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Crosstalk Between Apoptosis and Autophagy : BH3 Mimetics Activate Multiple Pro-Autophagic Pathways

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CANCÉROLOGIE

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FACULTY OF MEDICINE**

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CROSTALK BETWEEN APOPTOSIS AND AUTOPHAGY: BH3 MIMETICS ACTIVATE MULTIPLE PRO-AUTOPHAGIC PATHWAYS

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Discipline : **Biochimie, Biologie Cellulaire et Moléculaire**

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pour l'obtention du grade de
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dedicated to my parents
Zaineb Zahur Malik & Zahur Ahmad Malik

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SUMMARY

Macroautophagy (here after referred to as “autophagy”) is a conserved catabolic pathway that culminates in the degradation of old/damaged organelles, long-lived/aggregated proteins and portions of the cytosol. It consists in the stepwise engulfment of substrate into characteristic double-membraned “autophagosomes”, which mature by fusing with lysosomes to generate “autophagolysosomes” leading to the degradation of their luminal contents for metabolic recycling to maintain cellular homeostasis. Although autophagosomes are often found in the cytoplasm of dying cells, coining the term ‘autophagic cell death’, autophagy mostly subserves a cytoprotective function, and its suppression can precipitate the demise of stressed cells. Moreover, the absence of autophagy is frequently observed in many cancers and neurodegenerative diseases.

Beclin 1, a *bona fide* tumour suppressor, is the key autophagy regulatory protein that participates in autophagosome nucleation. Beclin 1 interacts with the anti-apoptotic proteins from the Bcl-2 family (Bcl-2, Bcl-X_L). In fact, Beclin 1 is a BH3-only protein that can interact with the BH3 receptor domain contained within Bcl-2 and its homologues and this interaction has an inhibitory check on autophagy. The disruption in this inhibitory interaction of Beclin 1 with Bcl-2 (or its close homologues) is an essential step for the initiation of the process of autophagy. Some pharmacological agents such as ABT737, referred to as ‘BH3 mimetics’, occupy the BH3-binding grooves to competitively disrupt the inhibitory interaction between Beclin 1 and Bcl-2/Bcl-X_L allowing Beclin 1 to maintain the class III phosphatidylinositol-3-kinase activity of Vps34 for the phagophore formation.

Autophagy is a complex process that is regulated by multiple protein complexes beyond that organized around Beclin 1. Thus, energy sensors including AMP-dependent kinase (AMPK), mammalian target of rapamycin (mTOR), Sirtuin1 (SIRT1) as well as stress-integrating pathways such as those involving the inhibitor of NF- κ B (I κ B) kinases (IKK) and the tumour suppressor protein p53, all have a major impact on the regulation of autophagy. In most of the paradigms of autophagic stimulation, they all seem to act upstream of the dissociation of Beclin 1-Bcl-2. Based on these premises, we decided to measure the possible effects of ABT737 on these multiple distinct autophagy regulators, i.e., whether artificial dissociation of the Beclin 1-Bcl-2 complex by BH3 mimetics would induce autophagy according to our expectation. Our results reveal that ABT737 stimulates multiple pro-autophagic pathways to be optimally efficient, thereby illustrating the complex inter-connections among autophagy-regulatory proteins. To our surprise, it turned out that inhibition of SIRT1, IKK, p53 and AMPK as well as

artificial activation of mTOR, all reduced autophagy induction by BH3 mimetics. These results place SIRT1, AMPK/mTOR, HDM2 and IKK downstream of the dissociation of the Beclin 1-Bcl-2 complex.

This study advocates that BH3 mimetics trigger multiple autophagy-stimulatory pathways maybe due to the high degree of connectivity that exists among autophagy-regulatory protein complexes meaning that a specific effect on the Beclin 1-interactome might affect other nodes in the autophagy-controlling network. These pathways cannot follow a linear hierarchy and rather must be interconnected in a complex circuitry, in which stimulation of autophagy by physiological triggers (such as starvation or organellar stress) induce an ensemble of intimately linked changes that are coupled to each other in positive feed forward loops. In this hypothetical scenario, the network properties of the system would constitute an indissociable ensemble that composes the autophagic switch. Understanding the molecular and functional properties of this switch by systems biology methods would then constitute the next frontier in autophagy research.

Key words: autophagy; Beclin 1; Bcl-2 family protein; BH3 mimetics; ABT737

RÉSUMÉ

La macro-autophagie (ci-après dénommée «autophagie») est une voie catabolique conservée dans l'évolution permettant la dégradation des organites endommagés ou vieux, des protéines à longue durée de vie ou agrégées et des portions du cytosol. L'autophagie débute par la séquestration de substrats dans des vacuoles caractéristiques à double membranes appelées «autophagosomes», qui après maturation fusionnent avec les lysosomes pour générer des «autolysosomes», conduisant à la dégradation de leur contenu luminal pour le recyclage métabolique afin de maintenir l'homéostasie cellulaire. Bien que les autophagosomes soient souvent retrouvés dans le cytoplasme des cellules mourantes, d'où le terme «mort cellulaire autophagique», l'autophagie a généralement une fonction cyto-protective, et sa suppression peut précipiter la mort des cellules stressées. En outre, l'absence d'autophagie est fréquemment observée dans de nombreuses pathologies incluant les cancers et les maladies neurodégénératives.

Beclin 1, un suppresseur de tumeur, est une protéine clé dans la régulation de l'autophagie qui participe à la nucléation de l'autophagosome. Beclin 1 interagit avec les protéines anti-apoptotiques de la famille Bcl-2 (Bcl-2, Bcl-X_L). Beclin 1 est une protéine «BH3-only» pouvant interagir avec le site de fixation au domaine BH3 présent dans la protéine Bcl-2 et de ses homologues et cette interaction inhibe l'autophagie. La perturbation dans l'interaction inhibitrice de Beclin 1 avec Bcl-2 (ou de ses homologues) est une étape essentielle pour l'initiation du processus d'autophagie. Certains agents pharmacologiques tels qu'ABT737, appelés «BH3 mimetics», occupent le site de fixation du domaine BH3 de façon compétitive pour perturber l'interaction inhibitrice entre Beclin 1 et Bcl-2/Bcl-X_L. Ceci permet à Beclin 1 de maintenir l'activité classe III phosphatidylinositol-3-kinase de Vps34 pour la formation du phagophore.

L'autophagie est un processus finement réglementé par de nombreux complexes protéiques. Les senseurs de la charge énergétique comme l'AMP-dépendante kinase (AMPK), la cible mammalienne de la rapamycine (mTOR), la Sirtuin1 (SIRT1) ou les voies d'intégration du stress telles que celles impliquant l'inhibiteur des kinases NF-κB (IκBα) (IKK) et le suppresseur de tumeur p53, ont tous un impact majeur dans la régulation de l'autophagie. Dans de nombreux paradigmes de stimulation autophagique, ils semblent tous agir en amont de la dissociation Beclin 1-Bcl-2. Sur la base de ces prémisses, nous avons décidé de mesurer les effets possibles d'ABT737 sur ces multiples régulateurs de l'autophagie, à savoir, si la

dissociation artificielle du complexe Beclin 1-Bcl-2 par les *BH3 mimetics* induirait l'autophagie selon nos attentes. Nos résultats révèlent qu'ABT737 stimule plusieurs voies pro-autophagiques pour obtenir une efficacité optimale, ce qui met en évidence l'interconnexion complexe entre les protéines régulatrices de l'autophagie. A notre grande surprise, il s'est avéré que l'inhibition de la SIRT1, IKK, p53 et AMPK ainsi que l'activation artificielle de la protéine mTOR, toutes réduisent l'induction de l'autophagie par *BH3-mimetics*. Ces résultats placent la SIRT1, AMPK / mTOR, HDM2 et IKK en aval de la dissociation du complexe Beclin 1-Bcl-2.

Cette étude démontre que les *BH3-mimetics* activent des voies multiples de stimulation de l'autophagie, peut-être en raison du degré élevé de connectivité qui existe entre les complexes protéiques de régulation de l'autophagie. Cela signifie qu'un effet spécifique sur l'interactome de Beclin 1 peut affecter d'autres voies dans le réseau du contrôle autophagique. Ces voies ne semblent pas suivre une hiérarchie linéaire, mais doivent être plutôt interconnectées dans un circuit complexe dans lequel la stimulation de l'autophagie par des déclencheurs physiologiques (tels que la carence en nutriments ou le stress des organites) induit un ensemble de changements intimement liés et impliqués dans une boucle de régulation positive. Dans ce scénario hypothétique, le réseau du système constituerait un ensemble indissociable composant le «*autophagy switch*». Comprendre les propriétés moléculaires et fonctionnelles de ce «*switch*» par des méthodes de biologie des systèmes constituerait alors la prochaine frontière dans la recherche sur l'autophagie.

Mots clé: autophagie; Beclin 1; Bcl-2 family protéine; les BH3 mimetics; ABT737

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA Carboxylase
ADP	Adenosine DiPhosphate
AIDS	Acquired Immune Deficiency Syndrome
AIF	Apoptosis-Inducing Factor
AMBRA1	Activating Molecule in Beclin 1-Regulated Autophagy 1
AML	Acute Myeloid Leukaemia
AMP	Adenosine MonoPhosphate
AMPK	AMP-dependent protein Kinase
APAF1	Apoptotic Peptidase Activating Factor 1
APO-1	Apoptosis antigen 1
ATF6	Activating Transcription Factor 6
<i>atg</i>	AuTophagy-related gene
Atg	AuTophagy-related protein
ATP	Adenosine TriPhosphate
AV	Autophagic Vacuole
Bad	Bcl-2-Associated Death promoter
BafA1	Bafilomycin A1
Barkor	Beclin-1-Associated autophagy-Related Key Regulator
Bax	Bcl-2-Associated X protein
Bcl-2	B Cell Lymphoma 2
Bcl-X _L	B-Cell Lymphoma-eXtra Large
BH	Bcl-2 Homology domain
Bid	BH3 Interacting-domain Death agonist
Bif-1	Bax-Interacting Factor-1
Bik	Bcl-2-Interacting Killer
BimEL	Bcl-2-like protein 11 (also known as BCL2L11)
BIR	Baculovirus IAP Repeat
Bmf	Bcl2 Modifying Factor
BNIP	Bcl2/adenovirus E1B 19 kd-Interacting Protein
BSA	Bovine Serum Albumin
CaM	CalModulin
CaMKK β	CalModulin Kinase Kinase β
CAMK β	CalModulin (CaM)-dependent Kinase β
cAMP	cyclic Adenosine MonoPhosphate
CCD	Coiled-Coil Domain
CD95	Fas receptor (also known as APO-1)
cisPt	CisPlatin
CMA	Chaperone-Mediated Autophagy

CNS	Central Nervous System
COX	Cytochrome c OXidase
CytC	Cytochrome C
DAPK	Death-Associated Protein Kinase
DED	Death Effector Domain
DIABLO	Direct IAP-Binding protein with Low PI
DiOC6(3)	3,3'-Dihexyl OxacarboCyanine Iodide
DISC	Death Inducing Signalling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	DeoxyriboNucleic Acid
DR	Death Receptor
DRAM	Damage-Regulated Autophagy Modulator
EBSS	Earle's Balanced Salt Solution
Egl1a	Programmed cell death activator in <i>C elegans</i>
eIF2 α	eukaryotic Initiation Factor 2 α
EndoG	Endonuclease G
ER	Endoplasmic Reticulum
FADD	Fas-Associated Death Domain
FIP200	<i>RB1CC1 gene product</i>
FK506	Tacrolimus, an immunosuppressant
FKBP12	FK506-Binding Protein 12, immunophilin family protein
FLIP	FLice Inhibitory Protein
FOXO3	Forkhead box O3 (also known as FOXO3a)
GABA	γ -AminoButyric acid
GABARAP	GABA(A) Receptor-Associated Protein, paralogue of LC3B
GAIP	G α -Interacting Protein
GAP	GTPase-Activating Protein
GAPDH	GlycerAldehyde-3-Phosphate DeHydrogenase
GATE-16	Golgi-associated ATPase Enhancer of 16 kDa
GCCR	Glycerol 3-phosphate Cytochrome-C Reductase
GDP	Guanosine DiPhosphate
GFP	Green Fluorescent Protein
GFP-LC3	GFP-LC3 fusion protein
GSK-3	Glycogen Synthase Kinase 3
GTP	Guanosine TriPhosphate
G β L	G-protein β -subunit-Like protein
HIF-1	Hypoxia-Inducible Factor-1
HIV	Human Immunodeficiency Virus
HMD2	Human orthologue of MDM2

HMGB1	High-Mobility Group Box 1
hsc70	Heat Shock Cognate 71 kDa protein
HTRA2	High Temperature Requirement protein A2
hVps34	Human Vacuolar Protein Sorting 34
IAP	Inhibitor of Apoptosis Protein
IKK	I κ B Kinase
IL-1	InterLeukin 1
IMS	Mitochondrial Intermembrane Space
IP3	Inositol 1,4,5-triPhosphate
IP3R	IP3 Receptor
IRE1	Inositol Requiring Enzyme 1
I κ B α	Inhibitor of κ B α
JNK	c-Jun N-terminal protein Kinase
LAMP1	Lysosome-Associated Membrane Protein 1
LAMP2	Lysosome-Associated Membrane Protein 1
LC3	microtubule-associated protein 1 Light Chain 3
LKB1	Liver Kinase B1
MAP1B	Microtubule-Associated Protein 1B
MAPK	Mitogen-Activated Protein Kinase
MAPK	Mitogen-Activated Protein Kinase
Mcl-1	Myeloid Cell Leukemia sequence-1
MDM2	Murine Double Minute 2
MDS	MyeloDysplastic Syndrome
MEFs	Mouse Embryonic Fibroblasts
mLST8	target of rapamycin complex subunit LST8 (also known as G β L)
MMP	Mitochondrial Membrane Permeabilization
MOMP	Mitochondrial Outer Membrane Permeabilization
mTOR	Mammalian Target Of Rapamycin
NAD ⁺	Nicotinamide Adenine Dinucleotide
NAF-1	Nutrient-deprivation Autophagy Factor-1
NASH	NonAlcoholic SteatoHepatitis
NEMO	NF- κ B Essential MOdifier
NF	Nutrient Free
NF- κ B	Nuclear Factor Kappa B
NF1	NeuroFibromin 1
NMR	Nuclear Magnetic Resonance
NOX	NADH Oxidase
NOXA	a BH3-only pro-apoptotic protein
OM	Mitochondrial Outer membrane

p14ARF	Alternate Reading Frame, homologue of murine p19ARF
p150	homolog of serine/threonine kinase Vps15
p300	E1A-binding protein, a histone acetyltransferase
p70S6K	ribosomal Protein S6 Kinase of 70 kDa
PAS	Phagophore Assembly Site
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PE	Phosphatidyl-Ethanolamine
PERK	PKR-like Eukaryotic initiation factor 2 α Kinase
PI	Propidium Iodide
PI3K	phosphatidyl-Inositol 3-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKR	double-stranded RNA-activated Protein Kinase
PRAS40	Proline-Rich AKT Substrate 40 kDa
PS	phosphatidyl-serine
PtdIns	Phosphatidyl-Inositol
PtdIns(3,4,5)P3	Phosphatidyl-Inositol-3,4,5-trisPhosphate
PtdIns(3)K	Phosphatidyl-Inositol 3-Kinase
PtdIns(3)P	Phosphatidyl-Inositol-3-Phosphate
PtdIns(4,5)P2	Phosphatidyl-Inositol 4,5-bisPhosphate
PTEN	Phosphatase and Tensin homolog
PTPC	Permeability Transition Pore Complex
PUMA	p53 Upregulated Modulator of Apoptosis
QCCR	Quinol Cytochrome C Reductase
Rab7	a ras-related protein (also known Rab7a)
Raptor	Regulatory-Associated Protein of mTOR
RB1CC1	RB1-inducible Coiled-Coil 1 (also known as FIP200)
Rheb	Ras Homolog Enriched in Brain, a small GTPase
Rictor	Iapamycin-Cnsensitive companion of mTOR
RIP	Receptor-Interacting serine-threonine kinase
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
RUBICON	RUN domain and cysteine-rich domain containing Beclin-1 interacting
SAPK	Stress-Activated Protein Kinase, a c-Jun N-terminal kinase
SAR	Structure-Activity Relationships
SCCR	Succinate Cytochrome C Reductase
SDS-PAGE	Sodium DodecylSulfate-PolyAcrylamide Gel Electrophoresis
SEM	Standard Error of the Mean

SIN1	SAPK-Interacting protein 1
siRNA	small-interfering RNA
SIRT1	Sirtuin 1
siUNR	Unrelevant control siRNA
SKD1	Suppressor of K ⁺ transport Defect 1
Smac	Small Mitochondria-derived Activator of Caspase
smARF	Short Mitochondrial ARF
SNARE	Soluble N-ethylmaleimide-sensitive factor-attachment protein Receptor
t-Bid	Truncated Bid
Tab	TAK1- Binding protein
TAK1	TGFβ-Activated Kinase 1
TBS	TRIS buffered saline
TEM	Transmission Electron Microscopy
TGFβ	Transforming Growth Factor β
TIGAR	TP53-Induced Glycolysis and Apoptosis Regulator
TM	Trans-Membrane
TNF	Tumour Necrosis Factor
TNFR	TNF Receptor
TOR	Target Of Rapamycin
TORC1	TOR Complex 1
TORC2	TOR Complex 2
TRADD	TNFR type 1-Associated Death Domain protein
TRAF 2	TNF Receptor Associated Factor 2
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TSC	Tuberous Sclerosis Complex
Ub	Ubiquitination
UCMD	Ullrich Congenital Muscular Disorder
ULK	Unc-51-Like Kinase
UPR	Unfolded Protein Response
UV	UltraViolet
UVRAG	UV irradiation Resistance-Associated tumour suppressor Gene
Vps	Vacuolar Protein Sorter
WT	Wild Type
XMEA	X-linked Myopathy with Excess Autophagy
Δψ _m	Mitochondrial transmembrane potential
4E-BP1	Eukaryotic initiation factor 4E Binding Protein 1 (known as PHAS-1)

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1

INTRODUCTION

In multicellular organisms, the control of tissue and organ homeostasis is accomplished through careful, meticulously coordinated and mutually interlinked processes of survival, proliferation and death working at single cell levels. Any deviation from the normal control of these processes in their mutual regulation can be a basis of the etiological determinant of a variety of human pathologies including cancer, diabetes, autoimmunity, immunodeficiency, inflammatory, neurodegenerative diseases and many others. Thus, a better understanding of the molecular mechanisms that regulate cell death and survival, equally in physiological and pathological scenarios, is crucial and imperative for the development of novel therapeutic tools. These highly regulated and coordinated processes encompass the entire life span of multicellular organisms, right from embryonic and post-embryonic development (their fundamental role during organogenesis, for example) to adult life (their critical role in numerous situations counting the regulation of tissue homeostasis, counteracting the cellular stress conditions, the elimination of toxic/harmful/infectious agents and numerous others). Apoptosis, a well established *bona fide* programmed cell death process, and autophagy, an emerging cell survival process, turns out to be the key mediators in most of these scenarios. Apoptosis is extensively studied and well documented process of programmed cell death whereas, the understanding of autophagic regulation is its infancy and its proposed manifold links to cellular growth, proliferation, apoptosis and senescence are on the verge of further exploration. Even though a proposed relationship between autophagy and cell death has been suggested since long, the recognition of the molecular players involved in the machineries of both apoptosis and autophagy has resulted in re-defining the interactions between these pathways in a context-dependent manner. Recent advances have provided further insights into the mutual control and relationship between autophagy and apoptosis, describing an intricate and complex crosstalk between these two processes. Interestingly, and in contrast to the preliminary description of “autophagic cell death”, accumulating evidence points to the antagonistic roles. Most commonly, the categorization of cell death modalities is based on morphological criteria. The morphological features defining apoptosis are chromatin condensation (pyknosis) along with rounding and reduction of cell volume, nuclear fragmentation (karyorrhexis), ultimately formation of apoptotic bodies (resulting from progressive cellular condensation and budding), and eventually engulfment by resident phagocytes (*e.g.*, epithelial cells or fibroblasts). ‘Autophagic cell death’ is morphologically defined (especially by transmission electron microscopy) as a type of cell death that occurs in the absence of the features of apoptotic cell death but is accompanied by a massive presence of autophagosomes, which are ultimately cleared by lysosomal compartment of the cell. In

physiologically relevant conditions, it is well established that the expression ‘autophagic cell death’ likely corresponds to cell death with autophagic features rather than to cell death by autophagy [1-3]. In most of these systems autophagy is induced/up-regulated in reaction to the death instigating signals and thereby to counteract death-inducing mechanisms. Continued investigation into the crosstalk between apoptosis and autophagy will elucidate the mechanisms controlling the equilibrium between survival and death processes under both normal physiological conditions and pathological conditions including cancer.

1.1 Apoptosis

1.1.1 Historical Notions and Introduction

Apoptosis is an evolutionary conserved multi-step and multi-pathway programmed cell death process that ignited unparalleled interests in this field of biological science after its discovery. German scientist Carl Vogt was the first to describe the principle of apoptosis in 1842 [4]. Lockshin and Williams used the concept of “programmed cell-death” for the first time in 1964 in relation to insect tissue development [5]. Kerr and colleagues coined the word “Apoptosis” in 1972 to describe a mechanism for committing suicide to differentiate such a fastidiously controlled and regulated cell death type from necrosis (which was still considered as a merely accidental instance of cell death) [6]. In ancient Greek, the word “*apoptein*” means “to fall away off” and describe the "dropping off" or "falling off" of petals from flowers, or leaves from trees in autumn, an image that was recalled by these scientist’s and stirred their mind as the situation mimics the morphological appearance of cells undergoing apoptosis.

The process of apoptosis has diverse roles and functions for the continued life process even though it’s a death inducing mechanism. It is through the process of apoptosis that redundant, useless, ectopic, aged, damaged, or mutated cells are eliminated during the processes of development in early life and tissue and organ homeostasis in adult life [7]. Its inhibition contributes a decisive and fundamental role in the initial stages of carcinogenesis; furthermore, most of the time resistance to cancer-chemotherapy is coupled to a resistance to apoptosis induction. Moreover, apoptosis is shown to participate in unwarranted, acute or chronic cell loss, in lot of pathologies such as myocardial ischemia, AIDS, stroke, septic shock, and neurodegenerative diseases (Figure 1) [8].

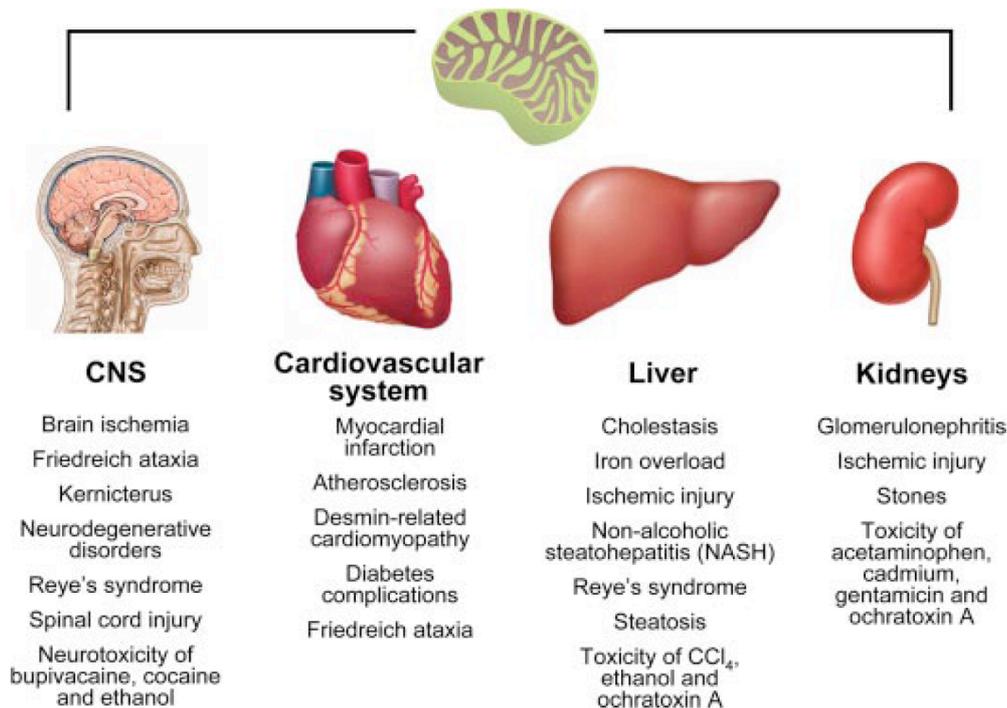


Figure 1 | Examples of the involvement of apoptosis in human pathologies.

Apoptosis has been implicated in a plethora of acute and chronic human diseases affecting several tissues and organs. The figure shows few examples of pathological conditions in which mitochondrial apoptosis plays a prominent role [9].

1.1.2 Morphological Features of Apoptosis

As mentioned above, apoptotic cells display dramatic morphological features including changes in both nuclear and the general cell architecture. All together the morphological features has been classically called “apoptotic phenotype”, and includes:

- (i) Chromatin condensation starting at the nuclear periphery (marginalization), then involving whole nucleus (nuclear pyknosis) and eventually followed by nuclear breakdown (karyorrhexis) [10, 11];
- (ii) General shrinkage of the cell (cytoplasmic pyknosis), accompanied by shape modifications (*e.g.* retraction of pseudopods, rounding up) [12];
- (iii) Shedding of the membraned vacuoles containing parts of the cytoplasm and/or undegraded organelles (blebbing of apoptotic bodies) [12];
- (iv) Clearance of apoptotic bodies by neighbouring cells with a phagocytic activity (*in vivo* and in some instances in cell culture) [13-15]; and
- (v) *In vivo*, the lack of an inflammatory response characterized by infiltrating cells from the

immune system [16].

1.1.3 Mechanisms of Apoptosis

1.1.3.1 The anatomy of apoptotic process

The process of apoptosis can be divided into four major steps/phases which follow each other successively in the course of apoptosis [17]:

- (i) Induction/initiation,
- (ii) Decision/integration,
- (iii) Degradation/execution, and
- (iv) Disposal.

Initiation is a highly heterogeneous phase, since it strictly depends on the specific stimulus that induces the apoptotic cascade. This stage involves a complex molecular interplay between pro- and anti-apoptotic signals activated either through the extrinsic or the intrinsic pathways to activate different initiatory mechanisms. Eventually, the decision/integration phase is the most crucial step deciding cell's fate for degradation or shifting the balance towards pro-survival mechanisms [18]. Once the so called "point-of-no-return" has been trespassed, an ensemble of catabolic processes mediate the dismantling of the cell via executioner mechanisms involving the activation of caspases and mitochondrial death effectors in a complex molecular interplay. These executioner mechanisms constitute essentially a post-mortem process involving morphological and biochemical alterations that are common to all distinct types of apoptosis despite different initiating stimuli [19-21]. During the disposal phase, apoptotic corpses are rapidly engulfed and degraded by neighbouring phagocytes, to avoid inflammation and the development of an autoimmune response [22, 23].

1.1.3.2 The biochemistry of apoptotic process

The biochemical signature of apoptosis has been classically associated with:

- (i) The activation of a specific class of cysteine-proteases cleaving after aspartate residues, named caspases, accounting (at least in part) for the structural degradation of apoptotic cells [24];
- (ii) Mitochondrial membrane permeabilization (MMP), leading to the liberation of toxic proteins into the cytosol and to the ultrastructural and functional breakdown of these organelles [9, 25, 26];

- (iii) Internucleosomal DNA cleavage mediated by both caspase-dependent [27, 28] and caspase-independent nucleases [29-31], which participates in chromatin condensation and nuclear breakdown [11, 12];
- (iv) Exposure of phosphatidylserine (PS) to the outer leaflet of plasma membrane, providing a recognition and internalization signal to macrophages and phagocytic cells [15, 16].

Importantly, cells succumbing from the activation of the apoptotic program do not always exhibit these biochemical hallmarks. Thus, in several settings apoptosis has been described to proceed without the involvement of caspases, *via* caspase-independent mechanisms mediated by some of the proteins released in the cytosol upon MOMP (*e.g.* apoptosis-inducing factor, *i.e.* AIF, endonuclease G, *i.e.* Endo G) [19, 26, 30].

1.1.4 Molecular Machinery of Apoptosis

Apoptosis can be executed by two fundamentally distinct (yet not totally independent) signalling pathways [9]:

- (i) The extrinsic pathway, which involves the activation of death receptors in response to ligand binding;
- (ii) The intrinsic pathway, involving subcellular organelles.

1.1.4.1 The extrinsic pathway

The extrinsic apoptosis pathway (death receptor pathway) initiates apoptosis by the ligation of the plasma membrane specific trans-membrane death receptors, TNF (Tumour Necrosis Factor) receptor super-family [32, 33]. Extracellular signals/ligands may include toxins, hormones, growth factors, nitric oxide or cytokines, that must transduce the effect response across the plasma membrane [34, 35]. The best characterised death receptors include CD95 (APO-1/Fas), TNF receptor and TNF-related apoptosis-inducing ligand (TRAIL) receptors; TRAIL-R1/DR4 (death receptor 4), TRAIL-R2/DR5 (death receptor 5) [32]. Activation of CD95 and TRAIL receptors leads to the clustering of the death domains such as Fas-associated death domain (FADD) which ultimately recruits the initiator caspase, caspase 8, via death effector domain (DED) to form the death inducing signalling complex (DISC) [32, 36]. The final outcome is either by the activation of the execution caspase 3 (type 1 signalling pathway) or by inducing MOMP (Mitochondrial outer membrane permeabilisation; type 2 signalling pathway) (Figure 2) [37, 38]. The later occurs by activated caspase 8 cleaving Bid, a pro-apoptotic member of the Bcl-2 family, activated Bid in turn activates Bax/Bak resulting in MOMP [39]. In contrast to

CD95 and TRAIL, TNF receptor activation results in the recruitment of TNF receptor associated death domain (TRADD) along with receptor interacting protein 1 (RIP 1) and TNF receptor associated factor 2 (TRAF 2) leading to the activation of nuclear factor kappa B (NF- κ B) which has anti-apoptotic and pro-apoptotic functions in context dependent manner [40-43]. For instance, apoptosis induction can lead to inhibition of NF- κ B pathway, as in TNF-induced apoptosis, in which activated-caspases cleave Inhibitor of I κ B kinase β (IKK β) at multiple sites to inactivate IKK activity [44] and likewise, caspase-3 cleaves I κ B α to generate a stable repressor form which inhibits NF- κ B [45]. In contrast, several reports indicate that NF- κ B can function, under certain circumstances, to promote apoptosis by transcriptionally regulating several pro-apoptotic proteins such as the BH3-only protein PUMA (Figure 2) [46, 47].

1.1.4.2 The intrinsic pathway

A diverse group of apoptosis promoting stimuli, such as DNA damage (cytotoxic drugs, radiation), heat shock, survival factor deprivation and other cellular stresses such as Ca²⁺ overload and over-generation of reactive oxygen species (ROS), can induce apoptosis *via* the intrinsic pathway [9, 48]. Although these stimuli may damage cells in a variety of different ways, all of these pathways engage the intrinsic apoptosis pathway (also known as mitochondrial pathway) by triggering MOMP [49, 50]. Loss of mitochondrial membrane potential hinders many mitochondrial and thereby cellular functions such as ATP generation, ion homeostasis and protein import. Moreover, the respiratory chains gets uncoupled, leading to ROS overproduction and hence mark as a point-of-no-return for cell survival. Following MOMP the mitochondrial inter-membrane space (IMS) proteins are released into the cytoplasm such as cytochrome c, apoptosis induced factor (AIF), small mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein (IAP)-binding protein with low PI (DIABLO) and endonuclease G to activate both caspase-dependent and caspase-independent mechanisms that eventually execute cell death [52, 53]. Irrespective of the molecular mechanisms that activate MOMP, the Bcl-2 (B Cell Lymphoma 2) family of proteins exerts a major part in the regulation of this process [54]. Of note, Bcl-2 family proteins are also decisive regulators of autophagy [55, 56] (discussed below).

1.1.4.2.1 Caspase-dependent apoptosis

Following MOMP, Cytochrome c (Cyt-c) is released from the mitochondria eventually binding to the adapter protein apoptosis inducing factor 1 (Apaf-1) (inducing conformational change and oligomerisation of Apaf-1) resulting in the formation of the apoptosome [57-59] which ultimately recruits initiator caspase 9 to initiate the caspase cascade, that involves parallel

activation of other executioner caspases 3 and 7. Caspase 3 appears to be the primary executioner caspase and is required for the initiation of multiple proteolytic events leading to the death of the cell [60, 61]. The other released proteins such as IAP (Inhibitor of apoptosis

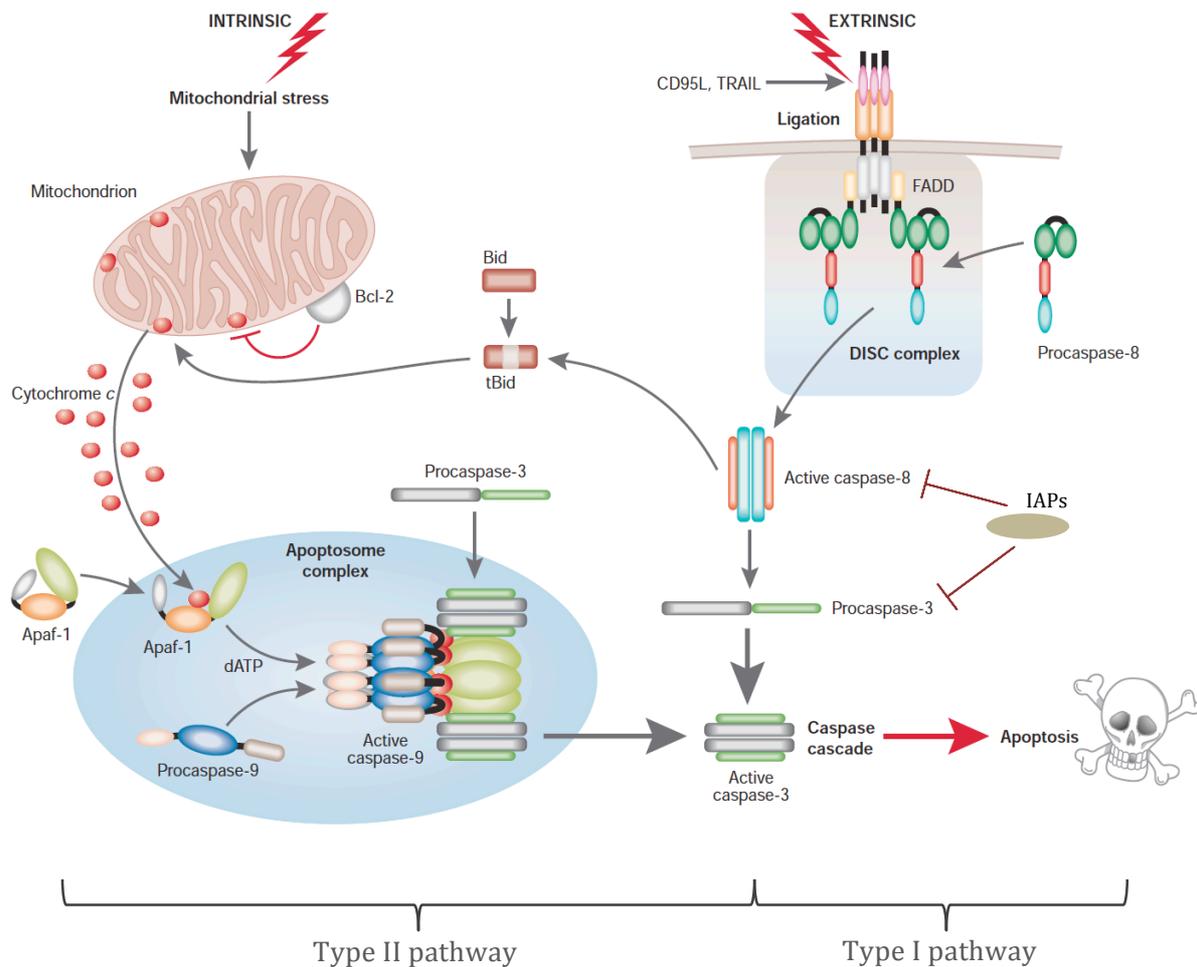


Figure 2 | The extrinsic and intrinsic (mitochondrial) pathways of apoptosis.

Two major apoptotic pathways are illustrated: one activated via death receptor activation (extrinsic) and the other by stress-inducing stimuli (intrinsic). Triggering of cell surface death receptors results in rapid activation of the initiator caspase 8 after its recruitment to adaptor molecules such as FAS-associated death domain protein (FADD) to form death inducing signalling complex (DISC) and leading to caspase cascade. In the intrinsic pathway, stress-induced apoptosis results in perturbation in mitochondrial function to ensue MOMP and the release of proteins, such as cytochrome *c*, whose release is regulated in part by Bcl-2 family members. Anti-apoptotic Bcl-2 proteins prevent MOMP by binding BH3-only proteins and activated Bax or Bak. Once released, cytochrome *c* binds to apoptotic protease-activating factor 1 (Apaf1), which results in formation apoptosome complex and activation of the initiator caspase 9 to start the caspase cascade. Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8 cleavage and activation of the BH3-only protein BH3-interacting domain death agonist (tBid) [51].

proteins) can block caspase activity/activation. Conversely, the anti-apoptotic activity of IAP can be blocked by other regulatory proteins such as Smac/DIABLO [62, 63]. IAPs, which are

the endogenous inhibitors of caspases, promote degradation of active caspases or render them inactive by keeping them away from their substrates. They inhibit caspase activity by binding their conserved BIR (baculovirus IAP repeat) domains to the active sites of caspases [64].

1.1.4.2.2 Caspase-independent apoptosis

MOMP can lead the cell to caspase-independent death due to irreparable and irrevocable loss of mitochondrial function and at the same time mitochondrial release of caspase-independent death effectors such as AIF [65] and endonuclease G [29]. After release, AIF translocate to the nucleus thereby triggering the chromatin collapse and digestion [65], and endonuclease G mediates its functions by enhancing DNA fragmentation [29] as shown in figure 3.

1.1.5 Bcl-2 Proteins and Regulation of Apoptosis

Bcl-2 family proteins are well studied and well described modulators of apoptosis and the interplay of their different subgroups determine the ultimate fate of the cell. MOMP is induced through different mechanisms such as reduced oxygen conditions in ischemic injury (changing the membrane potential and leading to swelling and disruption of the membrane) [52], however, this process is most commonly mediated by Bcl-2 family proteins. Bcl-2 is the prototype of a family of proteins containing at least one Bcl-2 homology (BH) region (Figure 4). The Bcl-2 protein family is composed of:

- (i) Anti-apoptotic multidomain members (*e.g.* Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, etc.), which contain four BH domains (BH1-4) [67];
- (ii) Pro-apoptotic multidomain proteins (*e.g.* Bax, Bak), which contain three BH domains (BH1-3, lacks BH4) [68];
- (iii) Pro-apoptotic BH3-only proteins (*e.g.* Bid, Bad) [69].

They possess the ability to incorporate themselves into the mitochondrial outer membrane (and other intracellular membranes) due to the additional C-terminal domain contained in them [27] and particularly, the specific set of BH domains contained in each Bcl-2 family member determines its profile of activity [70-72]. In this framework, early structure-function studies identified BH1, BH2 and BH4 as the prominent antiapoptotic determinants of Bcl-2 [70]. Conversely, the sole presence of the BH3 domain was found to suffice for apoptosis induction by Bax (as well as for heterodimerization with Bcl-2/Bcl-XL) [73, 74]. Later, numerous reports showed that the conserved trans-membrane (TM) domain and less-conserved, unstructured loops between BH domains also contribute to define the functional profile of Bcl-2 proteins,

either by acting as targeting signals for subcellular compartments (*e.g.* mitochondria, ER) or by modulating the overall tertiary structure [75-79]. Therefore, different combinations of Bcl-2 family proteins are expressed/anticipated in a cell type-, differentiation- and activation state-dependent fashion. Since multiple protein-protein interactions among each other and with other

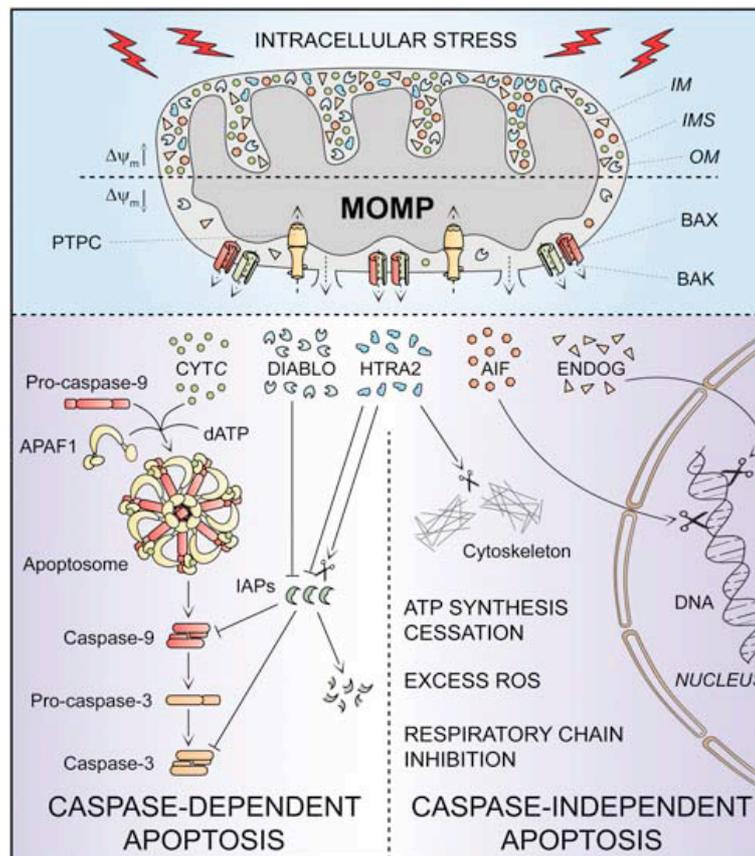


Figure 3 | Intrinsic pathway of apoptosis.

Multiple intracellular stress conditions converge to a mitochondrion-centered control mechanism. When lethal signals prevail, MOMP occur leading to release of proteins that are normally confined within the IMS which ultimately trigger the caspase cascade. DIABLO/SMAC and high temperature requirement protein A2 (HTRA2) facilitate caspase activation by neutralizing the functions of IAP family. On the contrary, AIF and endonuclease G (ENDO G) function in a caspase-independent fashion. Of note, the HTRA2 (serine protease) also contributes to caspase-independent apoptosis by cleaving a wide array of cellular substrates. IM, mitochondrial inner membrane; OM, outer mitochondrial membrane; PTPC, permeability transition pore complex [66].

cellular proteins are formed the stoichiometry of Bcl-2 proteins determines the propensity of cells to different apoptosis inducers including metabolic stressors, genotoxic insults, and the absence of obligate growth factors [9, 80].

Generally, BH1234 proteins (Bcl-2, Bcl-X_L, Mcl-1) reside in mitochondria, protecting these organelles against MOMP through several proposed mechanisms, including sequestration into inactive complexes of pro-apoptotic Bcl-2 family (Bax/Bak) proteins [9, 81], inhibitory interactions with permeability transition pore complex (PTPC) components and / or indirect

effects on endoplasmic reticulum Ca^{2+} stores [82]. In healthy (non-apoptotic) cells, Bax and Bak are in cytosolic inactive form with weak association with the outer mitochondrial membrane. Upon apoptotic stimuli, they undergo conformational modifications and are fully inserted into the outer mitochondrial membrane, thereby creating protein-permeable channels resulting in MOMP, and subsequently, results in the cytosolic leakage of IMS proteins followed by oxidative phosphorylation uncoupling, ROS overproduction and (eventually) mitochondrial transmembrane potential ($\Delta\psi_m$) dissipation [83]. Bax and Bak can be activated and inhibited by non-Bcl-2 family proteins such as tumour suppressor p53, which promotes apoptosis by inducing expression of PUMA (the BH3-only protein) [84]. p53 can also induce MOMP and apoptosis through direct activation of Bax [85] or Bak [86] or through binding and thereby blocking the activity of Bcl-2 and Bcl-X_L [85].

BH3-only proteins are pro-apoptotic which interact with the BH3 receptor domains contained in other Bcl-2 family proteins, hence activating BH123 (pro-apoptotic) proteins and/or by neutralizing BH1234 (anti-apoptotic) proteins [78]. BH3-only proteins can be grouped into two classes: activators (such as Bid) directly interact with (and hence activate) Bax, Bak and sensitizers (like Bad) that displace anti-apoptotic Bcl-2 proteins from inhibitory interactions with Bax, Bak thereby rendering them active [89]. They have different binding specificities for other Bcl-2 family proteins, for example, PUMA and Bim can bind to five antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1), Bad and Bmf interact with Bcl-2, Bcl-X_L, Bcl-w, but not with Mcl-1 or A1, while NOXA binds to only Mcl-1 and A1 (Figure 4) [90].

In recent years, many apoptosis-inducing and anti-apoptosis-inhibiting agents (used in several clinical and preclinical trials) have been a focus for cancer therapy for the targeted induction of apoptosis [91, 92]. Peptides and drugs that mimic the BH3-only proteins (BH3-mimetics such as ABT737, HA14-1) have been undertaken as the agents that can cause MOMP and apoptosis, or sensitize cells for death [93]. Whereas, conventional cancer chemotherapeutic drugs (such as cisplatin, etoposide and doxorubicin) induce MOMP in an indirect fashion by increasing pro-apoptotic signals for example by inducing p53 expression and/or by affecting Bcl-2 family members [94].

1.1.6 Apoptosis and cancer

Programmed cell death plays a fundamental role in the developmental processes and tissue homeostasis. Aberrations in this process are associated with a wide variety of human diseases such as developmental disorders, immune mediated pathologies, neuro-degeneration and cancer

[95]. In the view that programmed cell death operates as a natural barrier to cancer development, evasion of apoptosis (that may be intrinsic or acquired) is a crucial characteristic feature and hallmark of carcinogenesis and tumour progression as most of oncogenic mutations disrupt apoptosis (rendering the cancer cells resistant to internal and external death stimuli), leading to tumour initiation, progression or metastasis. Apoptosis is also the key culprit that circumvent and evade the anticancer effects of current treatment approach since the current

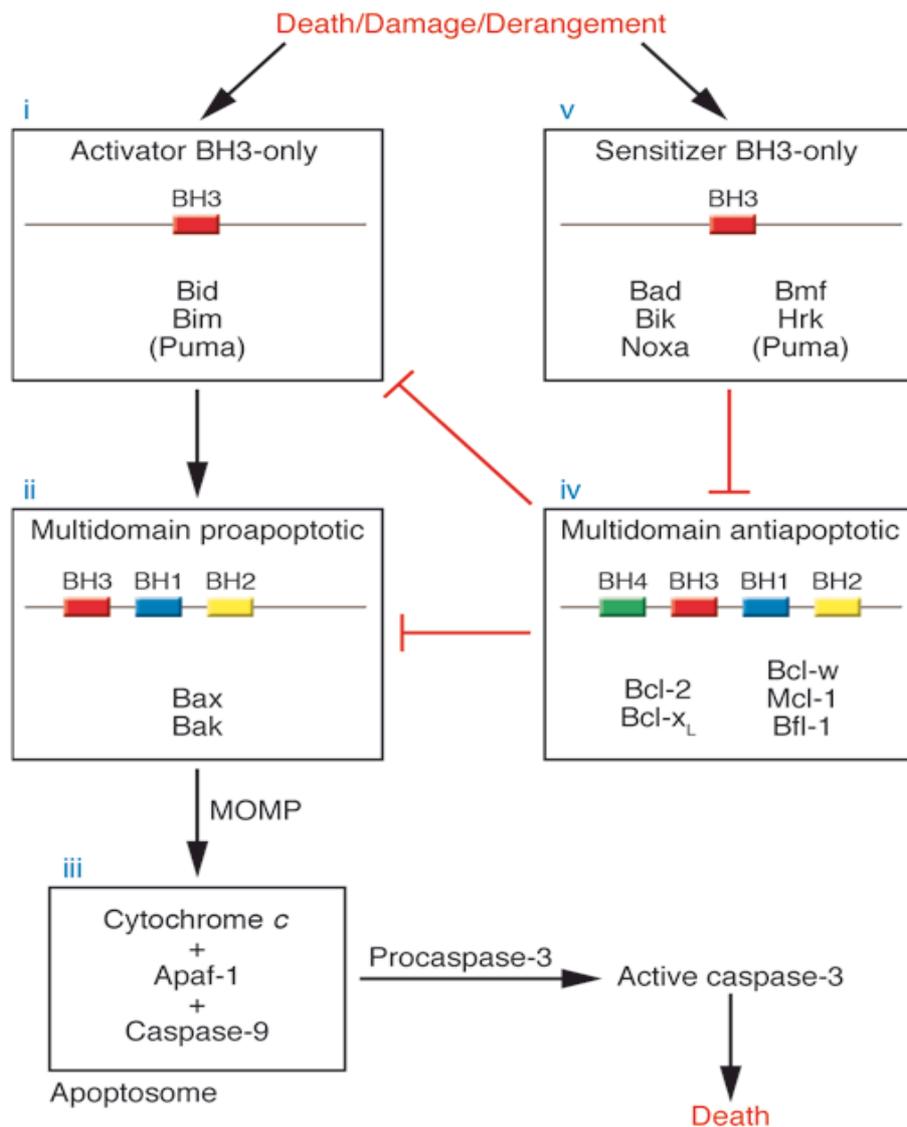


Figure 4 | A model of Bcl-2 family member control over programmed cell death.

The Bcl-2 family of proteins is divided into anti-apoptotic and pro-apoptotic members. In response to myriad death, damage, or derangement signals, BH3- only family members are activated (i). Activator BH3-only proteins interact with multidomain pro-apoptotic Bax and/or Bak (Bax/Bak), inducing their oligomerization (ii) and thus resulting in MOMP, release of cytochrome *c*, apoptosome formation, and caspase activation (iii). Bcl-2 and other multidomain anti-apoptotic proteins interrupt the death signal by binding and sequestering activator BH3-only family members, and perhaps also Bax/Bak (iv). Bcl-2 anti-apoptotic function may be antagonized by the competitive displacement of activator BH3-only molecules by sensitizer BH3-only proteins (v) [87, 88].

anticancer treatments (including chemo-, radio- and immuno-therapy) primarily act by activating cell death pathways including apoptosis in cancer cells [96]. Generally, the overall mechanism leading to disrupted apoptosis is an imbalance between pro-apoptotic and anti-apoptotic proteins [52]. The disturbed equilibrium between pro-apoptotic and anti-apoptotic partners may result due to; impaired upstream signals such as impaired death receptor signalling, mutations in associated proteins such as mutant p53 [97], impaired IAPs expression [64, 98] or the phenomena leading to altered downstream effectors' signalling such as caspase activation [99, 100].

In this scenario, the clinical strategies with the potential to eliminate cancer cells should restore apoptotic signalling pathways towards normality. Strategic target-based different potential anticancer drugs include; cell death receptor agonists, targeting the Bcl-2 family of proteins, p53-based treatments restoring its apoptotic function, IAPs antagonists and caspase activating drugs [101, 102]. Although, upcoming discoveries will generate new therapies and rational strategies to manipulate cell death programs, our current understanding of apoptosis offer new avenues for cancer diagnostics, prognosis and therapy.

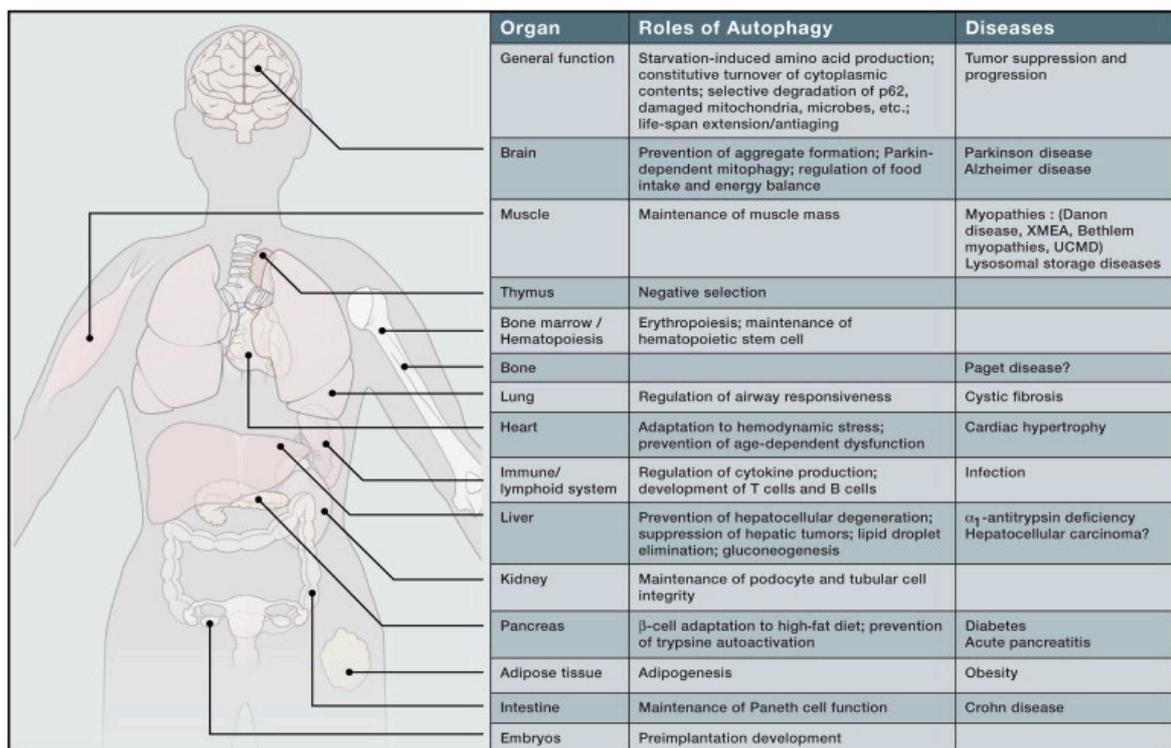
1.2 Autophagy

1.2.1 Historical Notions and Introduction

Eukaryotic cells respond to the changes in their environment as well as to the intracellular milieu by altering their anabolic and catabolic pathways and as one of the responses, living organisms from yeast to humans are capable of eating parts of themselves in order to survive in the conditions of nutrition scarcity, a process known as autophagy. Although research on this topic began in the late 1950s, substantial progress in the molecular study of autophagy has taken place during only the past 15 years as the study of autophagy in human health is a novel research field that has generated tremendous attention owing to the recognition of autophagy being involved in multiple developmental processes and various human diseases including cancer (Figure 5).

