Chapter 7
Oncogenes and Tumor Suppressor Genes in Autophagy

Gregor M. Balaburski, Anna Budina, and Maureen E. Murphy

Abstract The autophagy pathway has a clear, dual role in cancer development: autophagy inhibition increases tumor initiation, but for established tumors, autophagy inhibitors can potently suppress tumor progression. Given the role of autophagy in tumor initiation, it is perhaps not surprising that many oncogenes that are commonly found mutated in human cancer negatively regulate this process. Conversely a major tumor suppressor pathway, the p53/ARF axis, positively regulates autophagy. Notably, the majority of human tumors contain activated oncogenes that are predicted to lead to hyper-activation the PI3K/AKT/mTOR pathway. Additionally, most tumors contain inactivating mutations in the p53/ARF pathway. Therefore, it is reasonable to predict that cancer cells have an impaired ability to undergo autophagy, but at the same time a hyper-reliance on this pathway to promote survival during metabolic and hypoxic stress. As such, the autophagy pathway is likely to be an Achilles heel for cancer. Exploiting this weakness will be an important future goal for cancer researchers.

Keywords Autophagy • Beclin 1 • Bcl-2 • mTOR • p53 • ARF • PTEN • TSC1/2 • Oncogenes • Tumor suppressor genes • Chloroquine

1 The Role of Autophagy in Cancer

Autophagy has a significant but context-specific role in cancer. Autophagy encompasses the two most extreme functions in oncogenesis: tumor suppression and tumor promotion. Whether autophagy is tumor-promoting or tumor-suppressing to a

Gregor M. Balaburski and Anna Budina have equal contribution to this work.

G.M. Balaburski • A. Budina • M.E. Murphy (✉)
Program in Molecular and Cellular Oncogenesis, The Wistar Institute,
3601 Spruce Street, Philadelphia, PA 19104, USA
e-mail: mmurphy@wistar.org

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particular cancer appears to depend on the stage of the tumor. In the initiating stages of cancer development, autophagy blocks tumor initiation. Not surprisingly, several key autophagy regulatory genes are mutated in human cancer [for review see Kung et al. (2011)]. The tumor suppressive role of autophagy in cancer is best exemplified by the fact that many tumor suppressor genes positively regulate this process (p53, PTEN, TSC1/2, LKB1, $p14^{ARF}$), while an oncogenic pathway commonly activated in cancer, the mTOR/PI3K/AKT pathway, negatively regulates autophagy. Similarly, many key autophagy genes, including *Beclin 1*, are mutated in human cancer, and dampen the level of autophagy in tumor cells (Kung et al. 2011). Therefore, autophagy is clearly a significant pathway for tumor suppression.

The mechanism whereby autophagy is suppressive to the initiation of tumors was first elucidated by White and coworkers. This group first reported that tumors with decreased levels of Beclin 1, a key regulator of autophagy, had high levels of necrosis, and that this increased necrosis led to enhanced local inflammation. Inflammation is a known stimulant of tumor growth, and it has been proposed that this increased inflammation contributes to tumor development in cells with decreased autophagy (Degenhardt et al. 2006). White and colleagues also found that autophagy-deficient tumors had increased levels of reactive oxygen species (ROS), along with increased DNA damage foci, centrosome abnormalities, and indicators of gene amplification (Karantza-Wadsworth et al. 2007; Mathew et al. 2007). At present, the reason for the increased ROS in autophagy-defective cells is not clear: it might be the result of defective mitochondria (which would presumably be cleared normally by mitophagy, the selective autophagy of mitochondria). Alternatively, the increased ROS might be due to increased levels of misfolded proteins. In either case, the increase in genomic instability in tumors with decreased autophagy would be expected to accelerate tumor formation.

In addition to suppressing tumor initiation, autophagy can conversely be a critical survival pathway for established tumors. Indeed, while established tumors frequently exhibit decreased basal levels of autophagy in their cells, they appear to rely heavily on this basal level of autophagy in order to survive nutrient deprivation, hypoxia, and other stresses. Along these lines, for some tumor types, the level of autophagy can be a marker of poor prognosis (Lazova et al. 2012; Ma et al. 2011). Maintaining some level of autophagy is clearly necessary for tumor survival, as several groups have pioneered the use of autophagy inhibitors for treatment of cancer (Amaravadi et al. 2007; Carew et al. 2007).

