using the Compartmental Analysis BioApplication. These results suggest that the GFP-LC3 puncta formation assay is robust and suitable for high-throughput screening.

Because autophagy is a dynamic process, autophagosomes need to fuse with lysosomes to form autolysosomes where the enveloped contents are degraded. However, not only the autophagic cargos but also GFP-LC3 itself are degraded in the autolysosomes. Therefore, inhibition of lysosomal functions, such as with bafilomycin A1 and CQ, can also increase the number of GFP-LC3 puncta. Thus the increased number of GFP-LC3 puncta does not necessarily reflect autophagic degradation activity. Therefore, identified compounds that increase GFP-LC3 puncta need to be further examined by other biochemical assays, such as by the long-lived protein degradation assay.

Based on the high-content GFP-LC3 imaging screening followed by functional biochemical characterization, various compounds have been identified that can modulate autophagy (see Table 12.4). For example, in one screening using H4 human neuroglioma GFP-LC3 cells, eight compounds were identified to induce autophagy and seven of them are FDA-approved drugs. These drugs are able to degrade misfolded proteins with low cytotoxicity and may be used to treat expanded polyglutamine diseases such as Huntington's disease (Zhang et al. 2007). In another high-content GFP-LC3 imaging screening, four chemicals (perhexiline, niclosamide, amiodarone, and rottlerin) were identified from a collection of 3,584 drugs and pharmacologically active chemicals that can induce autophagy through inhibition of mTOR (Balgi et al. 2009) (see Table 12.4). This image-based autophagy screen has also led to the discovery of a novel autophagy inhibitor, specific and potent autophagy inhibitor 1 (Spautin-1). Spautin-1 inhibits autophagy by inhibiting ubiquitin-specific peptidase 10 (USP10) and USP13 which usually target to the Beclin 1–Vps34 complex resulting in enhanced degradation of the Beclin 1–Vps34 complex (Liu et al. 2011). Interestingly, the Beclin 1–Vps34 complex also regulates the stability of USP10 and USP13 which can control the levels of p53. This provided the molecular basis for why the levels of p53 are reduced in the tissues of *beclin1*$^{\pm}$ mice, which have increased tumorigenesis.

In addition, the high-throughput GFP-LC3 cell-based assay has been modified and used to identify Atg genes. The library of human cDNA clones (Castrillon et al. 2003) and genome-wide siRNA have both been used for this GFP-LC3 image-based screening (Tothova et al. 2007). Three genes (TM9SF1, TMEM166, and TMEM74) were identified that induce high levels of autophagosome formation when they are over-expressed from a library of 1,050 human cDNA clones. The genome-wide siRNA screen identified nine novel autophagy regulators including short coiled-coil protein (SCOC) and elongation protein zeta 1 (FEZ1). SCOC may form a complex with UVRAG and FEZ1 and may regulate ULK1 and Beclin 1 complex activities to regulate autophagy (Tothova et al. 2007).

4.2 BiFC–FRET Screening

Bimolecular fluorescence complementation–fluorescence resonance energy transfer (BiFC–FRET) is used to determine the interaction of three proteins in live cells whereas BiFC can only determine the interactions of two proteins. Autophagy is a highly regulated process which involves several stages including initiation, nucleation, elongation, docking, maturation, and degradation. Each step is regulated by complicated multiple protein complexes. For example, initiation is regulated by mTORC1 via the ULK1/2–Atg13–FIP200–Atg101 complex. Autophagosomal membrane nucleation is regulated by the Beclin 1–Atg14–Vps34–Vps15–UVRAG complex, and the elongation step is mediated by the Atg12–Atg5–Atg16 complex. These multiple protein complexes make them good candidates for BiFC–FRET screen. Dai et al. (2012) have elegantly established this screen by targeting the Atg12–Atg5–Atg16 complex. Briefly, Atg5 and Atg12 genes were fused with the N′- and C′-fragments of a red fluorescence protein (RFP), and Atg16 was cloned into a pEGFP-C1 plasmid. After co-transfection with these three plasmids, the interaction between Atg5 and Atg12 yielded an intact RFP signal, and this process was called BiFC. In contrast, FRET would occur due to the interaction between the Atg12–Atg5 heterodimer and Atg16. The interaction between Atg5, Atg12, and Atg16 can be determined using a microplate reader at 610 and 509 nm after excitation at 488 nm.

Using this screening method, 15 medicinal plants were identified that could inhibit the Atg12–Atg5–Atg16 complex from 83 types of traditional Chinese medicines. Furthermore, one compound evodiamine, which is the major active component from one of the 15 identified medicinal plants, was confirmed to inhibit the formation of the Atg12–Atg5–Atg16 heterotrimer resulting in autophagy inhibition. Because influenza A virus (IAV) requires the autophagy process to replicate, it was found that IAV replication was significantly repressed by evodiamine (Dai et al. 2012).