Etymologically, the word “autophagy” is derived from two Greek words; ‘auto’ meaning ‘self’ and ‘phagy’ meaning ‘eating’ and thus literarily, autophagy means self-eating. Autophagy is an

evolutionarily conserved process by which cytoplasmic cargo is sequestered inside characteristic double-membrane vesicles destined to the lysosomal degradation (Figure 6). This characteristic reflect the seminal observations made by Sam Clark Jr in 1957 while studying mouse kidneys cells under electron microscopy. In these studies, he observed mitochondria along with cytoplasmic parts within membrane-bound (concentrically lamellar) structures which he termed 'dense bodies' [103] and were subsequently shown to include lysosomal enzymes [104]. The word autophagy was first coined by the Belgian biochemist Christian de Duve who used this word to distinguish the phenomenon of lysosomal degradation of cell's own components from the lysosomal degradation of extracellular materials which he called 'heterophagy' [105, 106]. These early studies of autophagy from the 1950s to the early 1980s were based on morphological analyses. de Duve and others primarily examined the terminal stages of the process, the steps just before or after fusion with the lysosome, but more detailed insights into the molecular control of autophagy and its regulatory machinery, starting in the late 1990s, revolutionized the ability to detect and genetically manipulate this process, which allowed this field to grow at an extraordinarily fast pace uncovering and revealing that its actions in maintaining health and its aberrations in pathogenesis of disease are far more complex than merely cleaning and renewal.



Organ	Roles of Autophagy	Diseases
General function	Starvation-induced amino acid production; constitutive turnover of cytoplasmic contents; selective degradation of p62, damaged mitochondria, microbes, etc.; life-span extension/antiaging	Tumor suppression and progression
Brain	Prevention of aggregate formation; Parkin-dependent mitophagy; regulation of food intake and energy balance	Parkinson disease Alzheimer disease
Muscle	Maintenance of muscle mass	Myopathies : (Danon disease, XMEA, Bethlem myopathies, UCMD) Lysosomal storage diseases
Thymus	Negative selection	
Bone marrow / Hematopoiesis	Erythropoiesis; maintenance of hematopoietic stem cell	
Bone		Paget disease?
Lung	Regulation of airway responsiveness	Cystic fibrosis
Heart	Adaptation to hemodynamic stress; prevention of age-dependent dysfunction	Cardiac hypertrophy
Immune/ lymphoid system	Regulation of cytokine production; development of T cells and B cells	Infection
Liver	Prevention of hepatocellular degeneration; suppression of hepatic tumors; lipid droplet elimination; gluconeogenesis	α_1 -antitrypsin deficiency Hepatocellular carcinoma?
Kidney	Maintenance of podocyte and tubular cell integrity	
Pancreas	β -cell adaptation to high-fat diet; prevention of trypsin autoactivation	Diabetes Acute pancreatitis
Adipose tissue	Adipogenesis	Obesity
Intestine	Maintenance of Paneth cell function	Crohn disease
Embryos	Preimplantation development	

Figure 5 | Physiological and Pathological Roles of Autophagy.

This figure illustrates the physiological roles of autophagy in different tissues/organs in humans and the related diseases. These data have been obtained from both mouse and human. XMEA: X-linked myopathy with excess autophagy, UCMD: Ullrich congenital muscular disorder [107].

1.2.2 Types of Autophagy

There are three main types of autophagy in eukaryotic cells, which are mechanistically different (in terms of their mechanism for directing the cytoplasmic content to the lysosomes) from each other [108]:

- (i) Microautophagy,
- (ii) Macroautophagy,
- (iii) Chaperone-mediated autophagy (CMA).

Microautophagy is the direct sequestration of cytosolic components by lysosomes through invagination, protrusion, and septation of the lysosomal membrane. The function of this process in higher eukaryotes is not known, whereas it may serve to mediate the selective degradation of organelles in the yeast. In the case of macroautophagy, most frequently referred to simply as “autophagy”, autophagic cargoes are directly sequestered within characteristic double-membraned cytosolic vesicles, autophagosomes, ultimately delivered to lysosomes for bulk degradation (Figure 6). The sequestration can be either selective, targeting specific cargoes such as organelles or invasive microbes or it may be nonspecific, involving the engulfment of bulk cytoplasmic contents. CMA involves direct translocation of unfolded substrate proteins across the lysosomal membrane through the action of a cytosolic and lysosomal chaperone (like hsc70), and the integral membrane receptor LAMP2A (lysosome-associated membrane protein type 2A) (Figure 7) [109].

1.2.2.1 Subtypes of macroautophagy

Most of the scientific reports demonstrate an obligatory role of Beclin 1 (and dependency on Atg5 and Atg7) in the induction of autophagy (canonical autophagy) [110, 111] but some recent reports have also shown the presence of some subtypes of macroautophagy that are independent of Atg5 and Atg7 [112] or Beclin 1 (non-canonical autophagy) [113-115]. The regulation of Atg5/Atg7-independent macroautophagy involves ULK1, Beclin 1, and Rab9, an endosome-Golgi transport protein [112], whereas Beclin 1-independent macroautophagy is shown to bypass the beclin 1 and its main interactor, VPS34 (PI3K) [116]. Additional investigations will elucidate the molecular details of these subtypes of macroautophagy. Interestingly, latest evidence suggests that “basal” and “stress-induced/pathological” macroautophagy may emerge as two subtypes of macroautophagy that possibly involve distinct sets of macroautophagic machinery, each recruiting a unique set of autophagy regulators[117] .

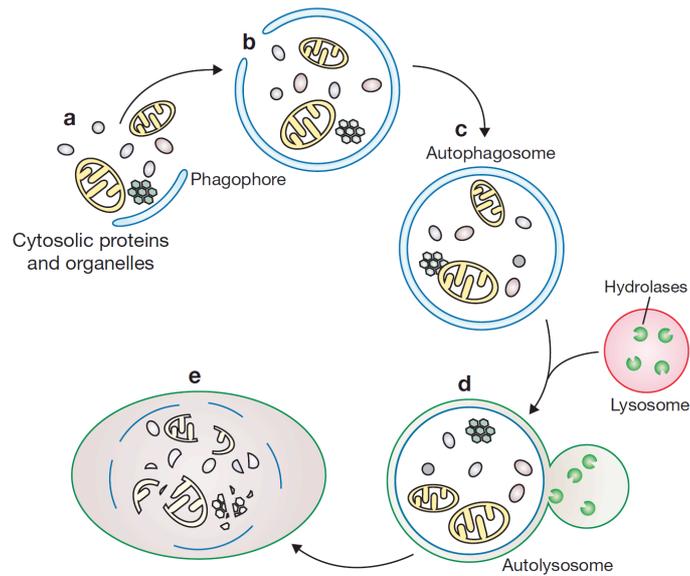


Figure 6 | Schematic simplified depiction of autophagy.

(a, b) Cytosolic material is sequestered by the phagophore, (c) resulting in the formation of a characteristic double-membrane vesicle, the autophagosome; (d) the outer membrane of the autophagosome subsequently fuses with the lysosome, exposing the inner single membrane of the autophagosome to lysosomal hydrolases; (e) the cargo-containing membrane compartment is lysed, and its contents are degraded [119].

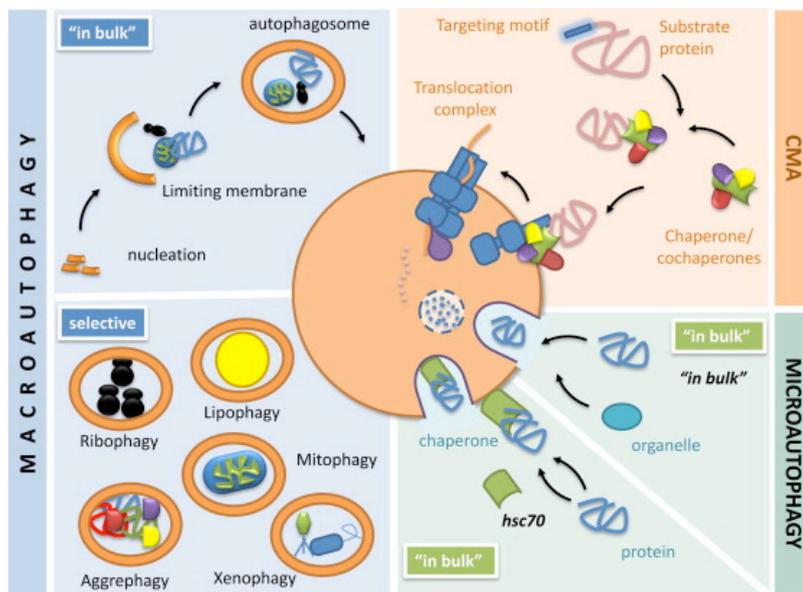


Figure 7 | Different types of autophagy in mammalian cells.

Three types of autophagy co-exist in all mammalian cells. Macroautophagy involves the sequestration of regions of the cytosol inside double membrane vesicles that become degradative compartments upon fusion with the lysosomes. Both in bulk and selective macroautophagy can take place (selective macroautophagy are highlighted on the left). Microautophagy occurs when cytosolic components are directly engulfed by lysosomes through invaginations of the lysosomal membrane. Chaperone-mediated autophagy, by contrast, initiates through the recognition by a chaperone of a targeting motif in the cytosolic proteins to be degraded. The chaperone/substrate complex reaches then the lysosome surface and the substrate is internalized through a translocation complex in the lysosomal membrane [120].

In addition to canonical and non-canonical macroautophagy, special types of macroautophagy directed to specific organelles have also been discovered, such as mitochondria (mitophagy) and peroxisomes (pexophagy), and to intracellular bacteria (xenophagy) (Figure 7) [118].

My thesis work focuses on macroautophagy, which hereafter will be referred to as 'autophagy'.

1.2.3 Autophagic Machinery and its Regulation

In the mammalian autophagy system, similar to yeast autophagy-related (*atg*) genes and several other non-Atg proteins, most of their homologues are shown to contribute to its regulation [121, 122]. The major breakthrough in the elucidation of autophagic molecular machinery came from yeast genetic screenings for autophagy-relevant genes. Until now more than 30 autophagy-related (*atg*) genes have been identified and most of them are found conserved in the course of evolution, emphasizing its importance in the process of evolution [123]. Among them, some genes are essential for autophagosome formation, referred to as 'core' *atg* genes, as they encode the fundamental machinery for the biogenesis of autophagy-related controlling proteins to initiate autophagosome formation, as well as several others in the nucleation, elongation, maturation, and degradation of autophagosomes. The core autophagic machinery of mammalian autophagy incorporates five functional sub groups [121, 122, 124]:

- (i) The Atg1/ *unc-51*-like kinase (ULK) complex including Atg13 and Atg17 kinase for autophagy induction,
- (ii) The class III phosphatidylinositol 3-kinase (PtdIns3K) complex, consisting of Vps34, Atg6/beclin 1 and Atg14, concerned with the formation of autophagosome,
- (iii) Two ubiquitin-like protein (Atg12 and Atg8/LC3) conjugation systems involved in the expansion of autophagosome membranes,
- (iv) Atg9 and its 'retrieval complex' that contributes to the delivery of membranes forming autophagosome, and
- (v) The proteins needed for the fusion between autophagosomes and lysosomes for ultimate degradation.

1.2.3.1 The *atg1/unc-51*-like kinase (ULK) complex

This complex is involved in the induction of autophagy and incorporates all the pathways that sense the changes that are obligatory for this process to commence. In normal (for example, nutrient-replete) conditions, mTORC1 possesses kinase activity and interacts with

ULK1 complex, which contains ULK1 Ser/Thr protein kinase (and possibly ULK2), mAtg13, and FIP200/Atg17, thus putting a check to the induction of autophagy. The phosphorylation of Atg13 and FIP200 by ULK1 is an important step in triggering autophagy when mammalian target of rapamycin (mTOR) complex 1 is inhibited (for example, in nutrient-depletion, pharmacological inhibition by rapamycin) (Figure 8, Box 1) [126, 127]. Recently a novel mammalian Atg protein, Atg101, has been found to interact with the ULK1–mATG13– FIP200 complex in an ATG13-dependent manner [128]. Additionally, Atg1/ULK complex is regulated by multiple other pathways including cAMP-dependent protein kinase A [129] and AMP-activated protein kinase [130, 131]. In yeast, the Atg1 complex is present at the ‘pre-autophagosomal structure’ for the formation of phagophore, the earliest morphological trait of autophagy [132-134], but there is no evidence for pre-autophagosomal structure in mammals [135]. Therefore, recent data demonstrate that the regulation of the Atg1-containing complex significantly differs between mammals and yeast [136].

1.2.3.2 The class III phosphatidylinositol 3-kinase-Beclin 1 complex

For the formation of initial phagophore membrane, its nucleation and assembly requires the class III phosphatidylinositol 3-kinase (PtdIns3K) complex including the essential autophagy protein Beclin 1 (also known Beclin 1 core complex) (Figure 8, Box 2). In line with its essential role in the induction of autophagy, the depletion of Beclin 1 by genetic means sensitizes mammalian cells to nutrient deprivation-induced cell death [125, 137] and similarly, over-expression of Beclin 1 stimulates autophagy, and this autophagy-stimulatory effect can be inhibited by Bcl-2 over-expression [138]. In this regard, Beclin 1 complex includes the hVps34 (human vacuolar protein sorting 34), p150 (a homolog of serine/threonine kinase Vps15), Atg14L (Barkor) and Beclin 1 (a homolog of atg6) [139, 140]. In the Beclin 1 core complex, few other Beclin 1 binding proteins have been recognized as disruption of their interaction with Beclin 1 affects autophagosome formation; the Beclin 1 binding partners that modulate autophagy include AMBRA1 (activating molecule in Beclin 1-regulated autophagy) [141], UVRAG (ultraviolet irradiation resistant-associated gene) [142], Bif-1 (Bax-interacting factor 1) [143] and Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1-interacting) [144, 145]. In the Beclin 1 core complex, the lipid kinase activity of Vps34 is indispensable for generating phosphatidylinositol (3)-phosphate (PtdIns(3)P) at the PAS (phagophore assembly site) for the recruitment of other Atg proteins (*e.g.*, Atg7 and Atg10) for the induction of autophagy [121, 146]. The possible role of Atg14L is to direct the class III PtdIns3K complex to the phagophore and to initiate the recruitment of Atg machinery as it localizes to both

phagophore and autophagosome in autophagy inducing conditions [139, 144]. Interestingly, Atg14 and UVRAG interact in the Beclin 1 complex in a mutually exclusive manner [139] and also Atg14 competes with UVRAG for Beclin 1 to promote autophagosome formation [140]. Moreover, UVRAG interacts with Bif-1 (also known as endophilin B1) for its recruitment to the complex to facilitate PIK3 activation for autophagy induction [143] whereas, UVRAG equally interact with Rubicon (hVPS34 inhibitor), which negatively regulate autophagosome maturation [144, 145]. The precise molecular mechanism of AMBRA1 is not much clear but is proposed to constitute a direct regulatory link between ULK1 and Beclin 1 core complex [147]. Moreover quite recently, HMGB1 (a chromatin-associated nuclear protein and extracellular damage-associated molecular pattern molecule), a novel Beclin 1-binding protein has been shown to be important in sustaining autophagy as deletion, depletion or inhibition of HMGB1 clearly diminishes autophagic activity [148, 149]. In mammalian cells, despite the confirmed role of Beclin 1, the source of initial phagophore membrane is controversial and may include; the endoplasmic reticulum [150], the trans-Golgi apparatus and endosomes [151, 152], the outer mitochondrial membrane [151, 153], the plasma membrane [154] and, in specific experimental settings, even the nuclear envelope [155].

These data suggest the existence of multiple beclin-1-containing complexes as Beclin 1 regulates autophagy in an intricate fashion, by binding through its different domains to different proteins forming multiprotein complexes in which each component binds to different regions of Beclin 1, possibly reflecting different affinities and/or different sub-cellular localizations in situational dependent context. Moreover, all these factors are likely to exert autophagy-unrelated functions, which so far have poorly been characterized. Thus, multiple Beclin 1-hVps34-complexes participate in the distinct steps of the autophagic controlling cascade from phagophore nucleation to the maturation of autophagosomes [121] and with the due advancement and research, more and more partners are being recognized making the control and working of this complex more intricate and complicated. Of note, anti apoptotic Bcl-2 protein family (such as Bcl-2 and Bcl-X_L) constitutively bind to Beclin 1 thereby disrupts Beclin 1-associated hVps34-PtdIns3K activity and hence inhibits autophagy (discussed later) [56].

1.2.3.3 Atg12 / LC3 (Atg8) conjugation systems

The hierarchical Atg proteins analysis reported these two (Atg12 / LC3 (Atg8) conjugation systems) highly conserved (though similar and functionally interconnected) ubiquitin-like conjugation systems acting at the late steps of autophagosome formation, i.e., elongation and

closure of the membrane [156-160] (Figure 8, Box 3).

Atg8 and its mammalian paralogs such as LC3B, GABARAP, and GATE-16 are ubiquitin-like proteins which are processed by proteolytic cleavage by the cysteine protease Atg4 [161, 162], activated by Atg7 (E1 enzyme), transferred to Atg3 (E2 enzyme), and finally covalently linked to an amino group of PE (lipidated LC3, LC3-II), a major membrane phospholipid [163] [164]. While the immature form of LC3 (also known as LC3-I) is cytosolic, lipidated LC3 (LC3-II) relocalizes to forming autophagosomes, thereby providing a specific marker of autophagy that can be monitored by immunofluorescence or immuno-blotting [165, 166]. Atg8/LC3-PE localizes on both the isolation membrane and the autophagosome [167]. In several *atg* mutants defective in isolation membrane formation, Atg8-PE (mammalian LC3-PE) still accumulates, which suggests that lipidation of Atg8 occurs without isolation membrane formation [168, 169].

Atg12, after synthesis, is activated by Atg7 (E1 enzyme), is transferred to Atg10 (E2 enzyme), and finally conjugated with its only target protein, Atg5 [170, 171]. The Atg12-Atg5 heterodimer non-covalently interacts with Atg16 (Atg16L in mammals) [172]. This Atg12-Atg5-Atg16 complex is formed constitutively in yeast, irrespective of nutrient conditions whereas in mammals, Atg12-Atg5-Atg16L1 predominantly localizes on the outer surface of the isolation membrane and dissociates from the membrane immediately before or after the completion of autophagosome formation [173].

Recent reports suggest the existence of a bidirectional interconnection in these closely related two conjugation systems as Atg12-Atg5-Atg16L is necessary for Atg8-PE formation in vivo. Moreover, in vitro reconstitution experiments have revealed that Atg12-Atg5 interacts with Atg3 and intensively facilitates the transfer reaction of Atg8 from Atg3 to PE, may be specifying the site of LC3 lipidation [172] [174]. In line these results, *atg3^{-/-}* mice fail to convert LC3-I into LC3-II (as expected), but also exhibit impaired Atg5-Atg12 conjugation, delayed dissociation of the Atg16L complex from phagophores and structural alterations of autophagosomes [175].

1.2.3.4 Atg9 and its recycling system (the retrieval complex)

Expansion of autophagosomes requires the delivery of phospholipids, possibly in the form of lipid bi-layers, and Atg9-positive compartments might function as the actual provider of phospholipids to nascent and elongating autophagosomes [176, 177]. Interestingly, integral membrane protein, Atg9 is shown to shuttle between the pre-autophagosomal structure (the

putative site of double-membrane vesicle biogenesis) and mitochondria in an Atg18-regulated fashion, having proposed role of transporting lipids required for the process of autophagy [178, 179] (Figure 8, Box 4). Moreover, in mammals, the Atg9 homolog Atg9L1 localizes to both autophagy-related structures (or omegasomes) as well as to the *trans*-Golgi network and endosomes [176, 180]. Atg9 is also reported for the recruitment of Atg12-Atg5-Atg16 and Atg8/LC3-II complexes to the autophagosomal membrane, and hence might act in concert with

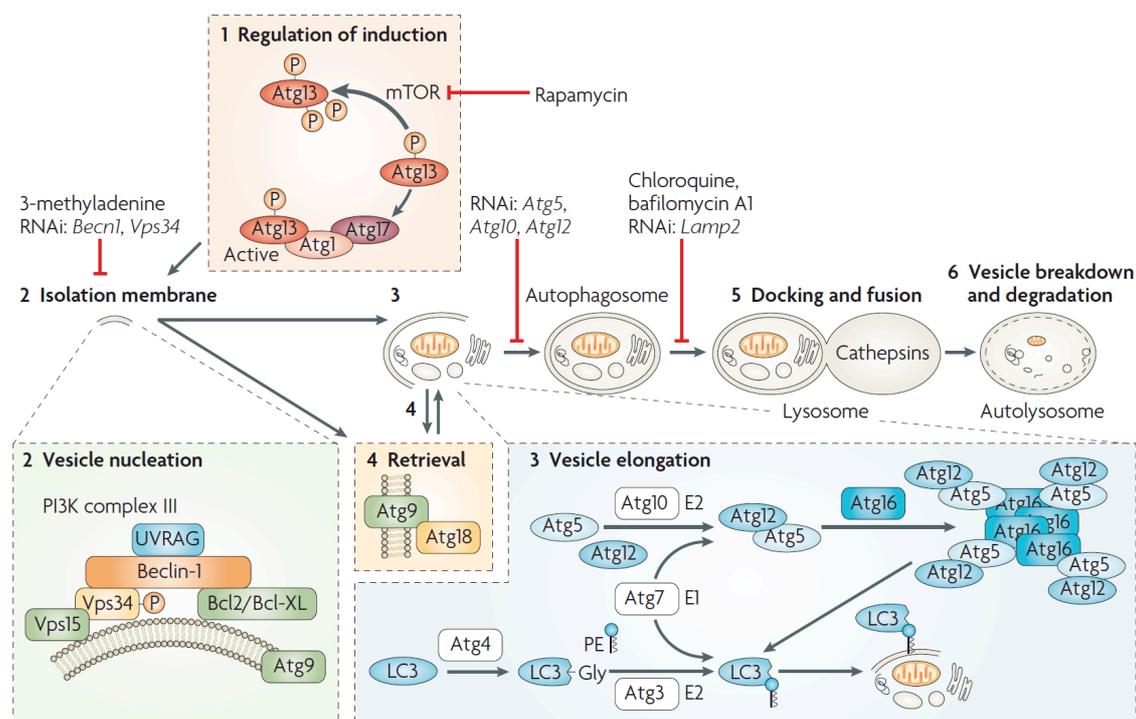


Figure 8 | Scheme of different steps and molecular control of autophagy.

Distinct stages of the process of autophagy: vesicle nucleation (formation of the isolation membrane/phagophore), vesicle elongation (growth and closure), fusion of the double-membraned autophagosome with the lysosome to form an auto(phago)lysosome, and degradation of the autophagosome inner membrane and contents inside the auto(phago)lysosome. At the molecular level, AuTophagy-related (Atg) proteins form different complexes that function in distinct stages of autophagy. Pharmacological inhibitors and small interfering RNAs that are capable of inhibiting distinct steps of this process are also shown [125].

these supra-molecular complexes to supply the expanding membrane with phospholipids [119, 174, 181]. Interestingly, Atg9 is absent from sealed autophagosomes suggesting that Atg9 is retrieved upon vesicle completion [182]. Furthermore, *atg9*^{-/-} mice die within 1 day of delivery, signifying that Atg9 is essential for survival during neonatal starvation [183] and it is also speculated that starvation-induced autophagy may rely on an alteration of the steady-state trafficking of mAtg9, in a Atg1-dependent manner (involving Atg17 being a subunit of the Atg1 complex) [180, 184].

1.2.3.5 Autophagosomal-lysosomal fusion

The final step of autophagy involves the fusion of matured autophagosome with lysosomal vesicles to form an autolysosome leading to the degradation and recycling of its contents (Figure 8). The fusion between autophagosomes and lysosomes or late endosomes is a highly coordinated membrane trafficking event and is well characterized in yeast [185, 186], but poorly studied in mammalian cells [187]. The regulators of this fusion events, between autophagosomes and endo-lysosomes, include Rab GTPases [188], the lysosomal membrane proteins LAMP1 and LAMP2 [189], the AAAtype ATPase SKD1 [190], the microtubular network [191], the luminal pH within lysosomes (which is also critical for the proper activation of acidic hydrolases) [192] and SNAREs [186, 193]. Rab7 is a key component in the regulation of autophagosomal maturation, facilitated by lysosomal membrane proteins LAMP1 and LAMP2. Membrane trafficking activity of Rab7 involves its cycling between GTP-bound (active) and GDP-bound (inactive) states [193].

1.2.4 Bcl-2 Family Proteins and Regulation of Autophagy

Beclin 1, is an essential autophagy protein and haplo-insufficient tumour suppressor, which possesses a coiled-coil (CC) domain that allows the interaction with membrane-anchored Vps15/p150 as well as an evolutionarily conserved domain that mediates the binding to Vps34/hVps34 [116]. Numerous other proteins interact with Beclin 1 to form different multiple Beclin-1-containing complexes, whose relative contribution to the regulation of autophagy may vary. In these complexes, Beclin 1 (originally isolated as a Bcl-2-interacting protein), which contain BH3-only domain essentially interacts with Bcl-2 family proteins (initially characterized as an apoptosis inhibitor) [55, 194].

Multi-domain anti-apoptotic Bcl-2 proteins possessing a hydrophobic cleft at their surface, the BH3-binding groove, can accommodate BH3 domains from pro-apoptotic Bcl-2 protein family members. The BH3 domains from pro-apoptotic proteins can be structurally defined as four-turn amphipathic α -helices that are contained in a sequence motif that lacks invariable amino acids: Hy-X-X-X-Hy-X-X-X-Sm-D/E-X-Hy, where Hy represents hydrophobic residues and Sm represents small residues, typically glycine [195]. The extreme variability of both pro-apoptotic BH3 domains, as well as that of the residues lining the BH3-binding groove of the anti-apoptotic proteins, determine and affect multiple protein-protein interactions and affinities among Bcl-2 family proteins, ultimately regulating the stoichiometry of these interactions [195].

Recently, a region including amino acids 114–123 from the autophagic regulatory protein Beclin 1 has been identified as a BH3 domain [56, 125, 196, 197]. This domain in Beclin 1 is the minimal sequence necessary for the interaction with the anti-apoptotic Bcl-2 family of proteins (in particular Bcl-2 itself, Bcl-X_L and Mcl-1). Although Beclin 1 is a *bona fide* BH3 protein, it has no pro-apoptotic effects [198]. Beclin 1 under physiological (resting) conditions constitutively interacts with anti apoptotic Bcl-2 protein family such as Bcl-2 and Bcl-X_L, by virtue of its BH3 domain, which inhibits its pro-autophagic activity [56]. Accordingly, the induction of autophagy by various triggers correlates with the dissociation of Beclin 1 from Bcl-2 or Bcl-X_L, thus allowing Beclin 1 to accomplish its autophagic stimulatory functions (Figure 9) [56, 122, 199].

Several distinct mechanisms have been proposed to account for the pro autophagic release of Beclin 1 from its inhibitory interactions with Bcl-2 or Bcl-X_L. One possibility is that BH3-containing proteins (such as BNIP3, Bad, Bik, NOXA, PUMA and BimEL) of the Bcl-2 family may competitively disrupt Bcl-2/Bcl-X_L interaction with Beclin 1 [197]. The studies on the pro-autophagic profile of ABT737, a small BH3-mimetic compound which disrupts the inhibitory interaction between Beclin 1 and Bcl-2/Bcl-X_L, (but not of Mcl-1, another anti-apoptotic member of the Bcl-2 protein family) provides evidence in the support of this model [56, 197]. ABT737-triggered autophagy could not be inhibited by Bcl-2/Bcl-X_L overexpression, yet was suppressed by knockdown of Beclin 1. In another model, pro-autophagic triggers phosphorylates either of these proteins (Beclin 1 and Bcl-2/Bcl-X_L), thereby dissociating their interaction. The fact that the activation of c-Jun N-terminal protein kinase (JNK) upon nutrient starvation promotes Bcl-2 phosphorylation on multiple residues (namely Thr69, Ser70 and Ser87) located in the non-structured loop between Bcl-2 N terminal BH4 and BH1 domains, ultimately supports this idea [200, 201]. Similarly, the phosphorylation of Beclin 1 within (or close to) its domain of interaction with Bcl-2 by death-associated protein kinase (DAPK) disrupts this interaction [202]. Therefore, post-translational modifications of Beclin 1 and/or Bcl-2 (and perhaps others) may affect their mutual interaction and although the two models that have been proposed to explain the dissociation of Beclin 1 from Bcl-2/Bcl-X_L during autophagy (competitive disruption by BH3-only proteins *versus* phosphorylation of Bcl-2 and/or Beclin 1) appear to be distinct, they may be functionally linked [55].

In conclusion, two scenarios can be visualized as described:

1. Competition: two distinct routes for the induction of autophagy might be anticipated, one relying on the activation of BH3-only proteins and the other one mediated by the

phosphorylation of Bcl-2 and/or Beclin 1, which would be engaged in a mutually exclusive fashion by specific autophagic triggers

2. Cooperation: these two molecular cascades might act in concert to promote autophagy. For instance, the phosphorylation of Bcl-2 in its non-structured loop might reduce the affinity for the BH3 domain of Beclin 1, and change Bcl-2 profile of selectivity for other BH3 domains. This might allow for the displacement of Beclin 1 from Bcl-2 by BH3-only proteins, in turn leading to Beclin 1-hVps34 complex formation.

Beclin 1 is the only BH3-only protein that does not have pro-apoptotic properties. The particular spatial organization of Beclin 1 may explain its lack of any pro-apoptotic activity; only ER-targeted (not mitochondrion-targeted) Bcl-2/Bcl-X_L can inhibit autophagy induced by Beclin 1, and only Beclin 1-Bcl-2/Bcl-X_L complexes present in the ER (not those on mitochondria) are disrupted by ABT737, suggesting that Beclin 1-Bcl-2/Bcl-X_L complexes that usually suppress autophagy are specifically located in the ER [56, 138, 197].

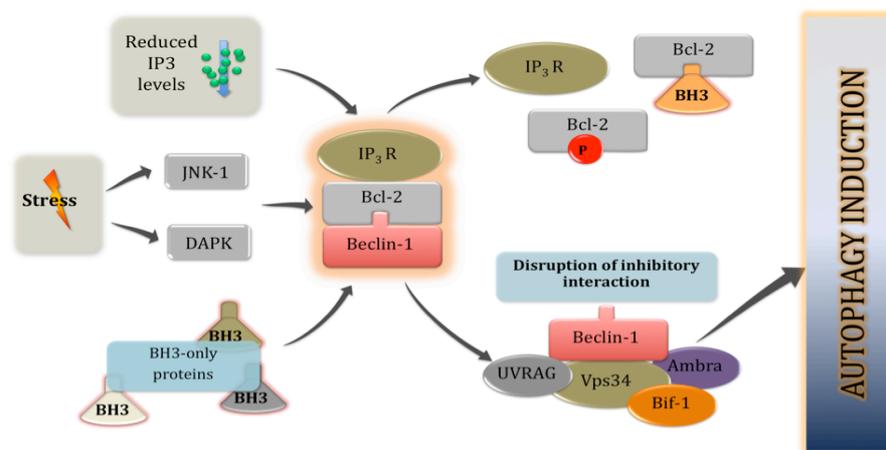


Figure 9 | Overview of the signal transduction pathways that regulate the Beclin 1 complex.

Beclin 1 is the component of autophagy machinery that function in vesicle nucleation/phagophore formation [122].

1.2.5 Autophagy Regulating Signalling Pathways

1.2.5.1 mTOR

Nutrients, including glucose but in particular amino acids, are the most relevant physiological regulators of autophagy, which is strongly up regulated when the supply of these metabolites is inadequate to meet the cell's bioenergetics and anabolic demands [203-206]. In turn, amino acids and nutrients generated by the degradation of autophagocytosed proteins act as feedback inhibitors of autophagosome generation. In this scenario, mTOR (similar to its yeast ortholog)

represents the crucial modulator of autophagy in mammalian cells [108] as many diverse signals, such as growth factors, amino acids, glucose, energy status, and different forms of stress, regulate the mTOR pathway [207]. The role of mTOR is not restricted to the control of autophagy but also expands its domains to the regulation of protein translation and cell-cycle progression depending upon growth factors and nutrient availability [208-210] and it is plausible that these processes might be linked with autophagy in the overall control of cellular processes. The mTOR pathway involves two functional complexes: a rapamycin-sensitive mTOR complex 1 (mTORC1), a regulator of autophagy, consisting of the mTOR catalytic subunit, raptor (regulatory associated protein of mTOR), GβL (G protein β-subunit-like protein; also known as mLST8), and PRAS40 (proline-rich Akt); and mTOR complex 2 (mTORC2), not a direct autophagy regulator, consisting of mTOR, Rictor (rapamycin-insensitive companion of mTOR), GβL, SIN1 (SAPK-interacting protein 1), and Proctor (protein observed with Rictor) [211, 212]. The activity of mTORC1 complex is mediated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress. Induction of autophagy (by starvation or by administration of rapamycin) leads to inhibition of mTORC1 which is accompanied by reduced phosphorylation of two of its downstream effectors, ribosomal protein S6 kinase-1 (also known as p70S6K) and translation initiation factor 4E binding protein-1 (4E-BP1, also known as PHAS-1) at Thr389/Thr421/Ser424 and Thr37/Thr46, respectively [207, 213]. As discussed before, ULK1 complex is downstream of mTOR in the signalling control of the autophagic machinery; in physiological conditions, mTOR is fully active (in a de-phosphorylated form) and prevents autophagy by maintaining the hyper-phosphorylation of proteins required (ULK1 complex) for the initiation of the autophagic cascade. Conversely, mTOR activity is rapidly inhibited in conditions of autophagy inducing stress, which allows the rapid up-regulation of autophagy by increasing the levels of the class III PI3K product, PI-3-P [214, 215].

A major signalling cascade controlling mTORC1 is the class I PI3K pathway that in contrast to the class III PI3K pathway inhibits autophagy [215, 216]. mTORC1 incorporates upstream activating signals that inhibit autophagy, such as insulin/IGF1 (insulin like growth factor 1), through the class I PtdIns3K and protein kinase B (PKB), also known as Akt pathway [217]. Activated PI3K, through the conversion phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-trisphosphate (PIP₃), phosphorylates and activates Akt, which in turn phosphorylates and inactivates tuberous sclerosis complex (TSC) 1/2, leading to activation of Rheb (Ras homologue enriched in brain, a small GTPase) and consequently mTORC1 [218].

Under normal conditions TSC1 and TSC2 form a dimer and function as a GTPase-activating protein (GAP) to inactivate the Rheb [219]. In this context, the tumour suppressor PTEN (phosphatase and tensin homologue) stimulates autophagy via PtdIns(3,4,5)P₃-phosphatase activity thereby antagonizing insulin/IGF1 pathway[215].

As a note, as compared to mTORC1, mTORC2 exhibits a reduced sensitivity to acute pharmacological inhibition by rapamycin (a lipophilic macrolide that is commonly employed in experimental settings to promote autophagy) [220, 221]. Moreover, rapamycin and its derivatives act by binding to the immunophilin FK506-binding protein 12 (FKBP12), which in turn becomes able to sequester TOR within an inactive molecular complex (Figure 10) [222, 223].

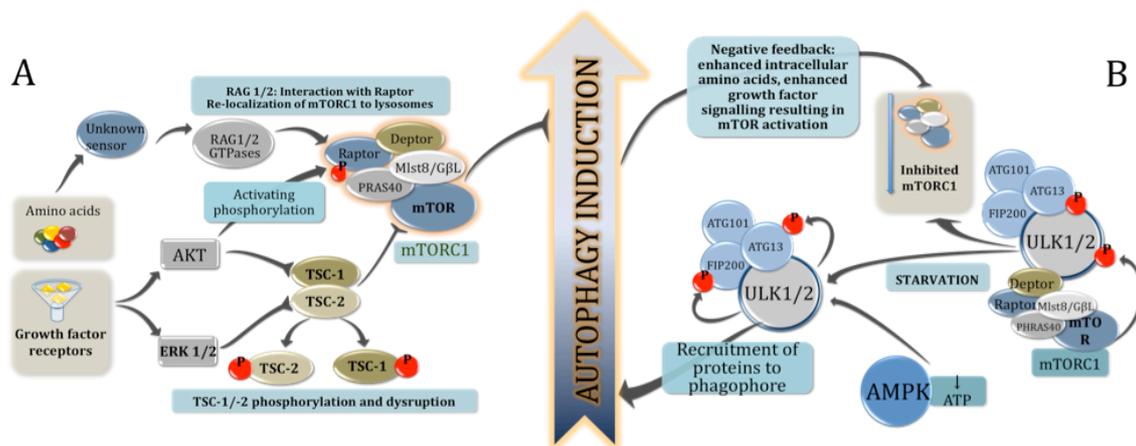


Figure 10 | Schematic overview of the autophagy signalling cascades involving mTOR and ULK1/2.

(A) mTORC1 (B) and ULK1 /2 . Multiple positive and negative feedback loops depicted in B, thereby depicting the regulatory process of autophagy at its induction step [122].

1.2.5.2 AMPK

AMPK is the cell's major energy-sensing kinase, activated by AMP, a sensitive indicator of the ADP/ATP ratio in the cell and upon its activation, AMPK activates a wide scale of catabolic processes such as glucose uptake and metabolism, while simultaneously inhibiting several anabolic pathways, such as lipid, protein, and carbohydrate biosynthesis [224]. Once activated, AMPK directly phosphorylates TSC2, thereby facilitating the priming phosphorylation for subsequent phosphorylation of TSC2 by glycogen synthase kinase 3 (GSK-3) to inhibit mTOR signalling. Acetyl-CoA carboxylase (ACC), a substrate to AMPK is essentially phosphorylated by activated AMPK thereby reducing its activity. AMPK is shown to mediate hypoxia-induced autophagy by down-regulating mTOR signalling [225-228]. Several upstream kinases, including liver kinase B1 (LKB1, which is activated by energy depletion), calcium/calmodulin

kinase kinase- β (CaMKK β , which is activated by cytosolic Ca²⁺), and TGF β -activated kinase-1 (TAK-1, which is also involved in IKK activation) can activate AMPK by phosphorylating a threonine residue on its catalytic α -subunit [229]. The recent identification of an interaction between AMPK and ULK1 raises the speculative possibility that AMPK may also act more directly on core components of the autophagy machinery to initiate autophagy (Figure 11) [230]. Moreover, in recent years, studies have demonstrated that Ca²⁺/calmodulin (CaM)-dependent kinase β (CAMK β), AMPK also transduces the pro-autophagic stimulus generated by increased cytosolic Ca²⁺ concentrations [171] and also ROS can modulate autophagy [231] through activation of AMPK [232].

1.2.5.3 Sirtuin1

Sirtuins are a family of NAD-dependent deacetylases that share a highly conserved central NAD⁺-binding and catalytic domain known as “sirtuin core domain” [233]. Among them, Sirtuin 1 has been reported to play a key role in a variety of physiological processes including metabolism, neurogenesis and cell survival due to its ability to deacetylate histones and numerous non-histone substrates. Sirtuin 1 (SIRT1) is also shown to require for the induction of autophagy by starvation and also, the transfection of cells with SIRT1 with intact deacetylase activity is sufficient to stimulate autophagy in cultured mammalian cells [234, 235]. Accordingly, *Sirt1*^{-/-} mice display a phenotype consistent with impaired autophagy, including increased levels of the autophagy substrate p62, accumulation of damaged organelles, disruption of energy homeostasis, and early perinatal lethality [234]. The role of the deacetylase SIRT1 in autophagy is further supported by the fact that many of the Atg proteins can be acetylated by p300 and knockdown of p300 (whereby reducing acetylation of Atg5, Atg7, Atg8, and Atg12) [236] as well as histone acetylase inhibition by spermidine (a natural polyamine) potently induce autophagy [237], suggesting a general role of protein acetylation in autophagy regulation (Figure 11).

1.2.5.4 p53

The p53 tumour suppressor, the “guardian of the cellular genome”, and associated molecular pathways are the most commonly mutated in human cancers, regulating apoptosis, autophagy, metabolism, and persistence in hypoxic environments [238]. Intriguingly, it has dual positive and negative regulatory roles in autophagy induction [239]. Nuclear p53 (transcription dependent) can induce autophagy through activation of AMPK (which in turn inhibits mTOR) [240] and up-regulation of (damage-regulated modulator of autophagy) DRAM and sestrin 2 [241, 242]. TIGAR is described as one of the few p53-induced genes that inhibit autophagy.

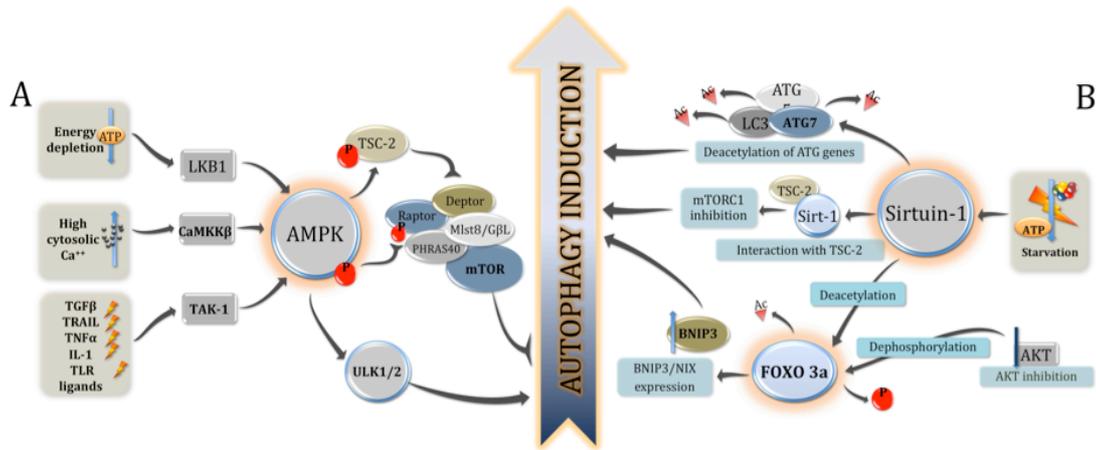


Figure 11 | Schematic overview of the autophagy signalling cascades involving AMPK and Sirtuin 1.

(A) AMPK (B) and Sirtuin 1 and FOXO 3. These pathways play a key role in nutrient sensing especially in starvation [122].

It is a fructose-2,6-bisphosphatase that stimulates the pentose phosphate shunt to lower intracellular ROS (Figure 12) [243]. Additionally, p53 differentially regulates autophagy through a direct molecular interaction with RB1CC1/FIP200 [244] and ULK1 [245], the critical proteins essential for the very apical step of autophagy initiation. Interestingly, key autophagy regulatory protein Beclin 1 is shown to control the levels of p53 for the optimal regulation of autophagy [246]. Recently, great attention has been paid to the transcription-independent functions of cytosolic p53; active p53 can accumulate in the cytosol and initiate mitochondrial cell death [9, 238, 247, 248], at the same time, cytosolic p53 is found to exert a tonic inhibitory action on the autophagic flow [249], depicting a crosstalk between two processes (discussed later). It has been shown that human, mouse and nematode cells subjected to knockout, knockdown or pharmacological inhibition of p53 manifest several signs of enhanced autophagy [250], both *in vitro* and *in vivo* [249]. Multiple distinct pro-autophagic stimuli including nutrient deprivation and mTOR inhibition by rapamycin were found to induce Mdm2-dependent proteasomal degradation of p53, suggesting the existence of a tonic inhibition of autophagy by p53. In line with this notion, p53 inhibition also promotes autophagy in enucleated cells, suggesting that the nuclear pool of p53 is not directly implicated in the suppression of autophagy [249]. Revealing the functional aspect of cytoplasmic p53 dependent inhibition of autophagy, it is discovered that HMGB1 complexes with p53 (their interactions in the nucleus serve to limit the level of cytosolic p53) to regulate its cytoplasmic localization and subsequent levels of autophagy and/or apoptosis [251]. Most of cancer-associated p53 variants/mutations inhibit autophagy and it is tempting to speculate, yet remains to be proven, that p53 mutants may stimulate carcinogenesis in a double (or even triple) fashion, by

combining defective transactivation of p53 transcriptional programs with suppressed autophagy (and in some instances also by interfering with the direct pro-apoptotic function of p53 at mitochondria) [9, 250].

1.2.5.5 NF- κ B

The stress responsive transcription factor, NF- κ B, and some of its upstream regulators (I κ B kinase, IKK), function to integrate diverse stress signals pathways with those of death (apoptosis) and survival (autophagy) pathways. IKK is a large multisubunit complex consisting of a regulatory subunit (NEMO/IKK- γ) and two catalytic subunits (IKK- β and IKK- α). IKK activates NF- κ B by phosphorylating a class of inhibitory molecules called inhibitor of κ B (I κ B), marking them for proteasome-dependent degradation. IKK-dependent phosphorylation of I κ B results in NF- κ B nuclear translocation and transcriptional activation of target genes [252]. NF- κ B has been reported to inhibit autophagy, for instance in the context of TNF α -induced cell death [253] or in macrophages infected by enterobacteria [254]. On the other hand, NF- κ B can induce autophagy by trans-activating the autophagy-triggering protein Beclin-1 [255] while on other hand, NF- κ B-incompetent cells are deficient in heat shock-induced autophagy [256]. In murine and human cells, IKK complex effectively triggers autophagy and optimal induction of autophagy by multiple different stimuli requires IKK activation. Of note, the induction of autophagy by IKK did not correlate with NF- κ B activation but relies on the activation of AMPK and JNK1, and, conversely, inhibition of NF- κ B failed to prevent IKK-induced autophagy [257, 258]. Thus, there may be both IKK-dependent, NF- κ B-independent as well as NF- κ B-dependent mechanisms for autophagy induction. Along similar lines, TGF β -activated kinase (TAK1), which is often required for IKK activation, underlies the optimal induction of autophagy in multiple settings [257]. TAK1 interacts with two structurally similar co-activators, TAK1-binding proteins 2 and 3 (TAB2 and TAB3) and in response to pro-autophagic stimuli, TAB2 and TAB3 dissociate from Beclin 1 to engage in stimulatory interactions with TAK1 [259, 260]. These results point to the existence of a direct molecular crosstalk between the canonical NF κ B activation pathway and the autophagic core machinery that guarantees the coordinated induction of these processes in response to stress (Figure 12).

1.2.5.6 Other autophagy regulating pathways

Additional important regulatory pathways of autophagy include:

- The transcriptional factor FOXO3, which has been shown to modulate autophagy, function downstream of Akt1 by trans-activating several *atg* genes in an mTOR independent fashion

[261] as well as involving the members of the death-associated protein kinase (DAPK) family (Figure 11).

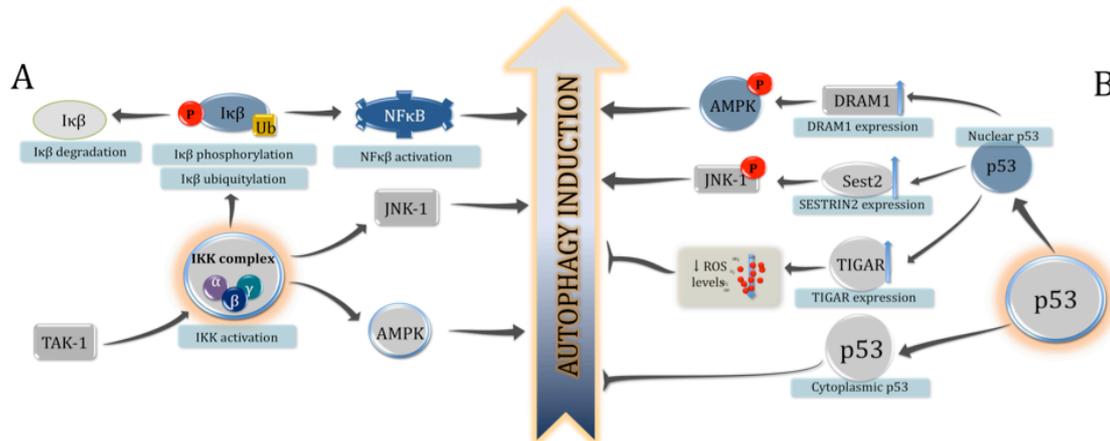


Figure 12 | Schematic overview of the autophagy signalling cascades involving IKK and p53.

(A) IKK complex (B) and p53. Note the dual control of autophagy by p53 via transcription dependent and independent ways [122].

- Hypoxia can induce autophagy *via* the HIF-1-mediated trans-activation of BNIP3 (BH3-only pro-apoptotic), but may also act in an HIF-1 and BNIP3-independent fashion, presumably by favouring the over-generation of pro-autophagic ROS [262, 263].

- DAPK-1, a calcium/CaM-regulated serine/threonine kinase normally associated with actin cytoskeleton, has been shown to stimulate autophagy by interacting with the microtubule-associated protein 1B (MAP1B) [264], and to be required for optimal autophagy induction by anti-estrogens, amino acid deprivation, or administration of interferon- γ [265].

- A dual role of Calcium is also suggested in the regulation of autophagy. The elevated Ca^{2+} may mediate autophagy involving activation of CaMKK- β (calcium/calmodulin-dependent kinase kinase- β), phosphorylation of AMPK and AMPK-dependent mTOR inhibitory signaling pathways [266], and at the same time it is shown that intracellular Ca^{2+} signals mainly via inositol 1,4,5-trisphosphate receptors (IP₃Rs) and suppress autophagy [267, 268]. IP₃R is a membrane glycoprotein complex acting as Ca^{2+} channel, activated by IP₃.

- Amino acid deprivation has also been shown to promote autophagy by favouring the phosphorylating activation of signal transducers belonging to the Ras/Raf-1/ERK1/2 signalling pathway, whose activity would converge on the phosphorylation of the G α -interacting protein (GAIP) [269, 270].

- ER stress pathway (including unfolded protein response) is a potent stimulus for the induction of autophagy [271] through phosphorylation of eIF2 α (eukaryotic initiation factor 2 α) by eIF2 α kinases like PKR (double-stranded RNA-activated protein kinase) and PERK (PKR-like eukaryotic initiation factor 2 α kinase) [272, 273]. Other ER stress mediators as ATF6 (activating transcription factor-6), or IRE1 (inositol requiring enzyme 1) activate and inhibit autophagy respectively [274].

1.2.6 Autophagy and Cancer

Many recent works show that autophagy inhibition favours tumourigenesis. At the same time, several tumour suppressor proteins have been reported to promote autophagy induction. Tumour suppressing properties of autophagy are exercised through limiting chromosomal instability, hampering oxidative stress and reducing local necrosis and inflammation. By contrast, enhanced autophagy helps to escape from excessive hypoxic, metabolic and therapeutic stress in the tumour cells[275].

Beclin 1, a crucial regulator of autophagy induction, is haplo-insufficient tumour suppressor gene found monoallelically deleted in a large number of breast, ovarian, prostate and other cancers and also inhibits tumourigenesis both in vivo and in vitro [110, 111, 276]. The convincing observations, for the role of autophagy as an oncosuppressor mechanism, comes from the studies showing Beclin 1- interacting autophagic genes like UVRAG [142] and Bif-1 [143, 277] are mutated in human cancers which seemingly leads to the altered autophagic activity in the mutant cells. The Bcl-2 family (that includes both oncoproteins and tumour-suppressor proteins) also control autophagy process [125]; the anti-apoptotic factors like Bcl-2, Bcl-X_L, and others suppress autophagy by inhibiting (binding of Vps34 to) Beclin 1 [56, 138] and, likewise, the pro-apoptotic BH3-only proteins like Bad, Bik, etc., promote the autophagic flow, by competitively inhibiting the inhibitory interactions with anti-apoptotic Bcl-2-like proteins with Beclin 1 [55, 56, 138, 197] showing a relation among autophagy, apoptosis and cancer[125]. Similarly, the loss of other important autophagy regulators such *atg4C* (it is required for the processing of LC3/Atg8) [278], DRAM [242], Atg5 [279, 280], Atg7 [280] render the atmosphere tumour-prone contributing to tumour development. p62/SQSTM1 (a multidomain scaffold protein, specifically turned over through autophagy) is preferentially accumulated in autophagy-defective cancer cells and its clearance by autophagy suppresses tumorigenesis [281, 282]. In cancer cells, the activity of mTOR kinase (the major negative regulator of autophagy in human cells) is also constitutively augmented owing to mutations that

inactivate up-stream negative regulators of the mTOR-PI(3)K signalling axis [283, 284]. Recent in vivo data reveals that mice lacking Atg7 in HSCs develop an atypical myeloproliferative morphology similar to human myelodysplastic syndrome (MDS) and converting to acute myeloid leukaemia (AML) [285]. In BCR-ABL-transformed cells, the PI3K-AKT-mTOR pathway is found constitutively active thereby conferring leukemogenic potential to hematopoietic cells in AML patients and in mouse and human [286-288] thereby showing that inhibition of autophagy as an essential link to pathogenesis of cancers.

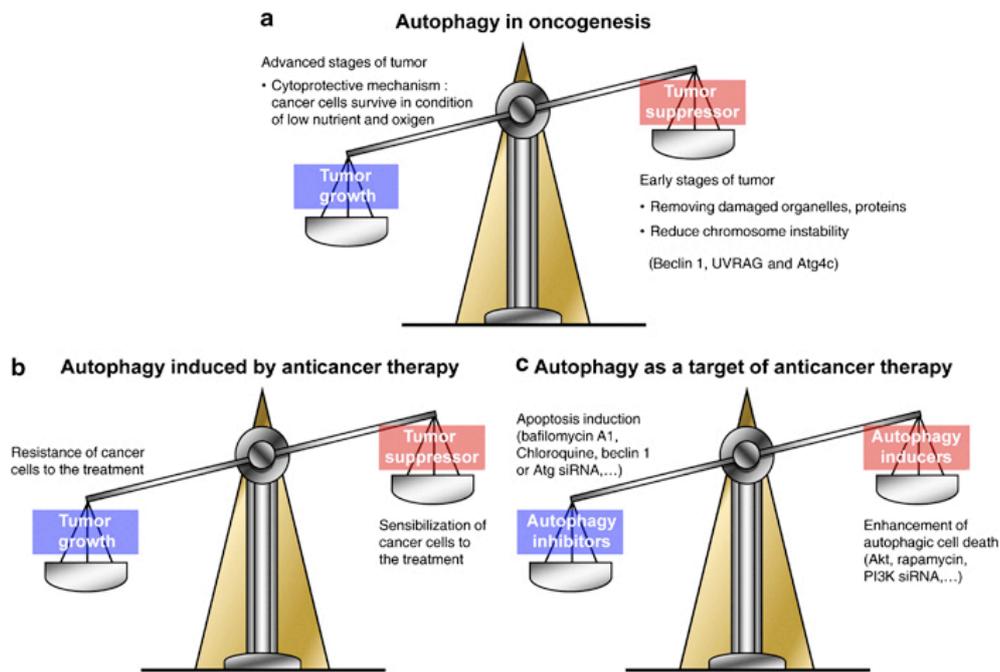


Figure 13 | Role of autophagy in oncogenesis and anticancer therapy.

