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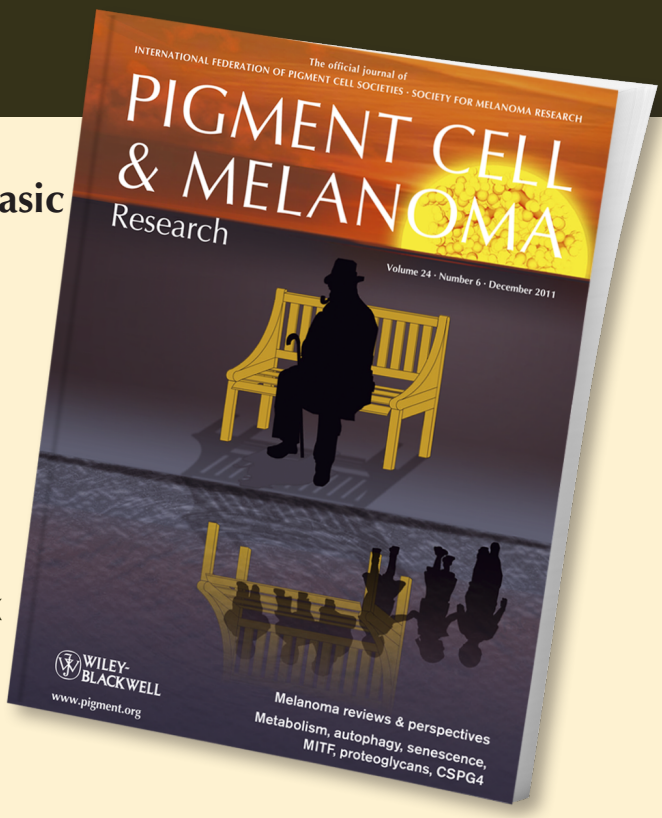

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REVIEW ARTICLE

The gluttonous side of malignant melanoma: basic and clinical implications of macroautophagy

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Summary

True to their inherent aggressive behavior, melanomas keep impressing the melanoma community with their ability to bypass tumor suppressor mechanisms. Name a pathway with the potential to control cell survival and melanoma cells will likely have it potentiated by multiple genetic or epigenetic alterations. In the context of progression and chemoresistance, large efforts have been dedicated to the identification of protective mechanisms associated with or linked to apoptotic death programs. These studies have guided the design of targeted anticancer strategies. Still, the promise for pro-apoptotic inducers as lead compounds for drug development has yet to come to fruition. It was then a question of time to identify alternative modulators of cell viability. An ideal candidate that is raising great expectations in the oncology field is autophagy, a catabolic process with multiple roles in cell homeostasis. Here we review the incipient literature on autophagy markers in melanocytic lesions. Intriguingly, histopathological studies are unveiling an intrinsic inter- and intratumor variability in the expression of autophagy modulators. Nonetheless, functional studies support a key role of autopaphagy programs in the response to a variety of stress factors. These include adaptive responses to nutrient deprivation, hypoxia and many anticancer agents, among other stimuli. Strategies are being also developed to mobilize the endocytic machinery and shift autolysosomes into death effectors. The opportunities that lie ahead in this field are exciting. Various authophagy mediators are potentially druggable. Moreover, animal models and the development of sophisticated screening methods offer a platform for multilevel academic–industrial collaborations. These efforts are expected to open avenues of research and, hopefully, lead to a more rational approach to melanoma treatment.

Introduction

Autophagy is a lysosomal-associated catabolic process which was first coined to describe the degradation of intracellular component within vesicular structures (reviewed in Yang and Klionsky, 2010). An ever-increasing number of reports outline key roles of autophagy well beyond a passive garbage disposal (Mizushima et al., 2008). Autodegradative processes are involved not only in the removal of protein aggregates and damaged organelles but also in adaptive responses to a variety of intracellular and extracellular stress signals (Kroemer et al., 2010). Depending on the route of delivery to the lysosome, these 'self-eating' processes can be assigned to three main subtypes: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy (Klionsky et al., 2007). CMA employs chaperones to translocate specific cytosolic proteins to the lysosome (Arias and Cuervo, 2010). In microautophagy, the cargo is incorporated directly into the lysosome upon direct invagination, protrusion or septation of the lysosomal membrane (Klionsky et al., 2007). Instead, macroautophagy involves the generation of double-membrane vesicles (autophagosomes) prior to the fusion to the lysosome (Xie and Klionsky, 2007). All these autodigestive processes can make a critical contribution to cell homeostasis. Nevertheless, deregulation of macroautophagy (herein referred to as autophagy, for

simplicity) is emerging as a consistent theme in a variety of cancers. In melanoma, the concept of autophagy is not foreign to pathologists. The presence of melanophages and vesicular structures associated with aberrant melanosomes have long been described in primary and metastatic lesions (Horikoshi et al., 1982). The field is now blossoming with expression and mechanistic studies of autophagy markers. Here we will summarize these recent studies, with a particular focus on the main unresolved questions.

Autophagosomes and autolysosomes

The machinery that controls and executes autophagy programs is extremely complex. In yeast, where this process has been described in great detail, 33 autophagy (ATG) genes have been directly linked to self degradation (Inoue and Klionsky, 2010). In mammalian cells, this number is notoriously higher, with recent high throughput screens identifying an intricate network of hundreds of interactions among more than 400 factors (Behrends et al., 2010; Lipinski et al. 2010). A series of excellent comprehensive reviews are available in the literature (Deretic, 2008; Galluzzi et al., 2008; Janku et al., 2011; Kroemer and Jaattela, 2005; Levine and Deretic, 2007; Maiuri et al., 2007; Rabinowitz and White, 2010). Here we will delineate the main common steps in autophagy programs (Figure 1), and their main molecular mediators (Figure 2) to present this knowledge in the context of available information in human melanoma.

Initiation of autophagy: vesicle induction

Classical autophagy programs are a result of cellular stress (see Figure 1, left panels). Typical inducers are protein aggregates or dysfunctional organelles which could be potentially harmful. Autophagy can also be evoked as an energy-generating process under situations of metabolic demand (growth factor or energy deprivation) or endoplasmic reticulum stress (Mizushima, 2007). Hypoxia, reactive oxygen species, DNA damage, and a variety of chemotherapeutic agents can also activate the autophagy machinery (Brahimi-Horn et al., 2010; Kroemer et al., 2010) (Figure 1). How these signals ultimately lead to the formation of autophagosomes is under intense scrutiny. Most of the knowledge of this step was identified in the context of the mTOR pathway (He and Klionsky, 2009). Thus, TORC1 negatively modulates the so-called ATG1 complex. This comprises ULK1/2 kinases, ATG13, FIP200, ATG101 and ATG20 (see summary in Figure 2). Nutrient deprivation relieves this inhibitory loop and allows the ATG1 complex to favor membrane nucleation. Although the mTOR pathway is a central blocker of autophagy programs, TORC1-independent autophagy programs also exist (Behrends et al., 2010; Sarkar et al., 2009). One of the questions pending in the autophagy field (and, by extension, in melanoma) is the identity of effectors of the ATG1 complex (Chen and Klionsky, 2011).