2 The mTOR/PI3K/AKT Pathway and Autophagy

mTOR is an atypical serine/threonine kinase with homology to the PI3 kinase family. This kinase is a key integrator of growth factor signaling, nutrient sensing and proliferation. mTOR is composed of two independent complexes, mTORC1 and mTORC2, which have different protein subunits as well as key differences in function. Key among the differences in protein subunits is the presence of RAPTOR in

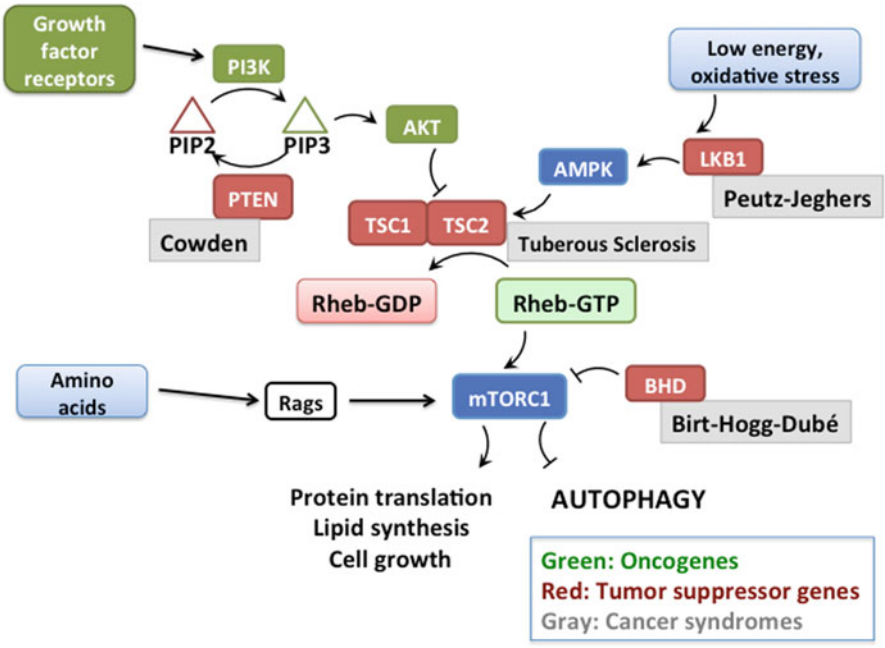

Fig. 7.1 Overview of the oncogenes and tumor suppressor genes involved in the regulation of mTOR and autophagy. Components of the mTOR signaling pathway that are dysregulated in many cancer types. In *green* are the oncogenes, mutation of which contributes to cancer and inhibits autophagy. In *red* are the tumor suppressor genes whose mutation leads to mTOR activation and impaired autophagy. In *grey* are the names of the cancer-prone syndromes caused by germ line mutations in tumor suppressor genes

mTORC1, and RICTOR in mTORC2. Because RAPTOR is required for the ability of mTOR to regulate autophagy, mTORC1 is the predominant species involved in autophagy regulation. mTORC1 is a master regulator of protein synthesis, lipid synthesis and energy metabolism, and, as noted above, it is also a critical negative regulator of autophagy [see for review Laplante and Sabatini (2012)].

In cancer, a wide variety of signaling molecules upstream of mTOR are mutationally activated (Fig. 7.1). These include receptor tyrosine kinases such as ERBB2 and EGFR, the small GTPases K-Ras and N-Ras, and the serine/threonine kinases phosphatidyl 3-kinase (PI3K) and AKT. These proteins converge on a GTPase called Rheb that positively regulates mTOR; the result is an increase in protein translation and cell proliferation, along with a concomitant decrease in autophagy. In addition to growth factor signaling and Rheb, amino acid levels are also sensed by mTOR and can regulate autophagy, but this signal is conveyed by GTPases called RAGs (Nicklin et al. 2009). There are a host of negative regulators of the mTOR pathway that are tumor suppressor genes, and that are mutationally inactivated in cancer; these include the mTOR inhibitors TSC1 and 2 (tuberous sclerosis complex

1 and 2), the kinase LKB1, and the phosphatase PTEN, which antagonizes PI3K. Whereas it is quite clear that mutations of these tumor suppressor genes results in concomitant decreases in autophagy, to date it has never been formally proven that the tumor suppressor function of these proteins relies on, or requires, their ability to regulate autophagy.

The mechanism whereby mTOR inhibits autophagy has been identified, and is conserved between yeast and mammals. Under normal growth conditions, mTORC1 (via RAPTOR) associates with the complex ULK1/2-ATG13-FIP200; this kinase is a master regulator of autophagy. Activated mTORC1 phosphorylates both ATG13 and ULK1/2, thereby suppressing the activity of this kinase (Ganley et al. 2009; Hosokawa et al. 2009; Jung et al. 2009). Inhibition of mTOR leads to rapid dephosphorylation of this complex, and translocation of the complex to the pre-autophagosomal membrane, where it functions to initiate autophagosome formation (Jung et al. 2009).

3 Targeting Autophagy in mTOR-Activated Tumors

It has been hypothesized that the low levels of autophagy present in many cancers (such as those with activating mutations in oncogenes in the mTOR pathway) may render these tumor cells more sensitive to energy imbalances and autophagy inhibition (Parkhitko et al. 2011). In support of this hypothesis, in a mouse model of PTEN-null tumors, AKT inhibitors showed limited efficacy, but when combined with the autophagy inhibitor chloroquine, this treatment led to marked tumor eradication (Degtyarev et al. 2008). Similarly, silencing of key autophagy genes sensitizes tumor cells to radiotherapy (Apel et al. 2008), enhances breast cancer cell death by tamoxifen (Qadir et al. 2008), and enhances the death of prostate cancer cells deprived of androgen (Li et al. 2008). Clinical trials using the autophagy inhibitor chloroquine or the mTOR inhibitor temsirolimus have been proposed for tumors with hyperactive mTOR, and initial promising results have been seen (Piha-Paul et al. 2011).