The role of autophagy in oncogenesis is manifold. At the early stages of tumour, autophagy acts as a tumour suppressor. During tumour progression, autophagy can contribute to tumour growth. Further studies are necessary to elucidate the molecular mechanisms of this switch (a). Autophagy as a response to cancer therapy can promote/suppress tumour (b). Autophagy can be considered as an unusual target to improve anticancer therapy (c) [293].

Considering autophagy as a survival mechanism in stress response, it is plausible to also consider it to be a mechanism of survival for established tumour cells under variety of stress conditions such as hypoxic and metabolic stress especially in the case of poorly vascularised tumours which show higher levels of autophagy in the core as compared with margins [289]. Autophagy may promote tumourigenicity of the cells expressing activated Ras oncogenes, as autophagy deficient cells with Ras-expressing onco-proteins displayed reduced tumour growth in mice xenografts showing the importance of autophagy during energetic and oxidative crisis

[290]. Moreover, inhibition of autophagy flux by genetic (by silencing atg genes) or pharmacological means cause vigorous tumour regression and ultimately prolonged survival in pancreatic cancer xenografts and their genetic mouse models [291], aggressive melanoma (showing high basal autophagy levels) cultures [134], and in colorectal tumours [292]. The tumour suppressor protein p53 controls autophagy in a dual fashion; nuclear p53 can induce autophagy via transcriptional regulation, whereas cytoplasmic p53 represses autophagy via transcriptional independent control, highlighting a context dependent critical role of autophagic process in tumour progression [293]. Collectively, the connection between autophagy and cancer depicts a very complex scenario owing to apparently contradicting data and needs further in-depth critical evaluation of autophagy especially to cater it for the design of targeted therapies (briefly discussed in figure 13).

1.3 Crosstalk Between Apoptosis and Autophagy

The death, degradation, proliferation and survival of cells are a normal facet of the healthy life of eukaryotic/multicellular organisms for maintaining both general organismal and tissue homeostasis. For example, cells divide giving rise to new daughter cells for fresh working pool of cells, and at the same time other cells has to die to maintain the population constant. Moreover, the cells (especially non-dividing cells) have to cope, adapt and counteract different internal and external cellular stress situations in order to function properly and survive. It is plausible that cellular response to stress might involves several integrated pathways regulating nutrient uptake and metabolism, cell cycle and growth/proliferation, cell fate and lineage decisions, and cellular survival and death programs. Therefore, it is not surprising that a close integration between signals that regulate these cellular processes. In this regard, two prominent mechanisms of self-destruction and self-survival have captured the attention over the last decades: apoptosis and autophagy. The role of autophagy in most physiologically relevant conditions suggests that autophagy serves a largely as cyto-protective process and the concept of “autophagic cell death” is abolishing with further evolution in the scientific knowledge. A bulk of circumstantial evidence suggests negative regulation of apoptosis by autophagy controlling system, and in turn, there is negative modulation of autophagy by apoptotic signalling. Several signals that trigger autophagy are also known as apoptosis inducers, such as starvation, oxidative stress. The balance between autophagy and apoptosis is regulated by various interlinked and mutually controlled signalling pathways. At multiple levels, the cross talk between these pathways determines cell fate in different settings of cellular stress (and

ultimately determines different etiological parameters in the pathogenesis of diseases including cancer) [1, 122, 125, 137, 294-296]. Therefore, autophagy and programmed cell death share a large number of components. This section will offer brief overview of these connections.

1.3.1 Bcl-2 Proteins

As discussed above, Bcl-2 proteins are best-known modulators of apoptosis, with BH3-only proteins functioning in apoptosis induction. Beclin 1 is also a novel member of the BH3-only branch of the Bcl-2 family whose enforced expression (as a full-length Beclin 1) fails to trigger MOMP (and apoptosis) although the introduction of a only the BH3 domain of Beclin 1 provokes Bax-dependent apoptosis [297], suggesting that some regions of Beclin 1 outside of the BH3 domain may exert an anti-apoptotic function. One explanation is that other domains of Beclin 1 by targeting Beclin 1 to the ER (or other non-mitochondrial sites) could neutralize “apoptogenic” potential of the BH3 domain, thereby hindering Beclin 1 from interacting with the apoptosis-regulatory mitochondrial pool of Bcl-2 or Bcl-X_L (Figure 14). Another possibility can be the relatively lower affinity of Beclin 1 BH3 domain for the Bcl-X_L/Bcl-2 BH3-binding groove than do BH3 domains from pro-apoptotic proteins and thereby, at physiologically relevant concentrations would not displace the pro-apoptotic proteins from the Bcl-2/Bcl-X_L BH3 binding groove to induce apoptosis [55]. Also recently, apoptosis induced by the pro-apoptotic protein Bax is shown to reduce autophagy by enhancing caspase-mediated cleavage of Beclin 1 [298].

One most captivating aspect about this regulation by BH3 domains relates to concepts of spatio-temporal organization and organelle specificity of the proteins. Although Bcl-2, Bcl-X_L, and Mcl-1 are largely localized to the mitochondria, where they serve to inhibit mitochondrial outer membrane permeabilization, only ER-targeted, but not mitochondrial-targeted Bcl-2 and Bcl-X_L suppress autophagy [137, 138, 299, 300]. One latest report suggests the involvement of the novel Bcl-2 binding partner nutrient-deprivation autophagy factor-1 (NAF-1), which functions to stabilize the Bcl-2-Beclin 1 interaction at the ER [301]. Adding interest to subcellular location of these key regulators, localization of AMBRA1 at mitochondrial level, and thereby its interaction with Bcl-2, is shown to regulate autophagy and modulate apoptosis [302]. Furthermore, the treatment of cells with ABT737 decreases the fraction of Beclin 1 that co-immuno-precipitates with ER-targeted Bcl-2 but does not affect the amount of Beclin 1 that co-immuno-precipitates with mitochondrion-targeted Bcl-2 [56]. Recently, it has been shown that only the ER-enriched pool of Bcl-2 is subjected to regulation of Beclin 1 binding by JNK1-

mediated phosphorylation [200]. Additionally, the essential autophagy protein Atg12 is shown as a positive mediator of mitochondrial apoptosis. Atg12 controls the apoptotic pathway by directly binding and hampering the function of pro-survival Bcl-2 family members (including Bcl-2 and Mcl-1) [303]. Similarly, UVRAG, which interacts with the Beclin-1 complex in the core autophagic control machinery, inhibits apoptosis by direct interaction with Bax (thereby blocking its translocation to mitochondria) [304] (Figure 14). These studies implies the probable presence of BH3 domains in Atg and other autophagy regulating proteins might dictate the crosstalk between these processes and future struggle in this direction with further elaborate this potential crosstalk.

1.3.2 p53

The biological effects of p53, the central integrator of numerous signaling pathways triggered by a numerous of cellular stress signals, are mediated by the combined activities of its nuclear and cytoplasmic localizations [238]. p53, a well-known activator of apoptosis, is responsible for up-regulating pro-apoptotic genes at the transcriptional level, such as *Bax*, *PUMA* and *NOXA*, and hampering the expression of anti-apoptotic gene products, such as *Bcl-2*. Whereas, cytoplasmic accumulation of p53 (by nuclear export) by transcription factor FOXO3 is considered as direct p53-mediated MOMP induction [305]. In the same line of action, it is discovered that HMGB1 complexes with p53 (their interactions in the nucleus serve to limit the level of cytosolic p53) to regulate its cytoplasmic localization and subsequent levels of apoptosis and/or autophagy [251]. Moreover, Cytosolic accumulation of p53 enhances Bax-mediated MOMP and ultimately apoptosis [85, 97]. p53 may play a dual role in autophagy regulation; transcription-dependent and transcription-independent, the later mechanism inhibits autophagy involving cytoplasmic localization of p53 (discussed above). Thereby, it is likely that the dual action of cytoplasmic p53, inhibition of autophagy and induction of MOMP, may signify a coordinated response for cell death induction. Transactivation of genes (such as DRAM and Sestrin 1 and 2) by p53 induces autophagy [242, 306] but interestingly, DRAM expression is also needed for apoptotic cell death from DNA-damaging agents [242]. Moreover, p53 is shown to mediate mTOR inhibition through AMPK and the tumour suppressors tuberous sclerosis-1 (TSC1) and TSC2) and thereby indirectly links to autophagy [240]. All this data clearly underscores the importance of mutual talk between apoptosis and autophagy.

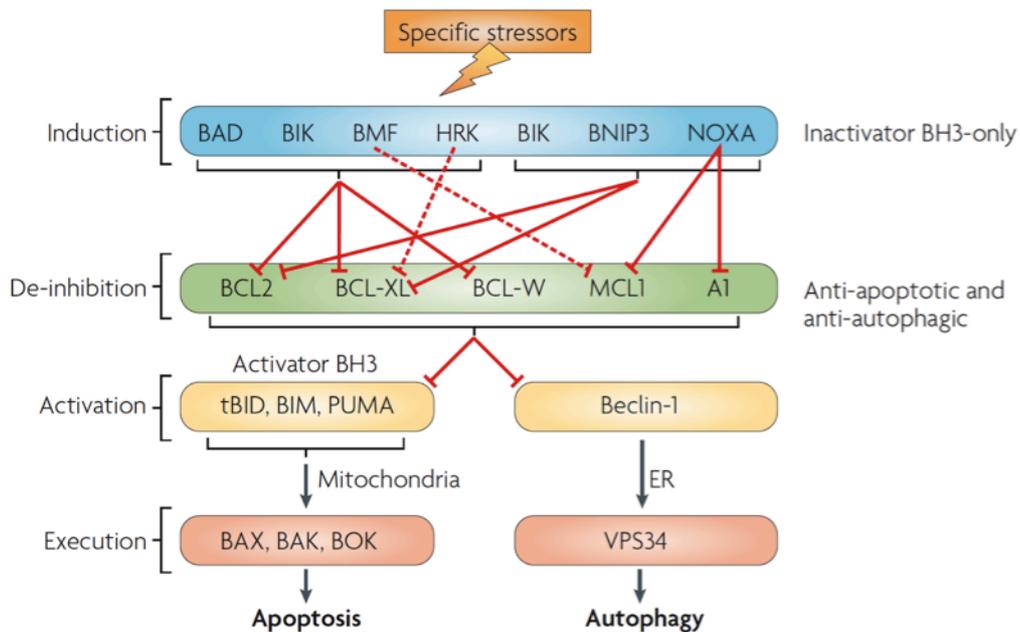


Figure 14 | Molecular crosstalk between apoptosis and autophagy.

Depending on their specificity (for pro- or anti-apoptotic and autophagy-inhibitory proteins that contain BH3 receptors), as well as their preferential sub-cellular localization (mitochondria or endoplasmic reticulum), BH3-only proteins can preferentially activate apoptosis or autophagy [125].

1.3.3 DAPK

DAPK (death-associated protein kinase) is a well-known pro-apoptotic serine/threonine kinase, whose activity is necessary for apoptotic cell death induced by death receptor activation [307, 308]. Recently, Beclin 1 has been shown to be a substrate of death-associated protein kinase (DAPK) and its subsequent phosphorylation reduces its binding to the Bcl-2 family members, thereby, suggesting a possible mechanism by which DAPK may also induce autophagy [202]. Furthermore, ectopic expression of DAPK has been shown to induce autophagic vesicle formation and membrane blebbing, the latter a characteristic of cells dying by apoptosis. Thus, DAPK may regulate both processes simultaneously, suggesting a critical role for DAPK as an integrator of apoptotic/autophagic cross talk. DAPK is also reported to up-regulate p53 through a mechanism that requires p19ARF (murine homologue of human p14ARF) [309] and as p53 can initiate either apoptosis or autophagy, this potentially links DAPK to both pathways.

1.3.4 Death related protease

Recent scientific literature has suggested the role of Atg proteins in mutual interacting processes of autophagy and apoptosis. Atg proteins can be the specific targets of death related

protease i.e., caspases and calpains, providing potential regulation of autophagy as well as uncovering their novel functions in the process of apoptosis.

1.3.4.1 Caspases

Caspases, a family of cysteine-aspartate-requiring proteases, play a central role in the process of apoptosis but their recent functions describe the mechanism by which apoptosis-triggering signals inhibit autophagy as different human Atg proteins are shown to be cleaved by different caspases [99]. Beclin 1 is cleaved by caspase 8 during chemotherapy-induced apoptosis leading to disruption of Bcl-2/Bcl-X_L-Beclin 1 interaction. The cleavage of Beclin 1 renders the protein non-functioning and cytosolic, losing its capacity to induce either autophagy or to affect apoptosis [310, 311]. Another study demonstrated that in response to interleukin-3 deprivation, cleaved Beclin 1 at C-terminus (Beclin 1-C) could acquire a new anti-autophagic and apoptosis-promoting function by translocating from the cytosol to the mitochondria and inducing apoptosis by releasing pro-apoptotic factors [312] (Figure 15). Furthermore, on induction of apoptosis by Bax overexpression renders Beclin 1 inactive by caspase 3-dependent cleavage [298]. Other Atg proteins have been implicated in apoptosis, for example, caspase 3 cleaves the human Atg4 family member Atg4D to generate a truncated product, ΔN63 Atg4D that, when overexpressed, induces apoptosis [313]. Likewise, the induction of Beclin 1/Atg7 dependent autophagy has also been demonstrated in response to the simultaneous inhibition of caspase-8 and a calpain-like protease.

1.3.4.2 Calpains

Calpains are calcium-dependent intracellular cysteine proteases that play a crucial role in the regulation of cell spreading, cell migration, programmed cell death, cell cycle progression and also the macroautophagy [310, 314]. Atg5 is a critical protein required for autophagy at the stage of autophagosome and is shown to act as an independent key player in both apoptosis and autophagy as it cooperates with Bcl-2 and Bcl-X_L to regulate both apoptosis and autophagy. Following death stimuli, Atg5 is cleaved by calpain, translocates to the mitochondria, interacts with Bcl-X_L and promotes cytochrome *C* release and caspase activation to promote mitochondria-mediated apoptosis [279]. Atg5 is also reported to interact with FADD (Fas-associated protein with death domain) and this interaction mediates interferon-induced cell death. Interestingly, Atg5 down-regulation reduced cell death whereas its ectopic expression increased cell death [315]. Likewise, the induction of Beclin 1/Atg7 dependent autophagy has also been demonstrated in response to the simultaneous inhibition of caspase-8 and a calpain-like protease.

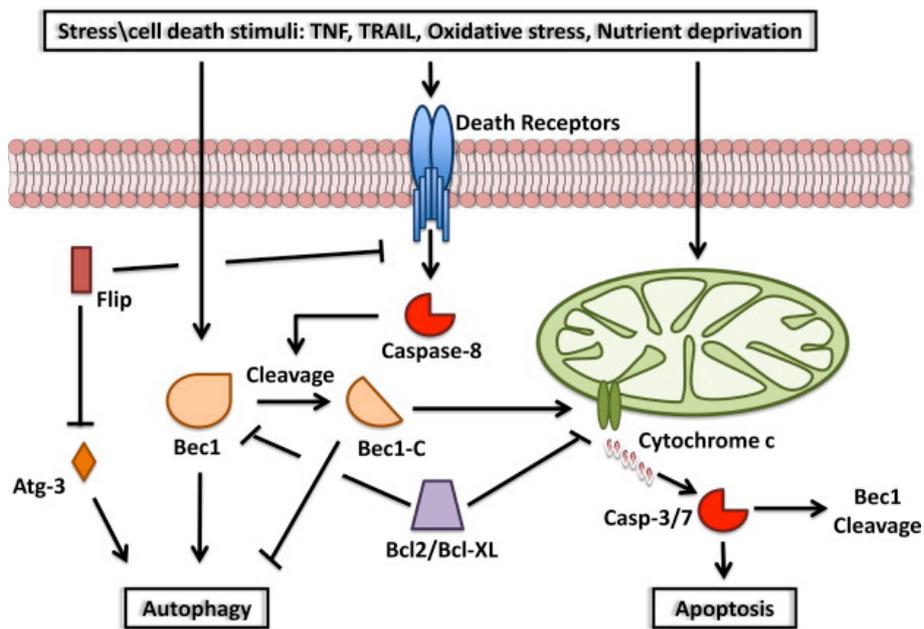


Figure 15 | Scheme of molecular interconnections between autophagy and cell death pathways.

Beclin 1 is a crucial point of interconnection between two processes. Caspase-dependent cleavage of Beclin 1 represses activation of autophagy and promotes apoptosis via the Bec1-C fragment. Bcl-2/Bcl-X_L act as both anti-autophagy and anti-apoptotic factors by specific inhibitory interactions. cFLIP (Flice inhibitory protein, an apoptosis inhibitor, also competes with LC3 for Atg3 binding) is able to repress the death receptor pathway and counteracts autophagosome elongation [316].

1.3.5 Calcium mediated signalling

Intracellular calcium mediated signalling underscore the degree to which the apoptotic cellular pathways and autophagy mediating pathways are interlinked at subcellular levels to control the induction of each other in mutually dependent manners. Increase in the cytosolic levels of Ca²⁺ controlled by ER and/or mitochondria comprise the induction of apoptotic signalling [317]. Similarly as discussed before, Ca²⁺ levels also regulate the process of autophagy; the elevated Ca²⁺ may mediate autophagy-involving activation of CaMKK-β and AMPK [266], but intracellular Ca²⁺ signals via IP₃ receptors and suppress autophagy [267, 268]. Moreover, Ca²⁺ also strongly activate calpains thereby manipulating the control of the two processes might be in reciprocal fashion.

1.3.6 NF-κB/IKK pathway

As discussed before, NF-κB activation has both anti- and pro-apoptotic functions thereby determining the outcome of specific apoptotic stimuli. The genes that are targeted to and also the level of the activation of signaling pathways leading of gene transcription determine pro- or

anti-apoptotic function of NF- κ B. For example, TRAF2 and IAP activation leads anti-apoptotic function of NF- κ B [42], while activation of p53, PUMA and caspases links a pro-apoptotic function of NF- κ B [43, 46]. Additionally, apoptosis induction can inhibit NF- κ B-related pathways. In fact, activated-caspases upon TNF-induced apoptosis cleave Inhibitor of I κ B kinase β (IKK β) to inactivate IKK activity [44]. Likewise, caspase 3 cleaves I κ B α to generate a stable, repressor form which inhibits NF- κ B [45]. Moreover, NF- κ B/IKK signalling also controls autophagy in dual mode; depending upon the framework of activation of signals can inhibit or activate autophagy (discussed above) [253-258]. Therefore, NF- κ B modulates both cell death and cell survival functions in context dependent manner and controls a crosstalk between autophagy and apoptosis at multiple levels.

1.3.7 p14ARF and their isoforms

p14ARF/INK4a (murine nucleolar p19ARF, a tumour suppressor protein and potent apoptotic inducers), is a known activator of p53 and works by antagonizing the function of Mdm2, whereas its short mitochondrial ARF (smARF) functions independently of Mdm2 and p53. It can work independent of the Bcl-2 family proteins to dissipate mitochondrial membrane potential without MOMP to induce caspase-independent apoptosis, which, interestingly, is diminished by knockdown of either Atg5 or Beclin 1. Moreover, both full-length ARF and smARF are proposed to induce autophagy by p53-dependent and p53-independent mechanisms, respectively. This entire complex cross-linking underscores the presence of delicate cross talk between the two processes [125, 318].

1.4 BH3 mimetics

Targeting the different cell death pathways has emerged as a promising new avenue for drug discovery and cancer therapy, as cancer cells may develop novel mechanisms for evading chemotherapy-induced apoptosis and autophagy-associated cell death pathways. Apoptotic pathway (regulated by the Bcl-2 family of proteins) and their defects play a major role in cancer development and also in conferring chemoresistance [80]. As discussed earlier, within this family, Bax and Bak play essential role in MOMP and pro-survival proteins (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1) oppose their molecular functions to promote cell survival. The BH3-only proteins such as Bim, Bad, PUMA and NOXA relieve the inhibition of pro-survival anti-

apoptotic proteins to relieve Bax and Bak. The clinical efficacy of most anti-cancer therapeutics primarily reflects their ability to induce apoptosis and the resistance to conventional anti-cancer therapeutics agents is often due to a failure to activate BH3-only proteins due to underlying mutations. Moreover, pathologic overexpression of pro-survival Bcl-2 family proteins is frequently found in many tumour types and in some scenarios, elevated Bcl-2 or Bcl-X_L expression correlates with poor responses to conventional therapies [319].

Hence, once it was understood that cancer cells depended on Bcl-2 or related anti-apoptotic proteins for their survival, it became apparently the need of great practical significance to identify and target these specifically identified Bcl-2 and/or Bcl-X_L as possible drug targets. The design of BH3 mimetics was based on the rationale that by acting as inhibitors of the anti-apoptotic Bcl-2 family members, these compounds would counter such apoptotic blocks to activate apoptosis. Actually, these pharmacological components, BH3 mimetics, can occupy the BH3-binding grooves of antiapoptotic proteins from the Bcl-2 family (such as Bcl-2, Bcl-X_L) to cater the ability to kill the cells activating pro-apoptotic proteins which are essential for the apoptotic mitochondrial membrane permeabilization [56, 93, 197]. Therefore, BH3 mimetics are a new class of cancer drugs that specifically target cancer cell survival mechanism to selectively kill cancer cells. Currently, several BH3 mimetics are either in pre-clinical development or have advanced to clinical trials [90, 320] including ABT737 and its oral analogues (ABT-263) (binds to Bcl-2, Bcl-X_L and Bcl-w but not to Mcl-1), gossypol and its analogues (targets Bcl-2, Bcl-X_L and Mcl-1), GX15-070 (Obatoclax, binds to all anti-apoptotic Bcl-2 family members), HA14-1 (inhibitor of Bcl-2 and is thought to disrupt Bax and Bcl-2 interactions), TW-37 (targets including Bcl-2, BCL-X_L, and Mcl-1)[321] (Figure 16). The potential of BH3 mimetics as inducers of autophagy has recently come to light, thereby point to the crosstalk between apoptosis and autophagy [56, 197].

1.4.1 ABT737

In my thesis work, I am mainly focused on ABT737, which is one of the most advanced Bcl-2 inhibitors in clinical development. It was discovered by Abott laboratories by a screening strategy using nuclear magnetic resonance, structure-based design and combinatorial chemical synthesis ('SAR by NMR') [93]. ABT737 binds to Bcl-2 and Bcl-X_L (but not Mcl-1) and can kill cells, provided that they express pro-apoptotic multi-domain proteins (such as Bax and Bak), which are essential for the apoptotic mitochondrial membrane permeabilization, as well as BH3-only proteins, which, once liberated from the BH3-binding cleft of Bcl-2 or Bcl-X_L,

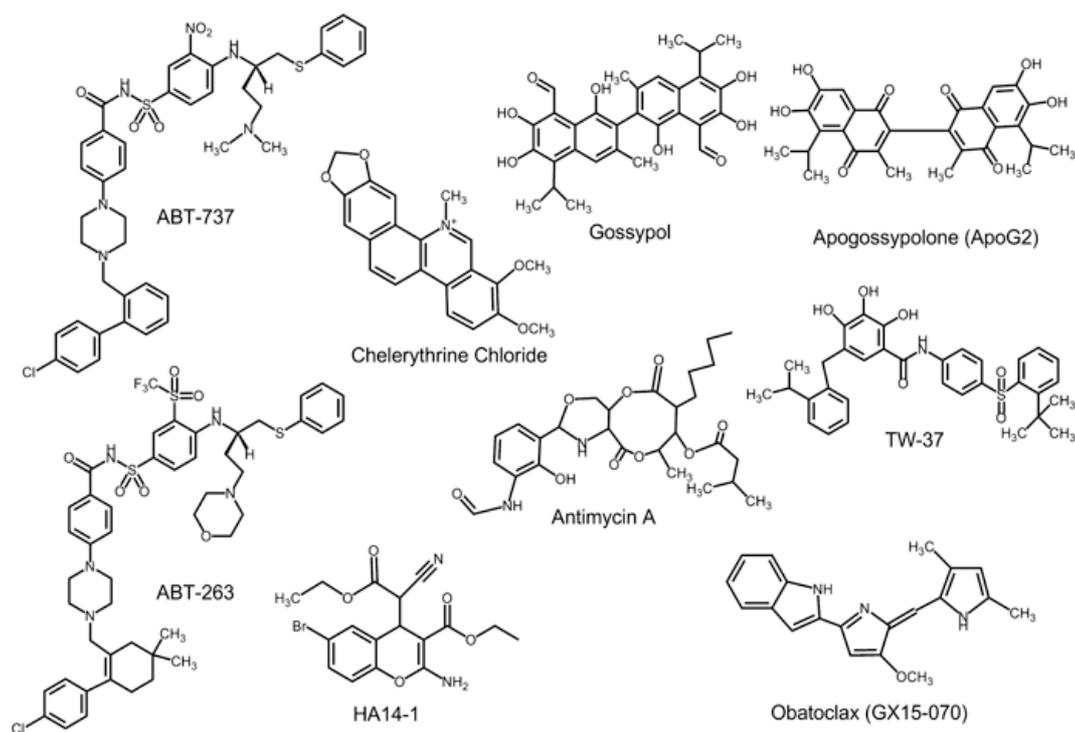


Figure 16 | Structures of some Bcl-2 inhibitors (BH3 mimetics).

BH3 mimetics are a new class of cancer drugs that specifically target a mechanism of cancer cell survival to selectively kill cancer cells [322].

can activate Bax and/or Bak. Therefore, ABT737 is effective at activating apoptosis solely through the Bcl-2 family, as apoptosis is deficient in the cells lacking both Bax and Bak [93, 323] (Figure 17).

It has been shown that BH3 mimetics induces autophagy. The studies on the pro-autophagic profile of ABT737 shows that ABT737 can induce autophagy because it liberates Beclin 1 from its inhibitory interaction with Bcl-2 or Bcl-X_L, thus allowing Beclin 1 to support the class III phosphatidylinositol-3-kinase activity of Vps34 (in cells that are resistant to its pro-apoptotic effect) [56, 197]. ABT737 was shown to inhibit the binding of a Beclin 1-derived BH3 peptide to Bcl-X_L or Bcl-2 in a competitive fashion, and to abolish the co-immunoprecipitation of Beclin 1 with Bcl-2 or Bcl-X_L (but not with Mcl-1). ABT737-triggered autophagy could not be inhibited by Bcl-2/Bcl-X_L overexpression, yet was suppressed by small-interfering RNA (siRNA)-mediated knockdown of Beclin 1 [197]. Moreover, only Beclin 1-Bcl-2/Bcl-X_L complexes present in the ER (but not those present on mitochondria) are disrupted by ABT737, suggesting that Beclin 1-Bcl-2/Bcl-X_L complexes that usually suppress autophagy are specifically located in the ER [56, 197]. Similar findings of autophagy induction have been obtained using other BH3 mimetics (such as HA14-1) [324-326].

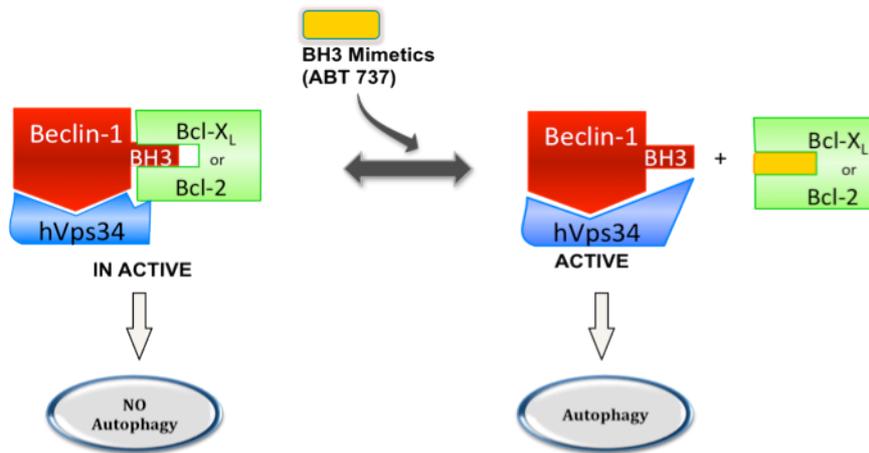


Figure 17 | BH3 mimetics act on the Beclin 1-Bcl-2 interaction to regulate autophagy.

Small molecules mimicking BH3 domains (BH3 mimetics, *i.e.*, ABT737) can bind to the BH3 receptor domain of Bcl-2 or Bcl-X_L and hence competitively disrupt the interaction between Bcl-2 or Bcl-X_L and Beclin 1 thereby inducing lipid kinase activity of the class III PI3K Vps34.

2

AIMS AND OBJECTIVES

2.1 Working hypothesis

ABT737 can induce autophagy because it liberates Beclin 1 from its inhibitory interaction with Bcl-2 or Bcl-X_L, thus allowing Beclin 1 to support the class III phosphatidylinositol-3-kinase activity of Vps34. ABT737 induces autophagy solely by its effects on the Beclin 1 interactome. Nonetheless, autophagy is a complex process that is regulated by multiple protein complexes beyond those organized around Beclin 1. In many paradigms of autophagic stimulation including nutrient starvation, positive regulators of autophagy (such as AMPK, SIRT1, IKK) must come into action while negative regulators (such as mTOR and cytoplasmic p53) must be inactivated for the optimal induction of autophagy. Based on these premises and incognita, we decided to measure the possible effects of ABT737 on multiple distinct autophagy regulators and hypothesized that:

“ABT737 activate multiple pro-autophagic pathways”

2.2 General objective

In this work we aim to get insight into the complex interconnections among the autophagy regulating the pathways and thereby revealing the

“network properties of autophagy-regulatory signalling cascades”

2.3 Specific objectives

1. To confirm that BH3-mimetic ABT737 is capable of increasing the autophagic flux and to check whether it stimulate different autophagic pathways;
2. To study the functional roles and implications of different pro-autophagic pathways in ABT737-induced autophagy;
3. To compare the results with the different BH3-mimetic HA14-1 to rule out off-target effects that might be due to non-specific functions of BH3-mimetics (ABT737).

3

MATERIALS AND METHODS

3.1 Cell lines and culture conditions

All cell lines were cultured at 37°C under 5% CO₂, in a medium containing 10% fetal calf serum, 10 mM Hepes buffer and 1mM sodium pyruvate. In addition, cell type-specific culture conditions include Dulbecco's modified Eagle's medium (DMEM) for human cervical carcinoma HeLa cells; DMEM containing 1% non-essential amino acids for WT and Bax^{-/-} Bak^{-/-} MEF (mouse embryonic fibroblasts); DMEM containing 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulphate for human osteosarcoma GFP-LC3 U2OS cells; McCoy's medium for HCT116 colon carcinoma cell lines (generous gift from Dr. B Vogelstein). All media and supplements for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). Cells were seeded in 6- or 12-well plates and grown for 24 h before treatment with ABT737 (2000, 1000, 100, 10 or 1nM; Selleck Chemicals, Houston, TX, USA), Bay11-7082 (1 µM; BioMol Research Laboratories, Lausen, Switzerland), EX527 (100 µM; Tocris Bioscience, Bristol, UK), bafilomycin A1 (BafA1; 1 nM), geldanamycin (2 µM), cisPt (10 and 20 µM), 17-allylamino-geldanamycin (2 µM), MG132 (10 µM; Sigma-Aldrich, St Louis, MO, USA), HA14-1 (1, 5, 10 and 20 µM), Nutlin-3 (10 µM) or RITA (10 µM; Alexis Biochemicals, Lausen, Switzerland) for the indicated period (12 h, unless otherwise stated).

3.2 Plasmids, transfection and RNA interference in human cell cultures

Transient transfections were performed with Lipofectamine 2000 reagent (Invitrogen) and cells were used 24 h after transfection unless specified differently. Cells were transfected with empty vector, Rheb D60K and Rheb Q64L [219] alone or together with green fluorescent protein (GFP)-LC3 [327]. Cells were cultured in six-well plates and transfected at 50% confluence with Oligofectamine reagent (Invitrogen), in the presence of 100 nM of siRNAs specific for human SIRT1 [235], Atg5 (Sigma), IKK α , IKK β , NEMO [258], AMPK α , Beclin 1, HMD2, p53, Vps34 [249], a scrambled siRNA or a siRNA targeting the unrelated protein emerlin [328]. siRNA-mediated protein downregulation was controlled by immunoblots.

3.3 Cytofluorometry

The following fluorochromes were employed to determine apoptosis-associated changes by cytofluorometry: 3,30-dihexyloxacarboxyanine iodide (40 nM) for quantification or the

mitochondrial trans-membrane potential (Dcm) determination and propidium iodide (1 mg/ml) for determination of cell viability (Molecular Probes). Cells were trypsinized and labelled with the fluorochromes at 37°C, followed by cytofluorometric analysis with a fluorescence activated cell sorter (FACS-Scan, Becton Dickinson, Franklin Lakes, NJ, USA).

3.4 Quantification of autophagy

Autophagy was quantified by counting the percentage of cells showing accumulation of GFP-LC3 in vacuoles, of a minimum of 100 cells per preparation in three independent experiments. Cells presenting a mostly diffuse distribution of GFP-LC3 in the cytoplasm and nucleus were considered as non-autophagic, whereas cells representing several intense punctuate GFP-LC3 aggregates with no nuclear GFP-LC3 were classified as autophagic. Two independent investigators read each GFP-LC3 staining. Cells cultured on coverslips were fixed in paraformaldehyde (4% w:v) for 30 min at room temperature for GFP-LC3 assays. Nuclei were labelled with Hoechst 33342 (10 mg/ml; Molecular Probes, Carlsbad, CA, USA). Fluorescence microscopy was performed with an IRE2 microscope (Leica Microsystems, Weitzler, Germany) equipped with a DC300F camera.

3.5 Automated high-content microscopy

A total of 10×10^3 U2OS cells stably expressing a GFP-LC3 chimera were seeded in 96-well imaging plates (BD Falcon, Oxford, UK) 24 h before treatment. The cells were cultured in the presence of ABT737 (1 μ M) under standard culture conditions (37 °C, 5% CO²) and subjected to pulsed observations (every 1 h for up to 36 h) with a BD pathway 855 automated live cell microscope (BD Biosciences, Oxford, UK). Images were further analyzed for the kinetics of GFP-LC3 sub-cytoplasmic count with the open source software Cellprofiler (freely available from the BROAD Institute at <http://www.cellprofiler.org>, Cambridge, MA, USA). Otherwise the videos of the kinetic GFP-LC3 were generated by Image J software.

3.6 Western blot analysis

Cells were washed with cold phosphate buffered saline at 4°C and lysed on ice using standard protocols [249]. In all, 40–50 μ g of proteins was loaded on 4–12% or 12% SDS–PAGE precast gels (Invitrogen) and transferred to Immobilon membrane (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h in phosphate-buffered saline-Tween-20 (0.05%) containing 5% non-fat milk. Primary antibodies including anti-SIRT1, -Beclin 1, -

p53, -p62, -NEMO, -P-NEMO (S376), -HDM2 (Santa Cruz, Santa Cruz, CA, USA), -STAT3, -P-STAT3 (Y705), -Akt, -P-Akt (T308 and T473), -mTOR, -P-mTOR (S2448), P-p53 (S392), -IKK α , -IKK β , -P-IKK α/β (S376/180), -AMPK α , -P-AMPK α (Y172), -Pyk2, -P-Pyk2 (Y402), -I κ B α , -P-I κ B α (S32/36), -p70S6K, -P-p70S6K (Y421/S424), -Atg5, -ACC, -P-ACC (S79) or anti-LC3 (Cell Signalling, Danvers, MA, USA) were incubated overnight at 4°C and revealed with the appropriate horseradish peroxidase labelled secondary antibodies (Southern Biotechnologies Associates, Birmingham, AL, USA) plus the SuperSignal West Pico chemoluminescent substrate (Pierce, Rockford, IL, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Billerica, MA, USA) or anti-actin (Abcam, Cambridge, MA, USA) were used to control equal loading.

3.7 Phospho-kinase array

The profile of phospho-kinase proteins was performed on HeLa cells treated with ABT737 (1 μ M) and HA14-1 (20 μ M) for 2, 6 and 12 h using a Proteome Profiler array–Phospho- Kinase array kit (ARY003) according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). Clustering was performed with MultiExperiment Viewer (MeV version 4, Boston, MA, USA). Hierarchical clustering was performed using Euclidean distance and average linkage algorithm based on their normalized phosphorylation intensities. Heatmap was generated by heatmap view integrated in MeV 4 software.

3.8 Mitochondrial respiratory chain activities

Respiratory chain activities of ABT737 and HA14-1-treated cells (HCT116 and HeLa cells) were measured at the various time points mentioned. The samples (after treatments) were kept frozen (–80° C) before assaying and analysed spectro-photometrically after permeabilizing and disrupting cells by means of freeze–thaw cycle. Briefly, a small aliquot of the cell-pellet (fewer than 1 million cells; 0.5–0.7 mg protein) was deep-frozen in 50 μ l PBS and subsequently thawed using 1 ml of ice-cold solution (medium A) consisting of 0.25M sucrose, 20mM Tris (pH 7.2), 40 mM KCl, 2 mM EGTA, and 1 mg/ml BSA, 0.01% digitonin (w/v), and 10% Percoll (v/v). After 10 min at ice temperature, cells were centrifuged, the supernatant discarded, and the pellet was washed with 1 ml of the same described solution (medium A) devoid of digitonin and Percoll. Again the pellet after centrifuge was re-suspended in 30 μ l of medium A and used for enzyme assays. The respiratory chain activities were analysed by three spectrophotometric assays (TSA) as described previously in the

literature.[329, 330].

3.9 ATP measurement

Intracellular ATP was measured using an ATP Assay kit (Calbiochem, Darmstadt, Germany) based on luciferin–luciferase conversion following the manufacturer’s instructions. After the stimulation with ABT737 (1 μ M) and HA14-1 (20 μ M), the cells were treated with lysis buffer containing 1% trichloroacetic acid for 1 min. The luciferin solution was added, and the luciferase activity was measured as an indicator for ATP content. For assessment of the chemoluminescent signals, the plates were read in a Fluostar luminometer (BMG Labtech).

3.10 Statistical analysis

Statistical analyses were carried out using the Prism software package (GraphPad Software Inc., San Diego, CA, USA) and the Microsoft Office 2003 Excel software package (Microsoft Corporation, Redmond, WA, USA). Mean values \pm S.D. or \pm S.E.M. were indicated and were compared using unpaired Student’s t-tests. For multiple comparisons, we used the one-factor (ANOVA) variance analysis corrected by the Bonferroni test.

4

RESULTS AND DISCUSSION

The results of this work are discussed under following points:

1. Confirmation of autophagy induction by ABT737
2. ABT737-induced autophagy involves the AMPK, mTOR and IKK pathways
3. Role of AMPK and mTOR in ABT737-induced autophagy
4. Functional implication of IKK in ABT737-induced autophagy
5. Implication of p53 in ABT737-induced autophagy
6. Implication of SIRT1 in ABT737-induced autophagy
7. Multiple signal-transduction pathways elicited by ABT737
8. Pro-autophagic activity of the BH3 mimetic HA14-1
9. Comparison of ABT737- and HA14-1-induced autophagy

4.1.2 Functional implication of Beclin 1 complex in ABT737-induced autophagy

Further we checked whether Beclin 1 complex is implicated in ABT737 induced autophagic flux. Consistent with the published finding that ABT737 induces autophagy via the Beclin 1 complex [56, 197] the knockdown of Vps34 (which is the enzymatic subunit of the Beclin 1 complex, a type 3 phosphatidylinositol-3 kinase) [195] inhibited ABT737-induced autophagy evaluated by LC3 lipidation and p62 degradation (Figure 19). This confirms that Beclin 1 mediated activity is necessary for ABT737 induced autophagy.

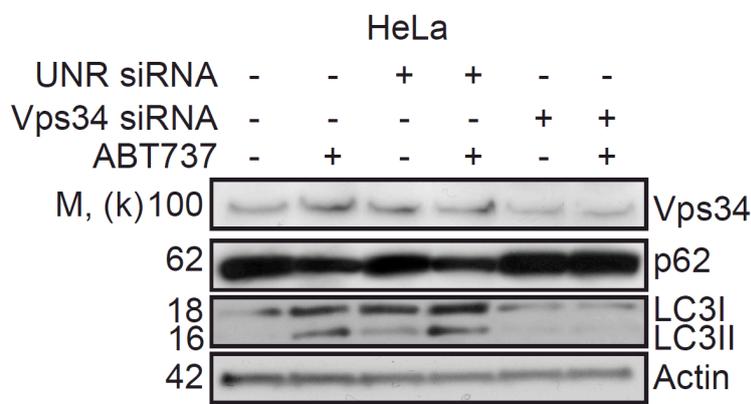


Figure 19 | Involvement of Vps34/Beclin 1-complex in ABT737-induced autophagy.

Immunoblot detection of the levels of p62 and LC3I/II. HeLa cells were transfected with a control siRNA or a siRNA specific for Vps34. After 48h, the cells were treated for 12h with ABT737 (1 μ M). Actin levels were assessed to ensure equal loading of lanes [331].

4.2 ABT737-induced autophagy involves the AMPK, mTOR and

IKK pathways

Next, we determined whether ABT737 stimulates additional pro-autophagic pathways by assessing its effects on the phosphorylation status of AMPK α and that of its substrate acyl-CoA carboxylase (ACC), the phosphorylation of mTOR and that of its substrate p70^{S6K}, as well as the phosphorylation of the IKK subunits, IKK α , IKK β and NEMO (IKK γ) in three different cancer cell lines namely HeLa, U2OS and HCT116 (Figure 20). Although the kinetics of these effects were different among the cell lines, ABT737 consistently induced the hyperphosphorylation of AMPK α and the three IKK subunits, as well as a consistent reduction in the

phosphorylation of mTOR. These changes were paralleled by alterations in the phosphorylation status of the AMPK α substrate ACC (which is also hyper-phosphorylated), the IKK substrate I κ B α (which is hyper-phosphorylated and ultimately degraded) and the mTOR substrate p70^{S6K} (which is hypophosphorylated upon ABT737 treatment), consistent with the interpretation that ABT737 stimulates the activation of AMPK, yet inhibits mTOR. Altogether, these results indicate that ABT737 has pleiotropic effects on several autophagy relevant signal-transduction pathways.

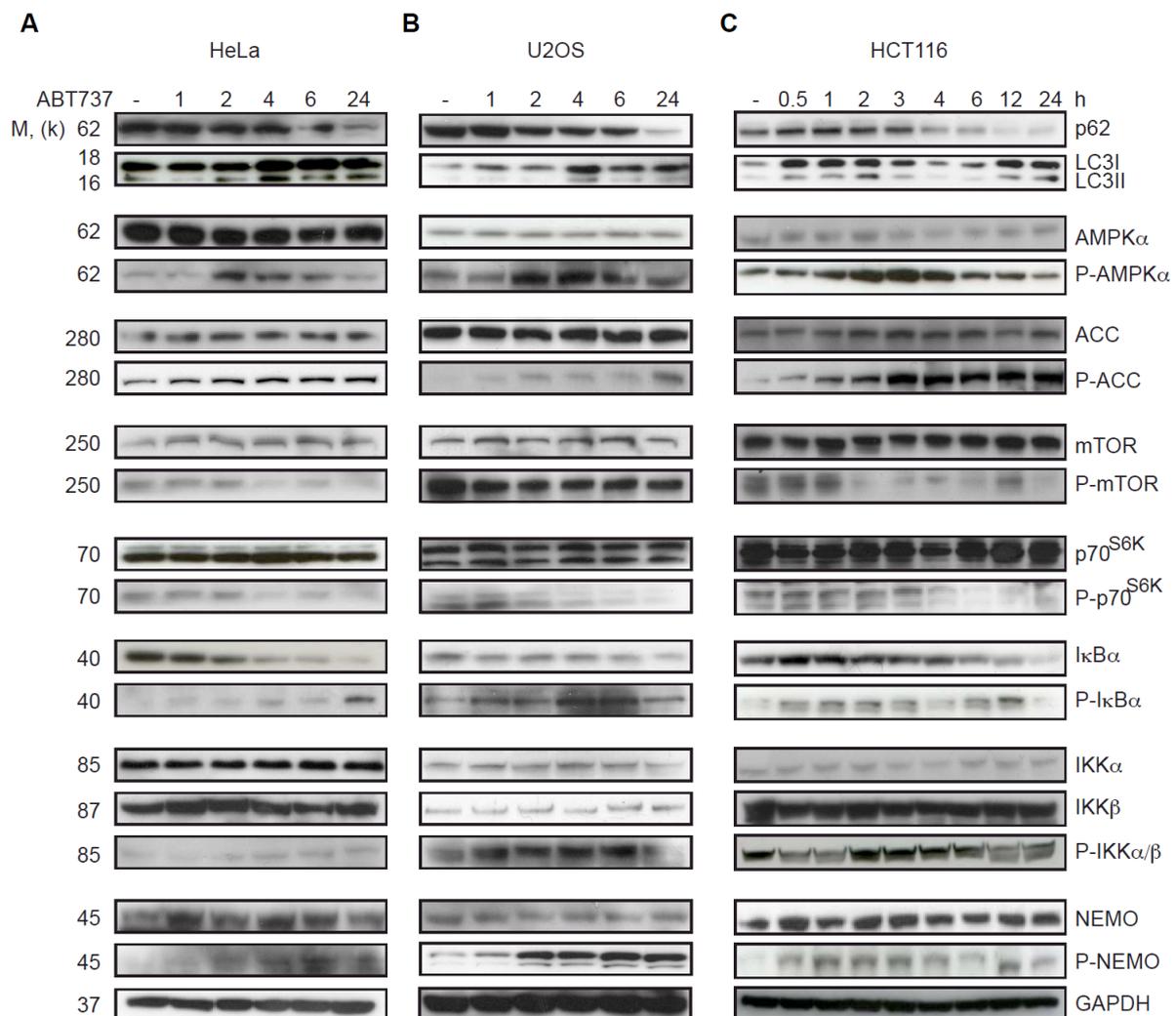


Figure 20 | Implication of AMPK, mTOR and IKK pathways in the autophagy induction by ABT737.

Immunoblot detection of the levels of p62 and LC3I/II as well as the phosphorylation status of AMPK α (Y172), ACC α (S79), mTOR (S2448), p70^{S6K} (Y421/S424), I κ B α (S32/36), IKK α/β (S176 or S180, respectively) and NEMO (S376) in different human cell lines (A-C) stimulated with ABT737 (1 μ M) for the indicated time. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were monitored to ensure equal loading. Data are representative of three independent experiments [331].

4.3 Role and functional implication of AMPK and mTOR in ABT737-induced autophagy

4.3.1 Role of AMPK and mTOR in ABT737-induced autophagy

ABT737 is able to induce the hyper-phosphorylation of AMPK α , as well as the phosphorylation of the AMPK α substrate ACC and autophagy at a dose around 1 μ M (Figure 21A). The concentrations of ABT737 needed to induce autophagy on cells are higher than those required for Bcl-2 blockade (in cell-free systems). Thus, we decided to analyze whether AMPK activation is required for the pro-autophagic activity of ABT737. Small interfering RNA (siRNA)-mediated depletion of AMPK α reduced the ABT737-induced LC3 lipidation and p62 degradation (Figure 21B) in each of the three cell lines tested (shown for HCT116 cells in Figures 21A and B). Moreover, ATP cellular levels progressively decreased after ABT737 treatment, which likely accounts for the observed ABT737-dependent AMPK activation (Figure 30A). Given that ABT737 also induced a consistent decrease in mTOR activity, we then investigated whether the inhibition of mTOR is a necessary step in the induction of autophagy by ABT737. For this purpose, we overexpressed a constitutive active form of Rheb (Rheb Q64L), which positively regulates mTOR, as showed by the phosphorylation status of the mTOR substrate p70S6K (which is hypo-phosphorylated upon ABT737 treatment) (Figure 21D). The expression of Rheb Q64L restored the activity of mTOR and prevented the autophagy induction by ABT737. In contrast, the expression of a dominant-negative form of Rheb (Rheb D60K) did not affect the level of autophagy induced by ABT737 (Figure 21C). Altogether these data confirm that ABT737-mediated stimulation of autophagy requires both AMPK activation and mTOR inhibition.

4.3.2 Functional implication of AMPK and mTOR in ABT737-induced autophagy

To get insights into the molecular pathways underlying the effects of ABT737 on different pro-autophagic signalling factors, we determined whether the knockdown of Vps34 or that of Beclin 1 would affect the hyperphosphorylation of AMPK (and that of its substrate ACC) or the hypophosphorylation of p70^{S6K}. Interestingly, although the inhibition of mTOR induced by ABT737 was inhibited by depletion of Vps34 or Beclin 1, the activation of AMPK was not affected by the knockdown of Vps34 or Beclin 1 (Figure 22), suggesting that ABT737 is able to modulate distinct and non-univocal pro-autophagic pathways.

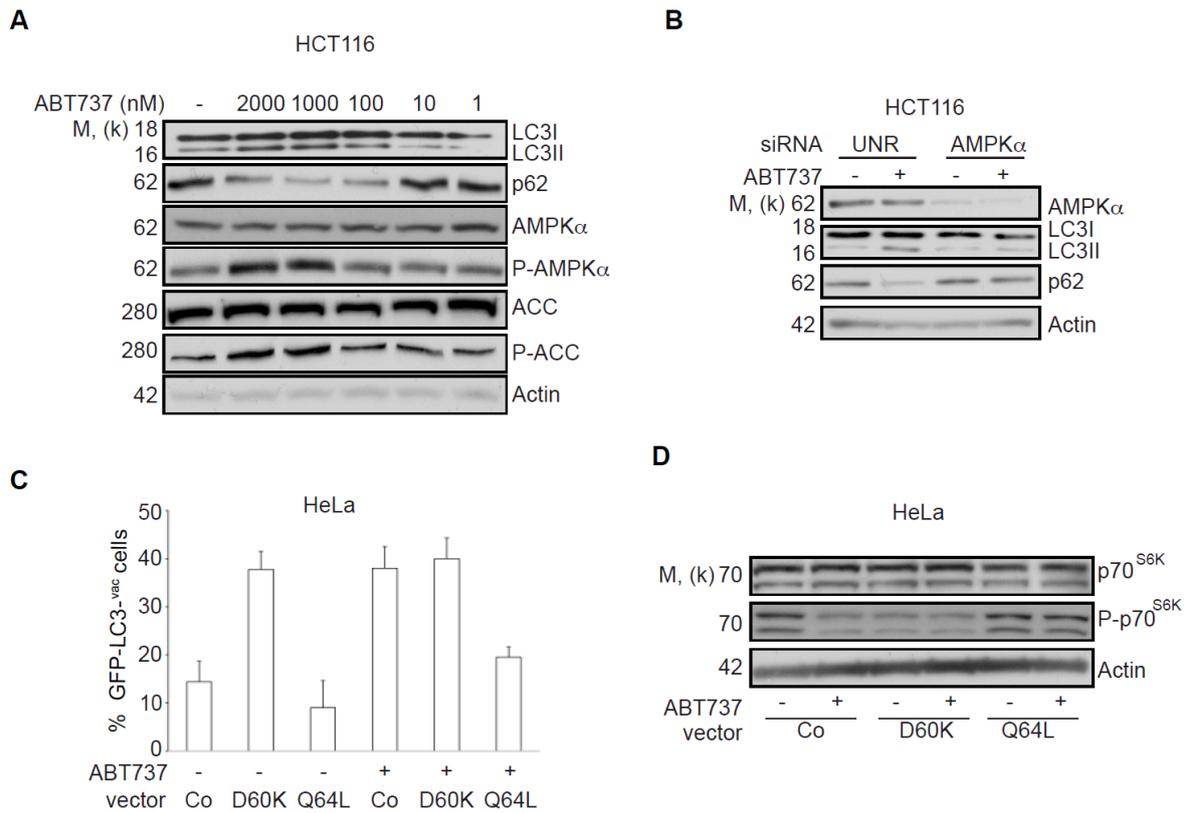


Figure 21 | Involvement of AMPK α and mTOR in ABT737-induced autophagy.

(A) Representative immunoblots showing dose-dependent induction of autophagy (depicted by LC3 lipidation and p62 degradation) by ABT737 and phosphorylation status of AMPK α (Y172) or ACC α (S79), performed on HCT116 cells treated for 12h. (B) Immunoblot detection of LC3 lipidation and p62 degradation in HCT116 depleted from AMPK α . 48h after the transfection, the cells were treated with ABT737 (1 μ M) for 12h. The efficiency of siRNA-mediated downregulation of the protein is also shown. (C) HeLa cells were simultaneously transfected with GFP-LC3 and equivalent amounts of plasmids coding for control vector, Rheb D60K (dominant-negative construct) or Rheb Q64L (constitutively active construct). After 24h, the cells were treated with ABT737 (1 μ M) for 12h. The percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is shown. (D) Representative immunoblots of p70^{S6K} (Y421/S424) phosphorylation status for the conditions indicated in C. Actin levels were monitored to ensure equal loading [331].

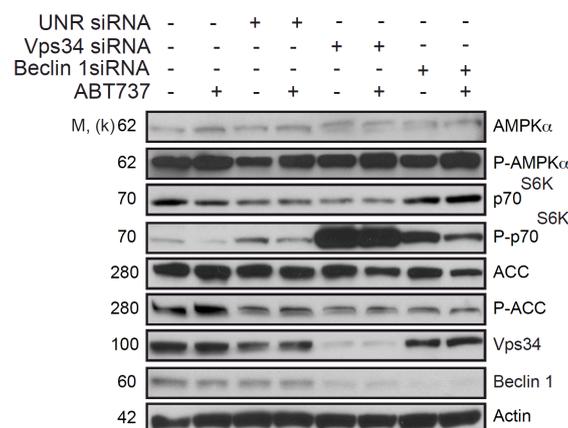


Figure 22 | Functional implication of AMPK α and mTOR pathways.

Effect of Vps34 and Beclin 1 downregulation on the ABT737-mediated activation of AMPK α and inhibition of mTOR. Phosphorylation status of AMPK α (Y172), ACC α (S79) and p70^{S6K} (Y421/S424) along with the

efficiency of siRNA-mediated downregulation of Beclin 1 and VPS34 is shown. Actin levels were monitored to ensure equal loading [331].

4.4 Role and functional implication of IKK in ABT737-induced

autophagy

4.4.1 Role of IKK complex in ABT737-induced autophagy

The IKK inhibitor Bay11-7082 [332], as well as the HSP90 inhibitors geldanamycin and 17-allylamino-geldanamycin, which can also inhibit IKK [333], were previously identified as inhibitors of starvation-induced autophagy [258]. We found that all three inhibitors also prevented the ABT737-stimulated lipidation of LC3 (Figure 23A) and the redistribution of GFP-LC3 to autophagic puncta in all three cell lines included in this study (Figure 23B). Accordingly, siRNA-mediated depletion of IKK α (Figure 23C), IKK β (Figure 23D) or NEMO (Figure 23E) reduced the ABT737-induced lipidation and inhibited the induction of GFP-LC3 puncta by ABT737 (Figure 23F). These results were obtained consistently in each of the three cells. These results underscore the obligate importance of IKK complex in the induction of autophagy by ABT737.