Vesicle nucleation

The next step in the autophagy program is the nucleation of the initiating membrane or phagophore (see Figure 1 for examples of this stage observed by electron

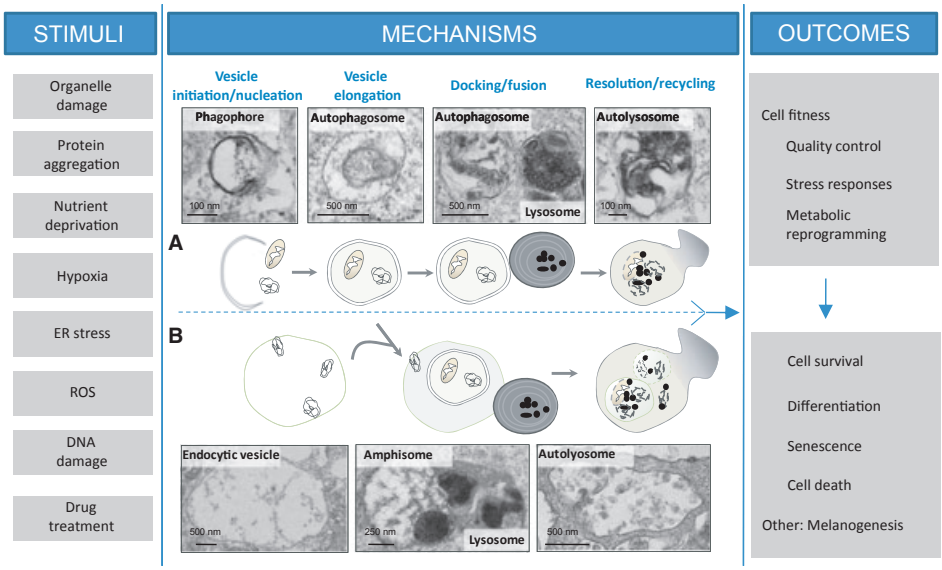

Figure 1. Inducers and outcomes of macroautophagy. Classical stress-associated factors (left) and their possible impact on cell physiology (right) after activation of autophagy programs. Two main pathways are described, involving the generation of autolysosomes directly from double membrane autophagosomes (A), or from hybrid structures generated after autophagosome/endosome fusion events (B). Electron micrographs correspond to representative images of classical autohagosomes, autolysosomes and amphisomes in the melanoma cell line SK-Mel-103 treated for 12 h with 0.5 μg/ml BO-110 (complexes of synthetic dsRNA packed with polycationic carriers). Note the variability in the size of the different autophagic structures).

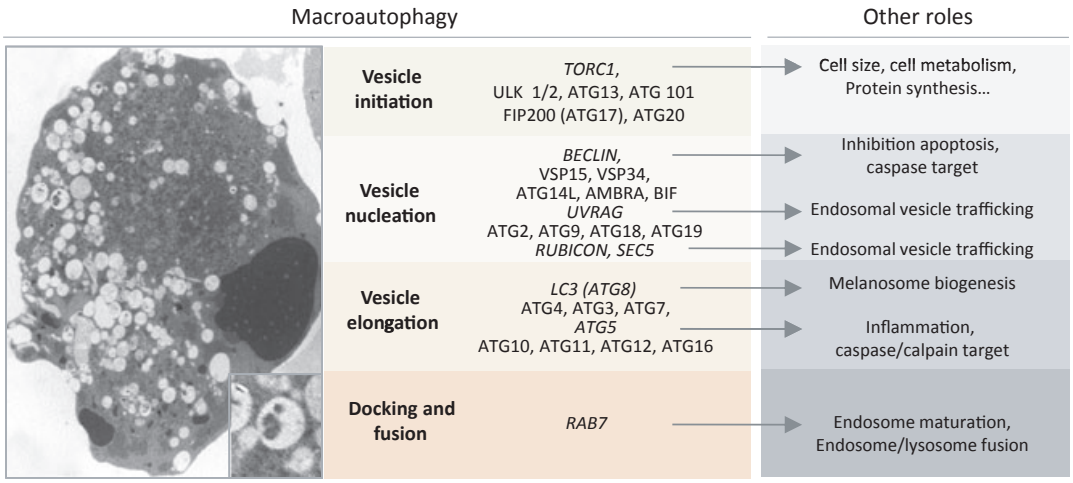

Figure 2. Pleiotropic roles of autophagy mediators. (Left) SK-Mel-103 melanoma cell line undergoing autophagy in response to cisplatin (50 μM, 24 h). Middle panels summarize the function of main autophagy factors and right panels their additional functions in other signaling cascades. In italics are proteins with roles in autophagy and other various cellular processes.

microscopy in melanoma cells). The particular membranes mobilized in melanocytic cells have not been described. In other systems, autophagosomes have been linked to the endoplasmic reticulum, the trans-Golgi network, the mitochondria or the plasma membrane (reviewed in Tooze, 2010). Early nucleation events involve the generation of phosphatidylinositol 3-phosphates (PI3P) that are associated with WIPI-1/2 phospholipid-binding effector. The process is modulated by class III PI3K (also known as VSP34), which interacts with Beclin 1 (BECN1) and VSP15 (Figure 2). This complex is positively regulated by AMBRA1, ATG14L, UVRAG, EXO84 and BIF1, and inhibited by RUBICON, SEC5 and antiapoptotic Bcl-2 proteins (BCL2 and BCLxL) (Bodemann et al., 2011; Deretic, 2008; Matsunaga et al., 2009; Mizushima et al., 2011).

Vesicle elongation: generation of autophagosomes

In yeast, this process occurs by the action of two ubiquitin-like conjugation systems, linked to Atg8 and Atg12 proteins, respectively (Geng and Klionsky, 2008; Xie and Klionsky, 2007). Atg8 (previously cleaved by the cysteine protease Atg4) is conjugated to the lipid phosphatidylethanolamine (PE). In turn, Atg12 is conjugated to Atg5 in a process that requires Atg16. In both cases, the E1-like enzyme is the Atg7 protein. The E2-like enzymes are Atg3 (for Atg8) and Atg10 (for Atg12) (Inoue and Klionsky, 2010). These two ubiquitin-like pathways have also been described in mammalian cells, although how this process is wired is not completely understood (Chen and Klionsky, 2011). For example, there are seven homologs of Atg8 (LC3A, LC3B, LC3C, GATE-16/GABARAP, GABARAPL1, GABARAPL2 and GABARAPL3) but it is unclear whether these proteins are functionally equivalent (Chen and Klionsky, 2011). The precise mechanisms by which pre-autophagosomal structures elongate their membranes are also not well understood, although homotypic fusion events associated with Atg16L1, Atg9 and various SNAREs (Soluble NSF attachment protein- receptor) have been described recently in yeast (Moreau et al., 2011; Nair et al., 2011).

Autophagosome–lysosome fusion

This is a hallmark of macroautophagy. In a still not well understood mechanism, autophagosomes slide on dynein motors along microtubules towards lysosomes for docking and fusion. These events give rise to autolysosomes, whereby cargo undergoes degradation by lysosomal hydrolases. The fusion of autophagosomes with lysosomes can be complete (i.e. giving rise to a single vesicular structure) or occurring via a kiss-and-run process, where the two vesicles are maintained (Burman and Ktistakis, 2010; Jahreiss et al., 2008; Noda et al., 2009; Roy and Debnath, 2010). The lysosomal LAMP1 and LAPM2, and the small GTPase RAB7 are involved in autolyosomal generation (Gutierrez et al., 2004; Saftig et al., 2008). Transcription factors that control lysosome biogenesis (Settembre et al., 2011), as well as RAB33B and its guanosine triphosphatase-activating protein (GAP) OATL1, have also been linked to this process (Itoh et al., 2011).

Retrieval/recycling

The last step in the autophagy process is the retrieval of ATG complexes from the autolysosomal membrane to be recycled for subsequent use. In yeast, this process is governed by Atg9 and modulated by Atg2, Atg18, Atg23 and Atg27 (Legakis et al., 2007; Reggiori et al., 2004). Interestingly, the human Atg18 homolog WIPI1 has recently been found to modulate the expression of the pigmentation genes MITF and TRP1 (Ganesan et al., 2008; Ho and Ganesan, 2011; see below). In melanoma cells, the role of these recycling proteins beyond melanosome maturation is still unclear.