4.3 High-Throughput Assay for Small-Molecule Inhibitors of Atg4

Among all the autophagy core proteins that have been identified, Atg4 is the only protein that has protease activity, which makes it an ideal target for developing high-throughput assays to measure protease activity. During autophagosome biogenesis, Atg4 cleaves Atg8 to allow its conjugation with phosphatidylethanolamine, which is a key step for the formation of autophagosomes. Furthermore, Atg4 can also regulate autophagy by deconjugating Atg8 from autophagosomal membranes. Therefore, compounds that affect the activity of Atg4 could be a target for therapeutic intervention by modulating autophagy.

A couple of high-throughput assays have been developed for measuring Atg4 activity. One assay uses LC3B fused to an assayable enzyme phospholipase A2

(PLA2) as an Atg4B substrate. PLA2 is inactive when it is expressed as a fusion protein when LC3B is fused on its N-terminus. When LC3B-PLA2 is cleaved by Atg4B, the released PLA2 then cleaves a fluorescence NBD-C6-HPC substrate to generate NBD fluorescence in a concentration-dependent manner, which can be measured using a plate reader. Four compounds were identified from a library of 3,282 bioactive molecules showing inhibitory effects against Atg4B; however, these compounds were not further characterized by other functional autophagy assays (Shu et al. 2011).

Another assay to determine Atg4 activity was developed using a FRET-based approach (Li et al. 2012). LC3B was fused with CFP and YFP at its N- and C-terminus, respectively, allowing FRET to occur. When the LC3B fusion was cleaved by Atg4, which separates the two fluorescence proteins, the FRET signals decreased when measured using a fluorescence spectrometer. Although this FRET-based assay to measure Atg4-specific activity is simple and easy to use, high-throughput screening has not been done using this assay.

4.4 Other Potential Methods for Autophagy Screening

In addition to the above assays, other assays for monitoring autophagy, which may be developed into high-throughput screening, have also been reported. One assay is to use the fluorescence-activated cell sorter (FACS) to quantify the turnover of GFP-LC3 in living mammalian cells (Shvets et al. 2008). The principle of this assay is based on the fact that during autophagy induction, the GFP-LC3 fluorescence intensity is decreased. One advantage of this assay is that FACS analysis can be used to evaluate more samples with much larger cell sample sizes compared to the image-based assays. Moreover, this assay can directly measure autophagy activity. Thus, this method may also be used to perform large-scale screens for identification of autophagy modulators.

To directly measure autophagic flux in real time in living cells, a luciferase-based reporter assay has also been established (Farkas et al. 2009). This assay is also based on the concept that LC3 fluorescent fusion partners are degraded within lysosomes. To quantitatively measure the dynamic change of LC3 fusion protein, LC3 is fused with the *Renilla Reinformis* luciferase (RLuc). The autophagy-dependent turnover of RLuc-LC3 can be measured using cell lysates or living cells for monitoring autophagic flux. Using this assay, a screen using a small-molecule kinase inhibitor library containing 80 compounds has led to the identification of 12 compounds as inducers of autophagic flux. In addition, three potent autophagy inhibitors were also identified from this screen (Farkas et al. 2011). KU559339 [a specific inhibitor of ataxia telangiectasia-mutated (ATM)], Gö6976 (a broad-spectrum protein kinase C inhibitor), and Janus 3 kinase (Jak3) inhibitor VI could all effectively inhibit rapamcyin-induced autophagy. Interestingly, subsequent studies revealed that KU55933 and Gö6976 directly and effectively inhibited PtdIns3K, suggesting that their effects on autophagy are independent of their known target kinases. Given the rapid

progress of autophagy research, there is no doubt that more assays for monitoring autophagy will be, or are being, developed. This will definitely lead to discovery of more agents that effectively modulate autophagy, and could be potentially used for treating cancer patients in combination with other chemotherapy drugs.

5 Concluding Remarks

It is now well known that autophagy plays a critical role in both tumorigenesis and growth of existing cancers. With the expanding understanding of molecular machinery and regulating signaling pathways for autophagy, more efforts have been put into the area of drug development for targeting autophagy. With many high-throughput screening assays available, more efficient and specific autophagy inducers or inhibitors will be identified. These agents will definitely provide a useful tool for treating cancer patients in combination with other chemotherapy drugs, in particular for cancers that are resistant to conventional chemotherapy drugs. However, many fundamental questions still remain to be answered before the autophagy modulation approach can be efficiently used for cancer prevention and therapy. How does autophagy mitigate the efficacy of mTOR inhibitor-induced inhibition on tumor cell growth? How does autophagy affect the immune response to cancer, and what would be the response to autophagy inhibition among different cancer cells that have different basal levels of autophagy? Nevertheless, identifying more agents that target different aspects of the autophagy pathway will definitely improve basic and translational research on autophagy and may also help to answer the above questions.

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