4.4.2 Functional implication of IKK complex in ABT737-induced autophagy

After knowing the obligate importance of IKK proteins in ABT737-induced autophagy, further looked into the molecular implication of this pathway by the knockdown of Atg5 or that of Beclin 1. Surprisingly, we noted that depletion of Atg5 or that of Beclin 1 abolished the hyperphosphorylation of IKK α/β , NEMO and I κ B α (Figure 24), suggesting an intimate link between IKK activation and autophagy as inhibitor of NF- κ B (I κ B) kinase is required for ABT737-induced autophagy, but autophagy is also necessary for IKK activation {Criollo, #298}.

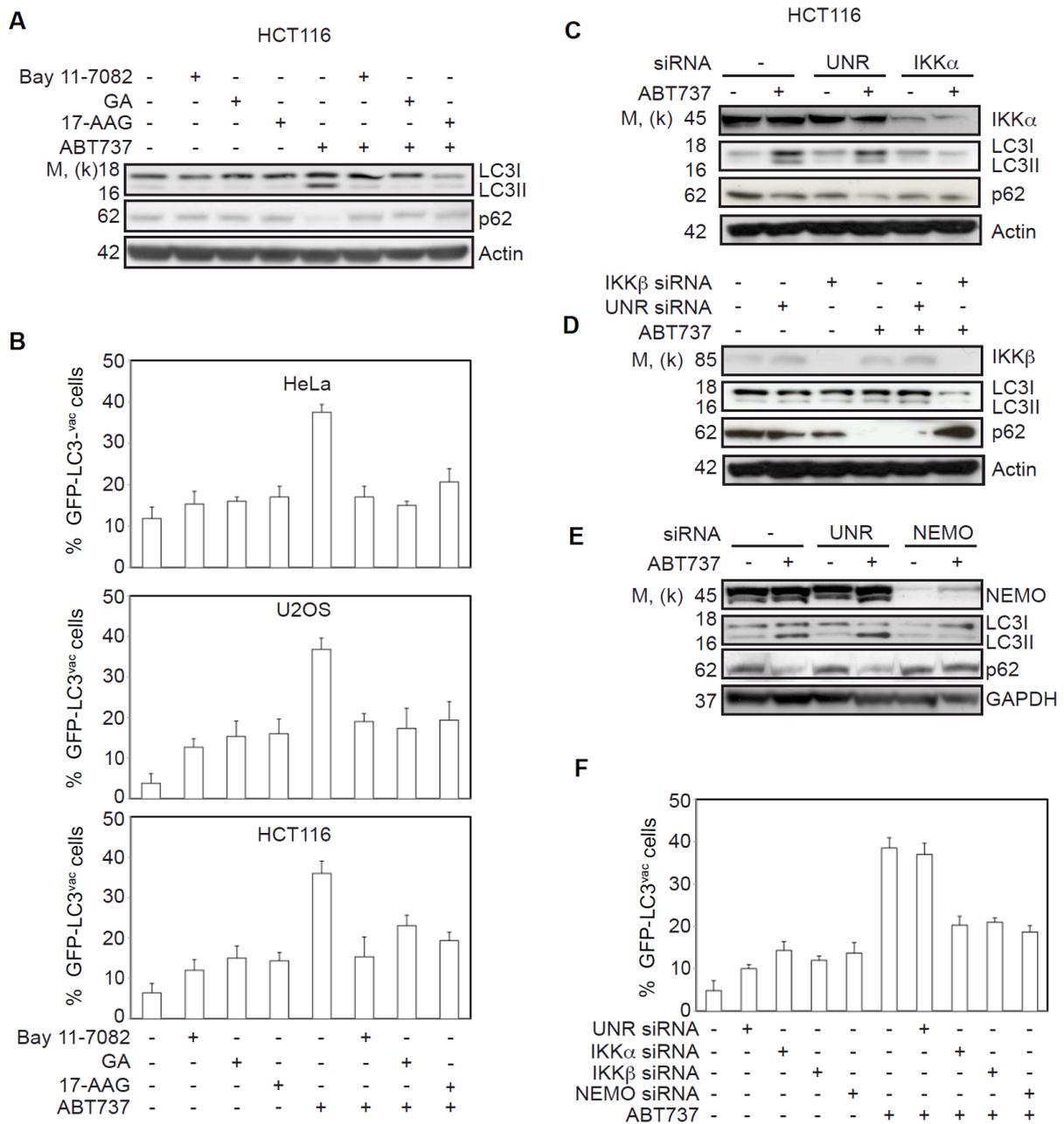


Figure 23 | Involvement of IKK in ABT737-induced autophagy.

(A, B) Pharmacological inhibition of IKK pathway reduces autophagy increased by ABT737. (A) LC3 maturation and p62 degradation in HCT116 treated with ABT737 (1 μ M) in the presence of Bay11-7082 (1 μ M), GA (geldanamycin; 2 μ M), 17-AAG (17-allylamino-geldanamycin; 2 μ M) for 12h. (B) GFP-LC3-expressing HeLa, U2OS, HCT116 cells were treated under the same conditions and the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is shown. (C-E) Immunoblot detection of LC3 lipidation, as well as p62 degradation in HCT116 subjected to knockdown of IKK α , IKK β , NEMO and treatment with ABT737 (1 μ M) for 12h. The efficiency of siRNA-mediated down-regulation of the protein is also shown. (F) HCT116 were transfected with unrelated (UNR) or a siRNA specific for the IKK complex subunits and subsequently (after 24h) with a plasmid for the expression of GFP-LC3, followed by culture in the presence of ABT737 (1 μ M) for 12h and the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is shown [331].

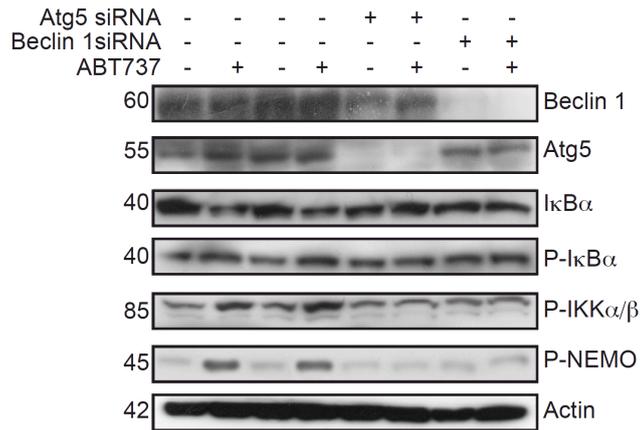


Figure 24 | Functional implication of IKK in ABT737-induced autophagy.

Immunoblot detection of phosphorylation status of IκBα, IKKα/β and NEMO. HCT116 cells were transfected with UNR or siRNAs specific for Atg5 and Beclin 1 as mentioned previously and treated with ABT737 (1μM) for 12h. The efficiency of siRNA-mediated downregulation of the proteins is also shown. Actin levels were assessed to ensure equal loading of lanes [331].

4.5 Implication of p53 in ABT737-induced autophagy

We investigated the contribution of p53 to ABT737- induced autophagy, based on the recent observation that cytoplasmic p53 must be ubiquitinated by MDM2 and then degraded by proteasomes to allow the induction of starvation-induced autophagy [249]. Pharmacological inhibition of MDM2 with RITA or Nutlin-3 prevented the degradation of p53 [249] and concomitantly prevented the induction of autophagy in HCT116 cells induced by ABT737 (Figure 25A). Similarly, proteasome inhibition by MG132 resulted in the maintenance of high p53 levels [249] and the inhibition of ABT737-induced autophagy (Figure 25A). RITA, Nutlin-3, MG132 all consistently inhibited the induction of GFP-LC3 puncta by ABT737 in HeLa, U2OS and HCT116 cells (Figure 25C). In accord with these results, which are based on the use of pharmacological inhibitors, knockdown of HDM2 by specific siRNAs also blunted the autophagic response of ABT737-treated cells (Figures 26B,C). These results show the complexity in pro-autophagic signal transduction pathway stimulated by ABT737, which involves the obligatory activation of the ubiquitin ligase MDM2.

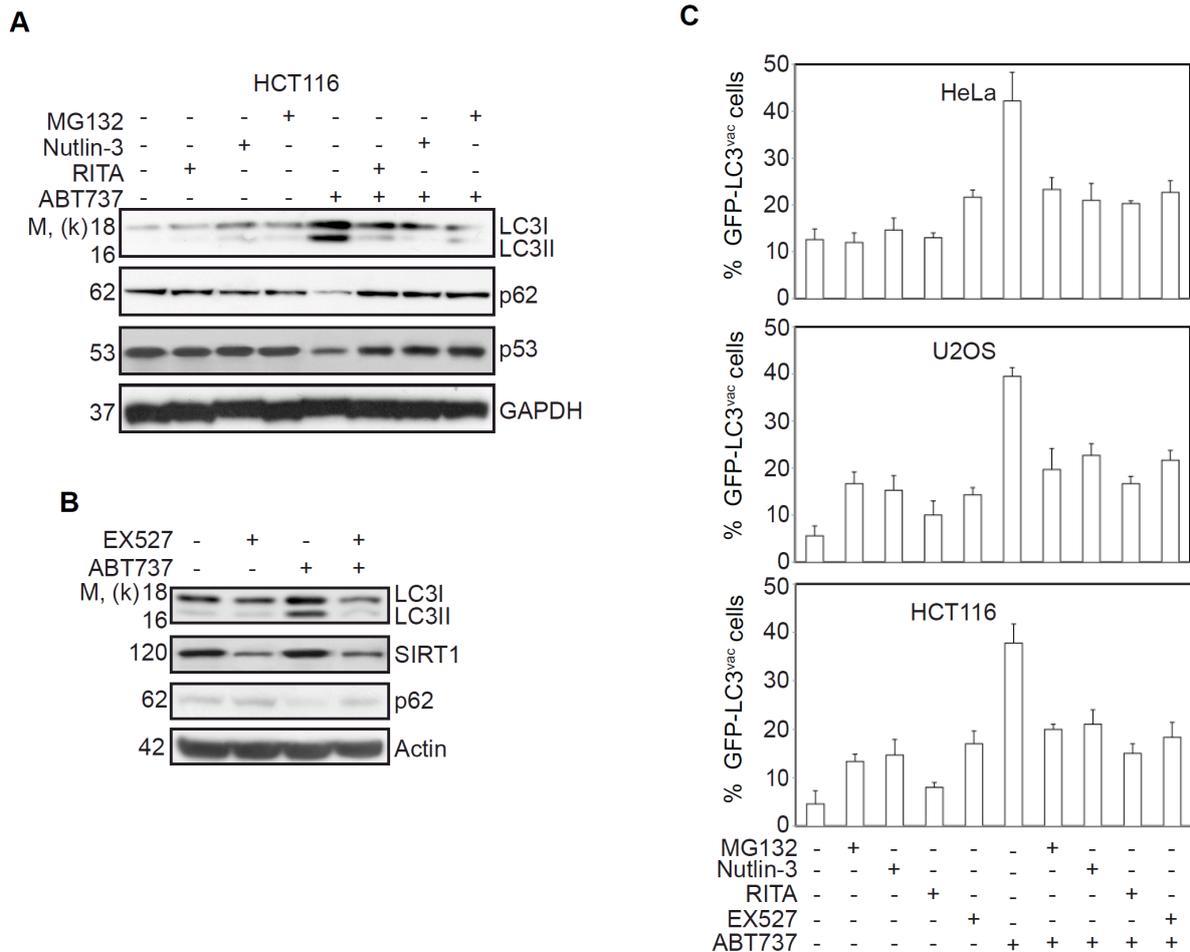


Figure 25 | Involvement of p53 and SIRT1 in ABT737-induced autophagy.

(A, B) Levels of LC3I/II and p62 expression in HCT116 treated with ABT737 (1 μ M) for 12h in the presence or absence of pharmacological inhibitors of MDM2 (RITA or Nutlin-3, both used at 10 μ M), proteasome (MG132, 10 μ M) and SIRT1 (EX527, 100 μ M). (C) GFP-LC3-expressing HeLa, U2OS or HCT116 cells were treated under the same conditions as in (A,B) and the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is reported. Actin and glyceraldehyde-3-phosphate dehydrogenase levels were assessed to ensure equal loading of lanes (means \pm s.e.m., $n=3$) [331].

4.6 Implication of SIRT1 in ABT737-induced autophagy

Sirtuin 1 (SIRT1) is required for autophagy induced by nutrient shortage [235]. SIRT1 inhibition by EX527 also prevented the induction of LC3 lipidation in ABT737-treated HCT116 cells (Figure 25B). EX527 consistently inhibited the induction of GFP-LC3 puncta by ABT737 in HeLa, U2OS and HCT116 cells (Figure 25C). In accord with these results, which are based on the use of pharmacological inhibitor, knockdown of SIRT1 by specific siRNAs also blunted the autophagic response of ABT737-treated cells (Figures 26 A,C). These results underscore the complex pro-autophagic signal transduction pathway stimulated by ABT737, which involves the obligatory activation of the deacetylase SIRT1.

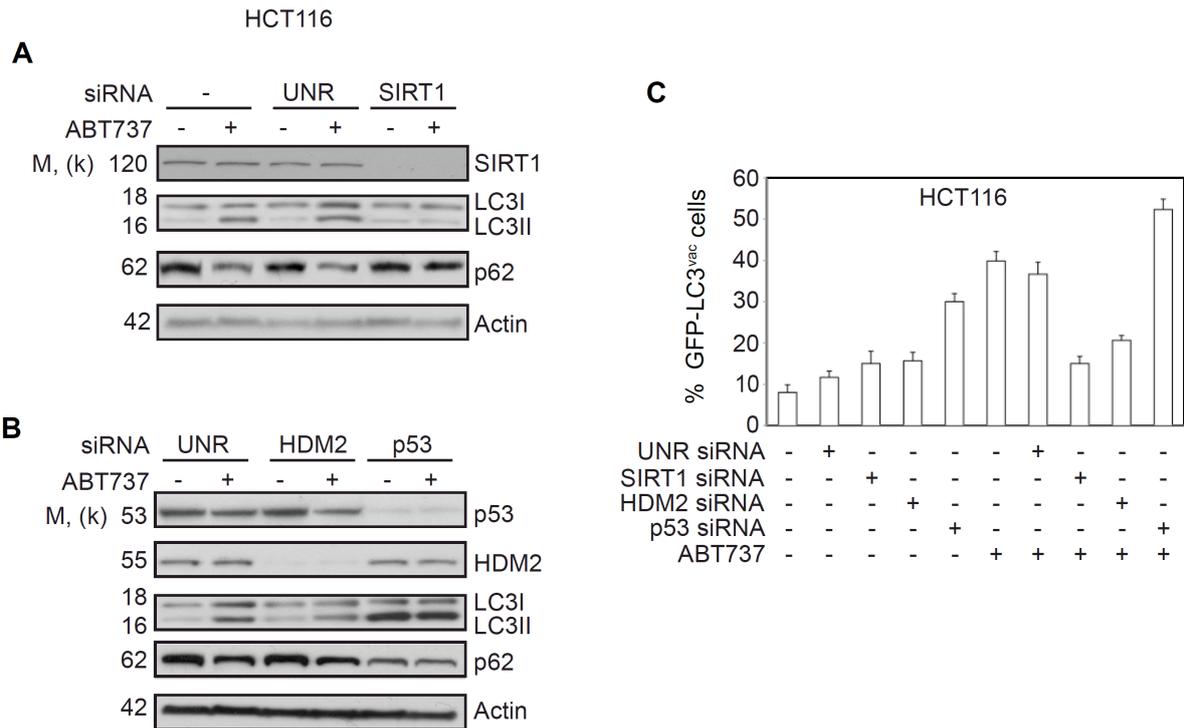


Figure 26 | Involvement of p53 and SIRT1 in ABT737-induced autophagy.

(A, B) Immunoblot detection of LC3 lipidation, as well as p62 degradation in HCT116 subjected to knockdown of SIRT1, HDM2 and p53. At 48h after the transfection, the cells were treated with ABT737 for 12h. The efficiency of siRNA-mediated downregulation of the proteins is also demonstrated. Glyceraldehyde-3-phosphate dehydrogenase levels were monitored to ensure equal loading. (C) Quantification of the percentage of GFP-LC3^{vac} in HCT116 transfected with siRNAs specific for SIRT1, HDM2 and p53 and stimulated for 12h with ABT737 (1 μ M) (means \pm s.e.m., $n=3$) [331].

4.7 Multiple signal-transduction pathways elicited by ABT737

To systematically assess the cellular signals elicited by ABT737, we determined changes in the phosphoproteome of HeLa cells treated for 2, 6 or 12 h with the BH3 mimetic ABT737, using a phosphoprotein-specific antibody array (Figures 27A and 27B). ABT737 caused the hyperphosphorylation of a few proteins such as Pyk2 (confirmed by immunoblot in Figure 31A) and AMPK α (confirmed in Figures 20 and 21), as well as that of other proteins (such as HSP27 and Paxillin). Moreover, ABT737 triggered the dephosphorylation of several proteins: p53 on three distinct serine residues (confirmed for S392 in Figure 31A), p70S6K on several residues (confirmed for threonine 421/seronine 424 in Figure 20), that of Akt (confirmed in Figure 31A), as well as that of numerous other proteins, including transcription factors of the STAT family (in particular STAT 1 and 4), ribosomal protein S6 kinase, the cyclin-dependent kinase inhibitor, p27 (on threonine 157), and endothelial nitric oxide synthase. These results hint to multiple effects of ABT737 on a plethora of signal-transduction pathways.

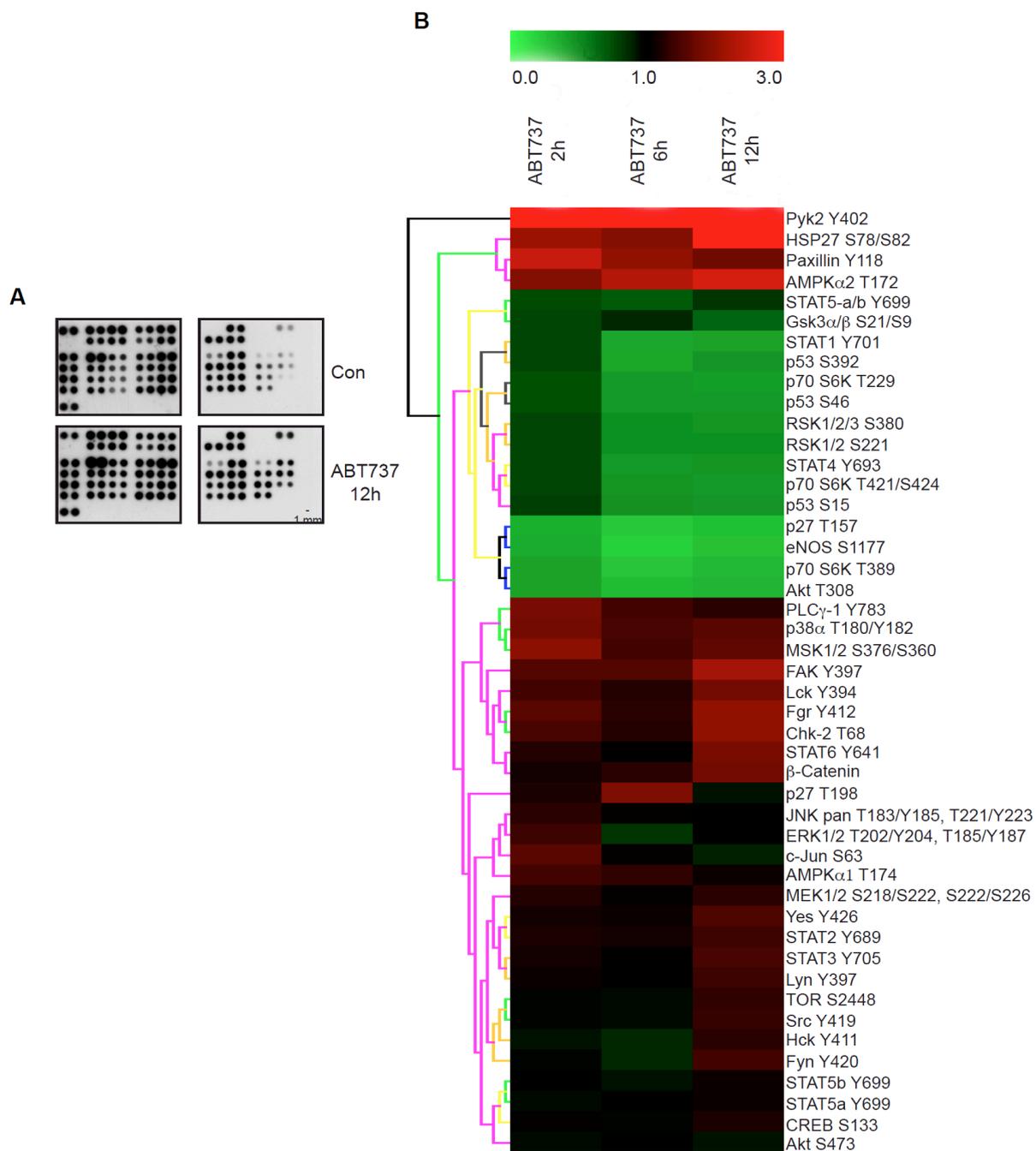


Figure 27 | Profile of phospho-proteome elicited by ABT737 in HeLa cells.

HeLa cells were treated with ABT737 (1 μ M) for 2, 6 or 12h. Cell extracts were then probed on human phosphoprotein arrays following manufacturer's instructions (see Materials and methods section). **(a)** Representative pictures of proteome profiler array. **(b)** Clustering analysis for the effect of BH3 mimetic ABT737 on protein kinase phosphorylation [331].

4.8 Pro-autophagic activity of the BH3 mimetic HA14-1

We assumed that the pro-autophagic activity of ABT737 might involve off-target effects. To address this possibility, we compared ABT737 with another structurally unrelated BH3 mimetic, HA14-1 [334]. In accord with another report [324], HA14-1 is highly efficient in inducing autophagy in GFP-LC3 U2OS (Figures 28A and 28B) as we checked the ability of HA14-1 to induce autophagy in time and dose dependent manners. Moreover, Immunoblot detection of LC3 lipidation, as well as p62 degradation in is also assessed in HeLa cells (Figure 28C). All the results argue against the possibility that ABT737 would induce autophagy through an off-target effect.

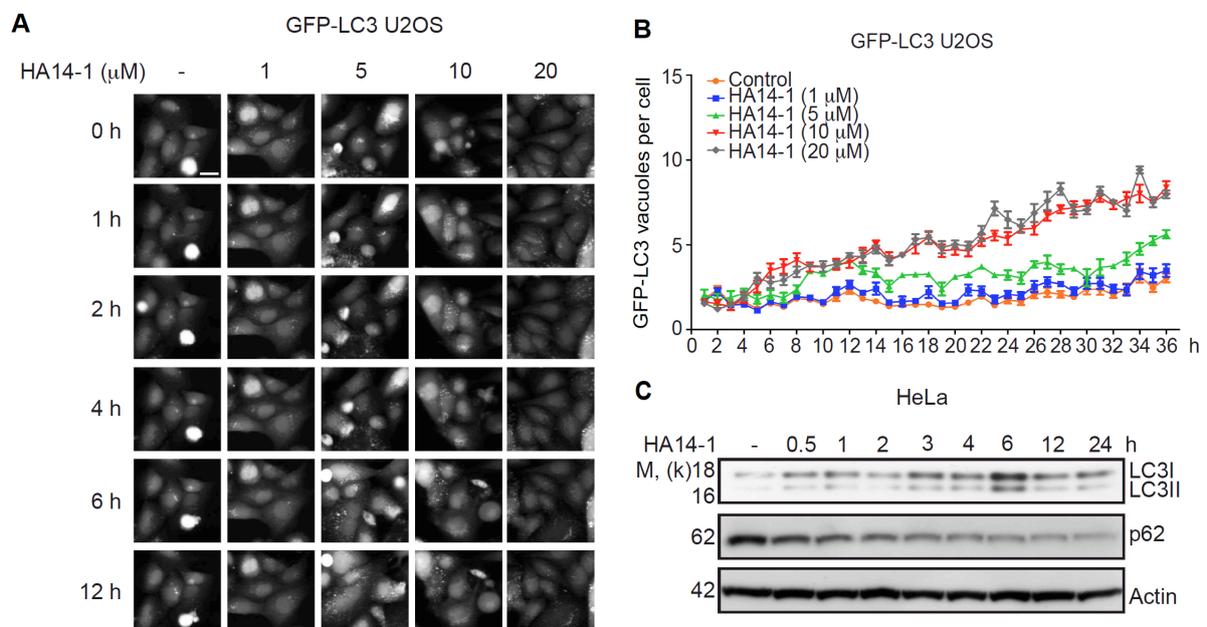


Figure 28 | Pro-autophagic activity of the BH3 mimetic HA14-1.

(A, B) U2OS cells stably expressing GFP-LC3 were treated with HA14-1 and the kinetics of puncta formation was determined by video-microscopy (C) Alternatively, HeLa cells were cultured in the presence of HA14-1 (20μM) at the time points mentioned, and the lipidation of LC3 and the expression of p62 were monitored by immunoblot. Actin levels were assessed to ensure equal loading of lanes (means±s.e.m., n=3) [331].

4.9 Comparison of ABT737- with HA14-1-induced autophagy

4.9.1 BH3 mimetics-induced autophagy and apoptotic machinery

To assess further for the possibility of off-target effects related to apoptotic signalling

pathways, we checked the ability of ABT737 or HA14-1 to induce autophagy in wild type (control) and *Bax*^{-/-}/*Bak*^{-/-} double-knockout MEFs. We found that they both induced autophagy in similar fashion as assessed by LC3 lipidation, p62 degradation and GFP-LC3 redistribution to autophagic dots (Figures 29A, B). Moreover, ABT737 and HA14-1 failed to induce a major loss of the mitochondrial trans-membrane potential (which may be associated with apoptosis or necrosis) (Figure 29C). Altogether our results show that both BH3 mimetics can stimulate autophagy without inducing cell death in the cancer cell lines used in this study.

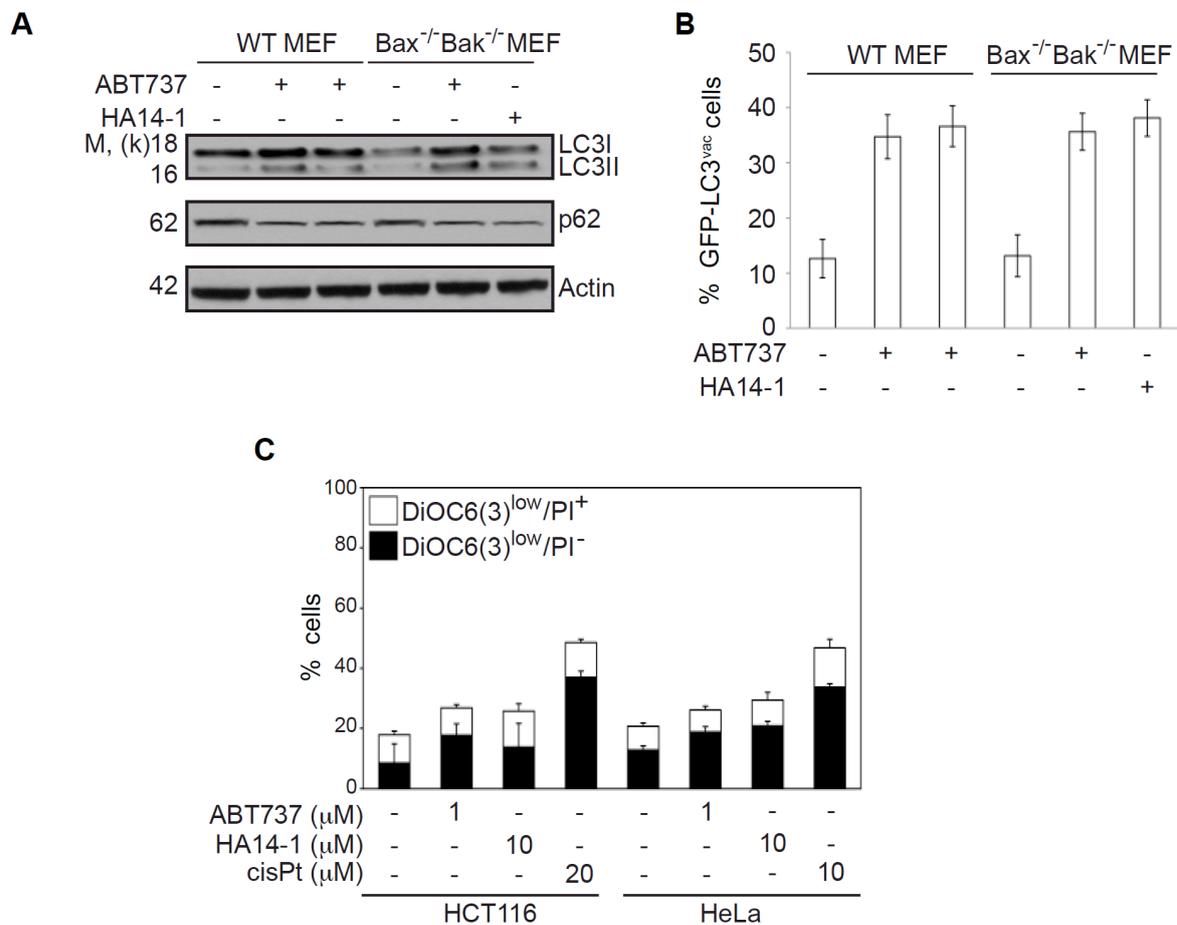


Figure 29 | BH3 mimetics-induced autophagy and apoptosis associated changes.

(A) WT and *Bax*^{-/-}/*Bak*^{-/-} MEF were cultured in the presence of ABT737 (1μM) or HA14-1 (20μM) overnight, and the lipidation of LC3 and the expression of p62 were monitored by immunoblot. Actin levels were assessed to ensure equal loading of lanes. Results are representative of three independent determinations. (B) In the same conditions as shown in A, the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}). (C) Cells (HCT116 and HeLa) were treated with ABT737, HA14-1 and cisPt overnight and stained with the $\Delta\psi_m$ -sensitive dye DiOC₆(3) and the vital die propidium iodine (PI). The black parts of the columns refer to the the DiOC₆(3)^{low} PI⁻ population and the remaining part of the column corresponds to DiOC₆(3)^{low} PI⁺ population (dead) [331].

4.9.2 Effects of BH3 mimetics on cellular ATP and mitochondrial oximetric functions

We additionally also evaluate and compare the changes in the mitochondrial oximetric axis on treatment with ABT737 and HA14-1. Interestingly, we found that the kinetic oximetric measurements of HeLa cells treated with an autophagy-inducing concentration of ABT737 or HA14-1 failed to reveal a major impairment of any of respiratory chain complexes. In comparison, a more than 50% decrease of complex III activity could be observed in HCT116 cells at later time points (12 and 24 h) after treatment with ABT737 or HA14-1. Yet, the activity of the succinate cytochrome c reductase (which channels electrons from succinate to added cytochrome c through both complex II and III) in these cells was not significantly affected, suggesting that electron flow in the respiratory chain is only poorly impacted by the observed complex III decrease, which would then have only a reduced biological relevance (Figure 30B). Moreover, cellular ATP levels progressively decreased after ABT737 treatment (which likely accounts for the observed ABT737-dependent AMPK activation) but in contrast to ABT737, HA14-1 failed to induce a major decline in intracellular ATP levels (Figure 30A). Altogether our results show that BH3 mimetics can stimulate autophagy both without inducing cell death and without impairing mitochondrial function.

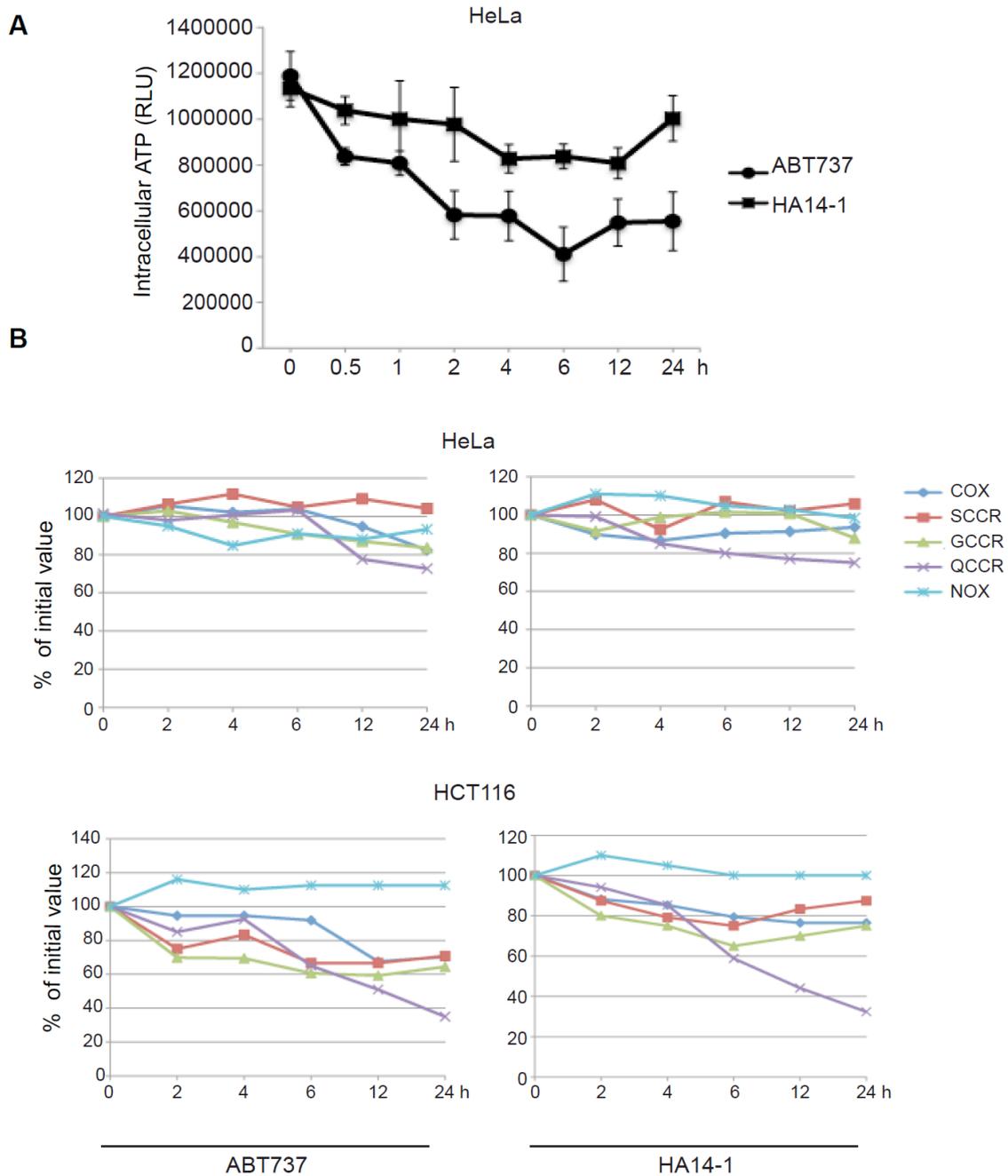


Figure 30 | Effects of BH3 mimetics on cellular ATP levels and mitochondrial functions.

(A) Concentrations of intracellular ATP in HeLa cells is measured after treatment with the BH3 mimetic, ABT737 (1 μ M) and HA14-1 (20M) at different time points using an ATP Assay kit (Calbiochem, Darmstadt, Germany) based on luciferin–luciferase conversion following the manufacturer’s instructions (RLU: Relative Luminescence Units). **(B)** Effects of ABT737 (1 M) and HA14-1 (20 M) treatment on respiratory chain activities of HCT116 and HeLa cells. Activities of complex IV (COX; cytochrome c oxidase), complex III (QCCR; quinol cytochrome c reductase), complex II and III (SCCR; succinate cytochrome c reductase), glycerol 3-phosphate dehydrogenase and complex III (GCCR; glycerol 3-phosphate cytochrome c reductase), and complex I, III and IV (NOX, rotenone-sensitive NADH oxidase) investigated in cells harvested at various time points [331].

4.9.3 BH3 mimetics and phosphorylation status of selected kinases

We confirmed the phosphorylation status of some selected kinases whose status was affected by ABT737 in phosphor-kinase array (Figure 27) and also compare the effect on those with HA14-1 treatment. Intriguingly, we found similar hypo-phosphorylation of mTOR and its substrate p70^{S6K}, p53 (serine 392) and Akt (threonine 308), as well as the hyper-phosphorylation of ACC and its substrate AMPK α and Pyk2 in line with specific (on-target) effects of the BH3 mimetics (Figures 31 A-C).

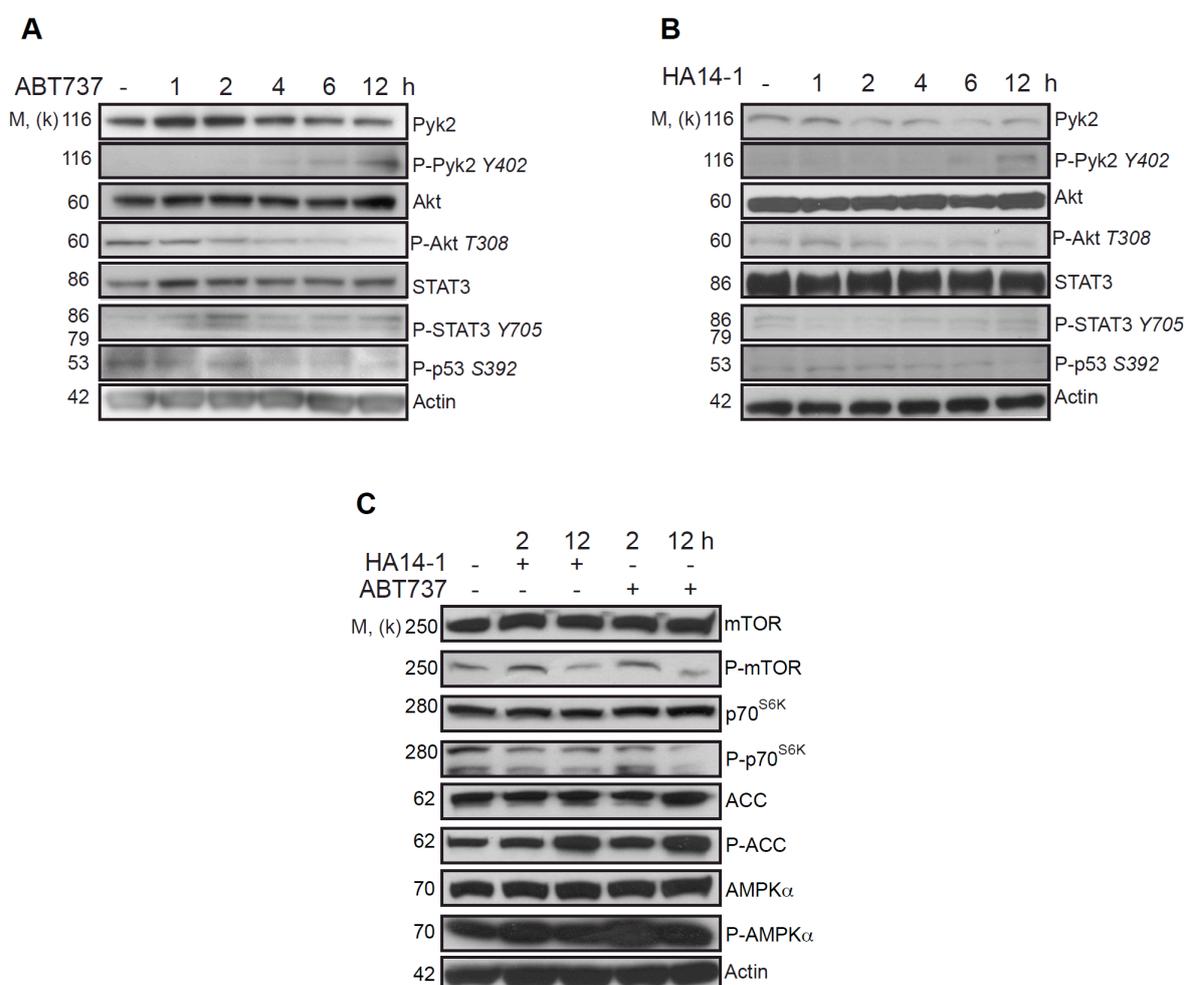


Figure 31 | BH3 mimetics and its effects on phosphorylation status of selected kinases.

(A,B) Representative immunoblots of selected kinases whose phosphorylation status was affected by ABT737 (1 μ M) or HA14-1 (20 μ M) treatment (Pyk2, Akt, STAT3 and p53) validating the phosphoprotein arrays. (C) Comparison of the effects of ABT737 (1 μ M) or HA14-1 (20 μ M) on the mTOR, ACC, AMPK and p70^{S6K} activation status. Actin levels were assessed to ensure equal loading of lanes [331].

5

CONCLUSIONS & PERSPECTIVES

In my thesis studies, we have demonstrated that ABT737 may stimulate multiple autophagy-stimulatory pathways due to the high degree of connectivity that exists among autophagy-regulatory protein complexes, meaning that a specific effect on the Beclin 1 interactome might affect other nodes in the autophagy-controlling network.

ABT737 is a highly specific BH3 mimetic, triggering apoptosis in cells in which neutralization of Bcl-2 is sufficient to de-inhibit the mitochondrial cell-death pathway. Moreover, ABT737 has been shown to induce autophagy by stimulating the dissociation of Beclin 1 from its inhibitory factors Bcl-2 and Bcl-X_L, thereby facilitating the Beclin 1-mediated allosteric activation of Vps34 and the consequent increase in phosphatidylinositol 3-phosphate-dependent autophagy [56, 197]. It appears that ABT737 activates multiple pro-autophagic signal transduction pathways. In this work, we show that ABT737 causes the activation of AMPK, the inhibition of mTOR, dephosphorylates (and hence likewise inhibits) p53, and deactivates the autophagy-inhibitory Akt kinase. These effects are obtained at comparatively high doses (in the μ M range) that can be attained *in vivo*, in animal experiments designed to evaluate the anticancer effects of ABT737. The ABT737 concentrations evaluated in this study may be clinically relevant, in spite of the fact that they are relatively high. We considered that some of these multiple consequences of ABT737 administration might be due to off-target effects, as ABT737 could affect proteins other than those bearing BH3 receptor domains (such as Bcl-2 and BCL-X_L) and/or act on signal-transduction pathways other than that controlled by the Beclin 1/Vps34 complex. However, ABT737 induced autophagy in cells that were fully apoptosis resistant (like MEF lacking both Bax and Bak) and failed to inhibit mitochondrial respiration before the induction of autophagy. Given this, it might be possible that a rather minor reduction in mitochondrial respiration induced by ABT737, that would be undetectable on whole cells in respirometric studies, would be perceived by homeostatic regulatory systems in the cells to stimulate autophagy, as well as a corrective increase in respiratory activity until the system decompensated. This remote possibility requires additional investigation. In spite of this caveat, knockdown of Beclin 1 or Vps34 fully suppressed ABT737-induced autophagy and also prevented the ABT737-mediated inhibition of mTOR (yet failed to affect AMPK activation induced by ABT737). In addition, several of the effects of ABT737 were shared by another structurally dissimilar BH3 mimetic, HA14-1, suggesting that the inhibition of mTOR and the dephosphorylation of p53 are specific (on-target) effects of BH3 mimetics.

In general, numerous signal transducers (including Bcl-2 family) involved in the cellular response to stress and damage also participate in the control of autophagy. The pro-apoptotic

multidomain proteins do not have any well-documented impact on the regulation of autophagy. In contrast, the multidomain proteins from the Bcl-2 family are potent autophagy inhibitors, while the BH3-only proteins have pro-autophagic properties. Indeed, the essential pro-autophagic protein Beclin 1 is a BH3-only protein. Beclin 1's BH3 domain usually engages in an inhibitory interaction with Bcl-2 and its close homologues, thereby preventing Beclin 1 from acting as an allosteric activator of the class III phosphatidylinositol 3-kinase (PI3KC3) also called Vps34, to generate phosphatidylinositol-3-phosphate (PI3P), which in turn is required for the initial steps of phagophore formation. Disrupting the inhibitory interaction of Beclin 1 with Bcl-2 (or its close homologues) is essential for the initiation of autophagy. The induction of autophagy by starvation is coupled to the dissociation of Beclin 1 from Bcl-2, which in part depends on the phosphorylation of Bcl-2 by JNK1, reducing its affinity for BH3 domains, and in part depends on the upregulation of the BH3-only protein Bad (which then competitively disrupts the interaction between Bcl-2 and Beclin 1). Accordingly, the absence of the sole BH3 domain protein encoded by the *Caenorhabditis elegans* genome; Egl1, results in the incapacity of nematodes to mount an autophagic response to starvation. The interaction between Beclin 1 and Bcl-2 is also lost upon induction of autophagy by several potent autophagy inducers that we have been characterizing. Thus, the pharmacological inhibition of p53 or the depletion of the cytoplasmic fraction of p53 potently induces autophagy at the same time that it dissociates the Beclin 1-Bcl-2 complex. Similarly, activation of the IKK complex (by transfection with constitutive active mutants of the IKK complex) induces autophagy while it activates JNK1 and disrupts the inhibitory interaction between Beclin 1 and Bcl-2. Starvation, ER stress and rapamycin all induce depletion of p53 that can be prevented by inhibiting or depleting HDM2, the p53-ubiquitinating enzyme targeting p53 for proteasomal destruction. Similarly, inhibition of IKK can prevent induction of autophagy by starvation, ER stress, rapamycin or p53 inhibition. Altogether these findings suggested that p53 inhibition/depletion is closely coupled to IKK activation (because both phenomena mutually stimulate each other) and that both these phenomena are closely linked to the activation of nutrient sensors (that is mimicked by rapamycin) yet occur upstream of the dissociation of the Beclin 1-Bcl-2 complex (Figure 32).

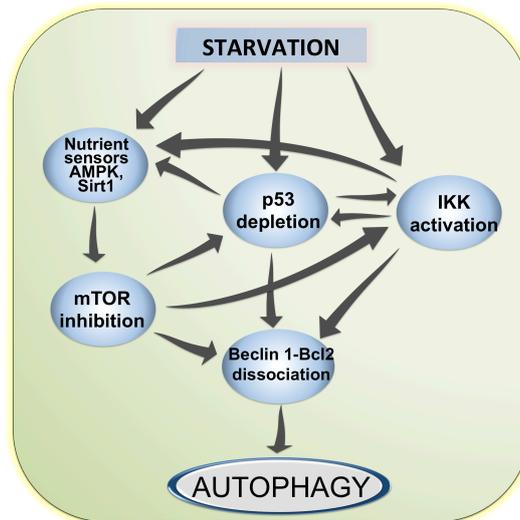


Figure 32 | Hypothetical hierarchy among autophagy-relevant events (Model 1).

The figure showing dissociation of Beclin 1 from Bcl-2-like proteins as a late event resulting from a series of upstream signals [335].

To examine this hypothesis, we have been investigating the question whether artificial dissociation of the Beclin 1-Bcl-2 complex by adding high concentrations of so-called BH3 mimetics, that is pharmacological ligands of the BH3 binding domain of Bcl-2, would induce autophagy according to our expectation (Fig 1a), that is without stimulating the activation of the nutrient sensors (sirtuin 1 and AMPK), without inhibiting mTOR (which is subordinated to AMPK), without depleting p53 and without activating IKK. To our surprise, we found that two different BH3 ligands, ABT737 and HA14-1, both induced all these changes. Control experiments corroborated that ABT737 and HA14-1 did neither inhibit oxidative phosphorylation nor reduce cellular ATP concentrations before the induction of autophagy, suggesting that they did not compromise general metabolism through off-target effects. Moreover, autophagy induction by ABT737 and HA14-1 was fully prevented by knocking down Beclin 1 or Vps34. To our surprise, it turned out that inhibition of sirtuin 1 (by means of the inhibitor EX527 or by a siRNA), inhibition of AMPK (by a siRNA specific for the $\alpha 1$ subunit), artificial activation of mTOR (by transfection with the constitutive active Rheb mutant, Rheb Q64L), inhibition of p53 depletion (by the pharmacological HDM2 inhibitors RITA or Nutlin-3 or by siRNA-mediated depletion of HDM2) or inhibition of IKK (by the inhibitors Bay11-7082 and 17-allylamino-geldanamycin or knockdown of any of the three IKK subunits, IKK α , IKK β , NEMO, all reduced autophagy induction by BH3 mimetics. These results place sirtuin 1, AMPK, mTOR, HDM2 and IKK downstream of the dissociation of the Beclin 1-Bcl-2 complex (Figure 33).

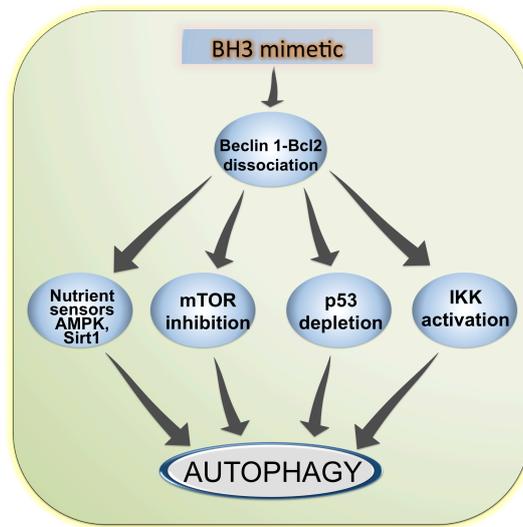


Figure 33 | Hypothetical hierarchy among autophagy-relevant events (Model 2).

Pro-autophagic signals stimulated by artificial dissociation of the Beclin 1-Bcl-2 complex using BH3 mimetics. Note that several signals that were thought to occur upstream of the liberation of Beclin 1 from Bcl-2 are also activated downstream of this event and are required for optimal autophagy induction [335].

How can these apparently contradictory results (compare Figure 32 and Figure 33) be reconciled? There is only one way out of the dilemma. The pathways cannot follow a linear hierarchy and rather must be interconnected in a complex circuitry, in which stimulation of autophagy by physiological triggers (such as starvation or organellar stress) triggers an ensemble of intimately linked changes that are coupled to each other in positive feed forward loops (Figure 34). Such self-amplificatory loops would interconnect the activation of nutrient sensors (that also result in mTOR inhibition), IKK, HDM2 (to deplete p53), and the Beclin 1/Vps34 complex (upon dissociation by Bcl-2 or its homologues), switching on the autophagic pathway at multiple levels. In this hypothetical scenario, the network properties of the system would constitute an indissociable ensemble that composes the autophagic switch. Understanding the molecular and functional properties of this switch by systems biology methods would then constitute the next frontier in autophagy research.

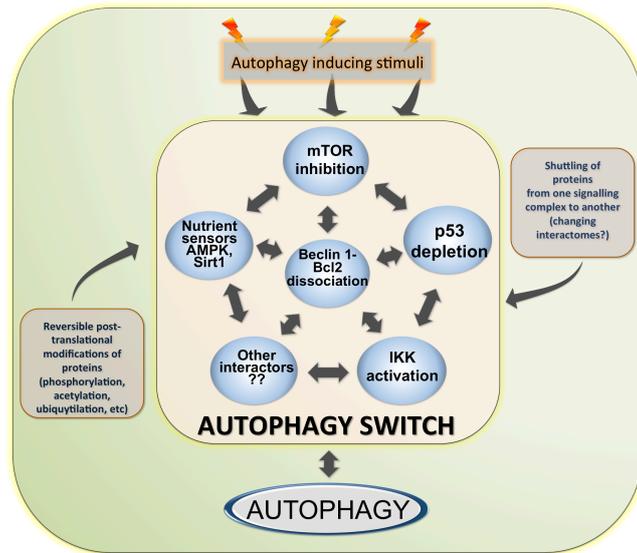


Figure 34 | Hypothetical hierarchy among autophagy-relevant events (Model 3).

Hypothetical central integrator of autophagic signalling. A series of molecular events occurs in a coordinated fashion, likewise due to the activation of self-amplifying feed forward loops. The exact nature of the signalling events that would be part of this central integrator, the probably complex interactions within this integrator, and the frontier between the integrator and stimulus-specific signals triggering autophagy remain to be defined [335].

7

PUBLICATIONS

The work performed during my stay in the Dr Kroemer's lab led to the publication of many scientific reports and reviews and the list of these publications is given below. The most relevant articles for this thesis are attached fully at the end in "ANNEXED ARTICLES".

- S. Shen, M. Niso-Santano, S. Adjemian, T. Takehara, **S. A. Malik**, H. Minoux, S. Souquère, G. Mariño, L. Galluzzi, O. Kepp, M. C. Maiuri, G. Pierron, H. Hikita, R. Kroemer, G. Kroemer. Cytoplasmic STAT3 represses autophagy by inhibiting PKR activity. *Molecular Cell*. (Accepted 2012).
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cytofluorometric system for monitoring the turnover of the autophagic substrate p62/SQSTM1. *Autophagy*. 2011 Aug;7(8):883-91. Epub 2011 Aug 1.

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8

ANNEXED ARTICLES

8.1 BH3 mimetics activate multiple pro-autophagic pathways.

ORIGINAL ARTICLE

BH3 mimetics activate multiple pro-autophagic pathwaysSA Malik^{1,2,3}, I Orhon^{1,2,3}, E Morselli^{1,2,3}, A Criollo^{1,2,3}, S Shen^{1,2,3}, G Mariño^{1,2,3},
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The BH3 mimetic ABT737 induces autophagy by competitively disrupting the inhibitory interaction between the BH3 domain of Beclin 1 and the anti-apoptotic proteins Bcl-2 and Bcl-X_L, thereby stimulating the Beclin 1-dependent allosteric activation of the pro-autophagic lipid kinase VPS34. Here, we examined whether ABT737 stimulates other pro-autophagic signal-transduction pathways. ABT737 caused the activating phosphorylation of AMP-dependent kinase (AMPK) and of the AMPK substrate acetyl CoA carboxylase, the activating phosphorylation of several subunits of the inhibitor of NF- κ B (I κ B) kinase (IKK) and the hyperphosphorylation of the IKK substrate I κ B, inhibition of the activity of mammalian target of rapamycin (mTOR) and consequent dephosphorylation of the mTOR substrate S6 kinase. In addition, ABT737 treatment dephosphorylates (and hence likewise inhibits) p53, glycogen synthase kinase-3 and Akt. All these effects were shared by ABT737 and another structurally unrelated BH3 mimetic, HA14-1. Functional experiments revealed that pharmacological or genetic inhibition of IKK, Sirtuin and the p53-depleting ubiquitin ligase MDM2 prevented ABT737-induced autophagy. These results point to unexpected and pleiotropic pro-autophagic effects of BH3 mimetics involving the modulation of multiple signalling pathways.