Amphisomes and the endocytic machinery in autophagy

Autophagosomes are not the only source of cytosolic structures to be degraded by lysosomes. In fact, multivesicular bodies and other late endocytic vesicles have long been proposed as a collecting station for material destined for lysosomal degradation (Seglen and Bohley, 1992). Endosomes can be targeted to lysosomes directly or after previous fusion with autophagosomes (to generate hybrid structures termed amphisomes). Functional studies in cells undergoing autophagy have revealed three main complexes involved in these processes. These are the ESCRT (endosomal sorting complex required for transport), HOPS (homotypic fusion and vacuole protein sorting) and SNARE (soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor) complexes (Luzio et al.; Metcalf and Isaacs, 2010; Rusten and Stenmark, 2009). Various RAB GTPases and vacuole protein sorting (Vps) genes add selectivity and specificity to the tethering processes (Nickerson et al., 2009). Of these, RAB7 and Vps34 (Class III PI3K) have emerged as key mediators in endo/lysosomal processes in various cell types.

In melanoma cells, electron microscopy and real time confocal microscopy have uncovered dynamic homotypic and heterotypic fusion events between endosomes, autophagosomes and lysosomes. Thus, in these cells, the presence of autophagosomes and amphisomes is notorious in resting conditions (A. Checinska, D. Alonso-Curbelo and M. S. Soengas, in preparation). Interestingly, endo/lysosomal trafficking can be exacerbated further by pharmacological agents. In particular, synthetic bioavailable particles consisting of dsRNA (BO-110 for simplicity) were found to be particularly efficient at RAB7-endosome and amphisome generation (Figure 1). These BO-110-driven amphisomes were able to recruit multiple lysosomes, ultimately resulting in cell autodigestion (Tormo et al., 2009a). Therefore, BO-110 represents a novel class of amphisome-generating compounds with therapeutic activity against melanoma cells (Kim and Ronai, 2009; Tormo et al., 2009b). Multiple anticancer agents were also highly efficient at mobilizing the endolysosomal machinery (A. Checinska and M. S. Soengas, in preparation; see inset in Figure 2 for autophagic vesicles generated by cisplatin in SK-Mel-103 cells). An increasing body of independent evidence (see Table 1, and additional information below), warrants detailed analyses of endolysosomal pathways in melanoma maintenance and drug response.

Caution in defining activation versus blockade of autophagy programs: pleiotropic roles of autophagy factors

The results summarized above have paved the way for the development of a variety of experimental tools for mechanistic assays of autophagy cascades. Caution should be observed, however, as most of these techniques are either static in nature or do not take into consideration other non-autophagy roles of ATG proteins (Klionsky et al., 2008; Virgin and Levine, 2009). A summarized review of the pros and cons of methods frequently used to assess autophagy programs is presented below, to put the current state of knowledge in the melanoma field into context.

Electron microscopy (EM) remains a defining strategy to assign endodegradative processes to autophagy (Eskelinen et al. 2011). However, there is some disagreement regarding the characteristics of *bona fide* double membrane autophagosomes (Kovács et al., 2007). Moreover, distinguishing single membrane autolysosomes from late endosomes can be problematic. It should also be taken into account that EM micrographs are snapshot images and therefore are not informative regarding the dynamics of autophagic flux. Thus autophagosomes can be visualized as a result an increased formation of phagophores and/or a reduction in autolysosome clearance.

Immunohistochemistry is very useful to define the levels and localization of autophagy factors in fixed cells and tissue specimens. For example, downregulation of Beclin1 in breast cancer cell lines and biopsies, reflecting allelic loss, supports the concept of this autophagy protein as a tumor suppressor (Funderburk et al., 2010; Liang et al., 1999). Visualization of focal aggregation of LC3B, and the autophagy target p62/Sequestosome1 in paraffin-embedded sections have also helped to uncover autophagosome formation during tumor development (Kirkin et al., 2009; Tanida et al., 2008). Note, however, that the presence of LC3 foci in cells cannot be considered to reflect increased autophagy (Kepp et al., 2011). Reduced autophagosome–lysosome fusion or defective proteolytic activity of lysosomes (i.e. decreased autophagy efflux) can also result in a transient accumulation of autophagosomes. Attention should be paid also to the role of Beclin-1 in apoptosis and cell cycle control (Funderburk et al., 2010; Kang et al., 2011), as well as of p62 as a scaffold in various autophagy-independent signaling cascades (Moscat and Diaz-Meco, 2009).

Immunoblotting is a convenient technique for cell-based assays of changes in the electrophoretic mobility of LC3B associated to its conjugation to phosphatidylethanolamine (PE) during the generation of the phagophore and the autophagosome. Nevertheless, estimating the ratio of unmodified LC3 (LC3-I) to PE-bound LC3 (LC3-II) may be complicated in cells with very active autophagy programs, as part of LC3-II is degraded in the autolysosome. Use of cathepsin inhibitors or blockers of lysosomal proteases have been used to favor the accumulation of LC-3 II and thus assess autophagosome influx and efflux (Kepp et al., 2011; Klionsky et al., 2008; Tasdemir et al., 2008).

Table 1. Summary of published studies on endogenous and treatment-induced autophagy programs in melanocytic cells, animal models and tissue specimens. ND. Not determined. Studies in which functional studies of specific autophagy factors have been directly addressed (i.e. by targeted depletion) are indicated.

Stimuli	Results	Outcome	Modulators	Molecular validation of autophagy	System	References
None (histological analysis of endogenous macromelanosomes and giant melanosomes)	ND	ND	ND	ND	Tissue specimens (nevocellular nevi, lentigo simplex and malignant melanoma)	Horikoshi et al. (1982)
None (endogenous LC3B)	No LC3B in melanocytes. Focal LC3B in invasive and metastatic melanomas	Tumor promoting. Melanoma cell-macrophage fusions	Correlation with Golgi markers	NO	Tissue specimens (superficial spreading melanoma)	Lazova et al. (2009); Pawelek (2007)
None (endogenous LC3A, BECN1)	No LC3A foci detected. Low BECN1 in deep, ulcerated melanomas, with markers of hypoxia	Biphasic role of BECN1 in cell death	ND	ND	Tissue specimens (nodular melanomas)	Sivridis et al. (2011)
None (endogenous LC3B, BECN1)	LC3B expression in melanocytes and nevi, heterogeneous in melanoma. BECN1 decreasing with progression	Tumor suppressor	ND	ND	Tissue specimens (nevi, RGP, VGP, metastatic melanoma)	Miracco et al. (2010)
Post treatment metastatic specimens (temozolomide and sorafenib clinical trial)	Variable content of autophagic vesicles	>6 autophagic vesicles per cell correlating with drug resistance. High AV content correlating with metastatic potential	ATG5	tet-shRNA ATG5	Stage IV melanomas from temozolomide and sorafenib clinical trial. Cell line C8181. Xenografts	Ma et al. (2011)
Endogenous autophagy factors. siRNA screening for new autophagy/ pigmentation genes	Requirement for pigmentation	Melanosome biology	LC3A, Beclin1, WIPI1, MITF, b-Catenin, TORC1	siRNA	Cultured cells: Melanocytes, MNT1 melanoma cells	Ho et al. (2011)
Monobenzone	Melanosome autophagy. Enhanced immunoattack of melanoma cells	Antitumor effect	ND	NO	Melanoma cell line 92.2	van der Boorn (2011a,b)
Ectopic BRAFV600E	Premature senescence and cell death in cells with mutated BRAF	Growth inhibition, cell death	mTOR	3-MA	Primary melanomas, lymph node metastasis, visceral metastasis.WM35 and 451 Lu melanoma cells. Xenografts	Maddodi et al. (2010)

Table 1. (Continued)