4 Beclin 1 and Autophagy

Beclin 1 was originally discovered as a Bcl-2 interacting protein (Liang et al. 1998). Beclin 1 is the mammalian homologue of the yeast protein Atg6, and this gene can substitute for Atg6 in yeast (Furuya et al. 2005; Kametaka et al. 1998). Beclin 1 is a 60 kDa protein with three functional domains: (1) a BH3-like only domain that interacts with Bcl-2, (2) a central coiled-coil domain, and (3) an evolutionary conserved domain (ECD) that enables it to interact with, and activate, the type III PI3K Vps34, which is a critical regulator of autophagosome formation. Notably, deletion of the ECD of Beclin 1 leads to loss of binding to Vps34, inability to enhance autophagy and loss of tumor suppressor function; this suggests a mechanistic link

between the autophagy-promoting function of Beclin 1 and its tumor suppressive function (Furuya et al. 2005).

The first indications that autophagy is tumor suppressive came from the finding that *Beclin 1* is a haplo-insufficient tumor suppressor gene. Specifically, mice with deletion of a single copy of the *Beclin 1* gene are predisposed to liver and lung tumors, lymphoma, and other tumor types (Qu et al. 2003; Yue et al. 2003). Additionally, *Beclin 1* is frequently mono-allelically deleted in human breast, ovarian, prostate and brain tumors (Aita et al. 1999; Miracco et al. 2007). Conversely, overexpression of Beclin 1 in MCF7 cells inhibits cell proliferation and tumorigenesis in a mouse xenograft model (Liang et al. 1999). Consistent with its tumor suppressive role, reduced levels of Beclin 1 can be found in many human tumors. Oddly, however, very few somatic mutations of *Beclin 1* are observed. Analysis of over 500 human tumors that included gastric, invasive breast ductal, colorectal, and hepatocellular carcinoma, as well as non small cell lung cancer and adult acute leukemia revealed that only 1.8 % of tumors contained somatic mutations in *Beclin 1*. Further, only 0.3 % of these mutations were within the *Beclin 1* coding region, and no tumors contained homozygous mutations (Lee et al. 2007). These data have led to the hypothesis that mechanisms other than mutation lead to the reduced level of Beclin 1 in human tumors; one possibility is promoter methylation and silencing (Li et al. 2010). Additionally, it has recently been shown that Beclin 1 expression is negatively regulated by micro-RNA-30a (mir-30a). Moreover, in preclinical mouse models, miR-30a plays a role in suppression of Beclin 1 mediated autophagy and sensitization of the tumor cells to platinum based chemotherapy (Zhu et al. 2009; Zou et al. 2012). The impact of miRNA-mediated regulation of Beclin 1 in clinical samples has yet to be explored.

Beclin 1 functions in autophagy by being a part of a highly conserved core complex with the key autophagy regulators Vps34 and Vps15. Beclin 1 is a positive regulator of Vps34 kinase activity (Furuya et al. 2005; Funderburk et al. 2010). This positive regulation is dependent in part on binding partners that directly interact with Beclin 1 and supplement the core complex. Two major positive regulators of Beclin 1 are UVRAG and Bif-1 (Fig. 7.2a). *Ultraviolet irradiation resistance-associated gene* (UVRAG) maps to chromosome 11q13 and like Beclin 1 is frequently mono-allelically deleted or mutated in many human cancers including gastric, colon and breast cancers (Bekri et al. 1997; Ionov et al. 2004; Kim et al. 2008a). UVRAG enhances Beclin 1 function by promoting its binding to Vps34. Over-expression of UVRAG results in increased autophagic flux and reduced proliferation of tumor cells, supporting the premise that UVRAG acts a tumor suppressor by virtue of its ability to regulate autophagy (Liang et al. 2006). Bif-1 (Bax-interacting factor-1) binds to UVRAG and enhances the ability of Beclin 1 to activate to Vps34 (Takahashi et al. 2007). Bif-1 null mice spontaneously develop tumors at a significantly higher rate than normal, suggesting that Bif-1 may also be a tumor suppressor. Consistent with this hypothesis, Bif-1 levels are greatly reduced in colon, prostate, urinary bladder and gastric cancers (Coppola et al. 2008a, b; Kim et al. 2008b; Lee et al. 2006). However, a comprehensive mutational analysis of Bif-1 has yet to be completed in human cancers.