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Introduction

Macroautophagy (that we refer to as ‘autophagy’) is a catabolic pathway that culminates in the degradation and recycling of cytoplasmic organelles or portions of the cytosol (Klionsky, 2007). Autophagy starts with the

formation of double-membrane structures that sequester parts of the cytoplasm and close to form autophagosomes. The subsequent fusion of autophagosomes with lysosomes leads to the degradation of their luminal content, and the end products of this catabolic reaction can then be recycled to maintain cellular homeostasis (Levine and Kroemer, 2008). Although autophagosomes are often found in the cytoplasm of dying cells, coining the term ‘autophagic cell death’ (Galluzzi *et al.*, 2007; Galluzzi *et al.*, 2008), autophagy mostly subserves a cytoprotective function, and its suppression can precipitate the demise of stressed cells (Boya *et al.*, 2005; Iwata *et al.*, 2005; Degenhardt *et al.*, 2006; Apel *et al.*, 2008). The functional link between autophagy and cell death, in particular Bcl-2-regulated apoptotic death, has been extensively characterized (Maiuri *et al.*, 2007a; Sinha and Levine, 2008). Beclin 1 regulates autophagy in a multiprotein complex, which includes class III phosphatidylinositol-3-kinase activity that participates in autophagosome nucleation. In this complex, Beclin 1 interacts with several proteins including Bcl-2, a protein initially characterized as an apoptosis inhibitor (Maiuri *et al.*, 2007a; Sinha and Levine, 2008).

Bcl-2 constitutes the prototype of a family of proteins containing at least one Bcl-2 homology (BH) region. The Bcl-2 family can be divided into anti-apoptotic multi-domain proteins (prototypes: Bcl-2, Bcl-X_L and Mcl-1), which contain four BH domains (numbered BH1–BH4), pro-apoptotic multi-domain proteins (prototypes: Bax and Bak), which contain three BH domains (BH1, BH2 and BH3), and the pro-apoptotic BH3-only protein family (which has more than a dozen members) (Petros *et al.*, 2004). The relative abundance of pro- and anti-apoptotic Bcl-2 proteins dictates the propensity of cells to undergo mitochondrial membrane permeabilization and to succumb to distinct apoptosis inducers (Huang and Strasser, 2000; Galluzzi *et al.*, 2008).

Multidomain anti-apoptotic Bcl-2 proteins possess a hydrophobic cleft at their surface, the BH3-binding groove, which can accommodate BH3 domains from pro-apoptotic Bcl-2 protein family members. The BH3 domains from pro-apoptotic proteins can be structurally defined as four-turn amphipathic α -helices that are contained in a sequence motif that lacks invariable amino acids: Hy–X–X–X–Hy–X–X–X–Sm–D/E–X–Hy, where Hy represents hydrophobic residues and Sm

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represents small residues, typically glycine (Sinha and Levine, 2008). The extreme variability of both pro-apoptotic BH3 domains, as well as that of the residues lining the BH3-binding groove of the anti-apoptotic proteins, determines a large heterogeneity in the affinities for each specific pair of interactions. These variable affinities, combined with the spatial and temporal variations in the concentration of each of these proteins, regulate the stoichiometry of these interactions. Thus, changing the concentration of just one BH3 domain-containing protein can affect multiple protein–protein interactions among Bcl-2 family proteins, thereby transmitting a pro-apoptotic signal throughout the network of Bcl-2-like proteins (Sinha and Levine, 2008). Some pharmacological components such as ABT737 are referred to as ‘BH3 mimetics’ because they occupy the BH3-binding grooves of anti-apoptotic proteins from the Bcl-2 family. ABT737 binds to Bcl-2 and Bcl-X_L (but not Mcl-1) and can kill cells, provided that they express pro-apoptotic multi-domain proteins (such as Bax and Bak), which are essential for the apoptotic mitochondrial membrane permeabilization, as well as BH3-only proteins, which—once liberated from the BH3-binding cleft of Bcl-2 or Bcl-X_L—can activate Bax and/or Bak (Oltersdorf *et al.*, 2005).

Recently, a region including amino acids 112–123 from the autophagic regulatory protein Beclin 1 has been identified as a BH3 domain (Oberstein *et al.*, 2007; Maiuri *et al.*, 2007b,c). This domain in Beclin 1 is the minimal sequence necessary for the interaction with the anti-apoptotic Bcl-2 family of proteins (in particular Bcl-2 itself, Bcl-X_L and Mcl-1). Although Beclin 1 is a BH3-only protein, it has no pro-apoptotic effects (Boya and Kroemer, 2009), perhaps because it has a low affinity for other Bcl-2 family members (Sinha and Levine, 2008). The BH3 domains of other BH3-only proteins such as Bad, as well as BH3-mimetic compounds such as ABT737, competitively disrupt the inhibitory interaction between Beclin 1 and Bcl-2/Bcl-X_L, thus allowing Beclin 1 to accomplish its autophagic stimulatory functions. Only ER-targeted (not mitochondrion-targeted) Bcl-2/Bcl-X_L can inhibit autophagy induced by Beclin 1, and only Beclin 1-Bcl-2/Bcl-X_L complexes present in the ER (but not those present on mitochondria) are disrupted by ABT737, suggesting that Beclin 1-Bcl-2/Bcl-X_L complexes that usually suppress autophagy are specifically located in the ER (Maiuri *et al.*, 2007b,c). This particular spatial organization may explain why Beclin 1 lacks any pro-apoptotic activity.

Hence, in cells that are resistant to its pro-apoptotic effect, ABT737 can induce autophagy because it liberates Beclin 1 from its inhibitory interaction with Bcl-2 or Bcl-X_L, thus allowing Beclin 1 to support the class III phosphatidylinositol-3-kinase activity of Vps34 (Maiuri *et al.*, 2007b,c). Similar findings have been obtained using other BH3 mimetics (Kessel and Reiners, 2007; Lian *et al.*, 2010). These results suggest that ABT737 induces autophagy solely by its effects on the Beclin 1 interactome. Nonetheless, autophagy is a complex process that is regulated by multiple protein complexes beyond that organized around Beclin 1.

Thus, energy sensors including AMP-dependent kinase (AMPK), mammalian target of rapamycin (mTOR) (Yang and Klionsky, 2010), Sirtuin1 (Lee *et al.*, 2008), as well as stress-integrating pathways such as those involving the inhibitor of NF- κ B (I κ B) kinases (IKK) (Criollo *et al.*, 2010) and the tumor suppressor protein p53 (Maiuri *et al.*, 2009) all have a major impact on the regulation of autophagy (Klionsky *et al.*, 2010; Madeo *et al.*, 2010). In many paradigms of autophagic stimulation including nutrient starvation, positive regulators of autophagy (such as AMPK, SIRT1, IKK) must come into action while negative regulators (such as mTOR and cytoplasmic p53) must be inactivated for the optimal induction of autophagy. At present, it is elusive whether ABT737 affects these pathways.

Based on these premises and incognita, we decided to measure the possible effects of ABT737 on multiple distinct autophagy regulators. Our results reveal that, in contrast to our initial expectation, ABT737 must stimulate multiple pro-autophagic pathways to be optimally efficient, thus illustrating the complex interconnections among autophagy-regulatory proteins.

Results

ABT737-induced autophagy involves the AMPK, mTOR and IKK pathways

In cells that are resistant to its apoptosis-inducing effect, such as U2OS cells (Figures 1a and b), HeLa cells (Figure 1e; Maiuri *et al.*, 2007b) and HCT116 cells (Figure 1c), ABT737 induces autophagy, as determined by following the redistribution of green fluorescent protein (GFP)-LC3 from a diffuse to a punctate pattern (Figures 1a–c) or by assessing the lipidation of endogenous LC3 protein by immunoblot (Figures 1d and 2a–c). ABT737 induced a further increase in GFP-LC3 redistribution and LC3 lipidation in the presence of bafilomycin A1 (Figures 1c and d), which inhibits the final stage of autophagic protein degradation, confirming that ABT737 increases autophagic flux. In accord with this conclusion, ABT737 reduced the abundance of the autophagic substrate p62 in all cell lines considered (Figures 2a–c). Consistent with the published finding that ABT737 induces autophagy via the Beclin 1 complex (Maiuri *et al.*, 2007b,c), the knockdown of Vps34 (which is the enzymatic subunit of the Beclin 1 complex, a type 3 phosphatidylinositol-3 kinase) (Sinha and Levine, 2008) inhibited ABT737-induced autophagy evaluated by LC3 lipidation and p62 degradation (Figure 1e).

Next, we determined whether ABT737 stimulates additional pro-autophagic pathways by assessing its effects on the phosphorylation status of AMPK α and that of its substrate acyl-CoA carboxylase (ACC), the phosphorylation of mTOR and that of its substrate p70^{S6K}, as well as the phosphorylation of the IKK subunits, IKK α , IKK β and NEMO (Figure 2). Although the kinetics of these effects were different among the cell lines, ABT737 consistently induced the

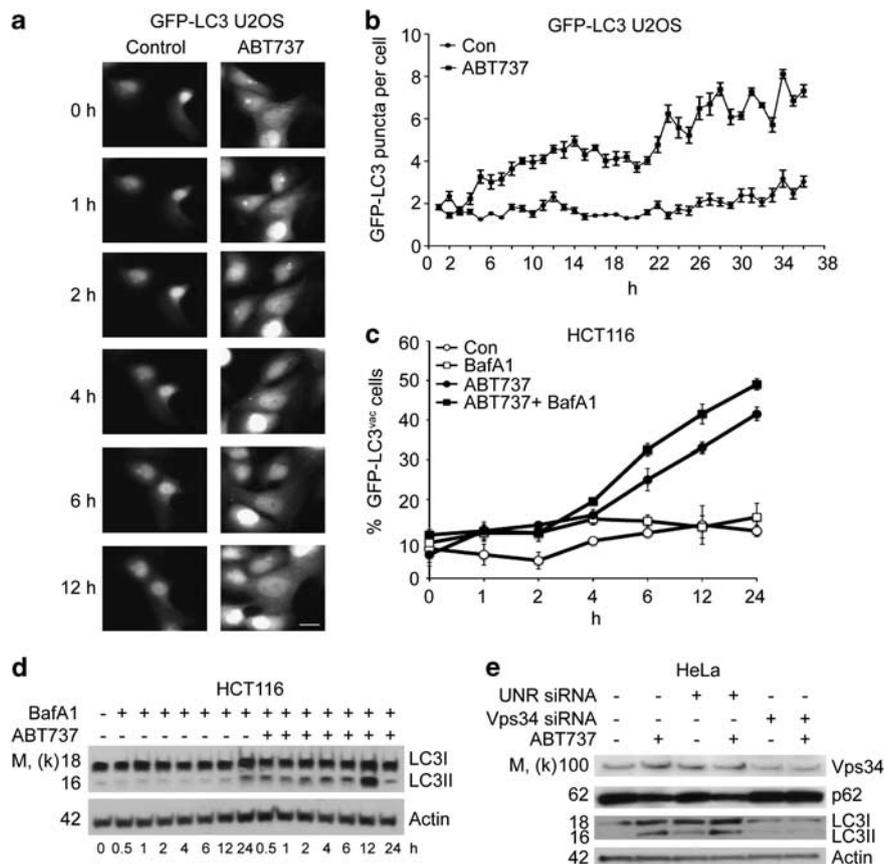


Figure 1 Autophagy stimulation by ABT737. (a, b) Levels of autophagy induced by ABT737 in U2OS cells stably expressing GFP-LC3 were measured in time-dependent manner as described in Materials and Methods section. Representative microphotographs of cells (size bar 50 mm) cultured in the presence of ABT737 (1 μ M) are shown in (a) and the number of GFP-LC3 puncta per cell (means \pm s.e.m., $n = 3$ separate experiments) are quantified in (b). (c, d) Effect of Bafilomycin A1 (Baf A1) on ABT737-induced autophagic vacuolization. GFP-LC3-expressing HCT116 cells were treated with Baf A1 (1 nM) for further 24 h. Panel c depicts the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) (means \pm s.e.m., $n = 3$). (d) Representative immunoblots ($n = 3$) of HCT116 cells treated with ABT737 and BafA1 for the indicated time. (e) Involvement of Vps34 in ABT737-induced autophagy. HeLa cells were transfected with a control siRNA or a siRNA specific for Vps34. After 48 h, the cells were treated for 12 h with ABT737 (1 μ M). Each experiment has been repeated at least three times, yielding similar results. (d, e) Actin levels were assessed to ensure equal loading of lanes.

hyperphosphorylation of AMPK α and the three IKK subunits, as well as a consistent reduction in the phosphorylation of mTOR. These changes were paralleled by alterations in the phosphorylation status of the AMPK α substrate ACC, the IKK substrate I κ B α (which are both hyperphosphorylated) and the mTOR substrate p70^{S6K} (which is hypophosphorylated upon ABT737 treatment), consistent with the interpretation that ABT737 stimulates the activation of AMPK, yet inhibits mTOR. Altogether, these results indicate that ABT737 has pleiotropic effects on several autophagy-relevant signal-transduction pathways.

Role of AMPK and mTOR in ABT737-induced autophagy

ABT737 is able to induce the hyperphosphorylation of AMPK α , as well as the phosphorylation of the AMPK α substrate ACC and autophagy at a dose around 1 μ M (Figure 3a). The concentrations of ABT737 needed to induce autophagy on cells are higher than those required

for Bcl-2 blockade (in cell-free systems). Thus, we decided to analyze whether AMPK activation is required for the pro-autophagic activity of ABT737. Small interfering RNA (siRNA)-mediated depletion of AMPK α reduced the ABT737-induced LC3 lipidation and p62 degradation (Figure 3b) in each of the three cell lines tested (shown for HCT116 cells in Figures 3a and b). Moreover, ATP cellular levels progressively decreased after ABT737 treatment, which likely accounts for the observed ABT737-dependent AMPK activation (Supplementary Figure 1A). Given that ABT737 also induced a consistent decrease in mTOR activity, we then investigated whether the inhibition of mTOR is a necessary step in the induction of autophagy by ABT737. For this purpose, we overexpressed a constitutive active form of Rheb (Rheb Q64L), which positively regulates mTOR, as showed by the phosphorylation status of the mTOR substrate p70^{S6K} (which is hypophosphorylated upon ABT737 treatment) (Supplementary Figure 1A). The expression of Rheb Q64L restored the activity of mTOR and prevented the

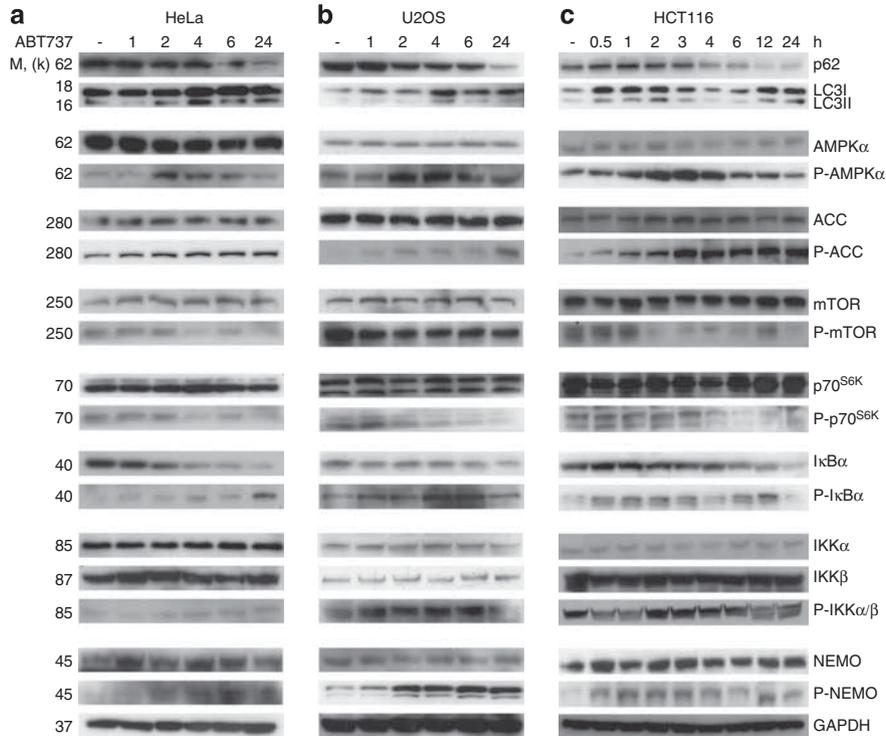


Figure 2 Implication of AMPK, mTOR and IKK in the autophagy induction by ABT737. Immunoblot detection of the levels of p62, LC3I/II, p53 and Sirtuin1, as well as the phosphorylation status of AMPK α (Y172), ACC α (S79), mTOR (S2448), p70^{S6K} (Y421/S424), I κ B α (S32/36), IKK α / β (S176 or S180, respectively) and NEMO (S376) in different human cell lines (a–c) stimulated with ABT737 (1 μ M) for the indicated time. Glyceraldehyde-3-phosphate dehydrogenase levels were monitored to ensure equal loading. Data are representative of three independent experiments.

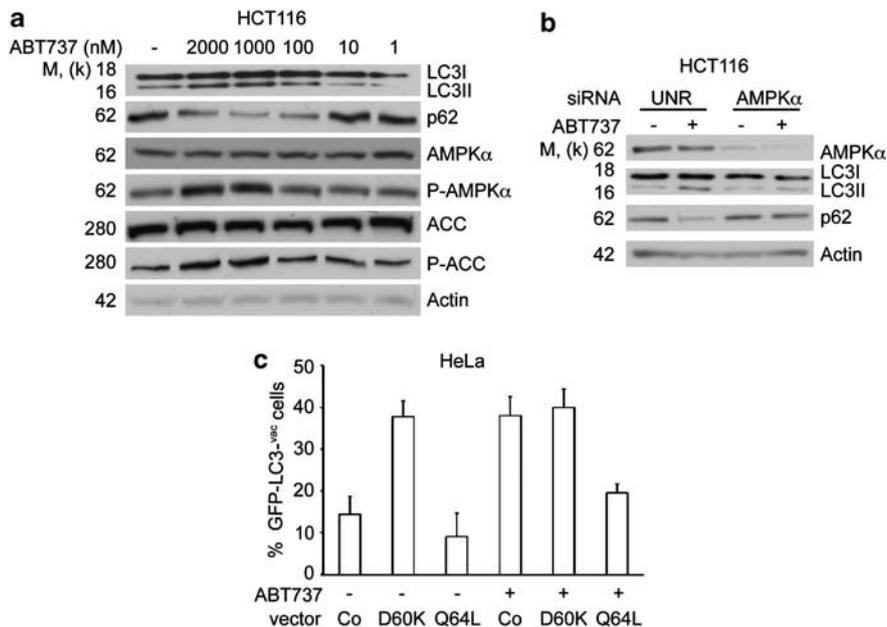


Figure 3 Involvement of AMPK α and mTOR in ABT737-induced autophagy. (a) Dose-dependent induction of autophagy by ABT737. Representative immunoblots of LC3, p62 and phosphorylated AMPK α (Y172) or ACC α (S79) were performed on lysates from HCT116 cells that were treated with ABT737 at the indicated concentrations for 12 h. (b) Immunoblot detection of LC3 lipidation and p62 degradation in HCT116 depleted from AMPK α . At 48 h after the transfection with the indicated siRNAs, the cells were treated with ABT737 (1 μ M) for 12 h. The efficiency of siRNA-mediated downregulation of the protein is also shown. Actin levels were assessed to ensure equal loading of lanes. Data in (a) and (b) are representative of three independent experiments. (c) Implication of mTOR in ABT737-induced autophagy. HeLa cells were simultaneously transfected with GFP-LC3 and equivalent amounts of plasmids coding for control vector, Rheb D60K (dominant-negative construct) or Rheb Q64L (constitutively active construct). After 24 h, the cells were treated with ABT737 (1 μ M) for 12 h. The percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is shown (means \pm s.e.m., three independent experiments).

autophagy induction by ABT737. In contrast, the expression of a dominant-negative form of Rheb (Rheb D60K) did not affect the level of autophagy induced by ABT737 (Figure 3c). Altogether these data confirm that ABT737-mediated stimulation of autophagy requires both AMPK activation and mTOR inhibition.

Functional implication of IKK in ABT737-induced autophagy

The IKK inhibitor Bay11-7082 (Pierce *et al.*, 1997), as well as the HSP90 inhibitors geldanamycin and 17-allylamino-geldanamycin, which can also inhibit IKK (Broemer *et al.*, 2004), were previously identified as inhibitors of starvation-induced autophagy (Criollo *et al.*, 2010). We found that all three inhibitors also prevented the ABT737-stimulated lipidation of LC3 (Figure 4a) and the redistribution of GFP-LC3 to autophagic puncta in all three cell lines included in this study (Figure 4b). Accordingly, siRNA-mediated depletion of IKK α (Figure 4c), IKK β (Figure 4d) or NEMO (Figure 4e) reduced the ABT737-induced lipidation and inhibited the induction of GFP-LC3 puncta by ABT737 (Figure 4f). These results were obtained consistently in each of the three cell lines (shown for HCT116 cells in Figures 4c–f). Surprisingly, we noted that depletion of Atg5 or that of Beclin 1 abolished the hyperphosphorylation of IKK α/β , NEMO and I κ B α (Figure 4g), suggesting an intimate link between IKK activation and autophagy. Inhibitor of NF- κ B (I κ B) kinase is required for ABT737-induced autophagy, but autophagy is also necessary for IKK activation.

Implication of p53 and SIRT1 in ABT737-induced autophagy

We investigated the contribution of p53 to ABT737-induced autophagy, based on the recent observation that cytoplasmic p53 must be ubiquitinated by MDM2 and then degraded by proteasomes to allow the induction of starvation-induced autophagy (Tasdemir *et al.*, 2008a). Similarly, SIRT1 is required for autophagy induced by nutrient shortage (Morselli *et al.*, 2010). Pharmacological inhibition of MDM2 with RITA or Nutlin-3 prevented the degradation of p53 (Tasdemir *et al.*, 2008a) and concomitantly prevented the induction of autophagy in HCT116 cells induced by ABT737 (Figure 5a). Similarly, proteasome inhibition by MG132 resulted in the maintenance of high p53 levels (Tasdemir *et al.*, 2008a) and the inhibition of ABT737-induced autophagy (Figure 5a). SIRT1 inhibition by EX527 also prevented the induction of LC3 lipidation in ABT737-treated HCT116 cells (Figure 5b). RITA, Nutlin-3, MG132 and EX527 all consistently inhibited the induction of GFP-LC3 puncta by ABT737 in HeLa, U2OS and HCT116 cells (Figure 5c). In accord with these results, which are based on the use of pharmacological inhibitors, knockdown of *SIRT1* and *HDM2* by specific siRNAs also blunted the autophagic response of ABT737-treated cells (Figures 5d–f). These results underscore the complex pro-autophagic signal-transduction pathway stimulated by ABT737, which

involves the obligatory activation of the deacetylase SIRT1, and the ubiquitin ligase MDM2.

Multiple signal-transduction pathways elicited by ABT737

To systematically assess the cellular signals elicited by ABT737, we determined changes in the phosphoproteome of HeLa cells treated for 2, 6 or 12 h with the BH3 mimetic, using a phosphoprotein-specific antibody array (Figures 7a and b). ABT737 caused the hyperphosphorylation of a few proteins such as Pyk2 (confirmed by immunoblot in Figure 7c) and AMPK α (confirmed in Figures 2 and 3), as well as that of other proteins (such as HSP27 and Paxillin). Moreover, ABT737 triggered the dephosphorylation of several proteins: p53 on three distinct serine residues (confirmed for S392 in Figure 7c), p70^{S6K} on several residues (confirmed for threonine 421/serine 424 in Figure 2), that of Akt (confirmed in Figure 7c), as well as that of numerous other proteins, including transcription factors of the STAT family (in particular STAT 1 and 4), ribosomal protein S6 kinase, the cyclin-dependent kinase inhibitor, p27 (on threonine 157), and endothelial nitric oxide synthase. These results hint to multiple effects of ABT737 on a plethora of signal-transduction pathways.

The pro-autophagic activity of ABT737 might involve off-target effects. To address this possibility, we compared ABT737 with another structurally unrelated BH3 mimetic, HA14-1 (Zhang *et al.*, 2009). In accord with another report (Kessel and Reiners, 2007), HA14-1 was highly efficient in inducing autophagy including in U2OS (Figures 6a and b) and HeLa cells (Figure 6c), arguing against the possibility that ABT737 would induce autophagy through an off-target effect. Furthermore, ABT737 or HA14-1 induced LC3 lipidation, p62 degradation and GFP-LC3 redistribution to autophagic dots in control and Bax^{-/-}Bak^{-/-} double-knock-out MEFs similarly (Figures 6d and e). ABT737 and HA14-1 failed to induce a major loss of the mitochondrial transmembrane potential (which may be associated with apoptosis or necrosis) (Supplementary Figure 1B). Moreover, kinetic oximetric measurements of HeLa cells treated with an autophagy-inducing concentration of ABT737 or HA14-1 failed to reveal a major impairment of any of respiratory chain complexes. In comparison, a more than 50% decrease of complex III activity could be observed in HCT116 cells at later time points (12 and 24 h) after treatment with ABT737 or HA14-1. Yet, the activity of the succinate-cytochrome *c* reductase (which channels electrons from succinate to added cytochrome *c* through both complex II and III) in these cells was not significantly affected, suggesting that electron flow in the respiratory chain is only poorly impacted by the observed complex III decrease, which would then have only a reduced biological relevance. Altogether our results show that BH3 mimetics can stimulate autophagy both without inducing cell death and without impairing mitochondrial function.

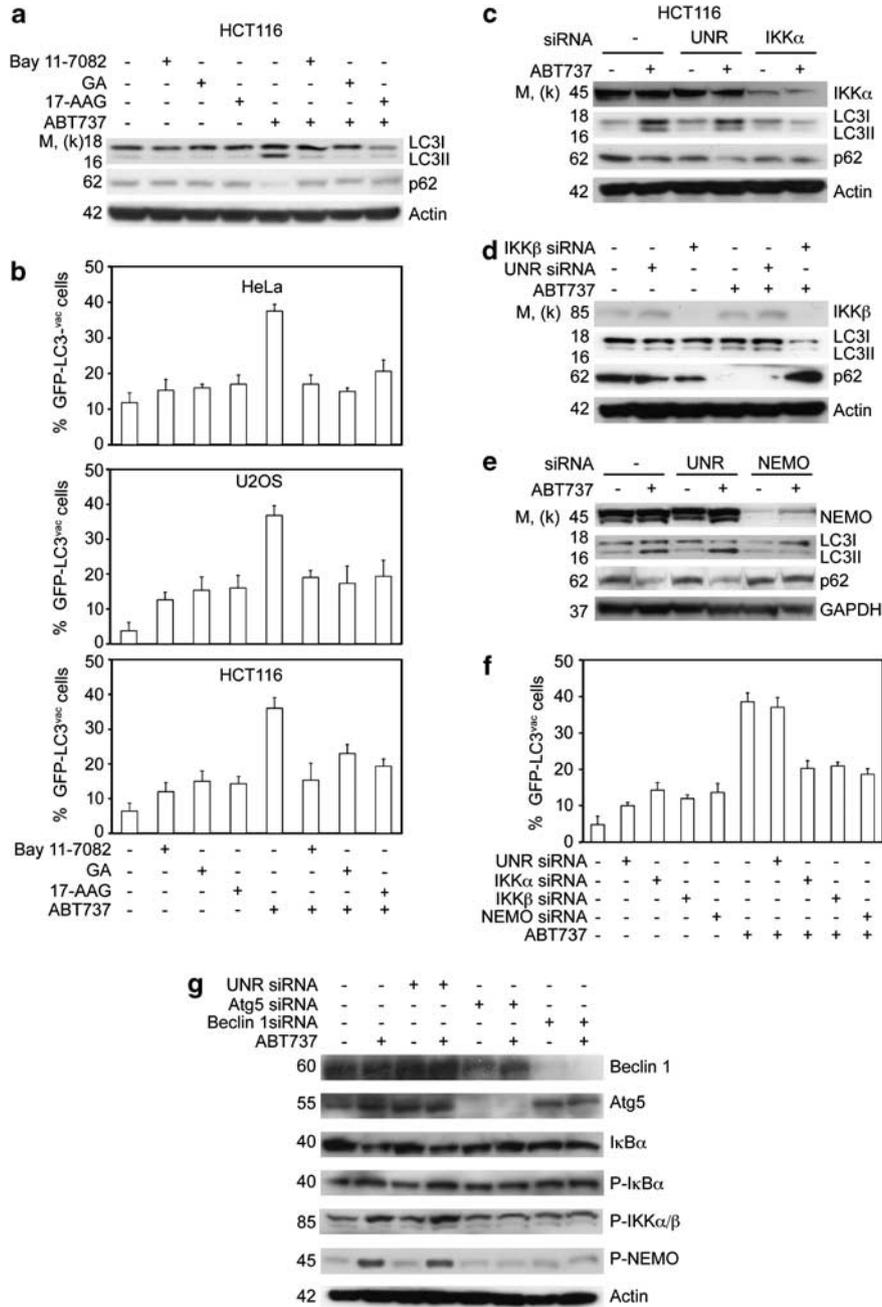


Figure 4 Involvement of IKK in ABT737-induced autophagy. **(a, b)** Pharmacological inhibition of IKK pathway reduces autophagy increased by ABT737. **(a)** LC3 maturation and p62 degradation in HCT116 treated with ABT737 (1 μ M) in the presence of Bay11-7082 (1 μ M), GA (geldanamycin; 2 μ M), 17-AAG (17-allylamino-geldanamycin; 2 μ M) for 12 h. Actin levels were assessed to ensure equal loading of lanes. Data are representative of three independent experiments. **(b)** GFP-LC3-expressing HeLa, U2OS, HCT116 cells were treated under the same conditions of **(a)** and the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is shown (means \pm s.e.m., $n = 3$). **(c–e)** Immunoblot detection of LC3 lipidation, as well as p62 degradation in HCT116 subjected to knockdown of IKK α , IKK β , NEMO. At 48 h after the transfection, the cells were treated with ABT737 (1 μ M) for 12 h. The efficiency of siRNA-mediated downregulation of the protein is also shown. Glyceraldehyde-3-phosphate dehydrogenase levels were monitored to ensure equal loading ($n = 4$). **(f)** HCT116 were transfected with unrelated (UNR) or a siRNA specific for the IKK complex subunits and subsequently (after 24 h) with a plasmid for the expression of GFP-LC3, followed by culture in the presence of ABT737 (1 μ M) for 12 h (means \pm s.e.m., $n = 3$). **(g)** Immunoblot detection of phosphorylation status of I κ B α , IKK α/β and NEMO. HCT116 cells were transfected with UNR or siRNAs specific for Atg5 and Beclin 1 as mentioned previously and treated with ABT737 (1 μ M) for 12 h. The efficiency of siRNA-mediated downregulation of the proteins is also shown. Actin levels were assessed to ensure equal loading of lanes. Data are representative of three independent experiments.

In contrast to ABT737, HA14-1 exerted less dramatic changes on the phosphoproteome (data not shown), failed to induce a major decline in intracellular ATP

(Supplementary Figure 1D) but induced a similar hypophosphorylation of p70^{S6K}, p53 on serine 393, Akt, as well as the hyperphosphorylation of AMPK α

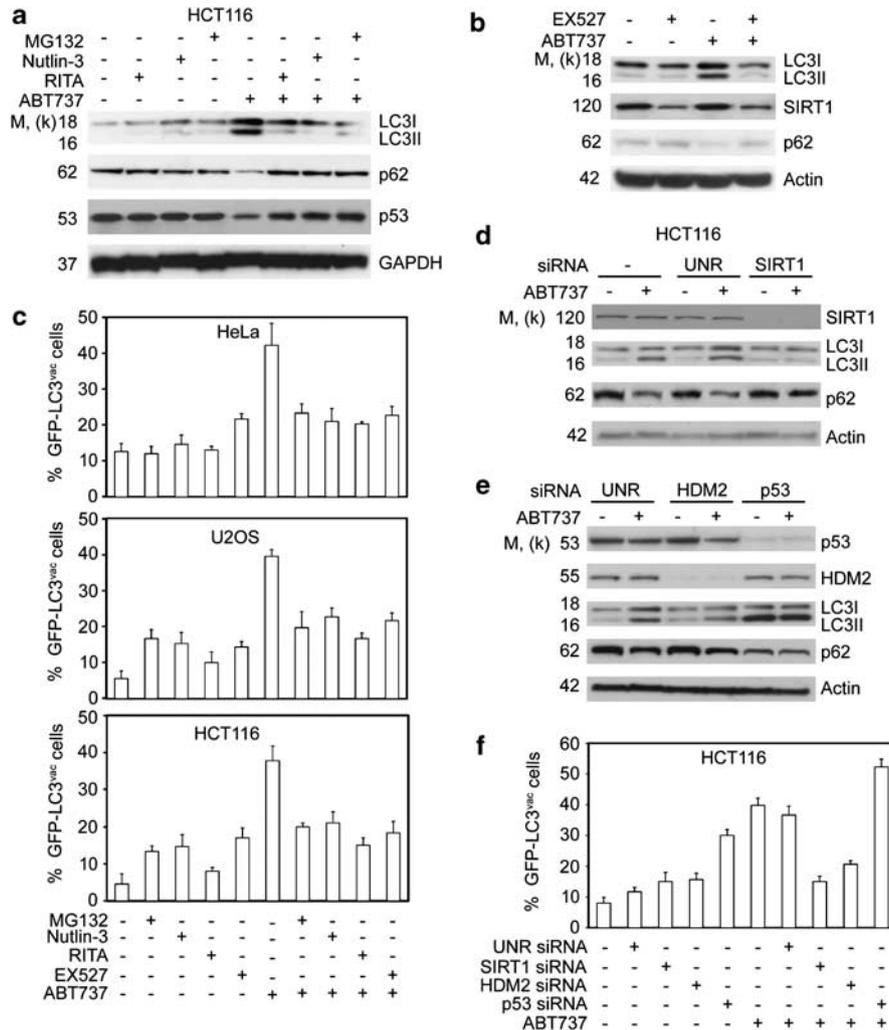


Figure 5 Involvement of p53 and SIRT1 in ABT737-induced autophagy. (a, b) Levels of LC3I/II and p62 expression in HCT116 treated with ABT737 (1 μ M) for 12 h in the presence or absence of pharmacological inhibitors of MDM2 (RITA or Nutlin-3, both used at 10 μ M), proteasome (MG132, 10 μ M) and SIRT1 (EX527, 100 μ M) ($n=3$). Actin and glyceraldehyde-3-phosphate dehydrogenase levels were assessed to ensure equal loading of lanes. (c) GFP-LC3-expressing HeLa, U2OS or HCT116 cells were treated under the same conditions as in (a, b) and the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is reported (means \pm s.e.m., $n=3$). (d, e) Immunoblot detection of LC3 lipidation, as well as p62 degradation in HCT116 subjected to knockdown of SIRT1, HDM2 and p53. At 48 h after the transfection, the cells were treated with ABT737 for 12 h. The efficiency of siRNA-mediated downregulation of the proteins is also demonstrated. Glyceraldehyde-3-phosphate dehydrogenase levels were monitored to ensure equal loading ($n=5$). (f) Quantification of the percentage of GFP-LC3^{vac} in HCT116 transfected with siRNAs specific for SIRT1, HDM2 and p53, as described in Materials and methods section, and stimulated for 12 h with ABT737 (1 μ M) (means \pm s.e.m., $n=3$).

or Pyk2 in line with specific (on-target) effects of the BH3 mimetics (Figures 7d and e). To get insights into the molecular pathways underlying the effects of BH3 mimetics on different pro-autophagic signalling factors, we determined whether the knockdown of Vps34 or that of Beclin 1 would affect the hyperphosphorylation of AMPK (and that of its substrate ACC) or the hypophosphorylation of p70^{S6K}. Interestingly, although the inhibition of mTOR induced by ABT737 was inhibited by depletion of Vps34 or Beclin 1, the activation of AMPK was not affected by the knockdown of Vps34 or Beclin 1 (Figure 7f), suggesting that ABT737 is able to modulate distinct and non-univocal pro-autophagic pathways.

Discussion

ABT737 is a highly specific BH3 mimetic (Oltersdorf *et al.*, 2005), triggering apoptosis in cells in which neutralization of Bcl-2 is sufficient to de-inhibit the mitochondrial cell-death pathway. Moreover, ABT737 has been shown to induce autophagy by stimulating the dissociation of Beclin 1 from its inhibitory factors Bcl-2 and Bcl-X_L, thereby facilitating the Beclin 1-mediated allosteric activation of Vps34 and the consequent increase in phosphatidylinositol 3-phosphate-dependent autophagy (Maiuri *et al.*, 2007b,c). It appears that ABT737 activates multiple pro-autophagic signal trans-

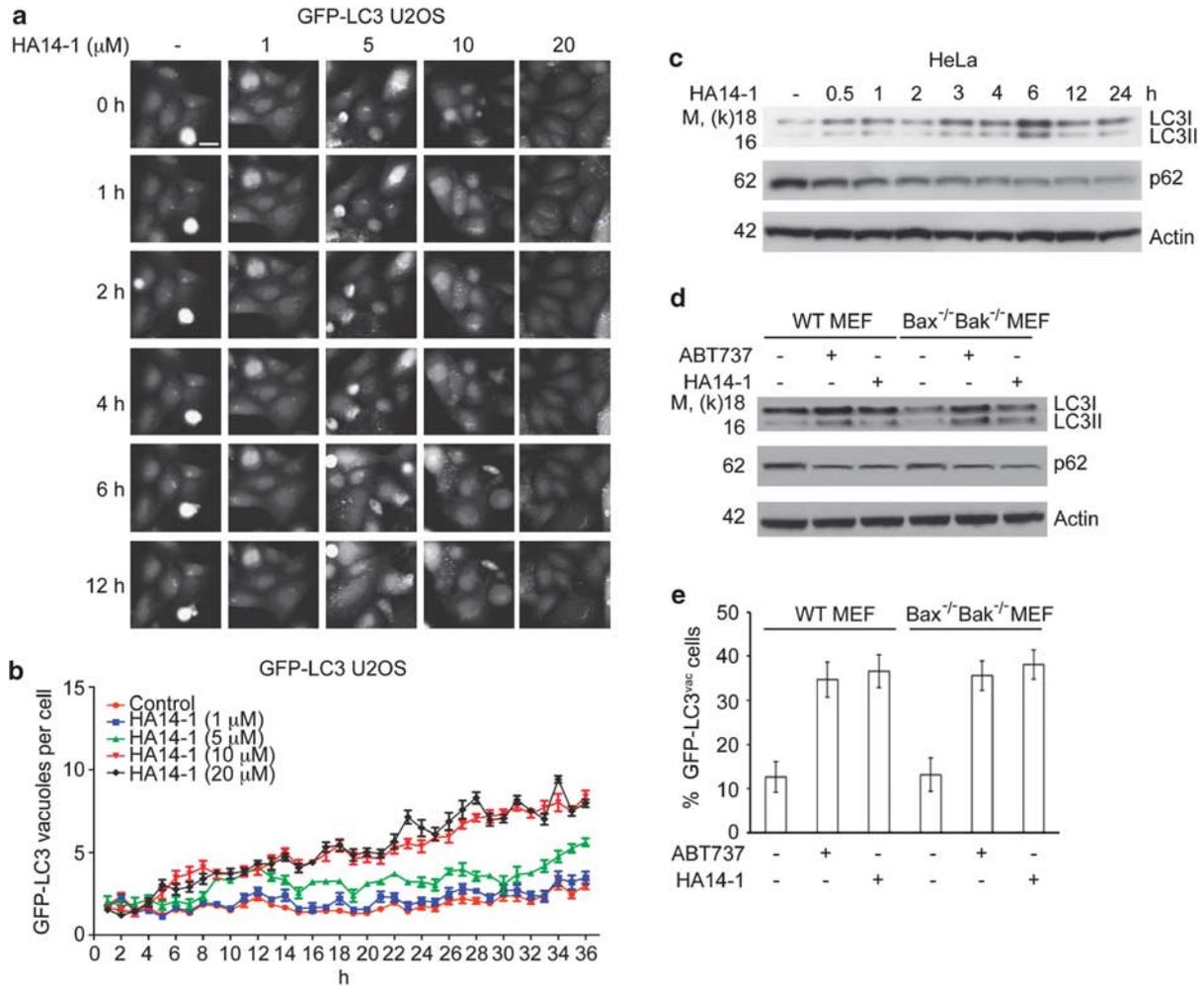


Figure 6 Pro-autophagic activity of the BH3 mimetic HA14-1. (a, b) U2OS cells stably expressing GFP-LC3 were treated with HA14-1 and the kinetics of puncta formation was determined by videomicroscopy (means \pm s.e.m., $n = 3$ separate experiments). (c) Alternatively, HeLa cells were cultured in the presence of HA14-1 (20 μ M) at the time points mentioned, and the lipidation of LC3 and the expression of p62 were monitored by immunoblot. Actin levels were assessed to ensure equal loading of lanes. Results are representative of three independent determinations. (d) WT and *Bax*^{-/-}*Bak*^{-/-} MEF were cultured in the presence of ABT737 (1 μ M) or HA14-1 (20 μ M) overnight, and the lipidation of LC3 and the expression of p62 were monitored by immunoblot. Actin levels were assessed to ensure equal loading of lanes. Results are representative of three independent determinations. (e) In the same conditions as shown in d, the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) is reported (means \pm s.e.m., $n = 3$).

duction pathways. In this article, we show that ABT737 causes the activation of AMPK, the inhibition of mTOR, dephosphorylates (and hence likewise inhibits) (Tasdemir *et al.*, 2008b) p53, and deactivates the autophagy-inhibitory Akt kinase. These effects are obtained at comparatively high doses (in the μ M range) that can be attained *in vivo*, in animal experiments designed to evaluate the anticancer effects of ABT737, and that induce the caspase-dependent destruction of platelets (Mason *et al.*, 2007). As a result, the ABT737 concentrations evaluated in this study may be clinically relevant, in spite of the fact that they are relatively high.

We considered that some of these multiple consequences of ABT737 administration might be due to off-target effects, as ABT737 could affect proteins other than those bearing BH3 receptor domains (such as Bcl-2 and BCL-X_L) and/or act on signal-transduction pathways other than that controlled by the Beclin 1/Vps34

complex. However, ABT737 induced autophagy in cells that were fully apoptosis resistant (like MEF lacking both Bax and Bak) and failed to inhibit mitochondrial respiration before the induction of autophagy. Given this, it might be possible that a rather minor reduction in mitochondrial respiration induced by ABT737—that would be undetectable on whole cells in respirometric studies—would be perceived by homeostatic regulatory systems in the cells to stimulate autophagy, as well as a corrective increase in respiratory activity until the system decompensated. This remote possibility requires additional investigation. In spite of this caveat, knockdown of Beclin 1 or Vps34 fully suppressed ABT737-induced autophagy and also prevented the ABT737-mediated inhibition of mTOR (yet failed to affect AMPK activation induced by ABT737). In addition, several of the effects of ABT737 were shared by another structurally dissimilar BH3 mimetic, HA14-1, suggesting that the

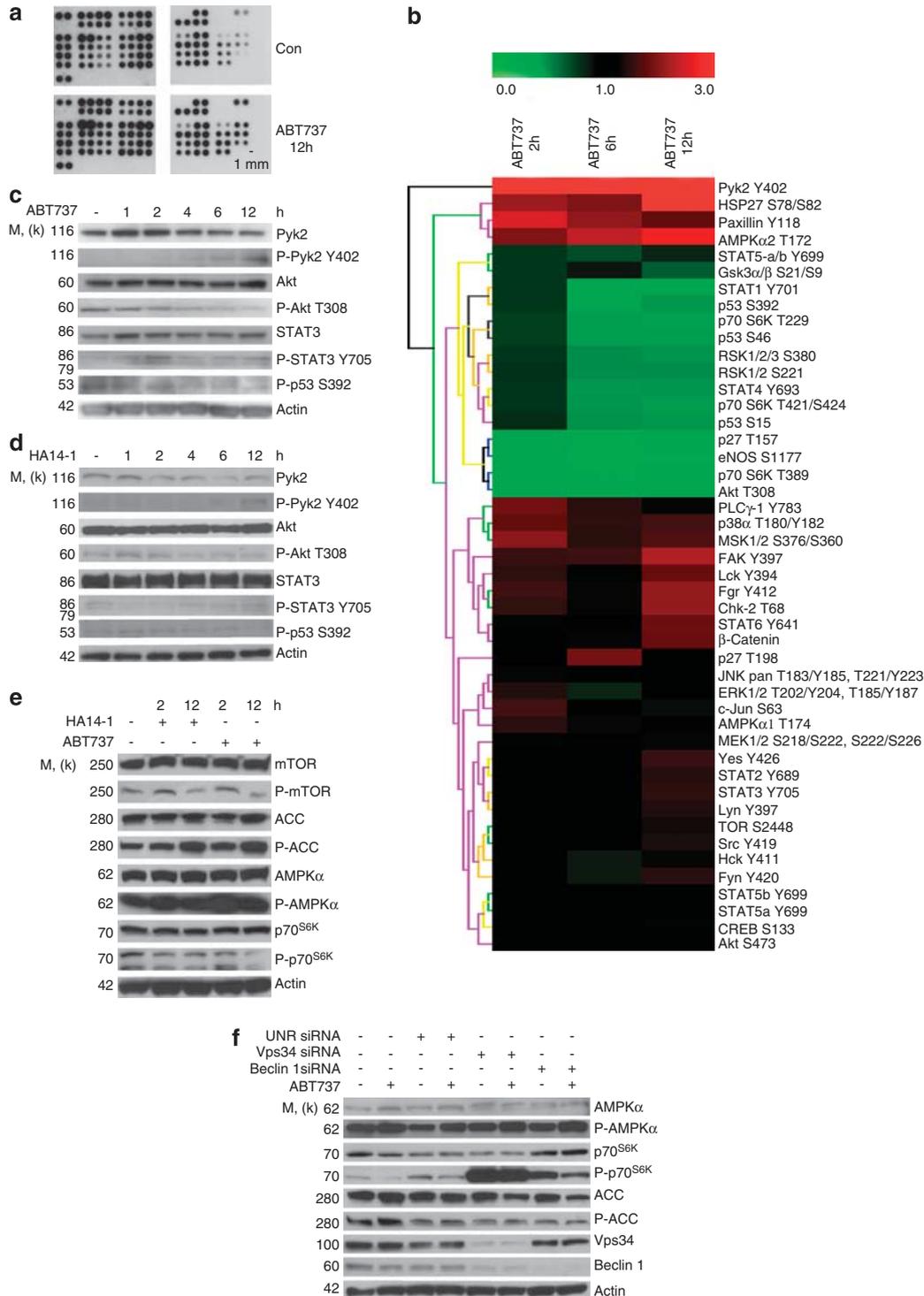


Figure 7 Profile of phospho-proteome phosphorylation status elicited in HeLa cells after ABT737 treatment. HeLa cells were treated with ABT737 (1 μ M) for 2, 6 or 12 h. Cell extracts were then probed on human phosphoprotein arrays following manufacturer's instructions (see Materials and methods section). **(a)** Representative pictures of proteome profiler array. **(b)** Clustering analysis for the effect of BH3 mimetic on protein kinase phosphorylation. **(c, d)** Representative immunoblots of selected kinases whose phosphorylation status was affected by ABT737 (1 μ M) or HA14-1 (20 μ M) treatment (Pyk2, Akt, STAT3 and p53) validating the phosphoprotein arrays ($n = 3$). **(e)** Comparison of the effects of ABT737 (1 μ M) or HA14-1 (20 μ M) on the mTOR, ACC, AMPK and p70^{S6K} activation status ($n = 3$). **(f)** Effect of Vps34 and Beclin 1 downregulation on the ABT737-mediated activation of AMPK α and inhibition of mTOR, as determined by immunoblot experiments ($n = 3$). **(c-f)** Actin levels were monitored to ensure equal loading ($n = 3$).

inhibition of mTOR and the dephosphorylation of p53 are specific (on-target) effects of BH3 mimetics. Thus, ABT737 may stimulate multiple autophagy-stimulatory pathways due to the high degree of connectivity that exists among autophagy-regulatory protein complexes, meaning that a specific effect on the Beclin 1 interactome might affect other nodes in the autophagy-controlling network.

Materials and methods

Cell lines and culture conditions

All cell lines were cultured at 37 °C under 5% CO₂, in a medium containing 10% fetal calf serum, 10 mM Hepes buffer and 1 mM sodium pyruvate. In addition, cell type-specific culture conditions include Dulbecco's modified Eagle's medium (DMEM) for human cervical carcinoma HeLa cells; DMEM containing 1% non-essential amino acids for WT MEF and Bax^{-/-}Bak^{-/-} MEF; DMEM containing 100 U/ml penicillin G sodium and 100 mg/ml streptomycin sulphate for human osteosarcoma GFP-LC3 U2OS cells; and McCoy's medium for HCT116 colon carcinoma cell lines (generous gift from B Volgelstein). All media and supplements for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). Cells were seeded in 6- or 12-well plates and grown for 24 h before treatment with ABT737 (2000, 1000, 100, 10 or 1 nM; Selleck Chemicals, Houston, TX, USA), Bay11-7082 (1 μM; BioMol Research Laboratories, Lausen, Switzerland), EX527 (100 μM; Tocris Bioscience, Bristol, UK), bafilomycin A1 (BafA1; 1 nM), geldanamycin (2 μM), cisPt (10 and 20 μM), 17-allylamino-geldanamycin (2 μM), MG132 (10 μM; Sigma-Aldrich, St Louis, MO, USA), HA14-1 (1, 5, 10 and 20 μM), Nutlin-3 (10 μM) or RITA (10 μM; Alexis Biochemicals, Lausen, Switzerland) for the indicated period (12 h, unless otherwise stated).

Plasmids transfection and RNA interference

Transient transfections were performed with Lipofectamine 2000 reagent (Invitrogen) and cells were used 24 h after transfection unless specified differently. Cells were transfected with empty vector, Rheb D60K and Rheb Q64L (Zhou *et al.*, 2009) alone or together with GFP-LC3 (Kabeya *et al.*, 2000). Cells were cultured in six-well plates and transfected at 50% confluence with Oligofectamine reagent (Invitrogen), in the presence of 100 nM of siRNAs specific for human SIRT1 (Morselli *et al.*, 2010), Atg5 (Sigma), IKKα, IKKβ, NEMO (Criollo *et al.*, 2010), AMPKα, Beclin 1, HMD2, p53, Vps34 (Tasdemir *et al.*, 2008b), a scrambled siRNA or a siRNA targeting the unrelated protein emerlin (Harborth *et al.*, 2001). siRNA-mediated protein downregulation was controlled by immunoblots.

Quantification of autophagy

Autophagy was quantified by counting the percentage of cells showing accumulation of GFP-LC3 in vacuoles, of a minimum of 100 cells per preparation in three independent experiments. Cells presenting a mostly diffuse distribution of GFP-LC3 in the cytoplasm and nucleus were considered as non-autophagic, whereas cells representing several intense punctuate GFP-LC3 aggregates with no nuclear GFP-LC3 were classified as autophagic. Each GFP-LC3 staining was read by two independent investigators. Cells cultured on coverslips were fixed in paraformaldehyde (4% w/v) for 30 min at room temperature for GFP-LC3

assays. Nuclei were labelled with Hoechst 33342 (10 mg/ml; Molecular Probes, Carlsbad, CA, USA). Fluorescence microscopy was performed with an IRE2 microscope (Leica Microsystems, Weitzler, Germany) equipped with a DC300F camera.

Automated high-content microscopy

A total of 10 × 10³ U2OS cells stably expressing a GFP-LC3 chimera were seeded in 96-well imaging plates (BD Falcon, Oxford, UK) 24 h before treatment. The cells were cultured in the presence of ABT737 (1 μM) under standard culture conditions (37 °C, 5% CO₂) and subjected to pulsed observations (every 1 h for up to 36 h) with a BD pathway 855 automated live cell microscope (BD Biosciences, Oxford, UK). Images were further analyzed for the kinetics of GFP-LC3 sub-cytoplasmic count with the open source software Cellprofiler (freely available from the BROAD Institute at <http://www.cellprofiler.org>, Cambridge, MA, USA). Otherwise the videos of the kinetic GFP-LC3 were generated by Image J software.

Western blot analysis

Cells were washed with cold phosphate buffered saline at 4 °C and lysed on ice as described previously (Criollo *et al.*, 2010). In all, 40–50 μg of proteins was loaded on 4–12% or 12% SDS-PAGE precast gels (Invitrogen) and transferred to Immobilon membrane (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h in phosphate-buffered saline-Tween-20 (0.05%) containing 5% non-fat milk. Primary antibodies including anti-SIRT1, -Beclin 1, -p53, -p62, -NEMO, -P-NEMO (S376), -HDM2 (Santa Cruz, Santa Cruz, CA, USA), -STAT3, -P-STAT3 (Y705), -Akt, -P-Akt (T308 and T473), -mTOR, -P-mTOR (S2448), P-p53 (S392), -IKKα, -IKKβ, -P-IKKα/β (S376/180), -AMPKα, -P-AMPKα (Y172), -Pyk2, -P-Pyk2 (Y402), -IκBα, -P-IκBα (S32/36), -p70^{S6K}, -P-p70^{S6K} (Y421/S424), -Atg5, -ACC, -P-ACC (S79) or anti-LC3 (Cell Signalling, Danvers, MA, USA) were incubated overnight at 4 °C and revealed with the appropriate horseradish peroxidase-labelled secondary antibodies (Southern Biotechnologies Associates, Birmingham, AL, USA) plus the SuperSignal West Pico chemoluminescent substrate (Pierce, Rockford, IL, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Billerica, MA, USA) or anti-actin (Abcam, Cambridge, MA, USA) were used to control equal loading.

Phospho-kinase array

The profile of phospho-kinase proteins was performed on HeLa cells treated with ABT737 (1 μM) and HA14-1 (20 μM) for 2, 6 and 12 h using a Proteome Profiler array-Phospho-Kinase array kit (ARY003) according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). Clustering was performed with MultiExperiment Viewer (MeV version 4, Boston, MA, USA). Hierarchical clustering was performed using Euclidean distance and average linkage algorithm based on their normalized phosphorylation intensities. Heatmap was generated by heatmap view integrated in MeV 4 software.

Flow cytometry

The following fluorochromes were employed to determine apoptosis-associated changes by cytofluorometry: 3,3'-dihexyloxycarbocyanine iodide (40 nM) for quantification or the mitochondrial transmembrane potential (Δψ_m) determination and propidium iodide (1 μg/ml) for determination of cell viability (Molecular Probes) (Maiuri *et al.*, 2007b). Cells were trypsinized and labelled with the fluorochromes at 37 °C,

followed by cytofluorometric analysis with a fluorescence-activated cell sorter (FACS-Scan, Becton Dickinson, Franklin Lakes, NJ, USA).