Stimuli	Results	Outcome	Modulators	Molecular validation of autophagy	System	References
Ecopic BRAFV600E in combination with fenretidine or bortezomib	Reduced LC3 foci by Fenretidine or Bortezomib	Drug resistance, survival	ATF4, BECN1, ATG7 ?	siRNA BECN1, ATG5	Cell lines (CHL-1, A375, WM266-4)	Amstrong et al. (2011)
Rapamycin	No effect on autophagy	Cell death by downregulation of av integrin	mTOR independent	3-MA, siRNA BECN1	B16 melanoma cells	Yang, Lei et al. (2009)
ADI-PEG20 (Arg depletion), in combination with MEK inhibition	Autophagy followed by cell death	Cytotoxicity (enhanced by MEK inhibition)	AMPK-dependent mTOR inhbition	siRNA BECN1	Melanoma cell line Mel1220	Savaraj et al. (2010)
Leucine deprivation, MEK inhibition, chloroquine	Defective autophagy in MEK-transformed melanocytes, or in melanoma cells	Cell death	ATG1, VPS34, MEK, sustained mTOR,	shRNA Vps34, ATG1	Immortalized and transformed melanocytes. Various melanoma cell lines. Xenografts	Sheen et al. (2011)
Hypoxia + Beclin depletion. Chloroquine. Trp2 vaccine	L3B in hypoxic areas	Survival. Blockade of autophagy favored cell death	BECN1	siRNA BECN1, ATG5	B16-F10 xenografts in Beclin +/− mice	Noman et al. (2011)
Hypoxia. Beclin deficient mice	Increased intratumoral angiogenesis	Enhanced tumorigenic potential	BECN1 in endothelial cells	Genetic modified mice, siRNA BECN1 (endothelial cells)	B16-F10 xenografts in Beclin +/− mice	Lee et al. (2011)
2-deoxy-D-glucose	Increased autophagy	Survival	ER stress/UPR (grp78 CHOP)	si ATG7 in 1420 cells (pancreatic)	MDA-MB-435 cells	Xi et al. (2010)
Compound C	Increased autophagy	Survival	mTOR downregulation (AMPK independent effects)	Bafilomycin	Melanoma cell line B16	Vucicevic et al. (2010)
Esomeprazole	Increased autophagy	Survival	mTOR, Beclin1, ATG5	siRNA BECN1, ATG5	Melanoma cell lines Me30966, Mel501, WM-793	Marino et al. (2011)
Cucurbitacin (CuB)	Induction of autophagy and upregulation of Bcl-2	Survival	c-Jun N-terminal kinase	ND	B16-F10 mouse melanoma cells	Ouyang et al. (2011)

Table 1. (Continued)

Stimuli	Results	Outcome	Modulators	Molecular validation of autophagy	System	References
Silibinin, MG132, mitomycin C	LC3 lipidation, Beclin1 induction	Survival when autophagy is on	p53 inhibition, NFkB induction	ND	Melanoma line A375-S2	Jiang et al. (2011)
FKBP51	Activation of autophagy, inhibition of apoptosis	Survival	NFkB	siRNA FKBP51	Cell lines, tissue specimens (normal skin, cutaneous melanomas). Xenografts	Romano et al. (2010)
Antitumoral roles. Innate immunity						
Hypericidin	Activation of autophagy	Cytoprotection at low doses. Kiling at high doses	ND	ND	Melanoma cell lines UCT Mel-1, A375	Davids et al. (2009)
Cysteamine, doxorubicin	Induction of autophagosomes, blockade of autolysosomes	Cell death	ND	3-MA, siRNA ATG5	Melanoma cell line B16. Allografts	Wan et a. (2010)
Metformin	Modest increase in LC3-II, caspase activation	Cell death (reduced in the presence of NH_4Cl)	ND	NH_4Cl	Melanoma cell line B16	Janjetovic et al. (2011)
Polygonatum cyrtonema lectin	Caspase activation followed by autophagy	Cell death	Mitochondria-mediated ROS-p38-p53 stress	ND	Melanoma cell line A375	Liu et al. (2009)
Oncolytic Herpes virus	Autophagy and caspase activation	Cell death	BECN1 induction, casp-1 activation	ND	Various human melanoma cell lines	Colunga et al. (2010)
dsRNA nanocomplexes	Autophagy and caspase activation	Amohisome formation, cell death, antimetastatic activity	RAB7, MDA5, NOXA, ATG5, IFN	3-MA, Bafilomycin, shRNA ATG5, time-lapse microscopy	Various human melanoma cell lines, xenografts. Genetically modified melanomas (Tyr:$NRAS^{Q61R}$; $INK4a/ARF^{-/-}$)	Tormo et al. (2009a,b)
TLR4-9 agonists	Autophagy	Antimetastatic immunotherapy	IFN	NH_4Cl, 3MA, Wortmanin, siRNA ATG12	B16F10 melanoma. Xenografts	Yan et al. (2011)
Cross presentation of melanoma cells by DC to T cells	Autophagosomes as MHCII antigen carriers	Autophagosomes favor, but autolysosomes inhibit MCHII presentation	BECN1, ATG12	siRNA BECN1, ATG12	B16-F10 mouse melanoma cells	Li et al. (2008)
IFN-g driven MHC-I antigen expression	Autophagy facilitating MHC-I expression	Autophagosome and autolysosomes required for MHC-1 induction by IFN-γ	ATG5, ATG7, BECN1	siRNA ATG5, ATG7, BECN1	B16-F1 mouse melanoma cells	Li et al. (2010)

Fluorescence microscopy for the detection of fluorescently labeled LC3, ATG5, ATG7, LAMP2 or RAB7 is a helpful strategy for kinetic analyses of autophagy in living cells. Fluorescent lysosomotropic agents such as LysoTracker or LysoSensor further increase the versatility of multispectral microscopy. In melanoma cells, real time imaging of mCherry-LC3, GFP-RAB7 and LysoTracker Blue, has revealed early and late autophagy events induced by chemotherapeutic agents (see below). However, validation studies should be considered when overexpressing autophagy factors, as these proteins can aggregate artificially (Ciechomska and Tolkovsky, 2007). Moreover, changing the endogenous levels of these proteins can affect autophagy flux substantially (Kepp et al., 2011).

Pharmacological modulators of mTOR and type III PI3K (i.e. rapamycin and 3-methyl adenine, respectively) are convenient reagents to blunt autophagosome formation. The lysosomotropic agent chloroquine is also an inexpensive reagent to assess autophagosome/lysosome and amphisome/lysosome fusion events (Kepp et al., 2011). Again, these reagents should be considered cautiously. mTOR-independent autophagy programs have been identified (Behrends et al., 2010). On the other hand, mTOR and PI3K are central effectors in signaling cascades that impact on cell cycle, cell metabolism, cell growth and protein synthesis (see Figure 2). Deregulation of these programs can artificially set tumor cells into autophagy. Chloroquine changes lysosomal pH, affecting other functions of this organelle (i.e microautophagy or chaperone-mediated protein degradation) (Klionsky et al., 2008).

RNA interference is becoming an obligatory step in functional studies of autophagy programs. In fact, current recommendations suggest that at least two independent ATG factors should be depleted for an unambiguous analysis of autophagy programs (Kepp et al., 2011; Klionsky et al., 2008). The requirement for this thorough analysis reflects the emerging complexity of ATG genes. In fibroblasts, ATG5 or ATG7 deficiency compromises, but does not completely abrogate, autophagosome formation (Nishida et al., 2009). From a therapeutic perspective, siRNA or shRNA-based gene transfer studies should also consider multiple points of cross-talk between autophagy and apoptosis (Laussmann et al., 2011). For example, cleaved forms of Beclin1, ATG4D and ATG5 have been identified that are inactive as autophagy mediators but can induce apoptosis (Yousefi et al., 2006). Beclin1 can modulate the levels of the caspase-9 and interfere indirectly with cell viability (Kang et al., 2011). Similarly, studies involving depletion of RAB proteins or PI3K complexes should pay attention to the involvement of these proteins in endocytic pathways which can impinge on cancer cell physiology at various levels (Furuya et al., 2010; Thoresen et al., 2010).