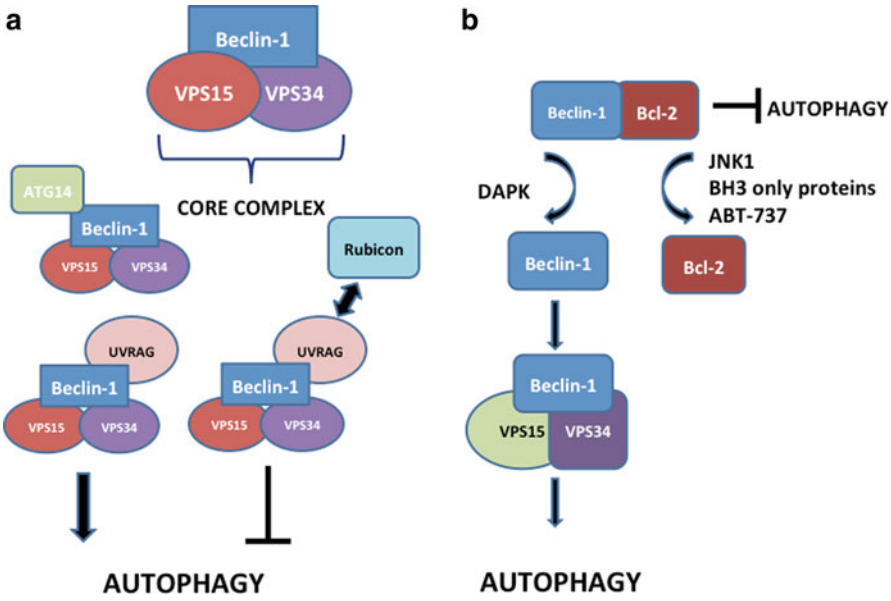

Fig. 7.2 Beclin 1 and the regulation of autophagy. (**a**) Beclin 1, in association with VPS34 and VPS15, is necessary for the formation of the core complex. This complex is positively regulated by independent associations with ATG14 and UVRAG, which enhance autophagy. Rubicon, a negative regulator, does not bind the core complex directly, but instead binds to UVRAG. (**b**) Bcl-2 sequesters Beclin 1 and inhibits autophagy. Modifications of Bcl-2 and Beclin 1 by JNK1 or DAPK, respectively release Beclin 1, resulting in initiation of autophagy. BH3-only proteins and small molecules such as ABT-737 can inhibit Bcl-2, to release Beclin 1 and positively regulate autophagy

5 Regulation of Autophagy by Bcl-2 Family Proteins

A critical negative regulator of Beclin 1, and autophagy, is the anti-apoptotic protein Bcl-2 (Pattingre et al. 2005). Knockdown of *Bcl-2* by antisense RNA or siRNA is sufficient to induce autophagy in human tumor cells (Erlich et al. 2007; Saeki et al. 2000). Based upon the finding that Bcl-2 interacts with Beclin 1 and inhibits autophagy, it was predicted that other Bcl-2 family members might likewise be involved in the regulation of autophagy. Indeed, the majority of the anti-apoptotic Bcl-2 family members, including Bcl-xl, Bcl-w and Mcl-1 can inhibit autophagy when overexpressed (Pattingre et al. 2005; Erlich et al. 2007; Maiuri et al. 2007a). Conversely, many of the pro-apoptotic Bcl-2 family members, including Bad, Noxa, Puma, BimEl, and Bik, can stimulate autophagy (Abedin et al. 2007; Rashmi et al. 2008).

The mechanism whereby Bcl-2 inhibits autophagy is by virtue of its ability to bind and sequester Beclin 1, thereby preventing interaction between Beclin 1 and Vps34. The interaction between Beclin 1 and Bcl-2 is highly regulated in cells; abrogation of this complex, and a concomitant increase in autophagy, can occur by two general mechanisms: (1) weakening of the Bcl-2-Beclin 1 interaction by phosphorylation of either protein or (2) competitive inhibition of the complex by

pro-apoptotic Bcl-2 family members, such as Puma or Noxa (Fig. 7.2b). The phosphorylation-mediated disruption of the Beclin 1/Bcl-2 complex occurs by phosphorylation of the amino-terminal loop of Bcl-2 or the BH3 domain of Beclin 1 by either jun-N-terminal kinase 1 (JNK1) or death-associated protein kinase 1 (DAPK1), respectively. In response to nutrient deprivation, JNK1 is responsible for multisite phosphorylation of Bcl-2, resulting in a disruption of the Bcl-2/Beclin 1 complex and induction of autophagy. Notably, phospho-mimetic mutants of Bcl-2 that cannot bind to Beclin 1 are unable to inhibit autophagy; conversely, non-phosphorylatable Bcl-2 mutants fail to release Beclin 1, and induce increased autophagy (Wei et al. 2008). Additionally, cells that are devoid of JNK1 show limited ability to induce starvation-mediated autophagy, and dominant-negative versions of JNK1 are potent inhibitors of autophagy (Wei et al. 2008). DAPK1 is a calcium/calmodulin-dependent serine/threonine kinase that phosphorylates Beclin 1 on threonine 119 in the BH3 domain and promotes the disruption of the Beclin 1/Bcl-xl complex. Overexpression of DAPK1 induces the formation of autophagic vesicles (Inbal et al. 2002), most likely by releasing Beclin 1 from Bcl-2 (Zalckvar et al. 2009a). In sum, the ability of Bcl-2 to negatively regulate autophagy is intimately tied to the activity of JNK, DAPK1, and possibly other, kinases.