Mitochondrial respiratory chain activities

Respiratory chain activities of ABT737 and HA14-1-treated cells were spectrophotometrically measured as previously described at various time points after disrupting cells by means of two freeze-thaw cycles (Rustin *et al.*, 1994; Benit *et al.*, 2006).

Statistical analysis

Statistical analyses were carried out using the Prism software package (GraphPad Software Inc., San Diego, CA, USA) and the Microsoft Office 2003 Excel software package (Microsoft Corporation, Redmond, WA, USA). Mean values \pm s.d. or \pm s.e.m. were indicated and were compared using unpaired Student's *t*-tests. For multiple comparisons, we used the one-factor (ANOVA) variance analysis corrected by the Bonferroni test.

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Conflict of interest

The authors declare no conflict of interest.

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8.2 BH3 mimetics reveal the network properties of autophagy-regulatory signaling cascades.

BH3 mimetics reveal the network properties of autophagy-regulatory signaling cascades

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Beclin 1 usually interacts with several autophagy-inhibitory proteins including the anti-apoptotic proteins from the Bcl-2 family (Bcl-2, Bcl-X_L and Mcl-1) and the inositol-1,4,5 triphosphate (IP₃) receptor, which interacts with Beclin 1 indirectly via Bcl-2. Beclin 1 possesses a BH3 domain that usually interacts with a hydrophobic cleft, the BH3 receptor domain, contained within Bcl-2 and its homologues. Dissociation of this interaction can be induced by phosphorylation or ubiquitination of the BH3 domain, by post-transcriptional modifications affecting the Bcl-2 protein, as well as by other BH3 domain-containing proteins that have a high affinity for Bcl-2 (or its homologues), and hence liberate Beclin 1 from its restraint. As a result, it has been thought that so-called BH3 mimetics, that is the pharmacological agents that occupy the hydrophobic cleft of Bcl-2, Bcl-X_L and Mcl-1, would induce autophagy solely by disrupting the interaction between Beclin 1 and its inhibitors. Unexpectedly, we found that two distinct BH3 mimetics, ABT737 and HA14-1, also stimulate other pro-autophagic pathways and hence activate the nutrient sensors Sirtuin 1 and AMPK, inhibit mTOR, deplete cytoplasmic p53 and trigger the IKK kinase. All these additional activities are required for optimal autophagy induction by BH3 mimetics, pointing to the existence of a coordinated autophagy-regulatory network.

Numerous signal transducers involved in the cellular response to stress and damage also participate in the control of macroautophagy (herewith referred to as “autophagy”). This applies for instance to the proteins from the Bcl-2 family, which can be divided into three categories, (i) the anti-apoptotic multidomain proteins (Bcl-2, Bcl-X_L, Mcl-1), (ii) the pro-apoptotic multidomain proteins (Bax, Bak) and (iii) the equally pro-apoptotic BH3-only proteins (exemplified by Bad, Noxa, Puma and a few others).

The pro-apoptotic multidomain proteins do not have any well-documented impact on the regulation of autophagy. In contrast, the multidomain proteins from the Bcl-2 family are potent autophagy inhibitors, whereas the BH3-only proteins have pro-autophagic properties. Indeed, the essential pro-autophagic protein Beclin 1 is a BH3-only protein. Beclin 1's BH3 domain usually engages in an inhibitory interaction with Bcl-2 and its close homologues, thereby preventing Beclin 1 from acting as an allosteric activator of the class III phosphatidylinositol-3-kinase (PtdIns3KC3) also called Vps34, to generate phosphatidylinositol-3-phosphate (PtdIns(3)P), which in turn is required for the initial steps of phagophore formation.

Disrupting the inhibitory interaction of Beclin 1 with Bcl-2 (or its close homologues) is essential for the initiation of autophagy. The induction of autophagy by starvation is coupled to the dissociation of Beclin 1 from Bcl-2, which in part depends on the phosphorylation of

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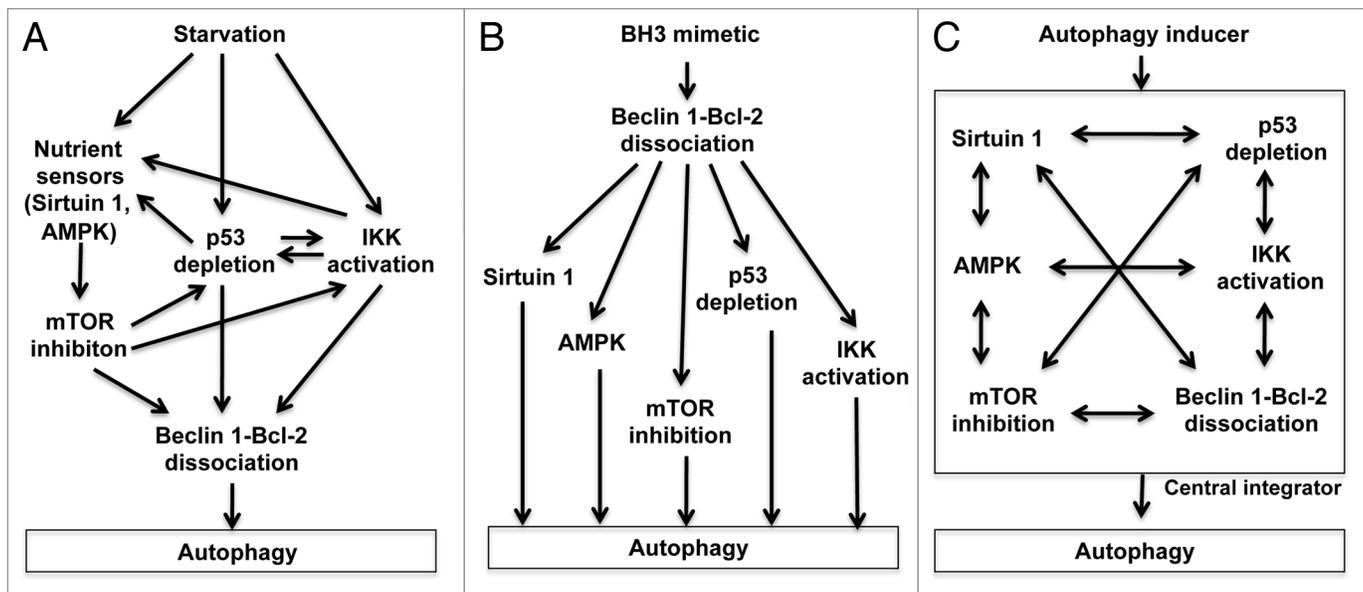


Figure 1. Hypothetical hierarchy among autophagy-relevant events. (A) Dissociation of Beclin 1 from Bcl-2-like proteins as a late event resulting from a series of upstream signals. (B) Pro-autophagic signals stimulated by artificial dissociation of the Beclin 1-Bcl-2 complex. Note that several signals that were thought to occur upstream of the liberation of Beclin 1 from Bcl-2 are also activated downstream of this event and are required for optimal autophagy induction. (C) Hypothetical central integrator of autophagic signaling. A series of molecular events occurs in a coordinated fashion, likewise due to the activation of self-amplifying feed forward loops. The exact nature of the signaling events that would be part of this central integrator, the probably complex interactions within this integrator, and the frontier between the integrator and stimulus-specific signals triggering autophagy remain to be defined.

Bcl-2 by JNK1, reducing its affinity for BH3 domains, and in part depends on the upregulation of the BH3-only protein Bad (which then competitively disrupts the interaction between Bcl-2 and Beclin 1). Accordingly, the absence of the sole BH3 domain protein encoded by the *Caenorhabditis elegans* genome, Egl1, results in the incapacity of nematodes to mount an autophagic response to starvation. The interaction between Beclin 1 and Bcl-2 is also lost upon induction of autophagy by several potent autophagy inducers that we have been characterizing. Thus, the pharmacological inhibition of p53 or the depletion of the cytosolic fraction of p53 potently induces autophagy at the same time that it dissociates the Beclin 1-Bcl-2 complex. Similarly, activation of the IKK complex (by transfection with constitutive active mutants of the IKK complex) induces autophagy while it activates JNK1 and disrupts the inhibitory interaction between Beclin 1 and Bcl-2. Starvation, ER stress and rapamycin all induce depletion of p53 that can be prevented by inhibiting or depleting HDM2, the p53-ubiquitinating enzyme targeting p53 for proteasomal destruction.

Similarly, inhibition of IKK can prevent induction of autophagy by starvation, ER stress, rapamycin or p53 inhibition. Altogether these findings suggest that p53 inhibition/depletion is closely coupled to IKK activation (because both phenomena mutually stimulate each other) and that both these phenomena are closely linked to the activation of nutrient sensors (that is mimicked by rapamycin) yet occur upstream of the dissociation of the Beclin 1-Bcl-2 complex (Fig. 1A). To examine this hypothesis, we have been investigating the question whether artificial dissociation of the Beclin 1-Bcl-2 complex by adding high concentrations of so-called BH3 mimetics, that is the pharmacological ligands of the BH3 binding domain of Bcl-2, would induce autophagy according to our expectation, that is without stimulating the activation of the nutrient sensors (Sirtuin 1 and AMPK), without inhibiting mTOR (which is subordinated to AMPK), without depleting p53 and without activating IKK. To our surprise, we found that two different BH3 ligands, ABT737 and HA14-1, both induced all these changes. Control experiments corroborated that ABT737 and HA14-1

neither inhibit oxidative phosphorylation nor reduce cellular ATP concentrations before the induction of autophagy, suggesting that they do not compromise general metabolism through off-target effects. Moreover, autophagy induction by ABT737 and HA14-1 is fully prevented by knocking down *Beclin 1* or *Vps34*.

To our surprise, it turned out that inhibition of Sirtuin 1 (by means of the inhibitor EX527 or by a siRNA), inhibition of AMPK (by a siRNA specific for the $\alpha 1$ subunit), artificial activation of mTOR (by transfection with the constitutive active Rheb mutant, Rheb Q64L), inhibition of p53 depletion (by the pharmacological HDM2 inhibitors RITA or Nutlin-3, or by siRNA-mediated depletion of HDM2) or inhibition of IKK (by the inhibitors Bay11-7082 and 17-allyl-amino-geldanamycin, or knockdown of the genes encoding any of the three IKK subunits, IKK α , IKK β , NEMO), all reduce autophagy induction by BH3 mimetics. These results place Sirtuin 1, AMPK-mTOR, HDM2 and IKK downstream of the dissociation of the Beclin 1-Bcl-2 complex (Fig. 1B).

How can these apparently contradictory results (compare **Fig. 1A and B**) be reconciled? There is only one way out of the dilemma. The pathways cannot follow a linear hierarchy and rather must be interconnected in a complex circuitry, in which stimulation of autophagy by physiological triggers (such as starvation or organellar stress) trigger an ensemble of intimately linked changes that are coupled to each other in positive feed forward loops (**Fig. 1C**). Such self-amplifying loops would interconnect the activation of nutrient

sensors (that also result in mTOR inhibition), IKK, HDM2 (to deplete p53) and the Beclin 1-Vps34 complex (upon dissociation from Bcl-2 or its homologues), switching on the autophagic pathway at multiple levels. In this hypothetical scenario, the network properties of the system would constitute a compact ensemble that composes the autophagic switch. Understanding the molecular and functional properties of this switch by systems biology methods would then constitute the next frontier in autophagy research.

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8.3 Neuroendocrine regulation of autophagy by leptin.

Neuroendocrine regulation of autophagy by leptin

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Key words: autophagy, leptin, obesity

The satiety hormone leptin plays a cardinal role in the pathophysiology of obesity and diabetes. Here, we show that pharmacological autophagy inducers like rapamycin, spermidine and resveratrol can reduce leptin concentrations in the serum of mice and that genetic inactivation of the leptin/leptin receptor system leads to an increase in autophagy in peripheral tissues including skeletal muscle, heart and liver. Paradoxically, intravenous or intraperitoneal administration of recombinant leptin protein also induced autophagy in these tissues. Moreover, leptin stimulated canonical autophagy in cultured human or mouse cell lines, a phenomenon that was coupled to the activation of adenosine monophosphate-dependent kinase (AMPK), as well as the inhibition of mammalian target of rapamycin (mTOR), and that was confirmed by autophagic flux measurements. These results suggest that leptin plays an important role in the neuroendocrine control of autophagy, underscoring the existence of novel links between metabolic control and autophagic flux that warrant further in-depth investigation.

Introduction

Leptin is an adipokine that is mostly produced by white adipose cells.¹ Thus, leptin serum levels are proportional to the overall adipose mass in both man and women, although the serum levels are 2–3 times lower in men than in women, even when adjusted for age and body-mass index. The best-known function of leptin is to regulate appetite.² Leptin acts on cells in the hypothalamus to reduce the production of orexigenic (appetite-stimulatory) neuropeptides and reciprocally enhance the secretion of anorectic peptides, thus controlling food intake. Mice deficient for leptin (ob/ob mice) or lacking one particular isoform of the leptin receptor, LRB (db/db mice) are characterized by hyperphagia, morbid obesity and type 2 diabetes.^{1,2} Although in humans with morbid obesity, loss-of-function mutations of leptin or its receptor are rare, it is thought that acquired resistance against leptin may play a major role in the pathogenesis of obesity.² Beyond its function as a satiety factor, leptin has metabolic functions that can be attributed to direct effects on a variety of peripheral tissues.²

Macroautophagy (here referred to as “autophagy”) plays a major role in the adaptation of cells to a plethora of distinct stressors including starvation.^{3,4} Thus, the culture of mammalian cells in the absence of nutrients and serum activates the autophagic program through a complex signal transduction pathway that involves the intertwined activation of nutrient sensors (such as adenosine monophosphate-dependent kinase, AMPK, and the deacetylase Sirtuin 1),^{5,6} the inhibition of mammalian target

of rapamycin (mTOR),⁷ the activation of other kinases such as c-Jun N-terminal kinase 1 (JNK1),⁸ or inhibitor of NFκB kinase (IKK)⁹ and the depletion of cytoplasmic p53.¹⁰ Similarly, mice deprived from nutrients for 24 h or more develop a systemic autophagic response in most tissues (with the exception of the brain) that have been examined with this respect.¹¹ Autophagy plays a major role in energy homeostasis, and its induction is indispensable for lipolysis,¹² a process that is also stimulated by leptin.¹

In vivo administration of leptin has been shown to stimulate the activating AMPK phosphorylation in peripheral tissues including muscle, yet inhibits AMPK in the hypothalamus¹³⁻¹⁶ suggesting that it may influence autophagy in a tissue-specific fashion. Driven by these considerations, we decided to determine whether external supply of recombinant leptin or genetic defects in leptin expression and leptin signaling might regulate autophagy.

Results and Discussion

In vitro induction of autophagy by leptin. When recombinant human leptin protein was added to HeLa cells, it stimulated a post-translational modification of LC3 that typically accompanies autophagy and that increases its electrophoretic mobility due to its proteolysis and lipidation close to its N-terminus.¹⁷ This capacity of leptin to convert LC3-I into LC3-II was lost upon its heat denaturation (Fig. 1A). Upon leptin supplementation, a

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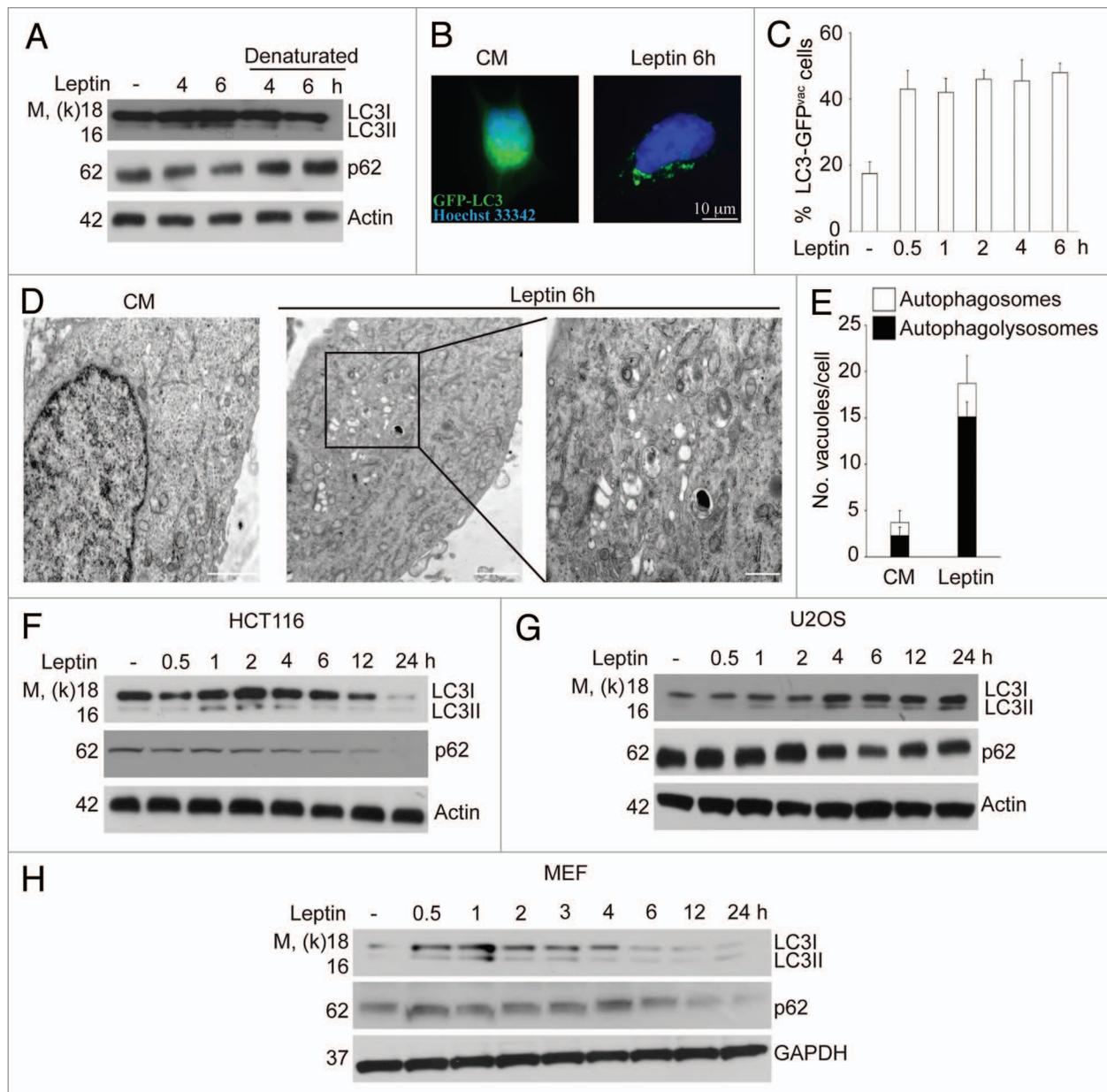


Figure 1. Induction of autophagy by leptin in vitro. (A–E) Signs of autophagy induced by leptin (3 nM) in HeLa cells. WT HeLa cells were cultured in the presence of intact or heat-denatured leptin for 4 or 6 h, followed by immunoblot determination (n = 3) of LC3 and p62 levels (A). Alternatively, GFP-LC3-transfected HeLa cells were cultured for the indicated period in complete medium (CM) or with leptin. Representative images (B) illustrating the subcellular redistribution of GFP-LC3 from a diffuse pattern (in controls) to cytoplasmic puncta (in treated cells) and quantitative results (means ± SEM, n = 3) (C) are shown. Transmission electron microscopy led to the identification of autophagosomes and autophagolysosomes after leptin treatment. Representative images of WT HeLa cells in absence or presence of leptin are shown (D), and quantitative results are plotted (E) (means ± SD are calculated for 50 cells for each experimental condition). (F–H) Signs of autophagy induced by leptin in HCT116 cells (F), U2OS cells (G) and mouse embryonic fibroblasts (MEF; H). Immunoblots were performed to detect two signs of autophagy, namely the generation of LC3-II and the depletion of p62/SQSTM1. Results are representative of at least three experiments yielding similar results. Actin or GAPDH levels were monitored to ensure equal loading.

GFP-LC3 fusion protein stably introduced into the cells redistributed from its usually diffuse location to defined cytoplasmic dots that constitute bona fide autophagosomes (Fig. 1B and C). Accordingly, addition of leptin caused the accumulation of autophagosomes and autophagolysosomes, as detectable by transmission electron microscopy (Fig. 1D and E). Leptin

stimulated the time-dependent lipidation of LC3 coupled to a diminution of the autophagic substrate p62/SQSTM1 in a variety of cell lines including human HCT116 colorectal cancer cells (Fig. 1F), human U2OS osteosarcoma cells (Fig. 1G) and mouse embryonic fibroblasts (MEF, Fig. 1H). GFP-LC3 dots induced by leptin rarely colocalized with the early endosome

marker EEA1 and frequently colocalized with the late endosome/amphisome marker Rab7, as well as with the lysosome/autolysosome marker LAMP2 (Fig. 2A), suggesting that leptin-elicited autophagosomes are matured by fusion with amphisomes and lysosomes to complete the autophagic process. Accordingly, autophagic flux experiments revealed that leptin induced more LC3-II in the presence than in the absence of bafilomycin A1, an inhibitor of autophagosome-lysosome fusion (Fig. 2B). In accord with established guidelines,^{18,19} these results indicate that leptin truly stimulates the formation and later maturation/turnover of autophagosomes. In addition, leptin stimulated the activating phosphorylation of AMPK α , the hyperphosphorylation of the AMPK substrate acetyl-coenzyme carboxylase (ACC), the dephosphorylation of mTOR, the dephosphorylation of the mTOR substrate p70^{S6K}, as well as the reduction of Sirtuin 1 expression (Fig. 2C). Moreover, leptin failed to induce autophagy in MEF lacking the essential autophagy genes Atg5 or Atg7, indicating that it stimulates a canonical autophagy pathway (Fig. 2D). Altogether, these results indicate that leptin can stimulate all hallmarks of macroautophagy in multiple cell lines in vitro.

In vivo modulation of autophagy by leptin. We determined whether leptin can activate autophagy in vivo, upon parenteral administration to mice. Intraperitoneal injection of leptin stimulated LC3 lipidation in skeletal muscle, myocardium, liver and kidney while it reduced the abundance of p62/SQSTM1 in a time-dependent fashion (Fig. 3A). Similarly, intravenous injection of leptin induced biochemical signs of autophagy in a dose-dependent manner (Fig. 3B). These in vivo effects of leptin were not abolished by co-administration of the LPS-neutralizing antibiotic polymyxin B (Fig. 3C), yet were lost upon its heat denaturation (not shown). Intraperitoneal leptin injections were similarly efficient in inducing autophagy as were injections of spermidine, resveratrol or rapamycin, which are also known to have an antiaging effect^{6,20-22} (Fig. 3D). Unexpectedly, these three agents reduced the concentration of leptin in the serum of treated mice (Fig. 3E), suggesting a complex relationship between autophagy and leptin at the level of whole-body physiology. Indeed, mice lacking endogenous leptin (*ob/ob* mice) exhibited an enhanced level of autophagy when compared with isogenic wild

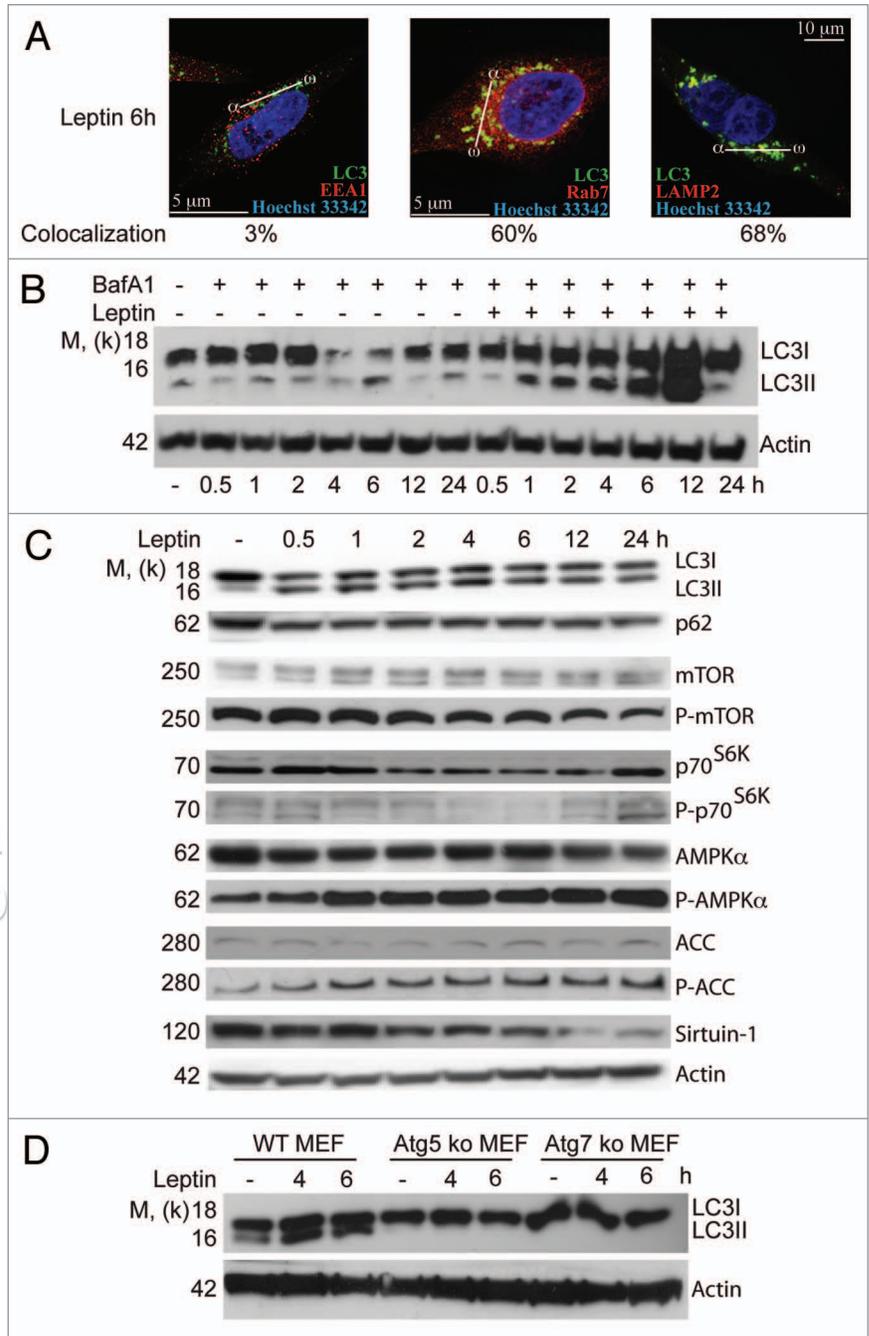


Figure 2. Mechanisms of autophagy induction by leptin in vitro. (A) Maturation of leptin-induced autophagosomes. HeLa cells stably expressing GFP-LC3 were cultured for 6 h in the presence of leptin, and cells were fixed, permeabilized and stained to detect markers of early endosomes, late endosomes/amphisomes and lysosomes/autophagolysosomes by immunofluorescence, as indicated. Representative images presenting the dominant phenotype are shown. Percentages refer to the percentage of the green fluorescence (GFP-LC3) colocalizing with the red fluorescent markers. (B) Autophagy flux induced by leptin. HeLa cells were cultured for various periods in the presence or absence of the indicated combinations of bafilomycin A1 and/or leptin, and LC3-II formation was assessed by immunoblotting. (C) Changes in the kinase activity of major autophagy regulators. After leptin treatment, HeLa cells were lysed and immunoblots were performed to measure the changes in the phosphorylation patterns of key autophagy-regulatory kinases and their substrates. (D) Dependency of leptin-induced autophagy on Atg5 or Atg7. Mouse embryonic fibroblasts (MEF) that were either wild type (WT) or derived from embryos deficient for Atg5 or Atg7 were cultured with leptin and the LC3-II formation was determined. Results are representative of at least three separate experiments. Actin levels were monitored to ensure equal loading.

Figure 3 (See opposite page). Regulation of autophagy by leptin in vivo. (A–C) Induction of autophagy by intraperitoneal (A and C) or intravenous (B) injection of recombinant leptin protein. Female adult C57Bl/6 mice were injected with leptin at distinct doses (1 mg/kg in A and C; 0.5 mg/kg or 1 mg/kg in B) alone or together with saturating amounts of polymyxin B (C). Then, different tissues were obtained at necropsy, snap-frozen and subjected to immunoblot determinations of LC3-II generation and p62/SQSTM1 depletion. (D and E) Effects of autophagy inducers on circulating leptin levels. C57Bl/6 mice received intraperitoneal injection of leptin, spermidine, resveratrol or rapamycin, and signs of autophagy were assessed by immunoblot after 6 h (D). Moreover, leptin levels were determined in the plasma in 24 h starved (NF) mice or six hours after injection of the indicated autophagy triggers (E). (F and G) Effects of the depletion of endogenous leptin or its receptor on autophagy. Tissues were obtained from wild type (WT) C57Bl/6 mice or isogenic mice bearing the *ob/ob* or *db/db* mutations, after normal feeding or starvation (F), as detailed in Materials and Methods. Moreover, *db/db* mice received the intraperitoneal injection of leptin (1 mg/kg). After six hours, organs were obtained at necropsy and signs of autophagy were determined (G). Note that each experimental group involved at least three mice, that experiments have been repeated at least three times, and that results are only shown for one representative animal in each group. GAPDH levels were monitored to ensure equal loading.

type (C57Bl/6) mice. This enhanced autophagy was detected in baseline conditions and was further augmented when the mice were kept in the absence of food (but allowed to drink water ad libitum) for 24 h. Mice lacking the principal leptin receptor isoform that mediated hypothalamic leptin signaling (*db/db* mice) manifested an increase in autophagy levels in muscle and liver (Fig. 3F), both in control and in starvation conditions. Of note, *db/db* mice did respond to intraperitoneal leptin injections in thus far that they upregulated autophagy in several peripheral tissues (muscle, heart, liver) (Fig. 3G).

Materials and Methods

Cell lines and culture condition. All cell lines were cultured at 37°C under 5% CO₂, in medium containing 10% fetal calf serum (FCS), 10 mM HEPES buffer and 1 mM sodium pyruvate. In addition, cell type-specific culture conditions include: Dulbecco's modified Eagle's medium (DMEM) for human cervical carcinoma HeLa cells; DMEM containing 1% non-essential amino acids for WT MEF, *Atg5^{-/-}* and *Atg7^{-/-}* MEF; DMEM containing 100 units/ml penicillin G sodium and 100 mg/ml streptomycin sulfate for human osteosarcoma GFP-LC3 U2OS cells; McCoy's medium for HCT116 colon carcinoma cell lines. All media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, USA). Cells were seeded in 6- or 12-well plates and grown for 24 h before treatment with human or mouse recombinant Leptin (3 nM; Invitrogen and R&D Systems, respectively) in presence or absence of bafilomycin A1 (BafA1; 1 nM; Sigma) for the indicated period (6 h, unless stated otherwise).

Plasmids transfection. Transient transfections were performed with Lipofectamine 2000 reagent (Invitrogen), and cells were used 24 h after transfection unless specified differently. Cells were transfected with empty vector alone or together with GFP-LC3.³⁴

Light and immunofluorescence microscopy. Cells were processed for immunofluorescence staining according to established protocols.⁹ Fixed and permeabilized cells were then incubated with primary antibodies (EEA1 and Rab7, Cell Signaling; LAMP2, SantaCruz) overnight and then revealed with the appropriate AlexaFluor® conjugates (Molecular Probes). Nuclei were counterstained with 10 µg/ml Hoechst 33342 (Molecular Probes-Invitrogen). Fluorescence and confocal fluorescence microscopy assessments (magnification 63x) were performed with an IRE2 microscope (Leica Microsystems, Wetzlar, Germany) equipped

with a DC300F camera and with a LSM 510 microscope (Carl Zeiss, Jena, Germany), respectively. To determine the percentage of colocalization, Image J software was used. Autophagy was quantified by counting the percentage of cells showing accumulation of GFP-LC3 in vacuoles (GFP-LC3^{vac}) among a minimum of 100 cells per preparation in three independent experiments. Cells presenting a mostly diffuse distribution of GFP-LC3 in the cytoplasm and nucleus were considered as non-autophagic, while cells exhibiting several intense punctuate GFP-LC3 aggregates with no nuclear GFP-LC3 were classified as autophagic. Each GFP-LC3 staining was read by two independent investigators.

Mouse experiments and tissue processing. C57Bl/6, *db/db* and *ob/ob* mice obtained from Charles River Laboratory were bred and maintained according to the Federation for Laboratory Animal Science Associations and the Animal Experimental Ethics Committee guidelines. Mice were housed in a temperature-controlled environment with 12 h light/dark cycles and received food and water ad libitum. Mice were kept 5 h without food (to avoid any metabolic alteration) but allowed to drink water ad libitum prior to treatment. Then, mice were injected i.p. with spermidine (50 mg/kg), resveratrol (25 mg/kg), rapamycin (5 mg/kg) and leptin (1 mg/kg) in the presence or absence of polymyxin B sulfate (1 mg/kg; Sigma) or i.v. with 0.5 or 1 mg/kg leptin at the indicated time points before anesthetization and sacrificing. For each experiment, at least 6 mice per genotype were used. Mice tissues were immediately frozen in liquid nitrogen after extraction and homogenized in a 50-mM Tris buffer, pH 7.4, containing 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, 300 mM sucrose and protease inhibitor cocktail (Complete; Roche). Tissue extracts were then centrifuged at 12,000 g at 4°C, and supernatants were collected. Protein concentration in the supernatants was evaluated by the bicinchoninic acid technique (BCA protein assay kit; Thermo Fisher Scientific).

Plasma leptin measurements. Mice were kept without food for 5 h to avoid any possible alteration in circulating parameters due to food intake prior to the measurements. Blood was extracted from the transverse sinus after anesthetizing mice with halothane. Blood was immediately centrifuged after collection at 3,000 g at 4°C, and the supernatant was collected and stored at -70°C until analysis. For plasma leptin measurements we used R&D Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). All protocols were performed according to manufacturer's instructions.

Western blot analysis. Cells were washed with cold PBS at 4°C and lysed on ice as previously described in reference 9.

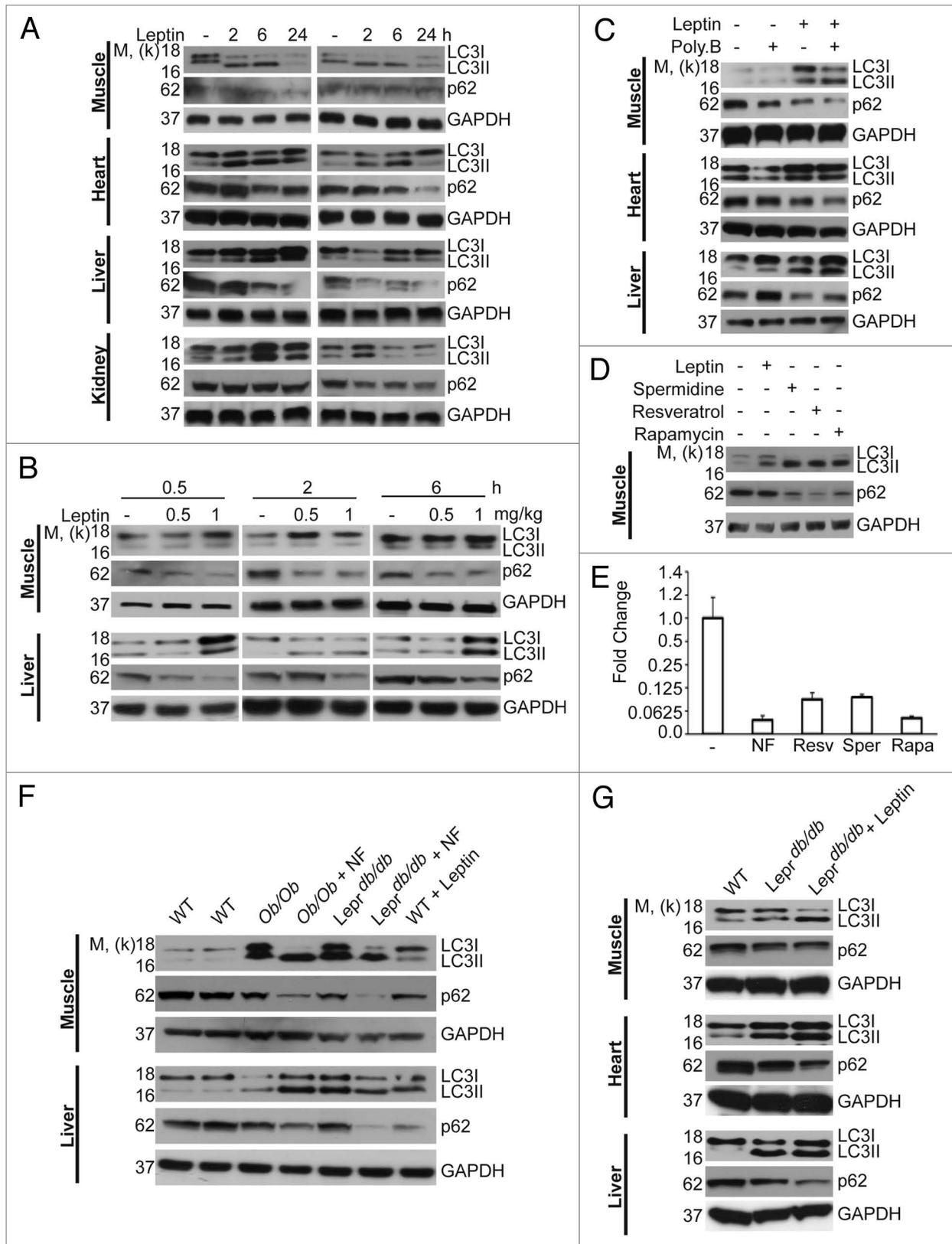


Figure 3. For figure legend, see page 2920.

Forty to 50 (20–25 for animal tissues) μg protein were loaded on 4–12% or 12% SDS-PAGE precast gels (Invitrogen) and transferred to Immobilon membranes (Millipore). The membranes were incubated for 1 h in PBS-Tween 20 (0.05%) containing 5% non-fat milk. Primary antibodies including anti-Sirtuin-1, -p62 (SantaCruz), -p62 (Abnova), -mTOR, -P-mTOR (S2448), -AMPK α , -P-AMPK α (Y172), -p70^{S6K}, -P-p70^{S6K} (Y421/S424), -ACC, -P-ACC (S79) or anti-LC3 (Cell Signaling) were incubated overnight at 4°C and revealed with the appropriate horseradish peroxidase-labeled secondary antibodies (Southern Biotechnologies Associates) plus the SuperSignal West Pico chemoluminescent substrate (Pierce). Anti-GAPDH (Chemicon, CA) or anti-actin (Abcam) were used to control equal loading.

Electron microscopy. Cells were fixed for 1 h at 4°C in 1.6% glutaraldehyde in 0.1 M Sörensen phosphate buffer (pH 7.3), washed, fixed again in aqueous 2% osmium tetroxide, stained in 2% uranyl acetate in 30% methanol and finally embedded in Epon. Electron microscopy was performed with a Tecnai 12 Spirit electron microscope (FEI, Eindhoven, The Netherlands), at 80 kV, on ultrathin sections (80 nm) stained with lead citrate and uranyl acetate.

Statistical procedures. Unless otherwise indicated, experiments were performed in triplicate and repeated at least twice. Figures show the results from one representative experiment (mean \pm SEM, $n = 3$ parallel samples). Data were analyzed with Microsoft Excel (Microsoft Co., Redmond, OR) and statistical significance was assessed by means of the two-tailed Student's *t*-test (* $p < 0.05$).

Concluding Remarks

Although leptin and its receptor (LR) structurally resemble the interleukin-6/interleukin-6 receptor system² (which inhibits autophagy),²³ and even though the LR can transduce multiple anti-autophagic signals including activation of type I PI3 kinases,² our present results indicate that administration of recombinant leptin injected into the peritoneal cavity or into the blood stream can induce autophagy in peripheral tissues including muscle, heart, liver and kidney, in accord with the previous observation that leptin stimulates AMPK activation in skeletal muscles.^{13–15} AMPK activation by leptin has been shown to account for increased fatty acid oxidation in muscle and liver,¹³ and AMPK activation induced by exercise or by administration of metformin is responsible of increased glucose uptake by muscle cells.²⁴ It remains to be investigated to which extent these AMPK effects might be linked to enhanced autophagy. Intriguingly, induction of autophagy by BH3 mimetics (which disrupt the

inhibitory interaction between the BH3 domain of Beclin 1 and Bcl-2-like proteins),²⁵ also can induce AMPK activation,²⁶ suggesting that there is a strong reciprocal link between AMPK and autophagy.²⁷ T cells adoptively transferred into leptin-deficient ob/ob mice exhibit reduced activation of mTOR, suggesting that physiological levels of leptin may be necessary to maintain mTOR activated (and probably to suppress autophagy). Besides, the activity of mTOR complex 1 is elevated in muscles from ob/ob mice.²⁸

In sharp contrast to the aforementioned results, recombinant leptin inhibits AMPK and consequently provokes the activation of mTOR in some cell types, for instance in hypothalamic neurons,¹⁶ primary vascular smooth muscle cells,²⁹ hepatic stellate cells,³⁰ as well as peritoneal macrophages,^{31,32} suggesting that leptin can inhibit autophagy as well. Accordingly, insulin-producing β cells from pancreatic islet exhibit increased autophagy in db/db mice.³³

The results reported here strongly suggest that physiological and pharmacological levels of leptin may control autophagy at multiple levels. While genetic inactivation of the leptin/LR system stimulates autophagy in peripheral locations, external application of leptin stimulates autophagy as well. Thus, any deviation from leptin homeostasis may lead to autophagy, underscoring the probable importance of neuroendocrine circuits that control autophagy in a leptin-dependent fashion. It can be anticipated, yet remains to be investigated, that eating disorders linked to aberrant leptin signaling are associated with a pathophysiologically relevant dysregulation of autophagy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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8.4 Inhibition of autophagy by TAB2 and TAB3.

Inhibition of autophagy by TAB2 and TAB3

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Autophagic responses are coupled to the activation of the inhibitor of NF- κ B kinase (IKK). Here, we report that the essential autophagy mediator Beclin 1 and TGF β -activated kinase 1 (TAK1)-binding proteins 2 and 3 (TAB2 and TAB3), two upstream activators of the TAK1-IKK signalling axis, constitutively interact with each other via their coiled-coil domains (CCDs). Upon autophagy induction, TAB2 and TAB3 dissociate from Beclin 1 and bind TAK1. Moreover, overexpression of TAB2 and TAB3 suppresses, while their depletion triggers, autophagy. The expression of the C-terminal domain of TAB2 or TAB3 or that of the CCD of Beclin 1 competitively disrupts the interaction between endogenous Beclin 1, TAB2 and TAB3, hence stimulating autophagy through a pathway that requires endogenous Beclin 1, TAK1 and IKK to be optimally efficient. These results point to the existence of an autophagy-stimulatory ‘switch’ whereby TAB2 and TAB3 abandon inhibitory interactions with Beclin 1 to engage in a stimulatory liaison with TAK1.

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Keywords: Beclin 1 interactome; mTOR; p53; pifithrin α ; rapamycin; stress response

Introduction

Macroautophagy (hereafter referred to as ‘autophagy’) is a catabolic pathway involving the sequestration of cytoplasmic material in double-membraned vesicles, the autophagosomes. Upon fusion with lysosomes, autophagosomes become autophagolysosomes and their content gets degraded by acidic hydrolases, allowing nutrients and macromolecules to fuel cellular metabolism or sustain stress responses (Klionsky, 2004; Mizushima *et al*, 2008). Multiple distinct perturbations of the cellular physiology can induce autophagy through a complex signalling network that crosstalks with several stress-response pathways (Kroemer *et al*, 2010; Green *et al*, 2011), including molecular cascades that are ignited by organellar damage as well as pathways leading to the activation of prominent transcription factors such as p53 (Tasdemir *et al*, 2008b; Scherz-Shouval *et al*, 2010) and NF- κ B (Herrero-Martin *et al*, 2009; Criollo *et al*, 2010; Comb *et al*, 2011).

Beclin 1 has been the first mammalian protein shown to play a critical role in the initiation of autophagy (Liang *et al*, 1999). Its complex interactome has a major influence on the positive and negative regulation of autophagy (Kang *et al*, 2011). One of the decisive events that ignites the autophagic machinery is the activation of the class III phosphatidylinositol 3-kinase (PI3KC3), also called VPS34, to generate phosphatidylinositol-3-phosphate, which is required for the initial steps of vesicle nucleation (Axe *et al*, 2008). Beclin 1 is an obligatory allosteric activator of VPS34, operating within the so-called ‘Beclin 1 core complex’ that involves Beclin 1, VPS15, VPS34 and, likely, AMBRA1 (He and Levine, 2010). Numerous additional proteins interact with Beclin 1. Pro-autophagic Beclin 1 interactors include ATG14 (also called ATG14L or BARKOR), UV radiation resistance-associated gene (UVRAG) and Bif-1/endophilin B1, which interacts with Beclin 1 via UVRAG. Autophagy-inhibitory interactors of Beclin 1 include RUN domain protein as Beclin 1 interacting and cysteine-rich containing (RUBICON), anti-apoptotic proteins from the BCL-2 family (BCL-2, BCL-X_L and MCL-1) and the inositol-1,4,5 trisphosphate receptor, which interacts with Beclin 1 indirectly via BCL-2 (He and Levine, 2010; Kang *et al*, 2011). Beclin 1 possesses a BH3 domain (residues 114–123) through which it constitutively interacts with BCL-2-like proteins (Maiuri *et al*, 2007), and autophagy induction requires the dissociation of Beclin 1 from such inhibitory liaisons (He and Levine, 2010; Kang *et al*, 2011).

The transcription factor NF- κ B is activated when the inhibitor of NF- κ B (I κ B) is phosphorylated by the I κ B kinase

(IKK), a multiprotein complex that is composed by one regulatory (IKK γ , also known as NEMO) and two catalytic subunits (IKK α and IKK β). IKK is activated in response to stressors as diverse as reactive oxygen species and DNA damage as well as by the ligation of death receptors (Baud and Karin, 2009). Frequently, the activation of IKK is mediated further upstream by yet another kinase, mitogen-activated protein kinase kinase 7 (MAP3K7), better known as TGF β -activated kinase 1 (TAK1) (Ninomiya-Tsuji *et al*, 1999; Takaesu *et al*, 2003; Landstrom, 2010). The phosphorylation of I κ B by IKK stimulates I κ B ubiquitination, thus targeting it for proteasomal degradation. In turn, I κ B degradation allows NF- κ B to translocate from the cytoplasm, where it is usually retained by I κ B, to the nucleus, where NF- κ B then becomes active as a cytoprotective and pro-inflammatory transcription factor (Baud and Karin, 2009). In both murine and human cells, the genetic inhibition of TAK1 or any of the IKK subunits (but not that of the NF- κ B subunit p65) prevents the induction of autophagy in response to a panoply of different stimuli including starvation, rapamycin, p53 inhibition and endoplasmic reticulum stress (Herrero-Martin *et al*, 2009; Criollo *et al*, 2010; Comb *et al*, 2011). Conversely, constitutively active IKK subunits potently stimulate autophagy through a pathway that does not necessarily involve NF- κ B, yet relies on the activation of AMPK and JNK1 (Criollo *et al*, 2010), as well as on several essential autophagy-related proteins including Beclin 1, ATG5 and LC3 (Comb *et al*, 2011).

Altogether, these results point to the existence of a major crosstalk between autophagy and the TAK1-IKK signalling axis, yet do not reveal the molecular mechanisms through which these pathways intersect. Here, we report the discovery that TAK1-binding protein 2 (TAB2) and TAK1-binding protein 3 (TAB3) function as tonic inhibitors of autophagy. We found that TAB2 and TAB3 constitutively interact with Beclin 1 and that this liaison is lost upon treatment with physiological inducers of autophagy, causing TAB2 and TAB3 to bind TAK1 instead of Beclin 1. Competitive disruption of the Beclin 1/TAB2/TAB3 complex suffices to induce Beclin 1- and TAK1-dependent autophagy, underscoring the importance of the inhibitory interaction between Beclin 1, TAB2 and TAB3.

Results

Identification of TAB2 and TAB3 as novel Beclin 1 interactors

To unveil a possible intersection between autophagy and the TAK1-IKK activation pathway, we identified Beclin 1 interactors in two yeast two-hybrid saturation screens based on a complex human random-primed cDNA library. This approach led us to identify 63 Beclin 1-interacting proteins. Beyond known interactors (such as UVRAG, ATG14 and ZWINT) (Behrends *et al*, 2010), our screens identified 11 new proteins that would bind Beclin 1 with an elevated Predicted Biological Score (Formstecher *et al*, 2005) (Figure 1A, also consultable at the following website <http://pim.hybrigenics.com/>). Among these 11 proteins, 1 (desmoplakin) has previously been described to bind multiple autophagy-relevant proteins including PIK3C3/VPS34 (but not Beclin 1), and two have been reported to interact with one single autophagy-related protein (PLEC1 with SQSTM1/p62 and SNX4 with RABGAP1

(Behrends *et al*, 2010). Of note, two among the putative Beclin 1 interactors, namely TAB2 and its close homologue TAB3, are known co-activators of TAK1 (Takaesu *et al*, 2000; Ishitani *et al*, 2003). Yeast two-hybrid technology allowed us to narrow down the domains that mediate the interaction of TAB2 and TAB3 with Beclin 1 to a C-terminal region that we called 'Beclin-binding domain' (BBD). The BBD spans from residues 526 to 657 in TAB2 and from residues 518 to 608 in TAB3 (Figure 1B). Co-immunoprecipitation experiments involving Beclin 1 and multiple TAB2 and TAB3 deletion constructs (Figure 1C) confirmed the interaction of full-length Beclin 1 with full-length TAB2 and TAB3, as well as with TAB2 and TAB3 fragments containing the C-terminal BBD. The binding of Beclin 1 to TAB2 and TAB3 was lost upon deletion of their BBDs (Figure 1D and E). Thus, TAB2 and TAB3 constitute novel *bona fide* members of the Beclin 1 interactome.

TAB2 and TAB3 dissociate from Beclin 1 upon autophagy induction

A His-tagged version of Beclin 1 was introduced together with epitope-tagged TAB2 or TAB3 into human cervical carcinoma HeLa cells, which were then driven into autophagy by culture in serum- and nutrient-free conditions (starvation), or by the administration of the mTOR inhibitor rapamycin or of the p53 inhibitor cyclic pifithrin α (PFT α). These three pro-autophagic stimuli all led to a decrease in the amount of TAB2 or TAB3 that co-immunoprecipitated with Beclin 1, as compared with control conditions (Supplementary Figure S1). Consistently, endogenous Beclin 1 co-immunoprecipitated with TAB2 or TAB3 (but not with TAK1) in control conditions, and this interaction was rapidly lost upon autophagy induction in HeLa cells (Figure 1F), as well as in other human cell lines (non small-cell lung cancer A549 cells, colorectal carcinoma HCT 116 cells) and mouse embryonic fibroblasts (MEFs, not shown). While in physiological conditions TAB2 and TAB3 failed to co-immunoprecipitate with TAK1, after the induction of autophagy with starvation, rapamycin or PFT α , both TAB2 and TAB3 were found to engage in such an interaction. Kinetic experiments revealed that TAB2 and TAB3 bind TAK1 as soon as they dissociate from Beclin 1 (Figure 1F). Inhibition of TAK1 by overexpression of a kinase-dead dominant-negative (DN) TAK1 mutant (TAK1^{K63W}) (Ono *et al*, 2003; Figure 2A and B) or by means of a specific small-interfering RNA (siRNA) (Supplementary Figure S2) prevented the relocalization of a green fluorescence protein (GFP)-LC3 chimera from a diffuse pattern to discrete cytoplasmic puncta (Tasdemir *et al*, 2008a) by starvation, rapamycin or PFT α (Figure 2C and D). Similarly, TAK1 depletion prevented the autophagy-associated redistribution of a red fluorescent protein (RFP)-tagged FYVE domain (FYVE-RFP) (Zhang *et al*, 2007) to intracellular membranes containing phosphatidylinositol-3-phosphate (Figure 2E and F).

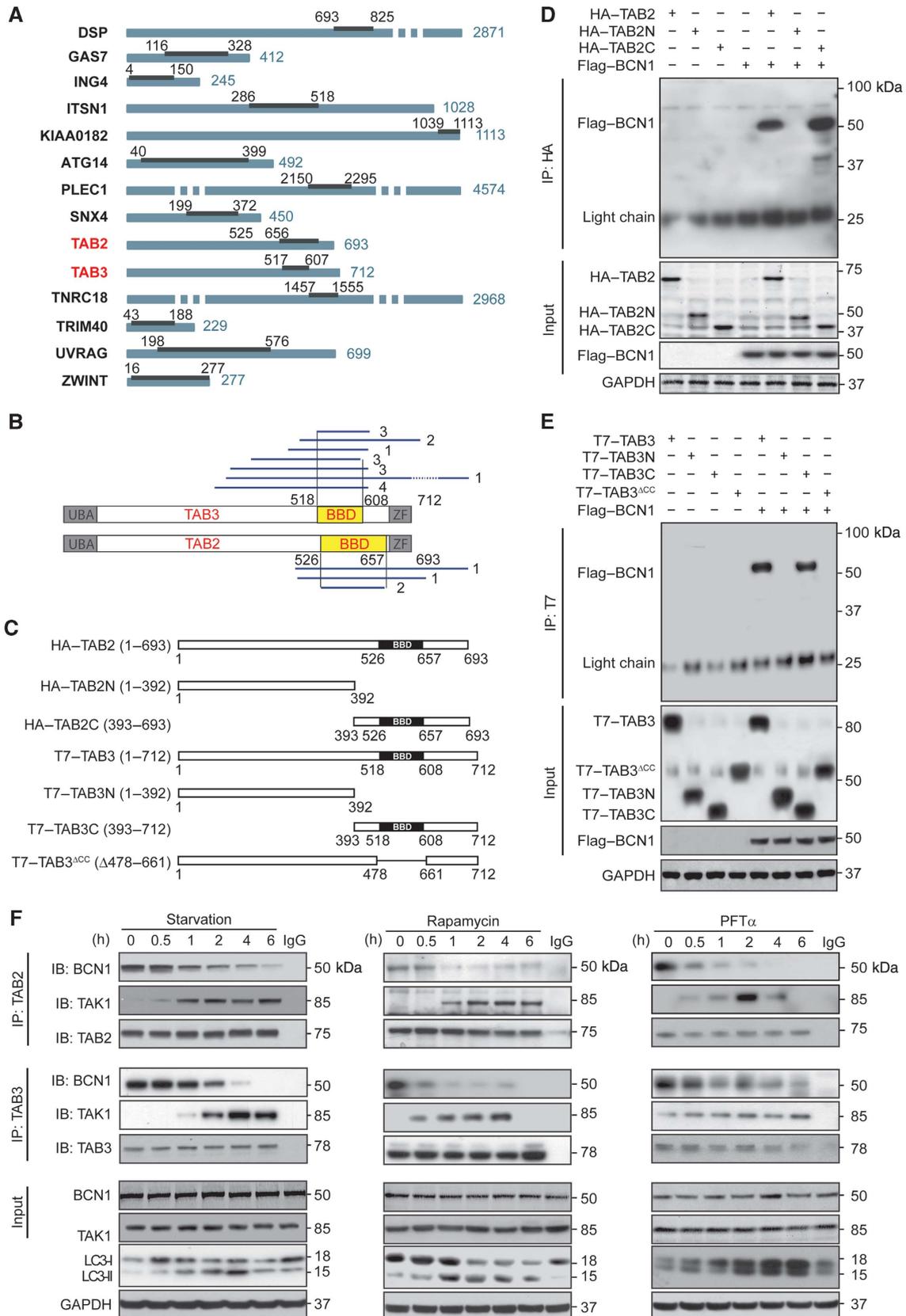
Altogether, these results support the idea that TAB2 and TAB3 dissociate from Beclin 1 to engage with TAK1 when autophagy is induced.