In summary, autophagy is a dynamic, multi-step process at the crossroads of multiple signaling cascades. A variety of techniques have been developed to visualize autophagosome formation and to assess the functional requirement of key autophagy modulators. When possible, these studies should be performed in vivo, with a detailed kinetic evaluation of early, intermediate and late stages of autophagosome formation and autolysosome clearance. Functional studies based on genetic depletion or pharmacological agents should be designed to consider pleiotropic roles of key components of macroautophagy programs. Guidelines for the analysis of autophagy in cells and tissue specimens have been outlined in an excellent recent review (Kepp et al., 2011).

Autophagy and melanogenesis

Electron microscopy has long-revealed phagocytic structures in melanocytic lesions (see Lazova and Pawelek, 2009 for a recent review). In the early 1980s, nevus, lentigo simplex and cutaneous melanomas had already been found to contain large melanosome complexes engulfed in vesicular structures and reflecting various states of degradation (Horikoshi et al., 1982). These melanized autophagosomes are now considered to account for the so-called coarse melanin, which is responsible for heavily hyperpigmented areas within melanoma specimens (Lazova et al., 2009). The molecular bases underlying the generation and, more importantly, the role of these melanin-containing autophagosomes are unclear. In a series of 12 superficial spreading melanomas, the Pawelek group reported that coarse melanin colocalized with LC3B, the Golgi 58K protein and β1,6-branched oligosaccharides, supporting a Golgi-associated autophagy process (Lazova et al., 2009). Intriguingly, hyperpigmented melanomas were found to present with a notable infiltration of melanophages which shared ultrastructural and immunohistochemical features with melanoma cells. Thus, phagocytic vesicles in these melanophages were stained with LC3B and Golgi 58K antibodies, as well as with leukocytic phytohemagglutinin (which recognizes β1,6-branched oligosaccharides). Based on these similarities and on the observation that melanoma cells can display a marked phagocytic phenotype (Pawelek, 2008), a model has been proposed whereby melanoma–macrophage fusion may favor melanoma progression (Pawelek, 2007; Pawelek and Chakraborty, 2008).

Different lines of investigation support an intricate interplay between pigmentation and autophagy genes. Mice deficient in Beclin1 or genes such as Fig4 or Vac14 that control autophagosome turnover show coat color defects (see Ho and Ganesan, 2011 for a recent review). In addition, chloroquine analogs that interfere with lysosomal functions induce depigmentation in artificial skin models (Ni-Komatsu et al., 2008). More definite evidence of mechanistic links between melanosome

biogenesis and autophagy genes has been uncovered recently by Ganesan and collaborators. These authors visualized LC3B in early and late stage melanosomes (Ho et al., 2011). Moreover, in a genome-wide RNA interference screening, they found LC3A, Beclin1 and WIPI1 as part of a series of 92 novel genes whose depletion compromised melanogenesis in normal melanocytes and in the melanotic melanoma line MNT-1 (Ganesan et al., 2008). Further depletion studies showed that WIP1 favors the maturation of Stage II to Stage IV melanosomes. This effect involves, at least in part, the inhibition of TORC1, stabilization of β-catenin, and a subsequent transactivation of MITF mRNA (Ho et al., 2011). In contrast to WIPI1, Beclin1 downregulation inhibited LC3 lipidation and melanosome accumulation but had no effect on MITF levels (reviewed in Ho and Ganesan, 2011). The signaling cascades underlying these differential functions of Beclin1 and WIPI1 on melanosome biogenesis have yet to be defined. In particular, it would be interesting to define the ultimate fate of LC3-decorated melanosomes. Does LC3 provide an element of quality control to eliminate aberrant melanosomes, as selective degradation of dysfunctional mitochondria or peroxisomes do in other systems? Conversely, how do LC3-bound melanosomes escape from lysosomal-dependent degradation so they can be secreted to surrounded keratinocytes during normal UV-induced pigmentation reactions? The Ganesan group (Ho and Ganesan, 2011) suggests that PI3K 'autophagy off' complexes (containing UVRAG or Sec5) favor the generation of early stage melanosomes. Instead, 'autophagy on' complexes (containing RalB and ATG14L) may be responsible for the elimination of melanosomes (Ho and Ganesan, 2011). Do the 'off' rheostats lead to coarse melanin, whereas the 'on' factors promote pathological depletion of melanosomes (i.e. in vitiligo patients)? Addressing these questions is likely to provide new insights on melanocyte and melanoma cell biology.

Despite the autophagy–pigmentation crosstalks described above, it should be mentioned that early studies addressing pigmentation features of melanoma specimens have already reported a lack of direct correlation between LC3 levels and melanin content. Moreover, classical inducers of autophagy, such as starvation, could mobilize Beclin1, LC3 and WIPI1 without affecting melanocyte pigmentation. These results illustrate the pleiotropic roles of autophagy regulators in and beyond autophagosome/autolysosome generation.

Autophagy markers in melanocytic nevi and melanoma specimens

To date, the localization and expression of key components of the autophagy machinery in melanocytic tumors have been addressed in four main studies. Surprisingly, the results obtained are rather variable, likely reflecting the intrinsic heterogeneity of melanoma tumors and the different techniques used to assess autophagy programs.

As indicated before, the Pawelek laboratory studied normal skin and 12 superficial spreading cutaneous melanomas. Using the LC3B antibody ab48394 (Abcam, Cambridge, USA), no staining was found in normal melanocytes. LC3B levels were low in thin lesions, but with a high focal staining in most (although not all) deep invading cases (Lazova et al., 2009). Instead, Miracco and collaborators (Miracco et al., 2010) reported that normal melanocytes and benign nevi showed moderate LC3B staining in the cytoplasm (and also in the nuclei). LC3B expression in melanomas was heterogeneous, undetectable in some cases but showing large foci in others. These studies were performed with an LC3B antibody from Sigma in 149 lesions that included benign and dysplastic nevi, radial growth phase (RGP), vertical growth phase (VGP) and metastatic melanomas. BECN1 was also analyzed in this study (antibody M-300; Santa Cruz, CA, USA). Normal melanocytes, 100% of benign nevi and 86.6% dysplastic nevi showed moderate-to-strong cytoplasmic BECN1 staining and nucleolar positivity. In melanoma specimens, cytosolic BECN1 was found to be more heterogeneous, decreasing with tumor progression, with only 54.6 and 26.7% of RGP and metastatic melanomas, respectively, scoring as highly positive. In contrast, non-cytoplasmic BECN1 was found to be more prominent in thin and ulcerated melanomas. Protein immunoblotting and mRNA expression analyses in some representative cases corroborated the trend found by immunohistochemistry. Miracco et al. (2010) support a reduced autophagy function during melanoma progression, consistent with a tumor suppressor role of this degradative program.

The Giatromanolaki group analyzed BECN1 (ab501031; Abcam) and LC3A expression (AP1805a, Abgent) in 79 cases of nodular cutaneous melanomas with a vertical growth pattern, but not radiologically detectable metastases (Sivridis et al., 2011). Both proteins had a diffuse and highly variable cytosolic expression. Low BECN1 correlated with depth of invasion, tumor ulceration and a sharp decrease in patient survival 5 yr after surgery. Curiously, high BECN1 was found in some specimens, particularly in hypoxic areas (i.e. with high HIF1α and LDH5 expression). This high reactivity was linked to an increased rate of early deaths. For LC3A, strong reactivity in more than 30% of cells was found in half the specimens, with no focal staining suggestive of autophagosome formation. LC3A levels did not seem to affect patient outcome (Sivridis et al., 2011).