Beclin 1-mediated autophagy is also regulated by pro-apoptotic members of the Bcl-2 family, the so-called BH3-only proteins. These BH3-only proteins compete with Beclin 1 for Bcl-2 binding, and they can release Beclin 1 from the inhibitory effects of Bcl-2. In general BH3-only proteins are believed to have overlapping function, but they tend to be induced in response to different stresses, such as nutrient deprivation, ER stress, and DNA damage (Happo et al. 2012); this fact may explain why most chemotherapeutic drugs induce autophagy in tumor cells.

6 The p53 Tumor Suppressor, a Dual Regulator of Autophagy

Since its discovery, p53 has been identified as a key tumor suppressor protein, and over 60 % of human tumors contain mutations in the *TP53* gene. In normal healthy cells, p53 is kept at low levels by the E3 ubiquitin ligase MDM2 (HDM2 in humans), which ubiquitylates p53 and targets it for proteasomal degradation. In response to various forms of stress, including DNA damage or hypoxia, phosphorylation of the amino terminus of p53 prevents interaction with MDM2, leading to p53 stabilization [see Kruse and Gu (2009) for review]. Activated oncogenes signal to p53 through transactivation of the ARF tumor suppressor, which binds to MDM2 and inhibits its activity (Tao and Levine 1999; Weber et al. 1999). Metabolic stress (nutrient deprivation) is known to induce p53 through phosphorylation on serine 15 mediated by the kinase AMPK, which responds to low AMP levels following ATP depletion (Feng et al. 2005; Jones et al. 2005). Once activated, p53 transactivates gene involved in cell cycle arrest and apoptosis, thereby eliminating premalignant cells (Vousden and Lane 2007; Zilfou and Lowe 2009).

Growing evidence suggests that, in addition to inducing either growth arrest or apoptosis, p53 is also capable of inducing autophagy in stressed cells. Several

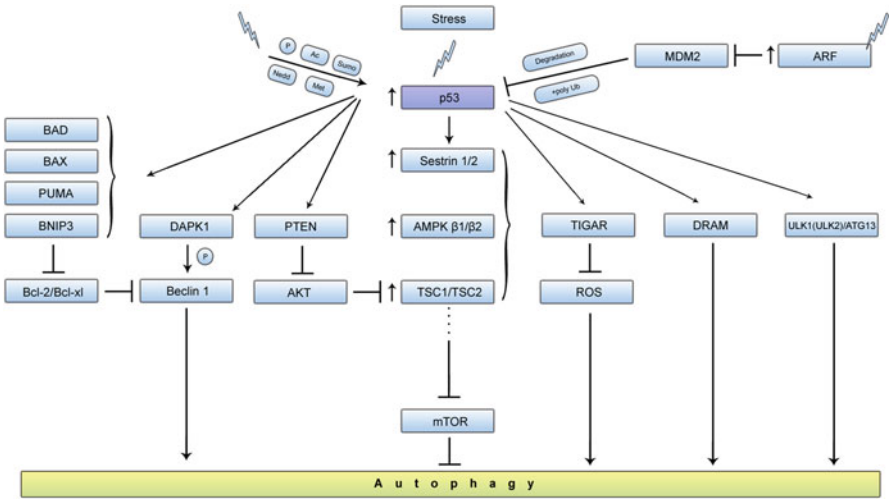

Fig. 7.3 Transcriptional targets of p53 in autophagy. In cells containing activated oncogenes, ARF binds to and inhibits MDM2, thereby stabilizing p53. In response to stresses, p53 is also activated through various posttranslational modifications, including acetylation (Ac), phosphorylation (P), neddylation (Nedd), and others. Nuclear p53 transactivates a number of genes that are positive regulators of autophagy, such as DRAM, DAPK1, ULK1(ULK2)/ATG13 and pro-apoptotic Bcl-2 proteins like BAD, BAX, PUMA, and BNIP3. Other p53 target genes induce autophagy via mTOR inhibition; these include Sestrin1 and 2, AMPKβ1 and β2, TSC2 and PTEN

mechanisms have been proposed to explain how p53 activates autophagy; the first is via regulation of mTOR activity (Fig. 7.3). In response to DNA damage p53 directly transactivates multiple negative regulators of mTOR (mammalian target of rapamycin), including the beta1 and beta2 subunits of AMPK, the tuberous sclerosis complex protein TSC2, and the phosphatase PTEN (Feng et al. 2007). All of these would negatively regulate mTOR, and therefore lead to induction of autophagy. Additionally, p53 transcriptionally regulates the genes *Sestrin1* and *Sestrin2*; the protein products of these genes bind to and activate AMPK, which in turn phoshophorylates TSC2 and therefore inhibit mTOR. The loss of Sestrin2 during nutrient deprivation (Budanov and Karin 2008) or pharmacological inhibition of AMPK (Feng et al. 2005) significantly reduces p53-mediated inhibition of mTOR, along with p53-mediated autophagy.