TAB2 and TAB3 are endogenous inhibitors of autophagy

Depletion of TAB2 or TAB3 by specific siRNAs induced the accumulation of GFP-LC3⁺ dots in HeLa and human osteosarcoma U2OS cells stably expressing GFP-LC3 (Figure

3A and B), in HeLa cells transiently transfected with a GFP-LC3-encoding plasmid as well as in HCT 116 cells (Supplementary Figure S3). Moreover, TAB2 or TAB3 knock-down stimulated the lipidation of endogenous LC3, an

autophagy-associated post-translational modification that enhances its electrophoretic mobility (shift from the LC3-I to the LC3-II form), and reduced the abundance of the autophagic substrate SQSTM1/p62 (Figure 3C), suggesting



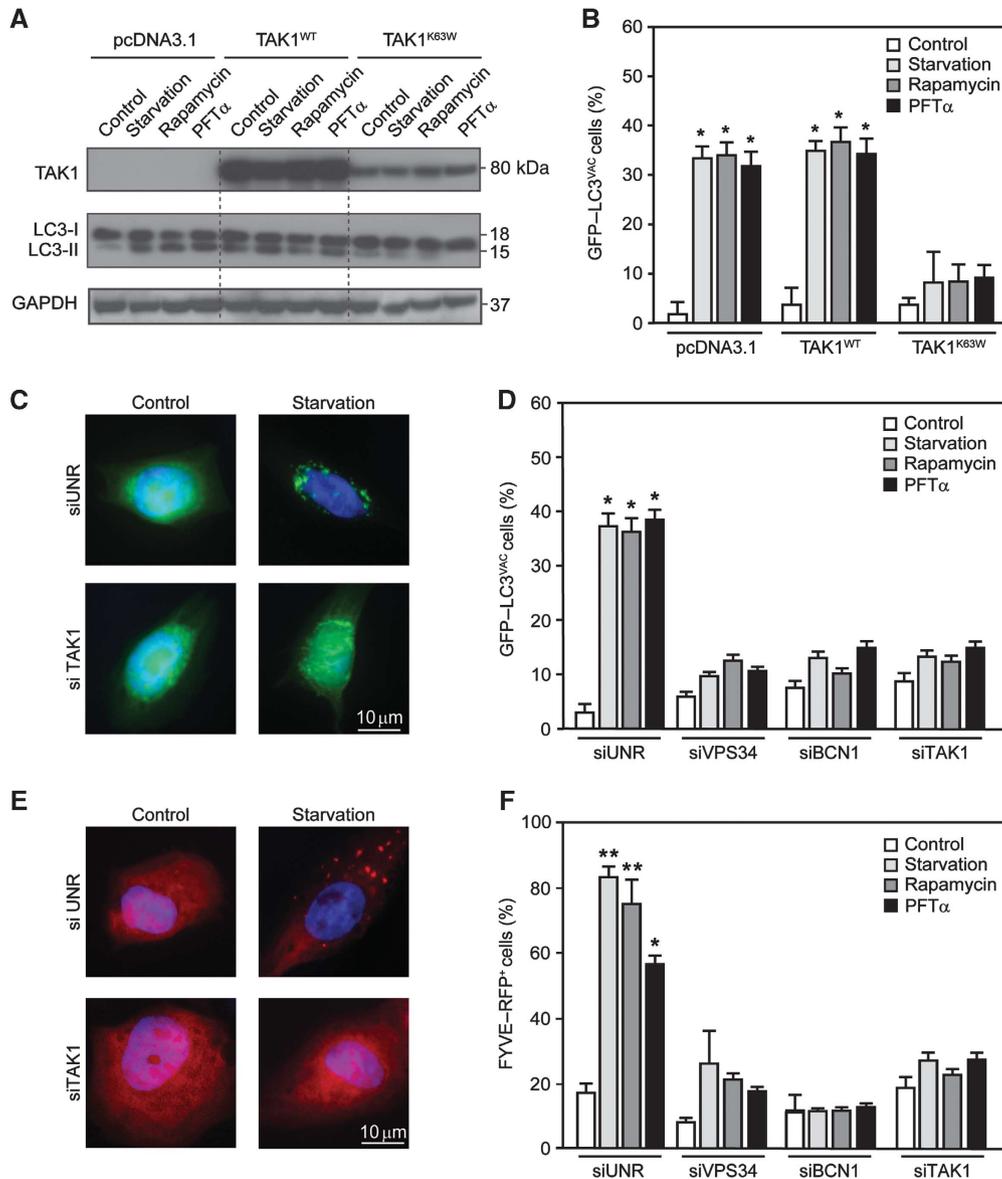


Figure 2 Reduced interaction between Beclin 1, TAB and TAB3 in conditions of autophagy induction. (A, B) Inhibition of autophagy by dominant-negative (DN) TAK1. HeLa cells were co-transfected with a GFP-LC3-encoding construct plus pcDNA3.1 (empty vector), or plasmids for the expression of WT TAK1 (TAK1^{WT}) or the DN TAK1^{K63W} mutant. One day later, cells were either left untreated (control) or driven into autophagy by starvation or by the administration of 1 μM rapamycin or 30 μM pifithrin α (PFTα), followed by immunoblotting for the detection of TAK1 and endogenous LC3 (A) or immunofluorescence microscopy for the quantification of cells with cytosolic GFP-LC3 puncta (GFP-LC3^{VAC} cells) (B) (mean values ± s.d., n = 3; *P < 0.01 versus control cells). GAPDH levels were monitored to ensure equal loading. (C, D) Inhibition of autophagy by knockdown of VPS34, Beclin 1 (BCN1) and TAK1. siRNAs that effectively deplete VPS34, BCN1 and TAK1 were co-transfected with a GFP-LC3-encoding plasmid in HeLa cells. Autophagy was then induced as in (A) and the frequency of GFP-LC3^{VAC} cells (mean values ± s.d., n = 3; *P < 0.01 versus control cells) was determined. (E, F) The same setting shown in (C, D) was performed with U2OS cells and FYVE-RFP (mean values ± s.d., n = 3; *P < 0.01, **P < 0.001 versus control cells).

Figure 1 Identification of novel Beclin 1 interactors. (A) Beclin 1 (BCN1) interactors identified by yeast two-hybrid technology. Proteins binding to BCN1 are listed and their interacting domains are indicated by black bars. Numbers refer to amino-acid positions. (B) Identification of TAB2 and TAB3 fragments interacting with BCN1 in the yeast two-hybrid system. Blue lines depict the fragments of TAB2 or TAB3 that were found to interact with BCN1 (numbers on the right refer to the amount of yeast clones identified for each fragment). The minimal domain required for the interaction is referred to as Beclin-binding domain (BBD). (C) Constructs derived from TAB2 and TAB3 used in this study. (D, E) Co-immunoprecipitation of TAB2 or TAB3 with BCN1. The indicated constructs, namely HA-tagged and T7-tagged TAB2, and TAB3 constructs in (D) and (E), respectively, and Flag-tagged Beclin 1 (Flag-BCN1) were transfected into HeLa cells alone or in combination. Twenty-four hours later, TAB2 and TAB3 were immunoprecipitated with antibodies specific for HA (D) or T7 (E) and the precipitate was separated by SDS-PAGE and revealed with an antibody specific for Flag. (F) Immunoprecipitation of endogenous BCN1 with endogenous TAB2 or TAB3. HeLa cells were subjected to autophagy induction with starvation conditions, 1 μM rapamycin or 30 μM pifithrin α (PFTα) for the indicated time and then processed for TAB2 or TAB3 immunoprecipitation followed by the immunodetection of BCN1, TAK1, TAB2 and TAB3. Results in (E) and (F) are representative for three independent experiments.

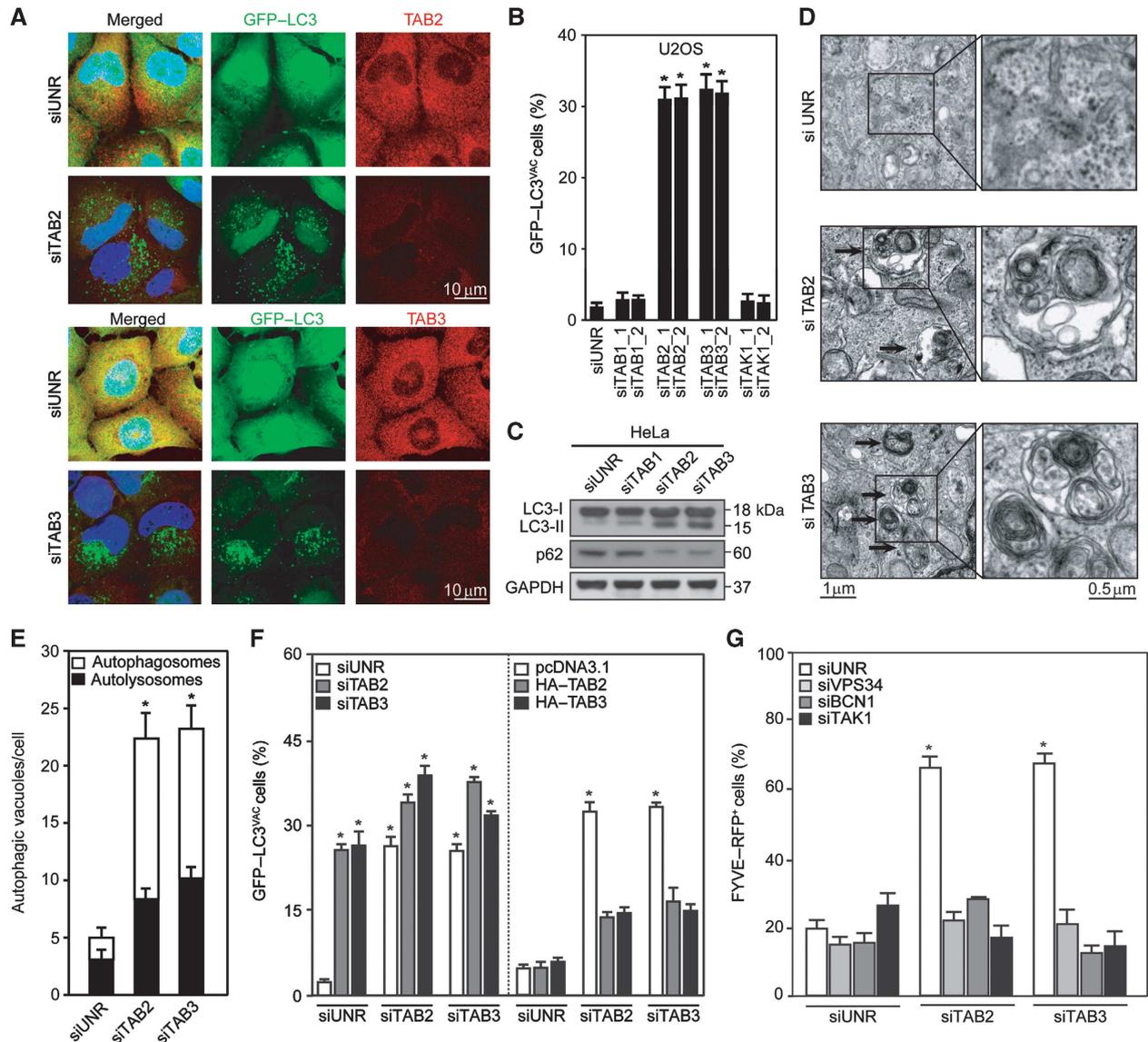


Figure 3 Induction of autophagosomes by depletion of TAB2 or TAB3. (A, B) Detection of autophagic GFP-LC3⁺ puncta. HeLa or U2OS cells stably expressing GFP-LC3 were transfected with siRNAs targeting TAK1, TAB1, TAB2 or TAB3 or with a control siRNA (siUNR). One day later, the subcellular localization and abundance of GFP-LC3 or immunostained TAB2 or TAB3 was determined by epifluorescence microscopy. Representative images are shown in (A) (HeLa cells) and quantitative results (mean values \pm s.d., $n = 3$; $*P < 0.01$ versus siUNR-transfected cells) are depicted in (B) (U2OS cells). (C) Lipidation of LC3 induced by TAB2 or TAB3 knockdown. Representative immunoblots showing the conversion of non-lipidated LC3 (LC3-I) to its lipidated variant (LC3-II) as well as SQSTM1/p62 protein levels are shown. GAPDH levels were monitored to ensure equal loading. (D, E) Quantification of autophagosomes and autophagolysosomes by transmission electron microscopy. Representative images of HeLa cells transfected with siUNR or with TAB2- or TAB3-targeting siRNAs are shown in (D), and quantitative results are depicted in (E) (mean values \pm s.d., $n = 3$; $*P < 0.01$ versus siUNR-transfected cells). (F) Epistatic analysis of the effects of TAB2 and TAB3 depletion on autophagy. HeLa cells stably expressing GFP-LC3 were transfected with siRNAs specific for TAB2 or TAB3 and/or with cDNAs coding for full-length HA-tagged TAB2 (HA-TAB2) or TAB3 (HA-TAB3). Twenty-four hours later, the frequency of cells exhibiting > 5 GFP-LC3⁺ cytosolic puncta (GFP-LC3^{VAC} cells) was determined. Results are mean values \pm s.d. ($n = 3$; $*P < 0.01$ versus siUNR-transfected cells). (G) U2OS cells stably expressing FYVE-RFP were transfected with siUNR, or with siRNAs specific for TAB2, TAB3, VPS34, Beclin 1 (BCN1) and TAK1, in the indicated combinations. Forty-eight hours later, the percentage of cells with RFP-FYVE⁺ puncta cells was determined. Results are mean values \pm s.d. ($n = 3$; $*P < 0.01$ versus siUNR-transfected cells).

that TAB2 and TAB3 act as endogenous inhibitors of autophagy. Accordingly, the depletion of TAB2 or TAB3 led to the accumulation of *bona fide* autophagosomes and autophagolysosomes, as determined by transmission electron microscopy (Figure 3D and E). Epistatic experiments revealed that the simultaneous knockdown of TAB2 and TAB3 induced only slightly more GFP-LC3⁺ puncta than the depletion of each of these proteins alone. Moreover, the induction of autophagy by TAB2 depletion was inhibited by transfection

of non-interferable TAB2 or wild-type (WT) TAB3 (and *vice versa* TAB2 transfection antagonized autophagy induction by TAB3 depletion), suggesting that both proteins inhibit the formation of autophagic puncta in a similar, overlapping manner (Figure 3F). Moreover, the depletion of either TAB2 or TAB3 induced the formation of FYVE-RFP⁺ puncta to similar extents (Figure 3G), suggesting that both TAB2 and TAB3 usually restrain the lipid kinase activity of the Beclin 1 complex.

The accumulation of autophagosomes may result from enhanced sequestration of cytoplasmic material (increased on-rate) as well as from reduced removal of autophagosomes by fusion with lysosomes (reduced off-rate). Therefore, we measured autophagosome formation induced by TAB depletion in the absence or presence of bafilomycin A1 (BafA1), which inhibited the colocalization of the autophagic marker GFP-LC3 and the lysosomal marker LAMP2, in accord with its known capacity to block the autophagosome-lysosome fusion (Mizushima *et al*, 2010) (Figure 4A–C). In the presence of BafA1, the depletion of TAB2 and TAB3 resulted in more GFP-LC3⁺ puncta than in its absence (Figure 4D). Similar flux determinations were performed in the presence of an alternative lysosomal inhibitor, ammonium chloride, or protease inhibitors (E64d plus pepstatin A) (Supplementary Figure S4). Also, MEFs lacking TAB2 expression due to homologous recombination (*Tab2*^{-/-}), but neither WT MEFs nor their *Tab1*^{-/-} counterparts, manifested increased LC3 lipidation, both in the absence and in the presence of BafA1 (Figure 4E). Autophagy induced by TAB2 or TAB3 knockdown followed the canonical pathway, as it was reduced upon depletion of essential autophagy proteins such as Beclin 1, its associated phosphatidylinositol 3-kinase VPS34, ATG5 and ATG7 (Figure 4F). Moreover, it involved the obligatory contribution of TAK1 and that of the three subunits of the IKK complex, as shown by additional experiments of siRNA-mediated knockdown (Figure 4G) or transfection with DN TAK1 (Figure 4H). In conclusion, TAB2 and TAB3 are endogenous inhibitors of the canonical autophagic pathway, which requires the action of kinases from the TAK1-IKK signalling axis.

Dissociation of TAB2 and TAB3 from Beclin 1 induces autophagy

Overexpression of full-length TAB2 or TAB3 inhibited starvation-, rapamycin- and PFT α -induced autophagy (Figure 5A), in line with the interpretation that both proteins are negative regulators of autophagic flux. TAB2 or TAB3 variants lacking the BBD (TAB2N, TAB3N, TAB3^{ACC}, Figure 1C) failed to modulate autophagy induced by various stimuli (Figure 5A and B). However, TAB2 and TAB3 deletion mutants containing their C-terminal moieties (TAB2C and TAB3C, Figure 1C), which include the BBD, had the striking property to induce the accumulation of GFP-LC3⁺ and FYVE-RFP⁺ puncta on their own, as well as to exacerbate autophagy induced by starvation, rapamycin or PFT α (Figure 5A and B). Accordingly, expression of TAB2C or TAB3C (but not that of TAB2N or TAB3N) reduced the interaction between endogenous Beclin 1 and TAB2 or TAB3 (Figure 5C). Moreover, the depletion of Beclin 1, ATG5, ATG7, TAK1, IKK α , IKK β or NEMO inhibited the generation of GFP-LC3⁺ puncta by TAB2C or TAB3C, suggesting that TAB2C- and TAB3C-induced autophagy is mediated by the canonical pathway and by TAK1-IKK activity (Figure 5D).

These results suggest that the dissociation of the multiprotein complex including the pro-autophagic protein Beclin 1 and the autophagy inhibitors TAB2 and TAB3 might be sufficient to induce autophagy. To further explore this possibility, we used yeast two-hybrid technology to determine the Beclin 1 domain that interacts with TAB2 and TAB3, finding it comprised between residues 150 and 278 (Figure 6A), a region that overlaps with Beclin 1 central

coiled-coil domain (CCD, residues 144–269) (Kang *et al*, 2011). In control conditions, such a TAB-binding domain (TBD) (Figure 6B) co-immunoprecipitated with TAB2 and TAB3, as did full-length Beclin 1 but not a Beclin 1 deletion mutant lacking the TBD (Beclin 1^{ATBD}) (Figure 6C). Moreover, recombinant TAB2 and TAB3 directly interacted with the TBD (but not with Beclin 1^{ATBD}) in pull-down experiments (Supplementary Figure S5), and transgene-driven TBD expression caused endogenous Beclin 1 to dissociate from TAB2 and TAB3 (Figure 6D and E). Of note, while WT Beclin 1 failed to induce autophagy upon transfection-enforced expression, TBD did so very efficiently (Figure 6F; Supplementary Figure S6), though slightly less than two gain-of-function mutants of Beclin 1 with a deleted or inactivated inhibitory BH3 domain (Figure 6F). TBD was also highly efficient in stimulating the formation of FYVE-RFP⁺ puncta (Figure 6G), suggesting that the disruption of the inhibitory interactions between Beclin 1, TAB2 and TAB3 stimulates the lipid kinase activity of the Beclin 1 complex. TBD-induced autophagy depended on endogenous Beclin 1, ATG5 and ATG7 as well as on the TAK1-IKK signalling axis, as demonstrated in knockdown experiments (Figure 6H) or upon transfection with DN TAK1 (Figure 6I). Taken together, these results indicate that the dissociation of multiprotein complex including Beclin 1, TAB2 and TAB3 potently stimulates autophagy.

Discussion

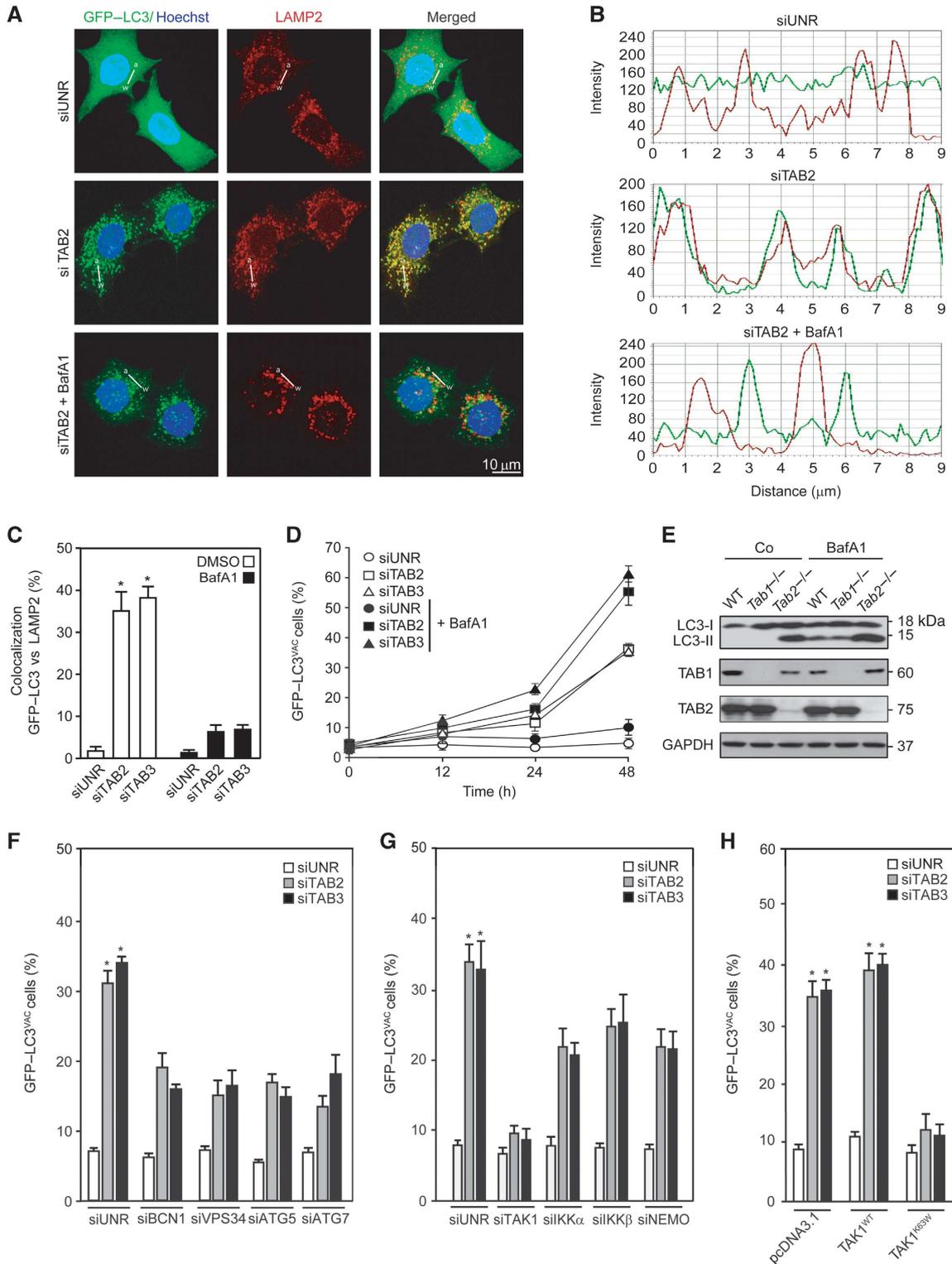
In this work, we provide evidence in favour of an unsuspected intersection between autophagy regulation and upstream portions of the canonical NF- κ B activation pathway. Under physiological conditions, when autophagy is off, Beclin 1 associates with several inhibitory factors that, as we discovered here, include TAB2 and TAB3. However, upon autophagy activation by several distinct stimuli including its most physiological inducer, starvation, as well as pharmacological triggers (rapamycin and PFT α), TAB2 and TAB3 dissociate from Beclin 1 and associate with TAK1. This latter association is well known to stimulate the kinase activity of TAK1 (Takaesu *et al*, 2000; Ishitani *et al*, 2003; Besse *et al*, 2007) and to ignite the IKK pathway (Jin *et al*, 2004; Kanayama *et al*, 2004).

TAB2 and TAB3 were found to interact with Beclin 1 in a direct fashion, using several complementary technologies (yeast two-hybrid screens and pull-down assays of recombinant proteins), and their interaction was confirmed by co-immunoprecipitation of tagged as well as endogenous proteins. These data add TAB2 and TAB3 to the ever-expanding list of Beclin 1 interactors that already comprises close to 20 confirmed proteins (including all four anti-apoptotic BCL-2 family proteins, AMBRA1, ATG14, HMGB1, nPIST, RUBICON, SLAM, PINK, Survivin, UVRAG, VMP1, VPS15, VPS34, etc.) (Kroemer *et al*, 2010; Kang *et al*, 2011). The detailed mechanisms through which TAB2 and TAB3 dissociate from Beclin 1 remain elusive. TAB2 and TAB3 are known to interact with K63-linked polyubiquitin chains through a highly conserved zinc finger domain (Kanayama *et al*, 2004). However, the BBD and the zinc finger domain do not overlap, and the latter (aa 684–710 in TAB3) is fully preserved in the TAB3^{ACC} mutant, which fails to interact with Beclin 1. Moreover, the Beclin 1 site that can undergo K63-linked

polyubiquitination is located in its N-terminal BH3-binding domain (Shi and Kehrl, 2010), and the fraction of Beclin 1 that co-immunoprecipitates with TAB2 or TAB3 does not manifest any shift in electrophoretic mobility that would be compatible with ubiquitination. Rather, TAB2 and TAB3 appear to engage in direct protein-to-protein interactions involving their CCDs (Ishitani *et al*, 2003), which overlap with their BBD and are required for TAK1 binding (Takaesu

et al, 2000; Ishitani *et al*, 2003), in line with the observation that the binding of TAB2 and TAB3 to Beclin 1 or TAK1 is mutually exclusive.

On the side of Beclin 1, the domain involved in TAB binding (residues 150–278) overlaps with the central CCD (residues 144–269), which is flanked by the BH3 domain on the N-terminal end (residues 114–123) and an evolutionarily conserved domain (ECD, residues 244–337) on the C-terminus.



Thus, TAB2 and TAB3 bind to the regulatory region of Beclin 1 that also mediates interactions with multiple autophagy-regulatory proteins including AMBRA1 (Fimia *et al*, 2007), ATG14 (Sun *et al*, 2008), nPIST (Yue *et al*, 2002), RUBICON (Zhong *et al*, 2009) and UVRAG (Liang *et al*, 2006). The question how TAB2 and TAB3 affect the binding of

these other factors to Beclin 1 will be the subject of future investigations.

Irrespective of the aforementioned molecular details, one fascinating aspect of autophagy regulation by TAB2 and TAB3 resides in its circuitry. Clearly, TAB2 and TAB3 are negative regulators of Beclin 1 (and the Beclin 1-dependent VPS34-

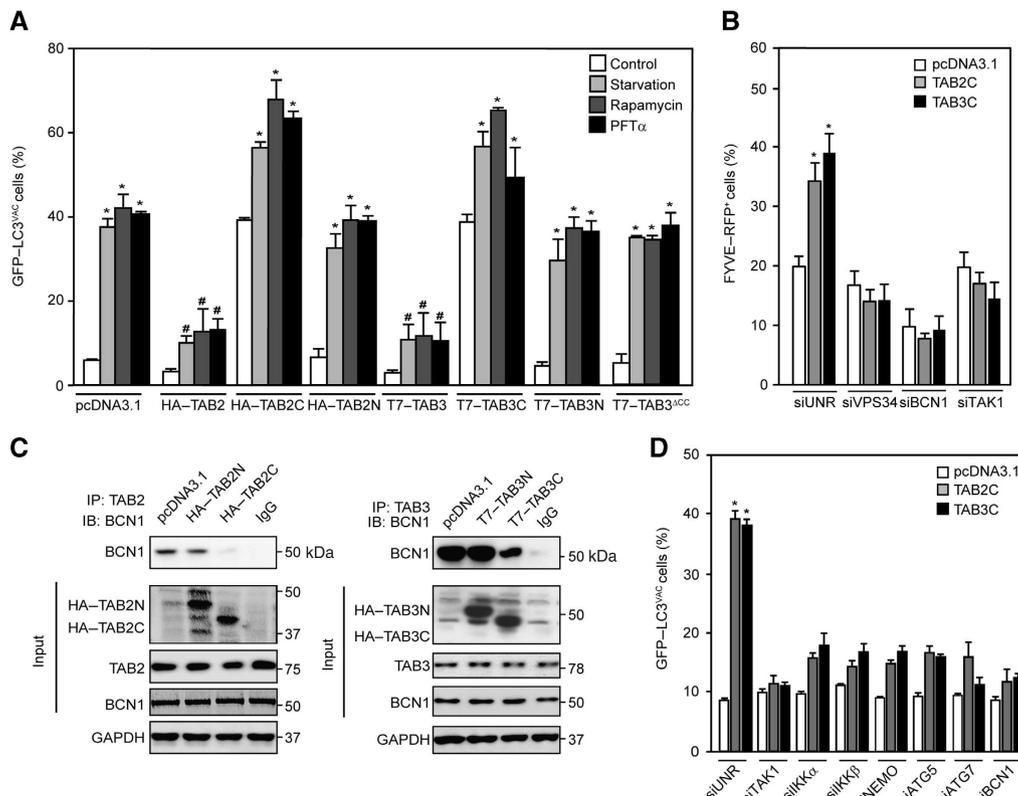


Figure 5 Inhibition of autophagy by full-length TAB2 and TAB3 but induction by their C-terminal fragments. (A) Effects of full-length TAB2 and TAB3 or their deletion mutants (as in Figure 1C) on autophagy. HeLa cells stably expressing GFP-LC3 were transfected with pcDNA3.1 (empty vector) or with plasmids encoding the indicated TAB2 and TAB3 variants for 24 h, then driven into autophagy by starvation or by the administration of 1 μ M rapamycin or 30 μ M pifithrin α (PFT α) for 4 h. Finally, the frequency (mean \pm s.d., $n = 3$) of cells with >5 GFP-LC3⁺ cytosolic puncta (GFP-LC3^{VAC} cells) was assessed ($*P < 0.01$ versus control cells transfected with the same plasmid; $^{\#}P < 0.01$ versus pcDNA3.1-transfected cells treated with the same pro-autophagic trigger). (B) U2OS cells stably expressing FYVE-RFP were co-transfected with pcDNA3.1 or with vectors encoding TAB2C or TAB3C together with a control siRNA (siUNR) or with siRNAs specific for VPS34, Beclin 1 (BCN1) or TAK1 for 24 h, followed by the quantification of cells exhibiting FYVE-RFP⁺ dots (FYVE-RFP⁺). Results are mean values \pm s.d. ($n = 3$; $*P < 0.01$ versus siUNR-, pcDNA3.1-transfected cells). (C) Inhibition of the interaction between endogenous TAB2, TAB3 and BCN1 by C-terminal fragments of TAB2 and TAB3. Forty-eight hours after transfection with pcDNA3.1 or plasmids coding for the indicated proteins, cells were lysed, TAB2 or TAB3 was immunoprecipitated and BCN1 was immunodetected. GAPDH levels were monitored to ensure equal loading. This experiment has been done three times, yielding comparable results. (D) Mechanisms of autophagy induction by the Beclin-binding domain (BBD)-containing C-terminal fragments of TAB2 and TAB3. HeLa cells were transfected with pcDNA3.1, TAB2C- or TAB3C-encoding plasmids in combination with the indicated siRNAs for 24 h, followed by the quantification of GFP-LC3^{VAC} cells (mean values \pm s.d., $n = 3$; $*P < 0.01$ versus siUNR-, pcDNA3.1-transfected cells).

Figure 4 Mechanisms of autophagy induced by depletion of TAB2 or TAB3. (A–D) Impact of bafilomycin A1 (BafA1) on the induction of GFP-LC3⁺ puncta by TAB2 and TAB3 depletion. HeLa cells stably expressing GFP-LC3 were transfected with a control siRNA (siUNR) or with siRNAs targeting TAB2 and TAB3 for 24 h. During the last 12 h of this period, BafA1 was optionally added. After fixation and permeabilization, LAMP2 was detected by immunofluorescence. Representative confocal microphotographs for the TAB2 siRNA are shown (A), together with the profiles of colocalization of fluorescent signals (B) along the indicated direction (α - ω). Columns in (C) represent the percentage of colocalization of GFP-LC3 and LAMP2 (mean values \pm s.d.; $*P < 0.01$ versus siUNR-transfected cells), as quantified in at least 50 cells for each condition. The frequency (mean \pm s.d.) of cells with >5 GFP-LC3⁺ cytosolic puncta (GFP-LC3^{VAC} cells) is plotted in (D). (E) Impact of BafA1 on LC3 lipidation. MEFs with the indicated genotypes were cultured in complete medium supplemented with BafA1 for 12 h and the proportion of LC3-I/LC3-II was determined by immunoblotting. GAPDH levels were monitored to ensure equal loading. (F, G) Impact of autophagy-relevant proteins and of the TAK1-IKK signalling axis on GFP-LC3 aggregation induced by the depletion of TAB2 or TAB3. HeLa cells stably expressing GFP-LC3 were transfected with siUNR or with siRNAs targeting the indicated proteins, alone or in combination, and 48 h later GFP-LC3^{VAC} cells were quantified (mean values \pm s.d., $n = 4$; $*P < 0.01$ versus siUNR-transfected cells). (H) Inhibition of autophagy by dominant-negative (DN) TAK1. HeLa cells stably expressing GFP-LC3 were co-transfected with pcDNA3.1 (empty vector) or with plasmids encoding WT (TAK1^{WT}) or a DN TAK1 variant (TAK1^{K63W}) together with the indicated siRNAs for 24 h, followed by the quantification of GFP-LC3^{VAC} cells (mean values \pm s.d., $n = 3$, $*P < 0.01$ versus siUNR-, pcDNA3.1-transfected cells).

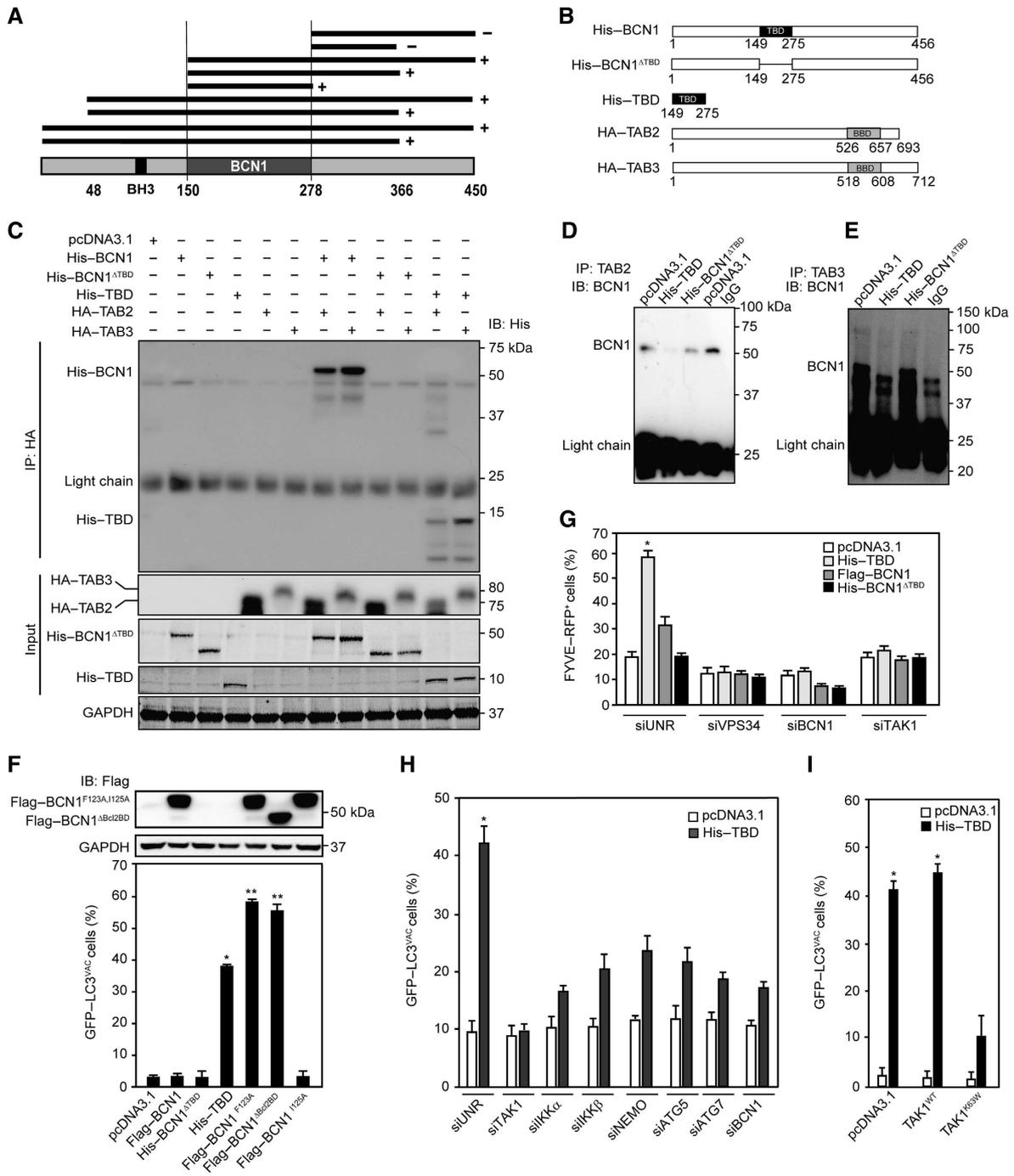


Figure 6 Induction of autophagy by a Beclin 1 fragment that disrupts the interaction between endogenous Beclin 1, TAB2 and TAB3. (A) Determination of the TAB-binding domain (TBD) within Beclin 1 (BCN1). Yeast two-hybrid technology was used to screen for positive (+) or negative interactions (-) between BCN1 fragments and full-length TAB2. (B) Schematic presentation of the constructs used in subsequent experiments (BBD, Beclin-binding domain). (C) Confirmation of the TBD by immunoprecipitation. The indicated constructs were transfected into HeLa cells, followed by lysis, immunoprecipitation of HA-tagged full-length TAB2 or TAB3 and detection of His-tagged constructs. (D, E) Inhibition of the interaction between endogenous BCN1, TAB2 and TAB3 by a BCN1 fragment corresponding to the TBD. HeLa cells were transfected with pcDNA3.1 (empty vector) or with plasmids encoding the TBD or a His-tagged Beclin 1 variant lacking the TBD (His-BCN1^{ΔTBD}), followed by co-immunoprecipitation of endogenous TAB2 (D) or TAB3 (E) and detection of BCN1. (F) Induction of autophagy by the TBD. HeLa cells stably expressing GFP-LC3 were transfected with pcDNA3.1 or constructs encoding the indicated BCN1 variants. After 24 h, Flag-tagged proteins were detected by immunoblotting and the frequency of cells with > 5 GFP-LC3⁺ cytosolic puncta (GFP-LC3^{VAC} cells) was assessed (mean values ± s.d., n = 3; *P < 0.01, **P < 0.001 versus pcDNA3.1-transfected cells). (G, H) Mechanisms of autophagy induction by the TBD. (G) U2OS cells stably expressing RFP-FYVE were transfected pcDNA3.1 or plasmids for the expression of TBD, BCN1 or BCN1^{ΔTBD} in combination with a control siRNA (siUNR) or siRNAs that effectively deplete VPS4, BCN1 and TAK1. Forty-eight hours later, the percentage of cells exhibiting FYVE-RFP⁺ dots (FYVE-RFP⁺) was assessed. (G). Alternatively, HeLa cells stably expressing GFP-LC3 were transfected with pcDNA3.1 or with a TBD-encoding plasmids plus siUNR or siRNAs specific the indicated proteins. Forty-eight hours later, the percentage of GFP-LC3^{VAC} cells was determined (H). Results are mean values ± s.d. (n = 3, *P < 0.01 versus siUNR-, pcDNA3.1-transfected cells). (I) Inhibition of TBD-induced autophagy by dominant-negative (DN) TAK1. Cells were co-transfected pcDNA3.1 or vectors encoding WT TAK1 (TAK1^{WT}) or a DN TAK1 variant (TAK1^{K63W}) alone or together with a TBD-encoding plasmid for 24 h, followed by the quantification of GFP-LC3^{VAC} cells (mean values ± s.d., n = 3; *P < 0.01 versus pcDNA3.1-transfected cells).

mediated production of phosphatidylinositol-3-phosphate), because knockdown of TAB2 or TAB3 induces autophagy, overexpression of TAB2 and TAB3 inhibits autophagy and fragments of Beclin-1 and TAB2 and TAB3 that contain the TBD or the BBD, respectively, induce autophagy as they disrupt the endogenous Beclin 1/TAB2/TAB3 complex. This inhibitory effect of TAB2 and TAB3 on autophagy appears to be dominant, as their deletion (by homologous recombination) or depletion (by siRNAs) induces an increase in the autophagic flux.

Interestingly, the induction of autophagy by TAB2 and TAB3 depletion or TBD transfection is not only inhibited by depletion of Beclin 1 (or VPS34) but also is limited by the knockdown of TAK1 or any IKK subunit. As our paper was under revision, another report was published that confirmed the role of TAB2 and TAB3 as negative regulators of autophagy (Takaesu *et al*, 2011). siRNA-mediated depletion of TAB2 and TAB3 was found to decrease the abundance of SQSTM1/p62 both in WT MEFs and in MEFs expressing a crippled version of TAK1 that lacks its ATP-binding site (due to the knockout of exon 2) (Takaesu *et al*, 2011). In sharp contrast, we found that the depletion of TAK1 or the overexpression of a DN TAK1 mutant inhibited autophagy in HeLa or U2OS cells responding to the depletion of TAB2 and TAB3 or to the overexpression of Beclin 1 TBD. Thus, TAK1 may either contribute to autophagy induction in a context-dependent fashion or do so through a mechanism that does not involve its catalytic activity.

The depletion of TAB2 or TAB3 induces autophagy, yet TAK1 appears to be necessary, at least to some extent, for this response, as shown here. We and others found that TAK1 is required for the optimal induction of autophagy by a number of stimuli including starvation, endoplasmic reticulum stress, rapamycin, PFT α , TGF β and the ligation of the death receptor TRAIL (Herrero-Martin *et al*, 2009; Criollo *et al*, 2010; Ding *et al*, 2010), suggesting a rather broad implication of TAK1 in autophagic signalling. However, it appears counterintuitive that depletion of TAB2 or TAB3 induces autophagy, as TAK1 normally relies on TAB2 and TAB3 as its obligatory cofactors (Takaesu *et al*, 2000; Ishitani *et al*, 2003; Besse *et al*, 2007). This discrepancy may either be explained by the kinetics of depletion (the pool of TAB2 and TAB3 that interacts with Beclin 1 might be less stable than the pool of TAB2 and TAB3 that is free to associate with TAK1) or by the existence of a TAB-independent mode of activation of TAK1 that will have to be explored in future studies.

Irrespective of these incognita, it is tempting to link the present data (which indicate that TAB2 and TAB3 act as negative regulators of the Beclin 1/VPS34 complex) and those published in the literature (showing that TAB2 and TAB3 act to stimulate TAK1) with our observation that TAB2 and TAB3 preferentially interact with Beclin 1 before, and with TAK1 after, the induction of autophagy. In a possible scenario, TAB2 and TAB3 would dissociate from Beclin 1 (thus unleashing the lipid kinase activity of the Beclin 1/VPS34 complex) and associate with TAK1 (thus activating the TAK1-IKK signalling axis), overall functioning as a 'switch' in the control of autophagy by molecularly linking a de-inhibitory reaction to a stimulatory one (Supplementary Figure S7).

The existence of such a molecular 'switch' (and likely others that remain to be discovered) may explain why

autophagy is accompanied by a series of stereotyped alterations in major stress-response signalling pathways, irrespective of the nature of the initiating stimulus. For instance, starvation stimulates well-known pro-autophagic pathways, including the activation of AMPK and of the Beclin 1/VPS34 complex, but also completely unexpected molecular cascades, such as those linked to the activation of IKK and to the depletion of cytoplasmic p53 (Criollo *et al*, 2010). Conversely, the inhibition, deletion or depletion of p53 as well as the activation of TAK1 or IKK (by transfection of constitutively active IKK subunits) stimulates AMPK-driven autophagy (Tasdemir *et al*, 2008b; Herrero-Martin *et al*, 2009; Criollo *et al*, 2010). Inhibition of p53 also leads to the activation of IKK, which is necessary for autophagy induction, and, *vice versa*, artificial stimulation of IKK causes p53 depletion (Criollo *et al*, 2010). Both p53 inhibition and IKK activation cause the dissociation of the BCL-2/Beclin 1 complex (Tasdemir *et al*, 2008b; Herrero-Martin *et al*, 2009; Criollo *et al*, 2010). However, BH3 mimetics that directly disrupt the BCL-2/Beclin 1 complex (Maiuri *et al*, 2007) also induce p53 depletion and IKK activation (Malik *et al*, 2011a). Cumulatively, these results suggest that the pathways underlying the regulation of autophagy cannot follow a linear hierarchy but must be interconnected in a complex network. In this setting, the stimulation of autophagy by physiological inducers (such as starvation or organellar stress) or pharmacological agents (such as rapamycin and PFT α) triggers an ensemble of intimately linked changes that are coupled to each other in self-amplificatory loops (Malik *et al*, 2011b). While the 'switch' that we postulate here (and that involves the dissociation of TAB2 and TAB3 from Beclin 1 and their association with TAK1) cannot explain all these coordinated alterations, it unveils some clues explaining the obligatory synchronization of autophagy induction with TAK1-IKK activation.

Materials and methods

Yeast two-hybrid system

Two different Beclin 1 baits have been screened. The first one was constituted by Beclin 1 residues 46–362, as previously identified in a screen for BCL-2-interacting proteins (Maiuri *et al*, 2007) fused in C-terminal to the lexA DNA-binding domain (pB28) (Formstecher *et al*, 2005). The second one was constituted by full-length Beclin 1 fused in N-terminal to the lexA DNA-binding domain (pB29) (Formstecher *et al*, 2005). A random-primed human placenta cDNA library based on the pP6 vector (Fromont-Racine *et al*, 2002) was transformed into the Y187 *Saccharomyces cerevisiae* strain. Ten million independent yeast colonies were collected, pooled and stored at -80°C as equivalent aliquot fractions of the same library. In both screens, >80 millions diploid cells have been tested. The first bait (Beclin 1 residues 46–432) has been screened with 1 mM 3-amino-1,2,4-triazole, while the full-length bait required 100 mM 3-amino-1,2,4-triazole, due to consistent autoactivation. Prey fragments from positive clones were PCR amplified and sequenced at their 5' and 3' junctions on a PE3700 Capillary DNA Sequencer (PE Applied Biosystems, Foster City, USA) (Fromont-Racine *et al*, 2002). The results were used to identify the corresponding genes in the GenBank database (NCBI). The baits used to map the Beclin-interacting domain have been obtained by recombination (gap repair) in the C-terminal of the lexA DNA-binding domain, using the following oligonucleotides: OLI6565 GGGCTGGCGGTTGGGGTTATTCGCAACGGCGACTGGCTGGAATTCACCACAGCCAGGCGAAACA; OLI6566 GGGCTGGCGGTTGGGGTTATTCGCAACGGCGACTGGCTGG AATTCGACACTCAGCTCAACGTCAC; OLI6567 GGGCTGGCGGTTGGGGTTATTCGCAACGGCGACTGGCTGGAATTCACCAACGCTTTAATGCAACC; OLI6568 AATCATAAATCATAAGAAATTCGCCCGGAATTA

GCTTGGCTGCAGGTCTCAAGTGACGTTGAGCTGAGTGTG; OLI6569 AATCATAAATCATAAGAAATTCGCCCGGAATTAGCTTGGCTGCAGG TCTCAGTGCCAGATGTGGAAGGTTG; OLI6570 AATCATAAATCATAA GAAATTCGCCCGGAATTAGCTTGGCTGCAGGTCTCATCAAACCTGT GTGCCAGAA.

Chemicals, cell lines and culture conditions

Unless otherwise indicated, chemicals were purchased by Sigma-Aldrich (St Louis, USA), and media and supplements for cell culture from Gibco–Invitrogen (Carlsbad, USA). Rapamycin was obtained by Tocris Bioscience (Ellisville, USA). All cells were maintained in standard culture conditions (37°C, 5% CO₂). Human cervical carcinoma HeLa and human osteosarcoma U2OS cells (and their GFP–LC3 and FYVE–RFP-expressing derivatives) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10 mM HEPES. Human colon carcinoma HCT 116 cells were maintained in McCoy’s medium supplemented with 10% FCS, 100 mg/l sodium pyruvate and 10 mM HEPES, while WT, *Tab1*^{-/-} and *Tab2*^{-/-} MEFs were cultured in DMEM supplemented with 10% FCS, 10 mM HEPES and 1% non-essential amino acids. For serum and nutrient starvation, cells were cultured in serum-free Earle’s balanced salt solution (EBSS) (Boya *et al*, 2005).

siRNAs, plasmids and transfections

siRNA transfections were performed by means of the Oligofectamine[®] reagent (Invitrogen), according to the manufacturer’s instructions. The following custom-designed siRNA duplexes were purchased from Sigma–Prologi (The Woodlands, USA): siBCN1 (sense 5′-CUCAGGAGAGCCAUUUdTdT-3′) (Boya *et al*, 2005); siVPS34 (sense 5′-ACGGTGTGATCATCTCCAAdTdT-3′); siTAB1_1 (sense 5′-GCCUUGCGUGAGAAGGCAAdTdT-3′); siTAB1_2 (sense 5′-GC AUUCAACAGACAGUUGAUUUdTdT-3′); siTAB2_1 (sense 5′-GGAA CGACUCAAAGAGAAdTdT-3′); siTAB2_2 (sense 5′-GGAACGACUUA CAAAGAGAAdTdT-3′); siTAB3_1 (sense 5′-GGUUGAAGUCUGAAGUU AAdTdT-3′); siTAB3_2 (sense 5′-GCACAUACCUCGUUAGUdTdT-3′); siTAK1_1 (sense 5′-GUAGAUCCAAGACUUTdTdT-3′); and siTAK1_2 (sense 5′-GAUGGUUAUUAUACCAAGUUdTdT-3′). An irrelevant siRNA duplex (siUNR, sense 5′-GCCGGUUGCCGGUUAAGUdTdT-3′) was employed as a negative control. Plasmid transfection was carried out by means of the Lipofectamine 2000[®] reagent (Invitrogen), as recommended by the manufacturer. A plasmid encoding a GFP–LC3 chimera (Kabeya *et al*, 2000) was co-transfected with the empty vector pcDNA3.1 (Invitrogen) or with mammalian expression vectors encoding HA–TAK1, Flag–TAK1^{WT}, Flag–TAK1^{K63W}, HA–TAB1, HA–TAB2, HA–TAB3 or T7–TAB3 (Takaesu *et al*, 2000). TAB2N (the HA-tagged N-terminus of TAB2, residues 1–392), HA–TAB2C (the HA-tagged C-terminus of TAB2, residues 393–693), T7–TAB3N (the T7-tagged N-terminus of TAB3, residues 1–392), T7–TAB3C (the T7-tagged C-terminus of TAB3, residues 393–712) and T7–TAB3^{ACC} were generated by PCR as previously described (Ninomiya–Tsuiji *et al*, 1999; Takaesu *et al*, 2000). Constructs encoding His-tagged Beclin 1 (His–BCN1), Flag-tagged WT Beclin 1 (Flag–BCN1) and Flag-tagged Beclin 1 mutants (Flag–BCN1^{F123A}, Flag–BCN1^{I125A} and Flag–BCN1^{Δbc12BD}) (Pattingre *et al*, 2005) were a generous gift of Dr B Levine (Texas Southwestern University, Dallas, USA). His–BCN1^{ATBD} was constructed by first inserting at positions 148 and 276 of the His–BCN1 coding vector two *EcoRI* restriction sites by site-directed mutagenesis (with the Quickchange[®] Lightning Site-directed mutagenesis kit, Agilent, Santa Clara, USA) based on the primers 5′-GAATGCAC AGATACTCTTTAGACCAGGAATTCACCTCAGCTCAACGCTCAGTAAAA TGA-3′ and 5′-CAACGCTCTTAATGCAACCTCCACATCGAATTCAGTG GACAGTTTGGCACAATCAATAAC-3′ and their exact inverse complementary sequences. The resulting plasmid was then digested with *EcoRI* and religated in the absence of the TBD-coding trait.

Immunoblotting and immunoprecipitation

For immunoblotting, cells were washed with cold PBS at 4°C and lysed as previously described (Criollo *et al*, 2007a,b). In all experiments, 50 μg of proteins was separated according to molecular weight on 4–12% SDS–PAGE precast gels (Invitrogen) and electrotransferred onto Immobilon membranes (Millipore, Bedford, USA). Unspecific binding sites were blocked by incubating membranes for 30 min in TBS supplemented with 0.05% Tween-20 and 5% w/v non-fat powdered milk. Primary antibodies recognizing the following proteins were employed: Beclin 1, SQSTM1/p62,

TAB2, TAB3 (from Santa Cruz Biotechnology, Santa Cruz, USA); LC3, TAB1, TAK1, VPS24, ATG7, His, HA (from Cell Signaling Technology, Danvers, USA); ATG5 (from MBL International, Woburn, USA); Flag (from Sigma–Aldrich); T7 (from Millipore). Upon overnight incubation at 4°C, primary antibodies were detected with the appropriate horseradish peroxidase-labelled secondary antibodies (Southern Biotechnologies Associates; Birmingham; UK) and the SuperSignal West Pico chemoluminescent substrate (Thermo Fisher Scientific Inc., Rockford, USA). A primary antibody that specifically recognizes glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from Millipore) was used to ensure equal lane loading. For immunoprecipitation, 7 × 10⁶ cells were lysed as previously described (Criollo *et al*, 2007b), and 300 μg of proteins was pre-cleared for 1 h with 30 μl of Pure Proteome[™] Protein G Magnetic Beads (Millipore), followed by incubation for 2 h in the presence of 2 μg of specific antibodies or IgG controls. Subsequent immunoblotting was carried out using TrueBlot[™]-HRP (eBioscience, San Diego, USA) secondary antibodies.