A different angle in clinical connections between autophagy and patient survival has been examined by the Amaravadi laboratory (Ma et al., 2011). These authors used electron microscopy to assess the autophagic index in 12 pretreatment cutaneous biopsies of Stage IV melanoma patients enrolled in a phase II clinical trial of

temozolomide and sorafenib. Again, EM revealed a marked heterogeneity in the number and size of autophagic vesicles (AV). With the caveat of a small sample population, a striking reduction of 4 months in patient disease-free survival after treatment was identified in cases with ≥6 AV/cell with respect to those with low autophagy content (<6 AV/cell). Whether the sorafenib/temozolomide combination altered the amount of autophagosomes and/or autolysosomes in vivo is a pending question that deserves attention. Nevertheless, inhibition of autophagy by ATG5 shRNA or with chloroquine significantly increased temozolomide-driven cell death in cultured melanoma cells (line C8161). Ma et al. (2011) also noted a higher tumorigenicity in mouse xenografts and an increased invasive potential of cultured melanoma cells if they had a high AV content. Only two non-invasive and two invasive cell lines were tested and, therefore, no global conclusions can be formally drawn. Still, these results certainly encourage a more in-depth analysis of autophagy as a key mechanism for melanoma maintenance and drug resistance, in line with other studies summarized below (see Table 1).

Context-dependent regulation of autophagy by Ras-BRAF-MEK and mTOR pathways

The histology analyses summarized above revealed a high heterogeneity in the expression and localization of autophagy markers in melanoma specimens. This variability also extends to the interplay with the classical melanoma oncogenes *BRAF* or *NRAS*. Automated quantitative immunohistochemistry of LC3B (SC-28266 from Santa Cruz) in 16 primary melanomas, 12 lymph nodes and 12 distant metastases suggested a higher focal staining in BRAF mutant specimens (Maddodi et al., 2010). In contrast, electron microscopy studies failed to reveal significant differences in the amount of autophagic vesicles in NRAS or BRAF mutated melanoma tumors (Ma et al., 2011). Moreover, the extent to which these autophagy-like phenotypes are activated to favor or to block melanoma initiation and progression remains an open question. In particular, the impact of the RAS-BRAF-MEK and mTOR pathways on autolysosome formation and melanoma cell viability seems to be highly dependent on experimental system and the cellular context.

Forced overexpression of activated BRAF promoted premature senescence and led to cell death by autophagy in cultured WM35 and 451Lu melanoma cells expressing $BRAF^{V600E}$, but not in SK-Mel-2, with wild-type BRAF (Maddodi et al., 2010). These authors then concluded that $BRAF^{V600E}$ can act as a pro-autophagy mediator. In a different setting, ectopically transduced $BRAF^{V600E}$ was reported to have the opposite role, namely, conferring resistance to autophagy (Armstrong et al., 2011). Here, experiments were performed in one cell line with wild-type BRAF (CHL-1), and two BRAF mutant cases (A375 and WM266-4). Treatments involved the synthetic retinoid fenretidine or the proteasome inhibitor bortezomib. Surprisingly, no explanation was provided for the lack of effect of depletion of endogenous BRAF on autophagy driven by bortezomib, and high levels of lipidated LC3B upon incubation in fenretidine (Armstrong et al., 2011). Therefore, additional studies are needed on a larger panel of melanoma cell lines or, better, under physiologically relevant conditions in vivo, to definitively assign positive or negative effects of the BRAF oncogene on basal or drug-induced autophagy programs.

Mechanistic analyses of autophagy also reveal a complex wiring of mTOR-dependent autophagy modulation in melanoma cells. In the mouse B16 cell line, rapamycin treatment was able to promote LC3B lipidation and inhibit metastatic dissemination in mouse xenografts. However, these therapeutic effects of rapamycin were linked not to autophagy but to downregulation of αv integrin expression and induction of apoptosis (Yang et al., 2009). This is consistent with rapamycin having minor effects on $BRAF^{V600E}$ (Armstrong et al., 2011) or leading to a transient accumulation of LC3 foci (Tormo et al., 2009b). The adaptation to TORC1 inhibition may account for the known lack of efficacy of rapamycin or other rapalogs as single agents against melanoma cells (Lasithiotakis et al., 2008; Marone et al., 2009; Werzowa et al., 2009). Nonetheless, autophagy induction by rapamycin can favor the effect of immunotherapy (Yan et al., 2011), particularly at early stages of melanoma development (Li et al., 2009).

Pro- and antiautophagic effects of amino acid deprivation in melanoma cells

Low nutrient content is one of the classical promoters of autophagy in mammalian cells (Mathew and White, 2011). Hence, it would be expected that these stimuli would activate autophagy programs in melanoma cells if proper mechanisms of response were in place. Depletion of arginine, an amino acid that most melanoma cells cannot synthesize (reviewed in Feun et al., 2008), did in fact promote LC3 lipidation in cell culture systems (Savaraj et al., 2010). This was achieved by treatment with a pegylated form of arginine deiminase (ADI-PEG20). The mechanisms underlying the effect of ADI-PEG20 are not completely defined but may involve, at least in part, the inactivation of mTOR via AMPK. In this case, autophagy was considered to be an attempt of cells to survive, as BECN1 shRNA increased cell death by ADI-PEG20. The authors hypothesized that ADI-PEG20-treated cells ultimately died once autophagy machinery could no longer replenish the pool of arginine. Inhibition of survival signals by MEK or blockade of lysosomal function with chloroquine enhanced ADI-PEG20 cytotoxicity in vitro (Savaraj et al., 2010).

A strict dependency of melanoma cells has been recently identified for the essential amino acid leucine (Sheen et al., 2011). Yet again, these cells defied preconceived notions on the wiring of autophagy programs. In an elegant study, the Sabatini laboratory has recently reported that low leucine levels can kill melanoma cells under conditions in which normal melanocytes remained viable (Sheen et al., 2011). However, in contrast to arginine, leucine defects promoted an apoptotic death because TORC1 remained active and autophagy was not properly induced. A main contributor to the inability of low leucine to activate autophagy was the Ras-MEK pathway. Consequently, MEK blockers favored autophagy and acted in a protective manner against leucine defects. Why MEK inhibition would favor killing by arginine deiminase but inhibit death upon leucine deprivation needs further investigation.

Given the complexity of MEK and mTOR signaling cascades, a key pending question is how to exploit the current knowledge on amino acid dependency to design effective treatments. Controling diet will be unlikely to suffice. Thus, dietary depletion of leucine did not significantly reduce melanoma growth in xenograft models (Sheen et al., 2011). A tractable liability may again be lysosome. Thus, inhibition of autophagy (and lyosomal activity) with chloroquine can promote cell death both when autophagy is partially *on* [arginine deaminase (Savaraj et al., 2010)] and when it is partially *off* [leucine deprivation (Sheen et al., 2011)]. A variety of experimental studies support the therapeutic use of chloroquine in combination studies (see below).

Hypoxia, autophagy and melanoma progression

Hypoxia has also been widely described as a potent tumor-associated proautophagy modulator (Brahimi-Horn et al., 2010). In melanoma, the correlation between autophagy and hypoxia has been better described in mouse models. In xenografts driven by B16-F10 cells, LC3B (visualized with an antibody from Agent, CA, USA) accumulated in pimonidazole-positive tumor areas. This induction of LC3B was not just a passive effect of tumor growth, as Beclin 1 silencing resulted in a significant induction of cell death and a concomitant decrease in tumor growth (Noman et al., 2011). Interestingly, B16-F10 cells grew better as subcutaneous xenografts in Beclin1 heterozygous mice than in their wild-type counterparts. Moreover, B16-F10 cells had an enhanced tumorigenic potential if the Beclin $1^{+/-}$ mice were placed under hypoxia, in part because of an increased intratumoral angiogenesis (Lee et al., 2011). Altogether, these results illustrate the impact of autophagy in the neoplastic cells and in their surrounding stroma.