In addition to controlling mTOR activity, p53 also directly regulates genes involved in autophagy. For example, the critical autophagy enzymes ULK1 and ULK2 are both upregulated in response to DNA damage, and are direct p53 target genes (Gao et al. 2011). p53 directly transactivates *DRAM1* (*d*amage-*r*egulated *a*utophagy *m*odulator1), which encodes multiple isoforms of a lysosome membrane protein that co-localizes with Cathepsin D and plays a role in autophagy (Crighton et al. 2006; Mah et al. 2012). Interestingly, silencing *DRAM1* inhibits both p53-mediated autophagy and -apoptosis (Lorin et al. 2009). This result suggests a

close connection between p53-mediated autophagy and apoptosis pathways. Finally, p53 can also regulate autophagic flux in cells by post-transcriptionally regulating LC3, which is a pivotal component of the autophagic machinery; this regulation of LC3 RNA levels by p53 is believed to confer survival in response to prolonged starvation (Scherz-Shouval et al. 2010).

Several other p53 target genes regulate the function of Beclin 1 in autophagy. For example, *death-associated protein kinase 1 (DAPK1)* is direct p53 target gene; the protein product of this gene binds to and inhibits the negative regulator of autophagy MAP1B (microtubule-associated protein 1B; an LC3-binding protein) (Harrison et al. 2008). As indicated above, DAPK1 also phosphorylates Beclin 1, thereby liberating it from inhibitory association with Bcl-xl/Bcl-2 (Zalckvar et al. 2009a, b). p53 also directly transactivates several BH3-only genes, including *Puma, Bad, Bax, Noxa* and *Bnip3*, all of which can control the formation of the Beclin 1/Bcl-2 complex (Yee et al. 2009; Maiuri et al. 2007b; Zhang and Ney 2009; Levine et al. 2008). Finally, DAPK1 can control the level and activity of $p14^{ARF}$, which induces autophagy in both a p53-dependent and -independent manner (Martoriati et al. 2005; Abida and Gu 2008; Pimkina et al. 2009).

7 Suppression of Autophagy by p53

Whereas stress-induced p53 clearly induces autophagy, under normal non-stressed conditions p53 inhibits the basal level of autophagy. As such, p53 can be viewed as a rheostat for the autophagy pathway, as it both positively and negatively controls stress-induced and basal autophagy, respectively. Kroemer and colleagues first demonstrated that knockout, knockdown, or pharmacological inhibition of p53 with the compound pifithrin-α resulted in an increased level of basal autophagy in the G1 and S phases of the cell cycle in human cancer cell lines, mice, and nematodes (Tasdemir et al. 2008a, b, c). Interestingly, this group found that inhibition of p53 elevated autophagy even in enucleated cells (cells without nuclei), supporting the premise that cytoplasmic p53 is responsible for this effect. It is of note that multiple triggers of autophagy, including rapamycin, nutrient deprivation, or ER stress, cause proteasome-mediated degradation of p53, indicating that degradation of this protein may be required for some forms of autophagy (Tasdemir et al. 2008b). The role of p53 in suppressing basal autophagy is evolutionarily conserved; work from Kroemer and colleagues showed that silencing the p53 orthologue CEP-1 in *C. elegans* enhances autophagy, and that this may contribute to increased longevity in this organism (Tavernarakis et al. 2008). Interestingly, p53 has been recently linked to the negative regulation of organismal aging (Donehower 2002), and one speculation is that its negative regulation of autophagy plays a role in this phenomenon.

The mechanism(s) whereby p53 negatively regulates autophagy, and the controls on this process, are still emerging. One mechanism whereby p53 may negatively regulate basal autophagy is through the posttranscriptional regulation of LC3 mRNA, described above (Liang et al. 2006). Alternatively, in response to nutrient

starvation p53 transcriptionally regulates the gene encoding TIGAR (TP53-induced glycolysis and apoptosis regulator). TIGAR inhibits autophagy by suppressing intracellular reactive oxygen species (Bensaad et al. 2006, 2009; Li and Jogl 2009). Recently, Androphy and coworkers have discovered that the SUMO E3 ligase PIASy binds to p53 and Tip60; this interaction promotes sumoylation of lysine 386, and leads to acetylation of lysine 120 the latter modification facilitates translocation of p53 to the cytoplasm, where it induces autophagy in a PUMA-independent manner (Naidu et al. 2012). Overall, there is a clearer picture for the mechanisms whereby p53 positively regulates autophagy, and less is known about how basal autophagy is negatively regulated by p53.