In human melanoma a detailed characterization of autophagy influx and efflux has not been performed in the context of hypoxia. Nevertheless, the presence of high BECN1 correlating with high HIF1α and LDH5 in some specimens is indicative of low oxygen effects on autophagy programs. This hypothesis is further supported by the observation that high BECN1 was linked to an increased rate of early deaths of melanoma patients (Sivridis et al., 2011).

Chemo- and radioresistance mediated by autophagy programs

An increasing body of evidence supports the induction of autophagy-associated proteins in response to a variety of therapeutic agents. Experimental systems, drugs used and the corresponding impact on autophagy mediators are summarized in Table 1. Most of these studies focused on limited established cell lines and detailed kinetic analyses of autophagosome formation, and autolysosome degradation have yet to be performed. Nevertheless, lipidation of LC3 and formation of LC3 foci are emerging as fail-safe mechanism(s) to maintain cellular viability. Protective roles of autophagy have been described in cultured melanoma cells in response to inducers of endoplasmic reticulum stress, such as tunicamycin or 2-deoxy-D-glycose (Hersey and Zhang, 2008; Xi et al., 2010). mTOR pathway downregulation by Compound C (Vucicevic et al., 2010) or by the proton pump inhibitor esomeprazole (Marino et al., 2011) also favored pro-survival autophagy. The triterpenoid Curcubitacin B can also promote a moderate LC3B II increase in B16 cells, at least in part via JNK (Ouyang et al., 2011). The triterpenoid silibinin, in turn, promoted an LC3B II accumulation in A375-S2 cells via p53 suppression and nuclear factor (NF)-κB activation (Jiang et al., 2011).

The impact of autophagy in the context of radioresistance has been suggested recently to be an effect of the immunophilin FKBP51. This compound elicits autophagosome formation while inhibiting apoptosis in a NF-κB-dependent manner (Romano et al., 2010). Regarding photodynamic therapy, 1 μM doses of the UVA-activated photosensitizer hypericin also induced protective autophagic vesicles, as determined by electron microscopy in the pigmented UCT Mel-1 and unpigmented A375 cells (Davids et al., 2009). In these systems where autophagosome formation is induced to eliminate damaged organelles, clear unfolded proteins, or provide nutrients under situations of stress, blockade of lysosomal function may provide a potent alternative to promote cell death (Ma et al., 2011; Sheen et al., 2011).

Autophagy modulators as viable drug targets?

Although additional studies are needed to dissect the wiring of autophagy pathways in vivo, it is clear that this program constitutes a key homeostatic mechanism in melanoma cells. True to the concept that 'too much'

can be as harmful as 'too little', activation of autophagy leading to the depletion of essential intracellular compartments holds also the potential to allow tumor self-degradation (Dikic et al., 2010; Hoyer-Hansen and Jaattela, 2008; Mathew et al., 2007). In this context, autophagy was initially referred to as Type II cell death (Type I corresponding to apoptosis). Nevertheless, as late stages of apoptosis can present with the generation of autophagosomes and activation of lysosomal proteases, an active debate in the oncology field centers on whether tumor cells can die *by* autophagy or *with* autophagy features (Kroemer and Levine, 2008). This controversy is further fueled by pleiotropic roles of autophagy modulators on the apoptotic machinery, and vice versa (Janku et al., 2011).

In cultured melanoma cells (shown for UCT Mel-1 and A375), high doses of hypericine (3 μM) can lead to a massive generation of endo-lysosomal vesicles followed by cell demise (Davids et al., 2009). Cysteamine in turn has been shown to sensitize B16 melanoma cells to doxorubicin by favoring autophagosome influx while blunting the generation of autolysosomes (Wan et al., 2010). Chemosensitizing effects associated with aberrant autophagosomes in melanoma cells have also been identified in response to cyclopamine (A. Checinksa and M. Soengas, in preparation). Nevertheless, an efficient exploitation of cytotoxic activities of autophagy is likely to require a situation of sustained cellular stress where the apoptotic machinery is also engaged. In this context, apoptotic caspases can be activated before, concomitantly with or after autophagy features are initiated. Examples of these three situations have been described in response to the *Polygonatum cyrtonema* lectin, oncolytic Herpes virus derivatives and dsRNA-based nanocomplexes (Colunga et al., 2010; Liu et al., 2009; Tormo et al., 2009a).

Comparative mechanistic analyses of pro-survival and pro-death inducers of autophagy revealed differences in the induction of caspases not only between these two types of compounds, but also on the pathways leading to autolysosomal generation. For example, rapamycin induced a transient accumulation of small LC3 foci reflecting classical autophagosomes. These were efficiently degraded after fusion with lysosomes and no effects on viability were observed (Tormo et al., 2009b). Instead, as mentioned above, BO-110, bioavailable long dsRNA packed with cationic nanoparticles for cytosolic delivery, promoted a massive mobililization of large endocytic vesicles (marked by the presence of the late endosomal protein RAB7). These endosomes recruited LC3 to form endosome–autophagosome hybrids (amphisomes) prior to lysosomal fusion (see examples of these structures in Figure 1). Persistent waves of amphisome generation and resolution ultimately resulted in tumor self-autodegradation (Tormo et al., 2009a,b). cDNA arrays revealed broad changes in the transcriptome profile of BO-110-treated cells (Tormo et al., 2009a). These included the activation of multiple interferon-associated genes and a variety of cytokines with proautophagic effects (Levine et al., 2011). RNA interference led to the identification of the RNA helicase MDA5 as the main sensor of BO-110, and a main effector of cell death, at least partly via the proapoptotic protein NOXA (Alonso-Curbelo and Soengas, 2009; Tormo et al., 2009a). These were rather unexpected results, as although MDA5 was best known for its functions in NF-κB and interferon (IFN) induction in immune cells (Kato et al., 2006; Takeuchi and Akira, 2008), no previous links to NOXA and autophagy had been reported in cancer. Importantly, the dual activation of autophagy and apoptosis by BO-110 was found in a broad spectrum of cell lines recapitulating the main defects in BRAF, NRAS, PTEN or p53. In addition, particularly effective was the inhibition of cutaneous melanomas driven by genetically modified mice (Tyr::NRAS^{Q61K}; INK4$^{a/ARF-/-}$) or lung xenografts induced by aggressive human melanoma cells (Tormo et al., 2009a). The efficacy and lack of measurable secondary effects of BO-110 in vivo point to dsRNA sensors as alternative targets for drug development in melanoma (Kim and Ronai, 2009). Of note, IFN-γ-driven responses may also favor autodegradative mechanisms in melanoma by agonists of Toll-like receptor (TLR)-4 and 9 (Yan et al., 2011), which, like MDA5, belong to the broad family of pathogen recognition receptors.

Tumor versus stroma: autophagy in immunotherapy

In addition to its classical roles in cell homeostasis, autophagy is emerging as a key modulator of inflammation and immune responses, which can impinge on tumor progression and drug response at multiple levels (Levine and Deretic, 2007; Levine et al., 2011; Virgin and Levine, 2009). Deregulated autophagy can have drastic effects on the number of B cells, CD4$^+$ T cells and CD8$^+$ T cells and thus determine the efficacy of immunosurveillance mechanisms (Levine et al., 2011). Interferon and inflammatory cytokines can be positively and negatively influenced by core autophagy factors, both in immune cells and in malignant compartments. Moreover, the crosstalk between cancer cells and antigen-presenting cells may also be influenced by core autophagy proteins (Levine et al., 2011; Virgin and Levine, 2009). This area of research remains relatively unexplored in melanoma but recent studies on murine cellular systems augur well for many fronts.

Using B16-F10 cells, tumor-generated autophagosomes were found to serve as major histocompatibility complex (MHC)-II antigen carriers for cross-presentation by DC to T cells. Cross-presentation was thus inhibited by shRNAs against BECN1 or ATG12, and was favored by autophagy inducers (such as rapamycin). Intriguingly, cross-presentation was enhanced by the blockade of

lysosomal function with NH_4Cl (presumably because antigen half-life was extended) (Li et al., 2008). Genetic or pharmacological inhibition of early or late autophagy events reduced MHC-I antigen expression induced by IFN-γ in melanoma cells (Li et al., 2009). Intriguingly, melanosome autophagy by the depigmentation agent monobenzone also favored the activation of dendritic cells and the subsequent attack of melanoma cells cytotoxic T cells (van den Boorn et al., 2011a,b). Defining the physiological impact of autophagosome and autolysosome formation in MHC-I and MHC-II antigens in human melanoma tumors will likely provide new insights into melanoma pathogenesis.

Open questions

As mentioned above, autophagy-associated phenotypes had been already described in melanoma patients in the early 1980s. After a lag phase that has been common in other pathologies, the field is now ripe for discoveries that may significantly change the way we understand and, likely, treat this disease. A series of areas of research that in our opinion deserve particular attention are presented below.

What is the molecular basis underlying the heterogeneous expression of autophagy factors in human melanocytic specimens?

As mentioned above, a puzzling inter- and intratumor variability has been observed in the expression and localization of core autophagy factors (particularly BECN1 or LC3B). Does this heterogeneity reflect technical artifacts or distinct cellular subpopulations with different stemness features and/or susceptibilty to cellular stress? Answer to these questions will require a concerted effort by pathologists, dermatologists and oncologists. Standardization of reagents and staining conditions is needed. Similarly, sufficiently large cohorts of clinically annotated specimens should be analyzed before definitive statements can be made on the physiological role of autophagy in melanoma initiation and progression. Adding genome-wide analyses to the repertoire of studies in melanoma could also be very informative, considering previously described genetic alterations in BECN1 and other autophagy genes in other cancers and pathological situations (Lauriola et al., 2011; Rioux et al., 2007; Walter et al., 2011; Yue et al., 2003). A point to consider is accumulating evidence regarding melanoma cell plasticity or phenotype switch (Eichhoff et al., 2011; Held and Bosenberg, 2010; Hoek and Goding, 2010; Roesch et al., 2010; Zipser et al., 2010). Ultimately, kinetic analyses of autophagosome formation and resolution should be addressed in physiologically relevant three dimensional settings. Specialists in intravital microscopy techniques in genetically modified mice may find here a new niche for research.

How is autophagy induced in vivo?

Hypoxia, DNA damage, ER stress, nutrients or amino acid deprivation have been all been linked to melanoma autophagy, but with various outcomes depending on the model used (Hersey and Zhang, 2008; Savaraj et al., 2010; Sheen et al., 2011). Comparative analyses of benign and malignant melanocytic cells may help to dissect signaling cascades in these two cell populations, and thus, identify potential targets for therapeutic intervention. Understanding the myriad of proteins, metabolites, extracellular factors and extrinsic perturbations that may affect autophagy influx and efflux may be the perfect challenge for computational or systems biologists. Transgenic GFP-LC3 mice (Mizushima et al., 2004), and genetically engineered mouse strains with conditional and inducible defects in *Beclin 1, Atg4, Atg5, Atg7, Atg16* and other autophagy factors are available (Hara et al., 2006; Komatsu et al., 2005; Marino et al., 2007; Qu et al., 2003; Saitoh et al., 2008; Yue et al., 2003). It would be very interesting to define what is the specific contribution of each of these genes to nevi generation and melanoma formation, for example in crosses with the Try::CreERT2:-$BRAF^{CA}$; $PTEN^{fl/fl}$ recently described by the groups of McMahon, Bosenberg, Marais and collaborators (Dankort et al., 2009; Dhomen et al., 2009).

What is the nature of the autophagosome cargo in melanoma cells?

As mentioned before, autophagy was first reported as a non-selective quality control mechanism for bulk degradation of cytosolic structures (Yang and Klionsky, 2010). However, selective autophagy has also been described, for example, for the clearance of mitochondrial subsets and specific cellular factors (Dikic et al., 2010; Geisler et al., 2010). In melanoma, this field remains virtually unexplored. This applies as well to other degradative processes (i.e. chaperone-mediated autophagy, microautophagy and proteasome-mediated degradation).

Does autophagy display pro- or antitumor effects in melanoma genesis?

This is a recurrent theme in all tumor systems where autophagy has been studied (Galluzzi et al., 2008; Hippert et al., 2006; Hoyer-Hansen and Jaattela, 2008; Janku et al., 2011; Kirkin and Dikic; Mathew et al., 2007). Tumor-suppressive roles of autophagy have been described in the context of oncogene-driven senescence (Young et al., 2009). Whether this is the case in senescent nevus cells has yet to be determined. It would also be interesting to determine whether autophagy factors contribute to metabolic reprogramming of melanoma cells, as has been described in other systems (Eng and Abraham, 2011).

How are tumor–stroma interactions modulated by and responding to autophagy inducers?

It has long been known that melanoma cells remodel and exploit their microenvironment to favor their sur-

vival. The impact of autophagy on MHC-I, MHC-II antigens and a variety of inflammatory cytokines (Levine et al., 2011) should be considered in the context of the classical immunoresistance of aggressive melanomas.

Do autophagy programs represent feasible targets for drug development?

From a translational perspective, this is a (multi)million dollar question. This is not just a figure of speech: BRAF-MEK inhibitors and CTL4-immunotherapy currently in active clinical use have the potential of impinging directly or indirectly in autophagy (Levine et al., 2011; Maddodi et al., 2010; Sheen et al., 2011). The synergistic effects of chloroquine and amino acid deprivation, or the various anticancer agents mentioned above, suggest that, at the minimum, autophagosme–lysosome fusion events can constitute points of vulnerability in melanoma cells. In fact, the anti-melanoma activity of chloroquine was already noted in the early 1990s. These potential therapeutic features of chloroquine were linked to the high affinity of this compound for melanin (Inoue et al., 1993). The results of ongoing clinical trials of hydroxychloroquine in combination with temozolomide in patients with advanced solid tumors (NCT00714181) are awaited with great interest. An alternative to lysosomal inhibition (which may affect viability of metabolically active cells) is a targeted activation of dual programs of apoptosis and exacerbated autophagy. In addition to chloroquine and derivatives, large efforts are being dedicated to the design and validation of alternative pharmacological blockers of autophagy (Fleming et al., 2010). These efforts will be aided by recent reports on the three dimensional structure of protein complexes with key roles in autophagy (Hanada and Ohsumi, 2005; Matsushita et al., 2007). Moreover, unbiased screenings are identifying compounds that maintain lysosomal function (maintaining the viability of metabolically active cells), but activating dual programs of apoptosis and exacerbated autophagy. Agents that mobilize the endosomal machinery in a tumor cell-specific manner may represent a new class of therapeutic agents (Tormo et al. 2009a). However, deciding on whether to interrupt or overactivate lysosomal activity will require a detailed analysis on the 'autophagic competency' of the tumors intended for treatment.

Concluding remarks

There is no life without eating (at least for multicellular organisms). Research is now demonstrating that tumors, and melanomas are no exception, are particularly gluttonous. Not only do they remodel their extracellular environment to ensure proper vascularization and nutrient supply, they spend an important fraction of their time and energy in self-degradative processes. The challenge is now how to translate the emerging information on autophagy programs into bedside use. The road ahead is exciting although not trivial. The endogenous stimuli that activate autophagy programs in melanocytic cells, the specific wiring of autophagy programs in normal and tumor cells in vivo, the influence of stromal compartments and, ultimately, the role of lysosomal-dependent degradation in drug response are some of many aspects that still have to be defined. Answering these questions will likely require concerted efforts between basic and clinical researchers, involving academic–industrial partnerships. We hope this review will encourage melanoma researchers and experts from other areas to join in, and help moving this field forward towards effective and durable antimelanoma responses.

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