8 The Autophagy Pathway Negatively Regulates p53 Function

There is considerable feedback regulation between the autophagy and p53 pathways (Fig. 7.4). For example, recent studies show that Beclin 1 can control the levels of p53 by regulating the activity of the ubiquitin hydrolases USP10 and USP13.

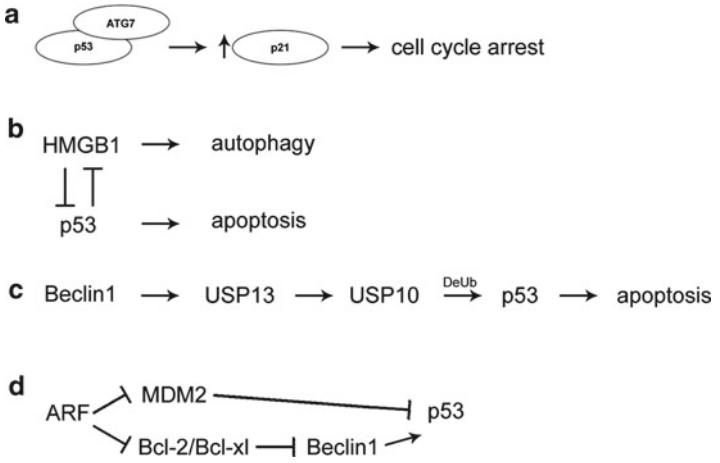

Fig. 7.4 Crosstalk between p53 and autophagy. (**a**) In nutrient-deprived cells, the essential autophagy protein ATG7 binds to p53 and promotes p21-mediated cell cycle arrest. (**b**) Under stressed conditions, HMGB1 and p53 interact in the nucleus and negatively regulate the nuclear export of each other. In the absence of p53, HMGB1 translocates to the cytoplasm and induces autophagy. In contrast, loss of HMGB1 caused increased cytosolic p53, which in turn promotes apoptosis and represses autophagy. (**c**) Beclin 1 modulates p53 activity via regulation of USP13 and USP10 ubiquitin hydrolases. Beclin 1 binds to and activates USP13, which then deubiquitylates USP10, allowing it to deubiquitylate and stabilize p53. (**d**) ARF can stabilize p53 via two mechanisms. In the p53-ARF-MDM2 pathway, ARF prevents p53 degradation through inhibition of MDM2 activity. ARF can also liberate Beclin 1 from its inhibitory association with Bcl-xl protein, leading to p53 stabilization

Specifically, Beclin 1 can bind and stabilize USP13. This increased USP13 binds and stabilizes USP10, which then can deubiquitylate and stabilize p53 (Liu et al. 2011). Therefore, cells with increased Beclin 1 have increased p53, and cells with decreased Beclin 1 have impaired p53 function. In agreement with this prediction, the levels and activity of p53 in the tissues of *Beclin 1*$^{+/-}$ mice are greatly reduced; this, along with the impairment of autophagy, may explain the increased tumorigenesis in *Beclin 1*$^{+/-}$ mice, and why *Beclin 1* is mono-allelically lost in some cancers (Qu et al. 2003; Liu et al. 2011).

Another mechanism whereby the autophagy machinery affects p53 function has emerged with the discovery that mouse embryonic fibroblasts (MEFs) from the *Atg7* knockout mouse have an impaired p53 pathway. Independent of its enzymatic activity, Finkel and colleagues found that the ATG7 could bind directly to p53 and serve as a co-activator of transcription, facilitating the transactivation of the p21 cyclin-dependent kinase inhibitor by p53 after nutrient withdrawal. Therefore, when nutrients are limiting, ATG7 negatively regulates p53 function (Lee et al. 2012).

HMGB1 (high mobility group box 1), another known inducer of autophagy, also negatively regulates p53. HMGB1 forms a complex with p53 following cell stress; this interaction sequesters the p53/HMGB1 complex within the nucleus, thus limiting the cytoplasmic localization of either protein. Knockout of *HMGB1* in mouse embryonic fibroblasts increases p53 cytosolic translocation, with subsequent inhibition of autophagy (Tasdemir et al. 2008b) and induction of apoptosis through the mitochondrial pathway (Chipuk et al. 2004, 2005; Livesey et al. 2012). Conversely, loss of p53 in HCT116 cells increases the level of HMGB1 in the cytoplasm, which then interacts with Beclin 1 to promote autophagy (Livesey et al. 2012). All of the above findings further confirm the existence of extensive crosstalk between p53 and components of the autophagy pathway, such that the fate of the cell clearly rests in the balance between these proteins.

9 ARF and Autophagy

The ARF tumor suppressor protein ($p19^{ARF}$ in mouse and $p14^{ARF}$ in humans) is encoded by an *a*lternative *r*eading *f*rame of the *Ink4a/ARF* locus (Quelle et al. 1995); this gene is transcriptionally induced in response to oncogene activation. When induced, much of ARF localizes to the nucleolus and nucleoplasm, where it binds and inhibits the p53-specific ubiquitin ligases MDM2 (Zhang et al. 1998; Pomerantz et al. 1998) and ARF-BP1 (Chen et al. 2005); this results in p53 stabilization, and concomitant cell cycle arrest and apoptosis. In addition to the regulation of the MDM2-p53 axis, ARF has tumor suppressive functions that are independent of p53. For example, ARF inhibits ribosome biogenesis by its interaction with nucleophosmin/B23 in the nucleolus (Sugimoto et al. 2003; Bertwistle et al. 2004) and it also interferes with the activity of several transcription factors, including c-myc, E2F1, and others (Sherr et al. 2005; Qi et al. 2004).

In addition to its multiple growth suppressive roles, both full-length ARF and a short isoform denoted smARF (short mitochondrial ARF) have been implicated in autophagy. smARF is translated from an internal methionine (Met45 in mouse, Met48 in human) of ARF, and consequently lacks the amino-terminal region containing the nucleolar-localization sequence, as well as the domain that interacts with MDM2. Kimchi and colleagues were the first to report that overexpression of smARF causes dissipation of mitochondrial membrane potential, activation of autophagy and subsequent p53- and caspase-independent cell death (Reef et al. 2006). Subsequently, the relevance of smARF, which accounts for less than 5 % of total ARF, to autophagy was questioned by data from Gu and colleagues, who showed that full-length ARF (containing a substitution at amino acid 45 and thus an inability to generate smARF) was capable of inducing autophagy in transfected cells, but smARF had reduced ability (Abida and Gu 2008). At present, whether smARF or full-length ARF is the major species relevant to autophagy induction is not yet clear.

10 The Mechanism Underlying ARF-Mediated Autophagy

Immunoprecipitation of ARF from highly purified mitochondria, followed by mass spectrometry of co-immunoprecipitated proteins, revealed the autophagy/apoptosis modulator Bcl-xl as an ARF-interacting protein at the mitochondria. Notably, ARF was shown to be able to disrupt the Beclin 1/Bcl-xl complex, thereby freeing Beclin 1 for autophagy induction (Pimkina et al. 2009). This proteomic analysis also revealed that the cytoplasmic stress-induced chaperone HSP70 is an ARF-interacting protein. In this case, a role for HSP70 in the trafficking of ARF to mitochondria was evident, as the HSP70 inhibitor phenylethynesulfonamide was shown to block ARF trafficking to mitochondria, and to block ARF-mediated autophagy (Pimkina and Murphy 2011). These findings support the premise that mitochondrial localization of ARF may be critical for its autophagy function.

One question that has existed is whether ARF-mediated autophagy is cytotoxic or cyto-protective to tumor cells. The original findings on smARF indicated that this protein was cytotoxic, but as this was made in transfected cells, it is likely that non-physiologically relevant levels of ARF were achieved. Our group silenced endogenous *ARF* in mouse embryo fibroblasts (MEFs) and tumor cell lines using two different short hairpins to *ARF* and showed that silencing *ARF* caused decreased autophagy, and decreased survival following nutrient deprivation. With the knowledge that many human tumor cell lines (particularly those with mutant forms of p53) have high levels of endogenous ARF, we then silenced this gene in tumor cells and showed that this inhibited the ability of these tumors to develop in vivo (Humbey et al. 2008). The combined data indicate that ARF-mediated autophagy is cytoprotective, at least for some tumors. In support of the notion that ARF and autophagy may promote tumor development, Kemp and colleagues found that ARF loss in a p53 knockout model of skin carcinogenesis impedes tumor development

(Kelly-Spratt et al. 2004); additionally, ARF loss impedes prostate tumor development in PTEN knockout mice (Chen et al. 2009). It should be noted that the contribution of ARF-mediated autophagy to tumor growth may be tumor- or tissue-specific; specifically, while silencing *ARF* limited the development of p53-null lymphomas, it enhanced the progression of p53-null sarcomas (Pimkina and Murphy 2009). These findings suggest that the role of ARF and autophagy in cancer may vary depending on tumor or tissue type, and the dependence of the cell on autophagy for survival.

11 Conclusions

There are two main pathways that regulate autophagy: these are the mTOR/PI3K/AKT axis and the p53-ARF axis. It is of note that the oncogenic mTOR pathway primarily negatively regulates autophagy, while the p53-ARF axis primarily positively regulates stress-induced autophagy. These data offer powerful support to the premise that, when it comes to the development of tumors, autophagy is clearly tumor suppressive. Notably, a hypothesis put forth by others is that the low levels of autophagy in transformed cells may be an Achilles heel for cancer; this suggests that inhibitors of autophagy should be considered as a novel avenue for cancer therapy. Along these lines, several groups, including ours, have tested autophagy inhibitors for cancer therapy in preclinical models and noted significant results (Amaravadi et al. 2007; Carew et al. 2007; Leu et al. 2009). The development and testing of other autophagy inhibitors, such as ULK1/2 kinase inhibitors and others, will be promising areas of future investigation.

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