Pull-down experiments with recombinant proteins

Recombinant full-length Beclin 1 (BCN1), the BCN1 TBD, a variant of BCN1 lacking the TBD (BCN1^{ATBD}) and the BBDs from TAB2 and TAB3 were all generated as GST-tagged proteins using the PGEX 6P1 vector (GE Healthcare Life Science, Piscataway, USA) and optionally liberated from GST using the PreScission Protease (GE Healthcare Life Science), following the manufacturer’s recommendations. Glutathione Sepharose[™] 4B beads (GE Healthcare Life Science) were washed in interaction buffer (100 mM Tris pH 7.4, 300 mM NaCl, 0.5% NP-40, 1% glycerol and Complete[™] Protease Inhibitor Cocktail Tablet, from Roche Applied Science, Indianapolis, USA) and then GST-tagged recombinant proteins (25–100 μg) were immobilized on 30 μl beads for 2.5 h at 4°C. Thereafter, beads were washed three times and diluted 1:10 in interaction buffer (final volume = 500 μl) followed by incubation for 1.5 h at 4°C on a roller with 50 μg of untagged recombinant proteins. Finally, beads were washed three times and proteins were eluted at 95°C for 5 min in the presence of SDS. In all, 5 μl of eluates was analysed by immunoblotting.

Light and immunofluorescence microscopy

Cells were processed for immunofluorescence staining according to established protocols (Criollo *et al*, 2007b; Bohrer *et al*, 2008). Fixed and permeabilized cells were incubated with primary antibodies overnight and then revealed with the appropriate AlexaFluor[®] conjugates (Molecular Probes–Invitrogen). Nuclei were counterstained with 10 μg/ml Hoechst 33342 (Molecular Probes–Invitrogen). Fluorescence and confocal fluorescence microscopy assessments were performed with an IRE2 microscope (Leica Microsystems, Weitzler, USA) equipped with a DC300F camera and with an LSM 510 microscope (Carl Zeiss, Jena, Germany), respectively. To determine the percentage of colocalization of fluorescent signals, the Image J software (freely available from the National Institute of Health, Bethesda, MD, USA at the address <http://rsb.info.nih.gov/ij/>) was used. Cells exhibiting >5 GFP–LC3⁺ or FYVE–RFP⁺ puncta were considered as positive (GFP–LC3^{VAC} and FYVE–RFP⁺, respectively).

Electron microscopy

Cells were fixed for 1 h at 4°C in 0.1 M Sörensen phosphate buffer (pH 7.3) supplemented with 1.6% glutaraldehyde, washed, fixed again in aqueous 2% osmium tetroxide, stained in 2% uranyl acetate in 30% methanol and finally embedded in Epon. Electron microscopy was performed with a Tecnai 12 Spirit electron microscope (FEI, Eindhoven, The Netherlands), at 80 kV, on ultrathin sections (80 nm) stained with lead citrate and uranyl acetate.

Statistical procedures

All experiments were performed in triplicate parallel instances and repeated at least three times. Unless otherwise specified, figures report the results from one representative experiment (mean values ± s.d., *n* = 3). Data were analysed with Microsoft Excel (Microsoft Co., Redmond, USA) and statistical significance was assessed by means of two-tailed unpaired Student’s *t*-tests.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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8.5 Direct molecular interactions between Beclin 1 and the canonical NF κ B activation pathway.

Direct molecular interactions between Beclin 1 and the canonical NFκB activation pathway

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General (macro)autophagy and the activation of NFκB constitute prominent responses to a large array of intracellular and extracellular stress conditions. The depletion of any of the three subunits of the inhibitor of NFκB (IκB) kinase (IKKα, IKKβ, IKKγ/NEMO), each of which is essential for the canonical NFκB activation pathway, limits autophagy induction by physiological or pharmacological triggers, while constitutive active IKK subunits suffice to stimulate autophagy. The activation of IKK usually relies on TGFβ-activated kinase 1 (TAK1), which is also necessary for the optimal induction of autophagy in multiple settings. TAK1 interacts with two structurally similar co-activators, TAK1-binding proteins 2 and 3 (TAB2 and TAB3). Importantly, in resting conditions both TAB2 and TAB3 bind the essential autophagic factor Beclin 1, but not TAK1. In response to pro-autophagic stimuli, TAB2 and TAB3 dissociate from Beclin 1 and engage in stimulatory interactions with TAK1. The inhibitory interaction between TABs and Beclin 1 is mediated by their coiled-coil domains (CCDs). Accordingly, the overexpression of either TAB2 or TAB3 CCD stimulates Beclin 1- and TAK1-dependent autophagy. These results point to the existence of a direct molecular crosstalk between the canonical NFκB activation pathway and the autophagic core machinery that guarantees the coordinated induction of these processes in response to stress.

Autophagy constitutes one of the major responses to extracellular and intracellular stress throughout the eukaryote phylogeny. In mammals, a number of additional cell-wide responses to adverse conditions have evolved, including those coordinated by the transcription factors NFκB and p53. The molecular cascades elicited by NFκB play cardinal roles in inflammation and cell survival, while p53-regulated processes participate in the adaptation of cells to oxidative damage, DNA repair, cellular senescence and tumor suppression. NFκB and p53 also influence autophagic responses, both in a direct fashion (by transactivating genes coding for regulators of autophagy) and indirectly, via transcription-independent mechanisms, as recently demonstrated by our laboratory.

The phylogenetically conserved initiation of autophagy involves the obligate activation of two kinases, a protein kinase (yeast Atg1 or its mammalian orthologs ULK1 and ULK2) and a lipid kinase (yeast Vps34 or its mammalian ortholog class III phosphatidylinositol 3-kinase, PtdIns3KC3). Both these kinases operate in multiprotein complexes that regulate their subcellular localization and enzymatic activity. While Atg1 phosphorylates multiple autophagy-relevant protein substrates, Vps34 acts as a phosphatidylinositol 3-kinase to influence the curvature of intracellular membranes and to stimulate the recruitment of specific proteins with affinity for phosphatidylinositol 3-phosphate (PtdIns(3)P) to them. The

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cytoplasmic pool of p53 can interact with RB1-inducible coiled-coil protein 1 (RB1CC1), also called FAK family kinase-interacting protein of 200 KDa (FIP200), which is the mammalian ortholog of yeast Atg17. Since Atg17/RB1CC1/FIP200 is essential for the activation of Atg1/ULK1/ULK2, this interaction may account for the autophagy-inhibitory functions of cytoplasmic p53. While in response to DNA damage p53 becomes stabilized and translocates from the cytoplasm to the nucleus, other stress conditions are also often accompanied by the depletion of cytoplasmic p53. Accordingly, there are multiple examples in which autophagy induction is linked to a reduction in the cytoplasmic pool of p53.

Autophagy is closely coupled to the activation of the canonical NF κ B activation

pathway. In this setting, TAK1 normally functions upstream of (hence activating) IKK, a multiprotein complex composed of one regulatory (IKK γ /NEMO) and two enzymatic (IKK α , IKK β) subunits. The IKK-mediated phosphorylation of I κ B ultimately triggers its ubiquitination and proteasome-mediated destruction, in turn allowing NF κ B to translocate from the cytoplasm to the nucleus. The induction of autophagy by multiple distinct stimuli (including starvation, mTOR inhibition with rapamycin, p53 inhibition with pifithrin α and the addition of the BH3 mimetic ABT737) is blunted when TAK1 or any of the components of the IKK complex are depleted or inhibited. Conversely, the expression of constitutively active IKK subunits stimulates autophagy through pathways that do not

necessarily require the activation of NF κ B, yet do involve the activation of JUN N-terminal kinase 1 (JNK1) and AMP-activated protein kinase (AMPK) (Fig. 1A).

The activation of TAK1 relies on its interaction with TAB2 and TAB3. Using several complementary methods (yeast two-hybrid screens, co-immunoprecipitation of tagged proteins, in vitro pull-down assays), we demonstrated that Beclin 1 interacts with the C-terminal coiled-coil domain of TAB2 and TAB3 (that we called "Beclin-binding domain," BBD) through its own coiled-coil domain (that we called "TAB-binding domain," TBD) (Fig. 1B). In resting conditions, when autophagy is switched off, TAB2 and TAB3 co-immunoprecipitate with Beclin 1 but not with TAK1. However, when

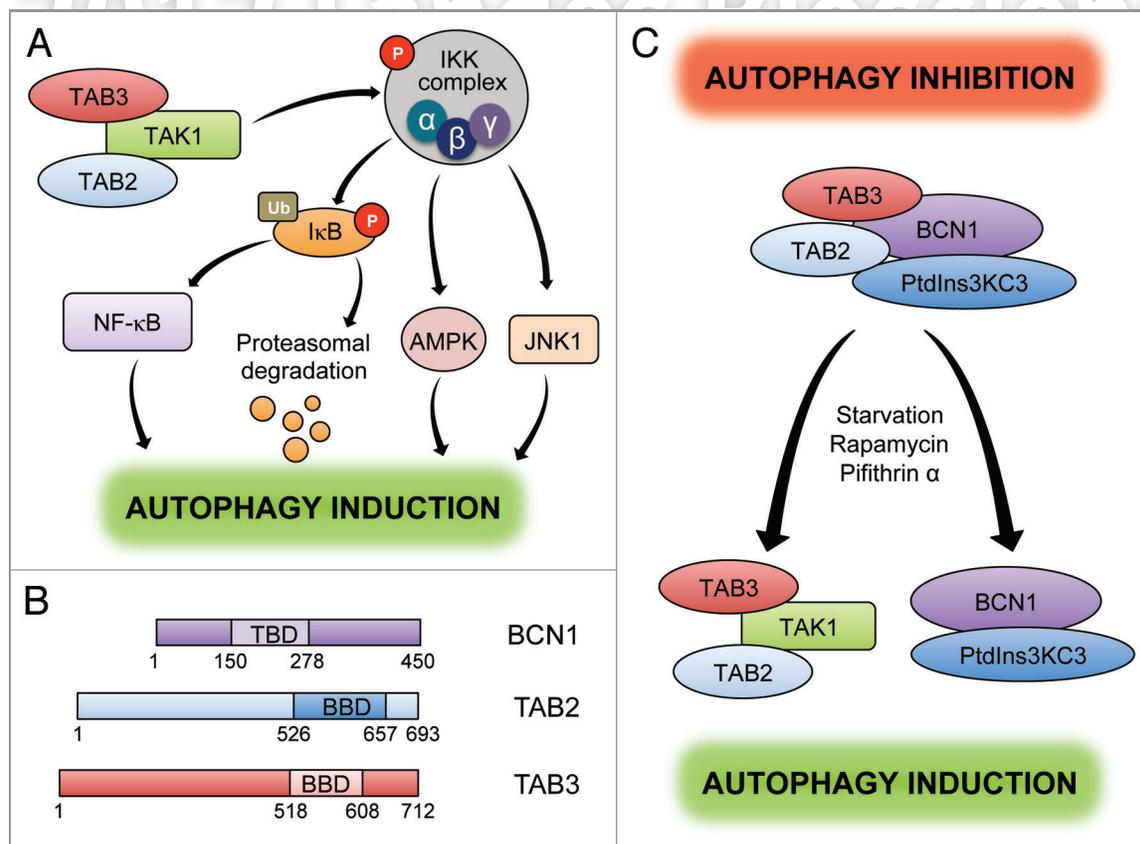


Figure 1. Contribution of TAB2 and TAB3 to a hypothetical autophagy regulatory switch. (A) Contribution of the canonical NF κ B activation pathway to autophagy. AMPK, AMP-activated protein kinase; I κ B, inhibitor of NF κ B; IKK, I κ B kinase; JNK1, JUN N-terminal kinase 1; P, phosphate; TAK1, TGF β -activated kinase 1; TAB2, TAK1-binding protein 2; TAB3, TAK1-binding protein 3; Ub, ubiquitin. (B) Schematic representation of the TAB-binding domain (TBD) in Beclin 1 (BCN1) and the Beclin 1-binding domain (BBD) in TAB2 and TAB3. Numbers indicate amino acid positions. (C) Possible dual function of TAB2 and TAB3 in autophagy regulation. In physiological conditions, TAB2 and TAB3 interact with the Beclin 1 complex, thereby repressing the class III phosphatidylinositol 3-kinase activity of Vps34/PtdIns3KC3. Upon induction of autophagy by starvation, mTOR inhibition (with rapamycin) or p53 inhibition (with pifithrin α), TAB2 and TAB3 dissociate from BCN1 and associate with TAK1. The (possibly) TAB2- or TAB3-dependent activation of the TAK1-IKK signaling axis then facilitates the induction of autophagy.

autophagy is induced by nutrient deprivation or pharmacological triggers (rapamycin or pifithrin α), TAB2 and TAB3 increasingly co-immunoprecipitate with TAK1 as they dissociate from Beclin 1. In contrast to the knockdown of TAK1 (which inhibits autophagy and reduces the lipid kinase activity of Vps34/PtdIns3KC3), the depletion of either TAB2 or TAB3 induces autophagy and stimulates the formation of PtdIns(3)P-enriched vesicles (which can be detected by means of a fluorescent FYVE peptide) as well as of LC3-positive autophagosomes. This suggests that both TAB2 and TAB3 operate as tonic inhibitors of autophagy. Accordingly, overexpression of TAB2 and TAB3 strongly blunts the autophagic responses to multiple stimuli. In contrast to full-length TAB2 and TAB3 (which suppress autophagy), truncated TAB2 and TAB3 mutants in which the C-terminal BBD is preserved stimulate the autophagic flux. Similarly, as compared with full-length Beclin 1 (which moderately induces autophagy), Beclin 1 TBD acts as a particularly efficient autophagic trigger. The expression of either the TBD or the BBD leads to the dissociation of endogenous complexes between TAB2, TAB3 and Beclin 1, correlating with an increase in the lipid kinase activity of Vps34/PtdIns3KC3. Of note, the transfection-enforced expression of the TBD or the BBD only results in the formation of PtdIns(3)P-containing vesicles and autophagosomes when endogenous Beclin 1

and Vps34/PtdIns3KC3 are present. Altogether, these results are compatible with the hypothesis that the pro-autophagic lipid kinase activity of the Beclin 1 complex is normally limited by inhibitory interactions between Beclin 1 and TAB2 or TAB3 (Fig. 1C).

Interestingly, TAK1 as well as the three subunits of the IKK complex are required for the optimal induction of autophagy by either the TBD or the BBD. Thus, the expression of a dominant-negative TAK1 variant as well as the siRNA-mediated depletion of IKK α , IKK β or IKK γ /NEMO reduce the formation of PtdIns(3)P-decorated vesicles and autophagosomes triggered by the TBD or the BBD. These results suggest that TAB2 and TAB3 have a dual role in the control of autophagy. In baseline conditions, TAB2 and TAB3 would interact with Beclin 1 and prevent it from acting as an allosteric activator of PtdIns3KC3. In contrast, upon induction of autophagy, TAB2 and TAB3 would dissociate from Beclin 1 and rather bind TAK1 to facilitate the pro-autophagic stimulation of the TAK1-IKK signaling axis (Fig. 1C). In this hypothetical scenario, several unknowns remain to be resolved. First, it remains to be determined through which mechanisms TAB2 and TAB3 dissociate from Beclin 1. Can this be explained by post-translational modifications? Does it involve the coordinated replacement of inhibitory interactions (including those with anti-apoptotic proteins of the

BCL-2 family and RUBICON) by stimulatory ones (including those with Atg14/BARKOR, UVRAG and BIF-1/endophilin B1)? Second, formal evidence that TAB2 and TAB3 are required as TAK1 activators during the autophagic response is missing. Irrespective of these details, it appears clear that autophagy and the canonical NF κ B activation pathway exhibit a consistent degree of crosstalk that is (also) mediated by direct molecular interactions between core constituents of these processes. The autophagic response of cells to stress is closely coordinated with the regulation of key transcription factors including NF κ B and p53 at multiple levels, some of which are on the point of being understood.

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8.6 Autophagy is required for the activation of NFκB.

Autophagy is required for the activation of NF κ B

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Key words: AMPK, A549, autophagosomes, DNA damage foci, HCT 116, LC3

Abbreviations: AMPK, AMP-activated protein kinase; BCN1, Beclin 1; DMEM, dulbecco's modified essential medium; EBSS, earle's balanced salt solution; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I κ B, inhibitor of NF κ B; IKK, I κ B kinase; JNK1, c-Jun N-terminal kinase 1; MEFs, mouse embryonic fibroblasts; mTORC1, mammalian target of rapamycin complex 1; PFT α , cyclic pifithrin α ; PI3K, phosphatidylinositol-3-kinase; siRNA, small-interfering RNA; TAK1, TGF β -activated kinase 1; TNF α , tumor necrosis factor α ; TRAIL, TNF-related apoptosis-inducing ligand; WT, wild type

It is well-established that the activation of the inhibitor of NF κ B (I κ B α) kinase (IKK) complex is required for autophagy induction by multiple stimuli. Here, we show that in autophagy-competent mouse embryonic fibroblasts (MEFs), distinct autophagic triggers, including starvation, mTOR inhibition with rapamycin and p53 inhibition with cyclic pifithrin α , lead to the activation of IKK, followed by the phosphorylation-dependent degradation of I κ B α and nuclear translocation of NF κ B. Remarkably, the NF κ B signaling pathway was blocked in MEFs lacking either the essential autophagy genes *Atg5* or *Atg7*. In addition, we found that tumor necrosis factor α (TNF α)-induced NF κ B nuclear translocation is abolished in both *Atg5*- and *Atg7*-deficient MEFs. Similarly, the depletion of essential autophagy modulators, including ATG5, ATG7, Beclin 1 and VPS34, by RNA interference inhibited TNF α -driven NF κ B activation in two human cancer cell lines. In conclusion, it appears that, at least in some instances, autophagy is required for NF κ B activation, highlighting an intimate crosstalk between these two stress-response signaling pathways.

Introduction

As they react and adapt to adverse conditions, cells modify their metabolism and repair damage. Such responses often involve the transcriptional reprogramming of the cell through the activation of stress-inducible transcription factors. Frequently, this is preceded by near-to-immediate (transcription-independent) adaptations that result in the formation of ultrastructurally detectable subcellular structures, such as DNA damage foci, stress granules¹ and autophagosomes.² Autophagosomes are double-membraned vesicles that mediate the first step of macroautophagy (herein referred to as "autophagy") by sequestering cytoplasmic organelles, long-lived proteins and/or portions of the cytosol. Autophagosomes are eliminated by fusing with lysosomes (thereby generating "autolysosomes"), causing the enzymatic digestion of their internal membrane and luminal content.^{2,3} Autophagy most often serves a cytoprotective function that allows cells to survive in response to multiple stress conditions and helps to protect multicellular organisms against degenerative, infectious, inflammatory and neoplastic diseases.³⁻⁵

The stress-responsive transcription factor NF κ B has prominent proliferative, anti-apoptotic and pro-inflammatory

functions. In the so-called canonical NF κ B pathway, NF κ B is activated when the inhibitor of NF κ B (I κ B) is phosphorylated by the I κ B kinase (IKK), a multiprotein complex that is composed by two catalytic (IKK α , IKK β) and one regulatory subunit (IKK γ , also known as NEMO). The IKK complex responds to multiple distinct perturbations, including the shortage of nutrient supply, reactive oxygen species, DNA damage or ligation of the tumor necrosis factor α (TNF α) receptor. I κ B phosphorylation results in its ubiquitination, targeting I κ B for destruction by the proteasome. The degradation of I κ B allows NF κ B to translocate from the cytoplasm (where it is usually retained by I κ B) to the nucleus.⁶ The genetic inhibition (by gene knockout or RNA interference) of any of the IKK subunits (but not that of the NF κ B subunit p65) prevents the induction of autophagy in response to various stimuli including endoplasmic reticulum (ER) stress, starvation, chemical inhibition of mTOR (with rapamycin) and p53 inhibition (with cyclic pifithrin α , PFT α).⁷ Along similar lines, TGF β -activated kinase (TAK1), which is often required for IKK activation, underlies the optimal induction of autophagy in multiple settings. Thus, depletion of TAK1 or transfection-enforced expression of a dominant-negative TAK1 variant prevents autophagy induction by starvation as well

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as by the administration of rapamycin, PFT α and TNF-related apoptosis-inducing ligand (TRAIL).^{8,9} Conversely, when introduced into human or murine cells, constitutively active IKK subunits potently stimulate autophagy through a pathway that does not necessarily involve NF κ B.⁷ Nonetheless, the activation of NF κ B can contribute to the stimulation of autophagy by upregulating Beclin 1 expression.¹⁰ Of note, IKK-dependent autophagy involves the induction of several other essential autophagy-related proteins, including ATG5 and LC3¹¹ as well as the activation of AMP-activated protein kinase (AMPK) and c-Jun N-terminal kinase 1 (JNK1).⁷

The aforementioned results underscore that the activation of IKK is intimately linked to the initiation of autophagy. Here, we addressed the question of whether the inverse would be true as well, i.e., whether autophagy would be required for the activation of NF κ B. As shown in three distinct cellular models, the nuclear translocation of NF κ B only occurs in the presence of a functional autophagic cascade.

Results and Discussion

Autophagic stimuli trigger the canonical NF κ B pathway in an autophagy-dependent fashion. Like other mammalian cells, mouse embryonic fibroblasts (MEFs) activate the autophagic machinery in response to starvation (culture in serum-free, nutrient-free Hank's balanced salt solution), rapamycin (which inhibits the mammalian target of rapamycin complex 1, mTORC1) or PFT α (a pharmacological inhibitor of p53). Thus, wild-type MEFs subjected to these pro-autophagic conditions manifested an increased conversion of the immature variant of LC3 (LC3-I) into its lipidated counterpart (LC3-II), a process that results in an increase in its electrophoretic mobility,^{12,13} and accumulated LC3-positive cytoplasmic vacuoles (Fig. 1A). Concomitantly, MEFs displayed a reduction in the abundance of I κ B α as well as an increase in its phosphorylation (Fig. 1A), leading, in turn, to the binding of the NF κ B subunit p65 to nuclear DNA, as determined by electrophoretic mobility shift assay (EMSA) (Fig. 1B). This result could be expected in view of the fact that starvation, rapamycin and PFT α all potently stimulate the activation of IKK.⁷ Unexpectedly, however, we found that, in contrast to WT MEFs (which are autophagy competent), MEFs lacking either of the essential autophagy proteins Atg5 or Atg7 were unable to phosphorylate and degrade I κ B α (Fig. 1A) and subsequently activate NF κ B (Fig. 1B) in response to pro-autophagic stimuli. Immunofluorescence detection of the subcellular localization of p65 confirmed that starvation, rapamycin and PFT α all induced the translocation of p65 from the cytoplasm to the nucleus in WT MEFs, but not in their *Atg5*^{-/-} or *Atg7*^{-/-} counterparts (Fig. 1C and D). Thus, at least in MEFs, autophagy induction is accompanied by the activation of the canonical NF κ B pathway, a process that requires autophagy-relevant proteins such as Atg5 and Atg7.

TNF α -induced NF κ B activation through the canonical pathway requires autophagy. It should be noted that autophagy-stimulating conditions are not universal NF κ B activators. Thus, starvation can trigger NF κ B activation in MEFs and human

non-small cell lung cancer A549 cells but only partially in human colorectal cancer HCT 116 cells and not at all in murine NIH3T3 fibroblasts and human cervical carcinoma HeLa cells in spite of the fact that all these cells mount a potent autophagic response.⁷ To investigate the impact of autophagy on a more general NF κ B activator, we took advantage of TNF α , which potently triggers the canonical NF κ B pathway in all the cell lines included in this study. Thus, TNF α caused the activating phosphorylation of IKK subunits as well as the degradation of I κ B α in WT MEFs (Fig. 1A), HeLa (Fig. 2A) and A549 cells (Fig. 2B). As expected, human TNF α also stimulated the nuclear translocation of the NF κ B subunit p65 in HeLa and A549 cells, while mouse TNF α did the same in WT MEFs. In addition, TNF α induced autophagy in both HeLa and A549 cells, as indicated by the increase in LC3-II and the accumulation of autophagosomes (Fig. 2A and B). At difference with other authors,¹⁴ we found that TNF α rapidly stimulates the lipidation of LC3 that accompanies autophagy.

Knockdown of multiple distinct autophagy-related proteins including ATG5, ATG7, Beclin 1 and the phosphatidylinositol 3-kinase (PI3K) VPS34, prevented the TNF α -mediated induction of autophagy (Fig. 2A and B) as well as the nuclear translocation of p65 in HeLa and A549 cells (Fig. 3A and B). Similarly, the knockout of *Atg5* or *Atg7* or the small interfering RNA (siRNA)-mediated depletion of ATG5, ATG7, VPS34 or Beclin 1 strongly inhibited p65 nuclear translocation and DNA binding (as induced by TNF α) in MEFs and HCT116 cells (Fig. 3A–C). Although the phosphorylation of IKK α and IKK β subunits was blunted upon ATG5, ATG7, Beclin 1 or VPS34 depletion, the addition of TNF α resulted in reduced I κ B α levels irrespective of the absence or presence of essential autophagy regulators (Fig. 2). This applied to all three cellular models studied here, namely MEFs, HeLa and A549 cells. We conclude that autophagy is required for the efficient translocation of the NF κ B subunit p65 to the nucleus.

Materials and Methods

Chemicals, cell lines and culture conditions. Unless otherwise specified, chemicals were obtained from Sigma-Aldrich and media and supplements for cell culture from Gibco-Invitrogen. Rapamycin was purchased from Tocris Bioscience. All cells were cultured in standard conditions (37°C, 5% CO₂). WT, *Atg5*^{-/-} and *Atg7*^{-/-} MEFs were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10 mM HEPES buffer and 1% non-essential amino acids (NEAA). Human cervical carcinoma HeLa cells, human non-small cell lung cancer A549 cells and human colorectal carcinoma HCT116 cells were cultured, in DMEM, DMEM-F12 and McCoy's 5A medium, respectively, all supplemented with 10% FCS and 10 mM HEPES buffer. For serum and amino acid starvation, cells were cultured in serum-free Earle's Balanced Salt Solution medium.¹⁷

siRNAs, plasmids and transfections. siRNA transfections were performed by means of the Oligofectamine[®] reagent (Invitrogen) according to the manufacturer's instructions. The following custom-designed siRNA duplexes were purchased

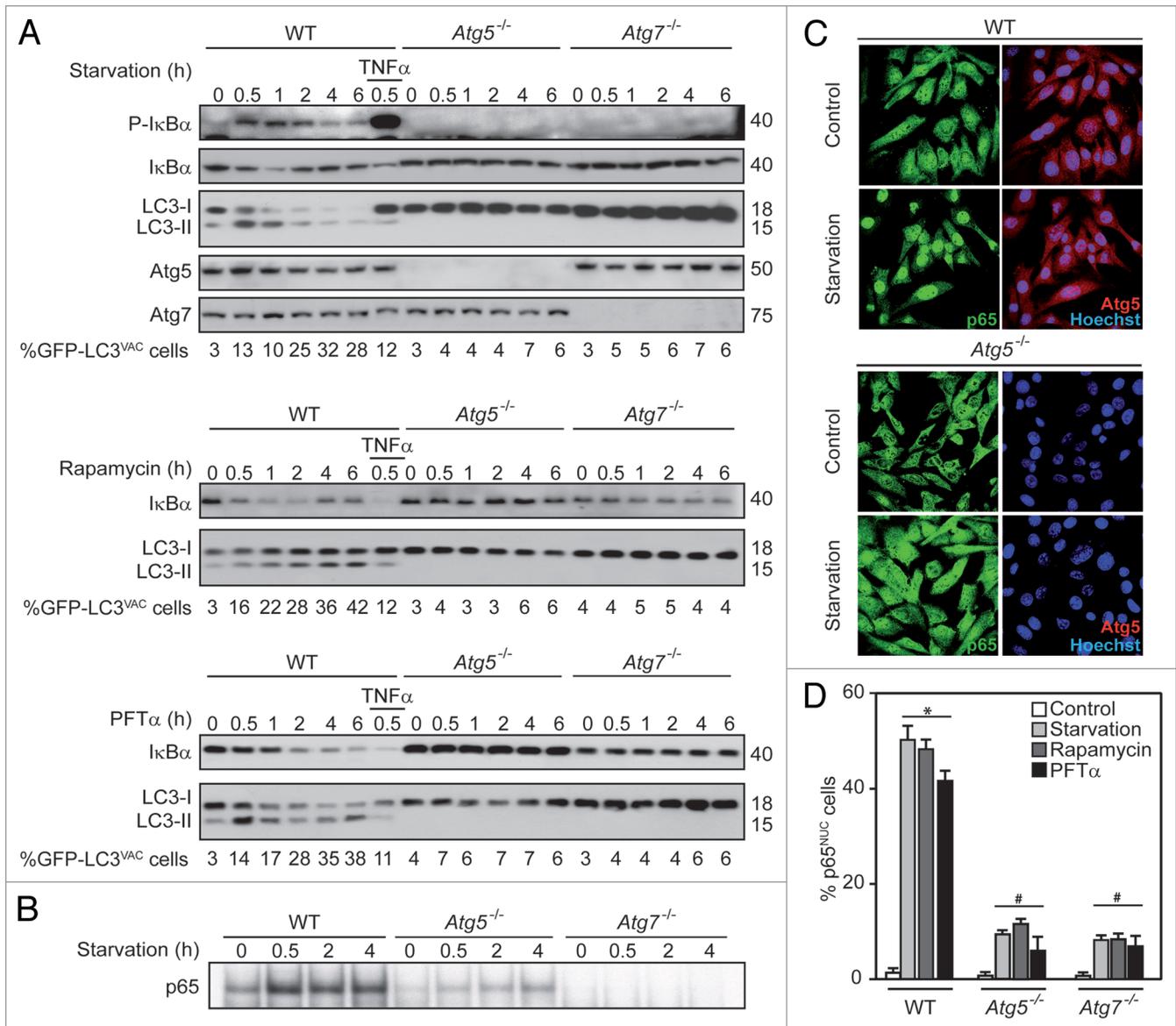


Figure 1. Activation of the canonical NF κ B activation pathway in autophagy-competent but not in autophagy-deficient mouse embryonic fibroblasts (MEFs). (A) Impact of autophagy-inducing conditions on I κ B levels. Wild-type (WT), *Atg5*^{-/-} and *Atg7*^{-/-} MEFs (previously transfected or not with a GFP-LC3-encoding plasmid, as appropriate) were kept for the indicated in the absence of serum and nutrients (starvation) or administered with 1 μ M rapamycin, 30 μ M pifithrin α (PFT α) or 1 ng/mL tumor necrosis factor α (TNF α), followed by immunoblotting for the detection of the indicated proteins or immunofluorescence microscopy for the determination of the percentage of cells exhibiting GFP-LC3 aggregation (GFP-LC3^{VAC} cells). (B) Impact of autophagy-inducing conditions on NF κ B activation. MEFs of the indicated genotype were subjected to starvation as reported, followed by EMSA for the detection of NF κ B subunit p65 binding to nuclear chromatin. (C and D) Translocation of the NF κ B subunit p65 to the nucleus in pro-autophagic conditions. WT, *Atg5*^{-/-} and *Atg7*^{-/-} MEFs were left untreated or subjected to starvation for 6 h and then processed for the immunofluorescence detection of p65 (revealed in green), Atg5 (revealed in red) and nuclear chromatin (stained in blue by Hoechst 33342). Representative pictures are shown in (C), while quantitative data on the percentage of cells exhibiting nuclear p65 (p65^{NUC}, means \pm SEM, n = 3, *p < 0.05 compared with WT MEFs, *p < 0.05, compared with the corresponding cell line under control conditions) are reported in (D).

from Sigma-Prologo or Thermo Fisher Scientific Inc.: siATG5 (sense 5'-UCU GAG CUA CCC GGA UAd TdT-3'), siATG7 (sense 5'-CCA CAG AUG GAG UAG CAd TdT-3'), siBCN1 (sense 5'-CUC AGG AGA GGA GCC AUU UdT dT-3'),¹⁸ siVPS34 (sense 5'-ACG GTG ATG AAT CAT CTC CAA dTd T-3'). An irrelevant siRNA duplex (siUNR, sense 5'-GCC GGU AUG CCG GUU AAG UdT dT-3') was employed as a negative

control. Transfection of a plasmid encoding a green fluorescent protein (GFP)-LC3 chimera¹⁹ was performed by means of the Lipofectamine 2000[®] reagent (Invitrogen), as recommended by the manufacturer.

Immunoblotting. For immunoblotting assessments, cells were washed with cold PBS and lysed as previously described in references 20 and 21. Fifty μ g of proteins were separated

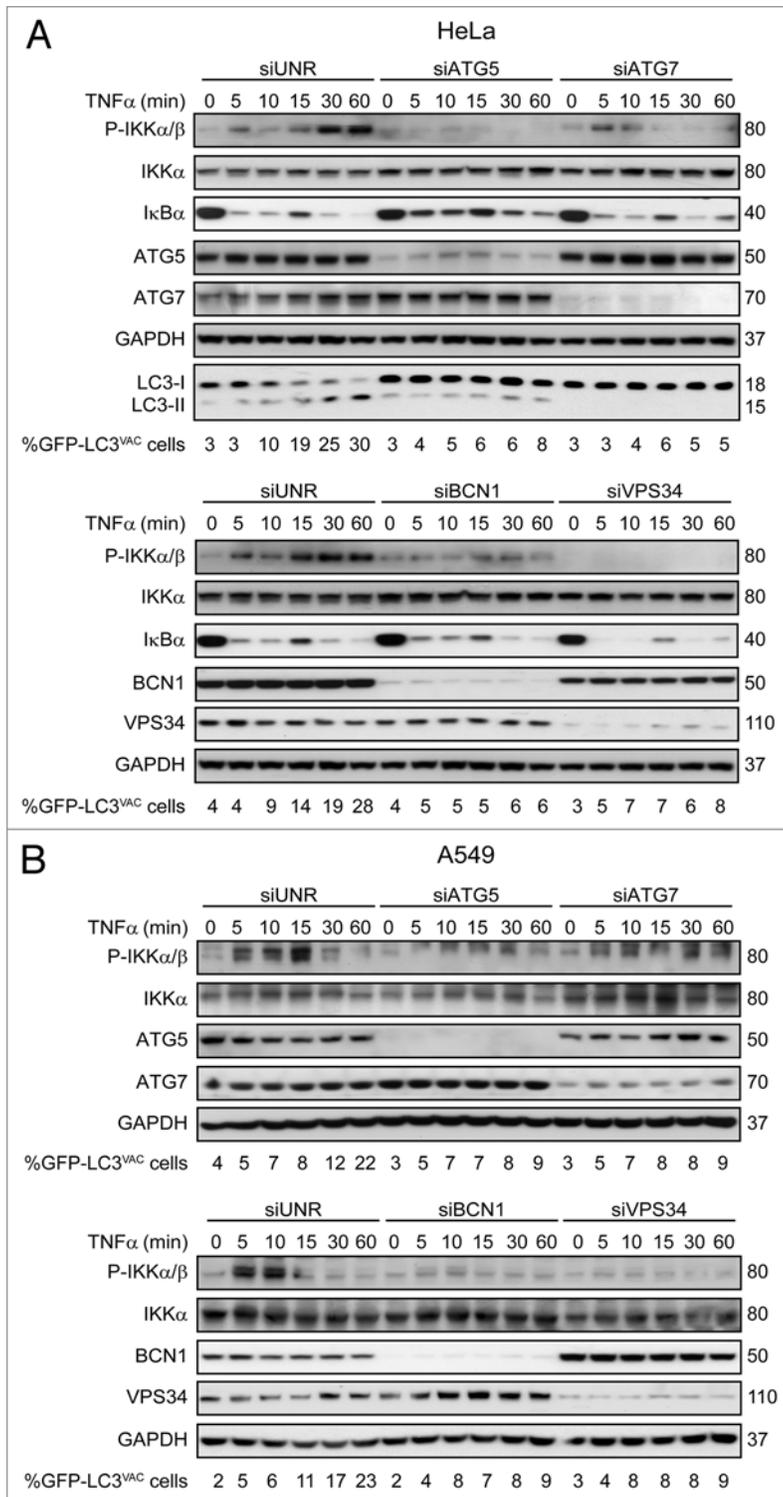


Figure 2. Activation of the canonical NF κ B pathway in HeLa and A549 cells responding to TNF α . Human cervical carcinoma HeLa (A) and human non-small cell lung cancer A549 cells (B) were transfected with a control siRNA (siUNR) or with siRNAs specific for the indicated proteins, alone or together with a GFP-LC3-encoding plasmid, for 48 h then treated with 1 ng/mL tumor necrosis factor α (TNF α) for the indicated time and lysed for the immunoblotting detection of the indicated proteins or subjected to immunofluorescence microscopy for the quantification of the percentage of cells exhibiting GFP-LC3 aggregation (GFP-LC3^{VAC} cells). GAPDH levels were monitored to ensure equal loading of lanes. Results are representative of three independent experiments.

according to molecular weight on 4–12% SDS-PAGE precast gels (Invitrogen) and electrotransferred to Immobilon membranes (Millipore). Unspecific binding sites were blocked by incubating membranes for 0.5 h in TBS supplemented with 0.05% Tween 20 and 5% w/v non-fat powdered milk. Primary antibodies recognizing the following proteins were employed: ATG5 (MBL International), ATG7 (Cell Signaling Technology), Beclin 1 (Santa Cruz Biotechnology), LC3 (Cell Signaling Technology), p65 (Santa Cruz Biotechnology), P-I κ B α , I κ B α , P-IKK α/β , IKK α and VPS34 (all from Cell Signaling Technology). After overnight incubation at 4°C, primary antibodies were revealed with the appropriate horseradish peroxidase-labeled secondary antibodies (Southern Biotechnologies Associates) and the SuperSignal West Pico chemoluminescent substrate (Thermo Fisher Scientific Inc.). An antibody specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from Millipore) was employed to ensure equal loading of lanes.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared, and 15 μ g of proteins were analyzed for DNA binding activity using the HIV-LTR tandem κ B oligonucleotide as a probe for NF κ B.²² Samples were then resolved by electrophoresis using acrylamide/bis-acrylamide/TBE gels.

Immunofluorescence microscopy. Cells were processed for immunofluorescence staining according to established protocols.^{23–25} Fixed and permeabilized cells were incubated with primary antibodies specific for ATG5, LC3 or p65 overnight and then detected with appropriate AlexaFluor[®]-conjugated secondary antibodies (Molecular Probes-Invitrogen). Nuclear counterstaining was performed with 10 μ g/mL Hoechst 33342 (Molecular Probes-Invitrogen). Confocal fluorescence microscopy assessments were done on a LSM 510 microscope (Carl Zeiss). Cells exhibiting more than 5 GFP-LC3⁺ cytosolic puncta were considered as positive.

Statistical procedures. All experiments were performed in triplicate instances and repeated at least twice, yielding similar results. Unless otherwise specified, the results from one representative experiment (means \pm SD, n = 3) are reported. Data were analyzed with Microsoft Excel (Microsoft Co.), and statistical significance was assessed by means of unpaired two-tailed Student t-tests.

Concluding Remarks

In the present work, we reveal an unexpected and intimate relationship between the activation of NF κ B and autophagy. It has been established that IKK, which operates upstream of NF κ B in the canonical pathway, is also required for the initiation of autophagy in

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8.7 Stimulation of autophagy by the p53 target gene Sestrin2.

Report

Stimulation of autophagy by the p53 target gene Sestrin2

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Key words: autophagy, sestrins, p53, dram

The oncosuppressor protein p53 regulates autophagy in a dual fashion. The pool of cytoplasmic p53 protein represses autophagy in a transcription-independent fashion, while the pool of nuclear p53 stimulates autophagy through the transactivation of specific genes. Here we report the discovery that Sestrin2, a novel p53 target gene, is involved in the induction of autophagy. Depletion of Sestrin2 by RNA interference reduced the level of autophagy in a panel of p53-sufficient human cancer cell lines responding to distinct autophagy inducers. In quantitative terms, Sestrin2 depletion was as efficient in preventing autophagy induction as was the depletion of Dram, another p53 target gene. Knockout of either Sestrin2 or Dram reduced autophagy elicited by nutrient depletion, rapamycin, lithium or thapsigargin. Moreover, autophagy induction by nutrient depletion or pharmacological stimuli led to an increase in Sestrin2 expression levels in p53-proficient cells. In strict contrast, the depletion of Sestrin2 or Dram failed to affect autophagy in p53-deficient cells and did not modulate the inhibition of baseline autophagy by a cytoplasmic p53 mutant that was reintroduced into p53-deficient cells. We conclude that Sestrin2 acts as a positive regulator of autophagy in p53-proficient cells.

Introduction

Macroautophagy (which we refer to as “autophagy”) is a catabolic process in which portions of the cytosol or cytoplasmic organelles are sequestered within two-membraned vesicles, autophagosomes, and then targeted for bulk degradation through the fusion with lysosomes.^{1,2} This process can be elicited by multiple types of cellular stress, in particular nutrient depletion to which cells adapt by activating macroautophagy and by catabolizing macromolecules, allowing them to generate substrates for glycolysis and oxidative phosphorylation.³ The molecular pathway that links nutrient depletion to autophagy involves activation of the energy

sensor AMP-responsive protein kinase (AMPK, which responds to low ATP levels), which in turn inhibits the cardinal autophagy-repressive kinase, mammalian target of rapamycin (mTOR). Hence, inhibition of mTOR by addition of rapamycin is one of the most potent mechanisms to stimulate autophagy.⁴ Autophagy can also be elicited by alternative mechanisms, namely depletion of inositol-3-phosphate by addition of lithium or endoplasmic reticulum (ER) stress.⁵⁻⁸ Autophagy is indispensable for the adaptation of cells to stress, for the removal of damaged organelles, and for the maintenance of genomic stability.^{3,9,10} The invalidation of the autophagic program can accelerate tumor progression^{9,10} and disabled autophagy may constitute a near-to-general hallmark of developing cancer.^{11,12}

The oncosuppressor protein p53 plays an important role in the regulation of autophagy, at two radically distinct levels. At the first level, p53 can stimulate autophagy when it is present in the nucleus and transactivates autophagy-stimulating genes. Thus, the activation of p53 by genomic stress¹³ or an inducible p53 transgene^{14,15} can stimulate autophagy through transcriptional mechanisms. One p53-elicited gene product, Dram (for “damage-regulated autophagy modulator”) has been shown to be essential for p53-induced autophagy and apoptosis,^{14,15} as well as for autophagy induced by the p53 homolog p73.¹⁶ At a second level, p53 potently inhibits autophagy when it is present in the cytoplasm, where it inhibits AMPK and activates mTOR.¹⁷⁻²⁰ Thus, deletion, depletion or pharmacological inhibition of p53 triggers autophagy in normal and cancer cells.

Recently, Michael Karin and co-workers reported that the products of two p53 target genes, Sestrin1 and Sestrin2, inhibit mTOR through an indirect mechanism that involves the stimulation of AMPK.²¹ In view of the role of mTOR in the control of autophagy, we decided to investigate whether Sestrins might control autophagy. Here, we report that Sestrin2 acts as a new positive regulator of autophagy in p53-proficient but not in p53-deficient cells.

Results and Discussion

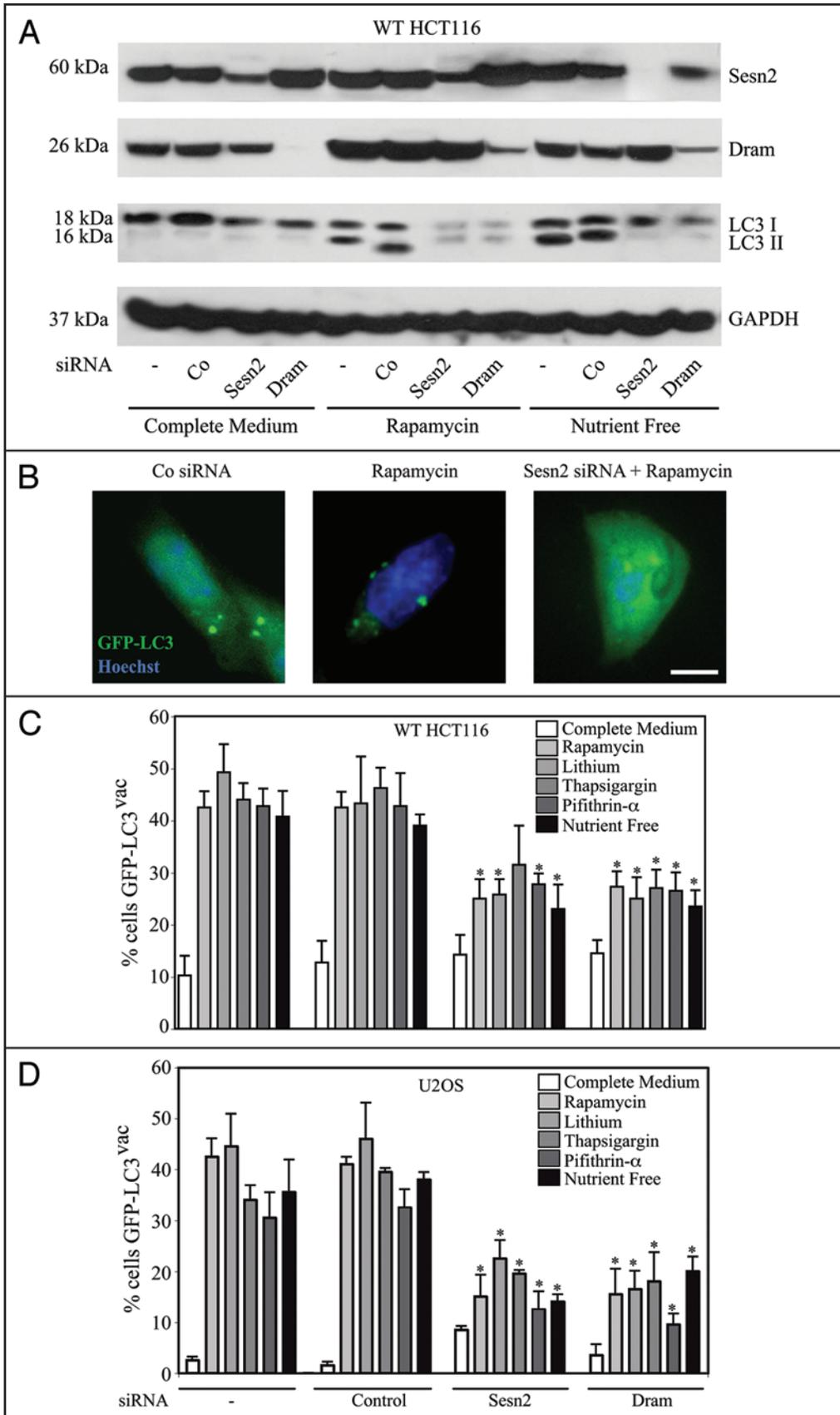
Contribution of sestrin2 and dram to the induction of autophagy in p53-proficient cells. To assess the possible contribution of Sestrins and Dram to the induction of autophagy, we

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Figure 1. Contribution of Dram and Sestrin2 to the induction of autophagy in p53-sufficient cells. (A) Immunoblot detection of LC3 conversion in HCT116 cells. Cells were subjected to the knockdown of Dram or Sestrin2 by siRNAs and 48 h later cells were stimulated overnight with rapamycin or culture in nutrient-free (NF) conditions for induction of autophagy. Representative immunoblots of Sestrin2 (Sesn2), Dram and LC3 are shown. GAPDH was detected as a loading control. (B and C) Fluorescence microscopic detection of GFP-LC3 aggregation in HCT116 cells. Cells were transfected with the indicated siRNAs, followed by transfection with GFP-LC3 at 24 h and overnight stimulation of autophagy starting at 48 h with the indicated agents. Representative fluorescence microphotographs are shown in (B) (the bar indicates 10 μ m) and quantitative results (means \pm SEM, n = 3) given in (C). Asterisks refer to significant (p < 0.01) inhibitory effects of Sestrin2 or Dram-specific siRNAs as compared to control siRNA. (D) Fluorescence microscopic detection of GFP-LC3 aggregation in U2OS cells. Identical setting as in (C) with the exception that U2OS cells were used.



took advantage of commercially available siRNAs that were used to efficiently deplete the respective proteins in a panel of cell lines comprising HCT116 colon cancer cells (Fig. 1A–C), U2OS osteosarcoma cells (Fig. 1D) or HeLa cervical carcinoma cells (not shown). This depletion did not affect the viability of cells, as determined by the combined assessment of plasma membrane permeability (with propidium iodine) and the mitochondrial transmembrane potential (with DiOC₆(3)) (data not shown).²² Once the depletion of Sestrin2 (Sesn2) or Dram was efficient, 48 hours after transfection, the cells were stimulated to activate the macroautophagic program by a series of different inducers, namely culture in nutrient-free (NF) conditions, addition of the quintessential mTOR inhibitor rapamycin, or addition of alternative pharmacological autophagy inducers: lithium,⁵ thapsigargin²³ or cyclic pifithrin- α (which is a chemical inhibitor of p53, yet may have additional off-target effects).^{24,25} Both nutrient depletion and pharmacological induction of autophagy resulted in an increased expression of Sestrin2 and Dram (Fig. 1A).

Autophagy was measured by two alternative methods, namely by immunoblot detection of the lipidation of LC3 (which leads to the conversion of the LC3 I isoform to the electrophoretically more mobile LC3 II isoform) or by assessing the redistribution of GFP-LC3 from a diffuse pattern to cytoplasmic puncta (which are indeed autophagosomes or autophagolysosomes the membranes of which are decorated by lipidated LC3).^{26,27} Using this technology, we found that depletion of Sestrin2 or knockdown of Dram were similarly efficient in preventing LC3 lipidation induced by nutrient deprivation or rapamycin (Fig. 1A). Similarly, we observed that the knockdown of Sestrin2 or Dram reduced the GFP-LC3 aggregation in cytoplasmic dots that was induced by the entire panel of chemical autophagy inducers (Fig. 1B–D). Similar results were obtained in three different p53-proficient cell lines, namely HCT116 cells (Fig. 1A–C), U2OS cells (Fig. 1D) and HeLa cells (not shown). In conclusion, both Sestrin2 and Dram contribute to the induction of autophagy in p53-proficient cells.

Absent contribution of sestrin2 and dram to autophagy induction in p53-deficient cells. We have shown in the past that distinct cell lines from murine or human origin activate the macroautophagic program when p53 is removed by homologous recombination or RNA interference.^{17–20} Accordingly, p53-deficient (p53^{-/-}) HCT116 cells exhibited a strong baseline level of autophagy, as indicated by the massive presence of LC3 II (Fig. 2A) and GFP-LC3-positive puncta (Fig. 2B). This level of autophagy could not be further enhanced by the addition of rapamycin or culture of cells in nutrient-free conditions (Fig. 2B). Transfection of p53^{-/-} HCT116 cells with wild type (WT) p53 caused an inhibition of autophagy. Similarly, a p53 mutant that lacks the nuclear localization sequence (NLS) and hence is confined to the cytoplasm was able to suppress the increased baseline autophagy of p53^{-/-} HCT116 cells. In contrast, a p53 mutant that lacks the nuclear export signal (NES) and hence is sequestered in the nucleus was incapable to inhibit autophagy, in accord with the notion that cytoplasmic (but not nuclear) p53 suppresses autophagy.^{17–20} In these experimental conditions p53^{-/-} HCT116 cells expressed significant levels of Sestrin2 and Dram without that WT, NLS⁻ or NES⁻ p53 would affect their abundance (Fig. 2A). Knockdown of

Sestrin2 or Dram failed to affect the levels of autophagy, as quantified by immunoblot (Fig. 2A) or fluorescence microscopy (Fig. 2B). Altogether, these data indicate that Sestrin2 and Dram do not participate in the regulation of autophagy by cytoplasmic p53.

Concluding Remarks

p53 regulates autophagy through two distinct mechanisms. On the one hand, the nuclear pool of p53 can induce autophagy through the transcriptional activation of specific target genes that include Dram (as previously shown by Kevin Ryan and collaborators)¹⁴ and Sestrin2 (as shown here). On the other hand, the cytoplasmic pool of p53 can inhibit autophagy by non-transcriptional mechanisms that are not fully understood. In this work, we depleted Dram or Sestrin2 from p53-proficient and p53-deficient cells to discover that both proteins contribute to the induction of autophagy, but only in p53-proficient cells. Thus, p53-proficient cells depleted from Dram or Sestrin2 exhibited a reduction in autophagy as compare to control cells, irrespective of whether autophagy was induced by the most physiological stimulus, nutrient depletion, or by pharmacological stimulators such as rapamycin. In strict contrast, we observed that Dram or Sestrin2 depletion failed to affect the increased autophagy of p53-deficient cells and had no effect on the inhibition of autophagy by cytoplasmic p53 that was re-introduced into p53-minus cells.

We conclude from these data that Dram and Sestrin2 are positive modulators of autophagy in p53-positive cells responding to distinct types of autophagy inducers. Indeed, when Dram or Sestrin2 were depleted, a whole series of distinct autophagy inducers elicited suboptimal responses. This applies to rapamycin but also to a variety of agents that cause autophagy through radically distinct mechanisms: lithium, thapsigargin and cyclic pifithrin- α . It should be noted that none of these inducers (nutrient depletion, rapamycin, lithium, thapsigargin or pifithrin- α) are known to activate p53, suggesting that other (transcriptional or post-transcriptional) p53-independent mechanisms likewise contribute to the modulation of Sestrin2 and Dram levels by autophagy inducers. At first glance, it may appear contradictory that the depletion of Dram or Sestrin2 reduces autophagy induction by the p53 inhibitor pifithrin- α , yet fails to inhibit autophagy induced by p53 deletion. This contradiction may be resolved by either assuming that pifithrin- α exerts off-target effects²⁶ or by speculating that the mechanisms of autophagy induction by acute pharmacological inhibition of p53 or deletion of the p53 gene from the genome may be distinct.

Irrespective of these theoretical considerations, our results indicate that Sestrin2 is a novel positive regulator of autophagy. In quantitative terms, Sestrin2 contributes to autophagy as much as does Dram, at least in the panel of human tumor cells investigated in this study. Hence, the elevation of Sestrin2 protein levels elicited via p53 or p53-independent mechanisms can efficiently stimulate autophagy.

Materials and Methods

Cell culture and autophagy induction. U2OS (osteosarcoma) and HeLa (cervical carcinoma) cells were cultured in DMEM containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate

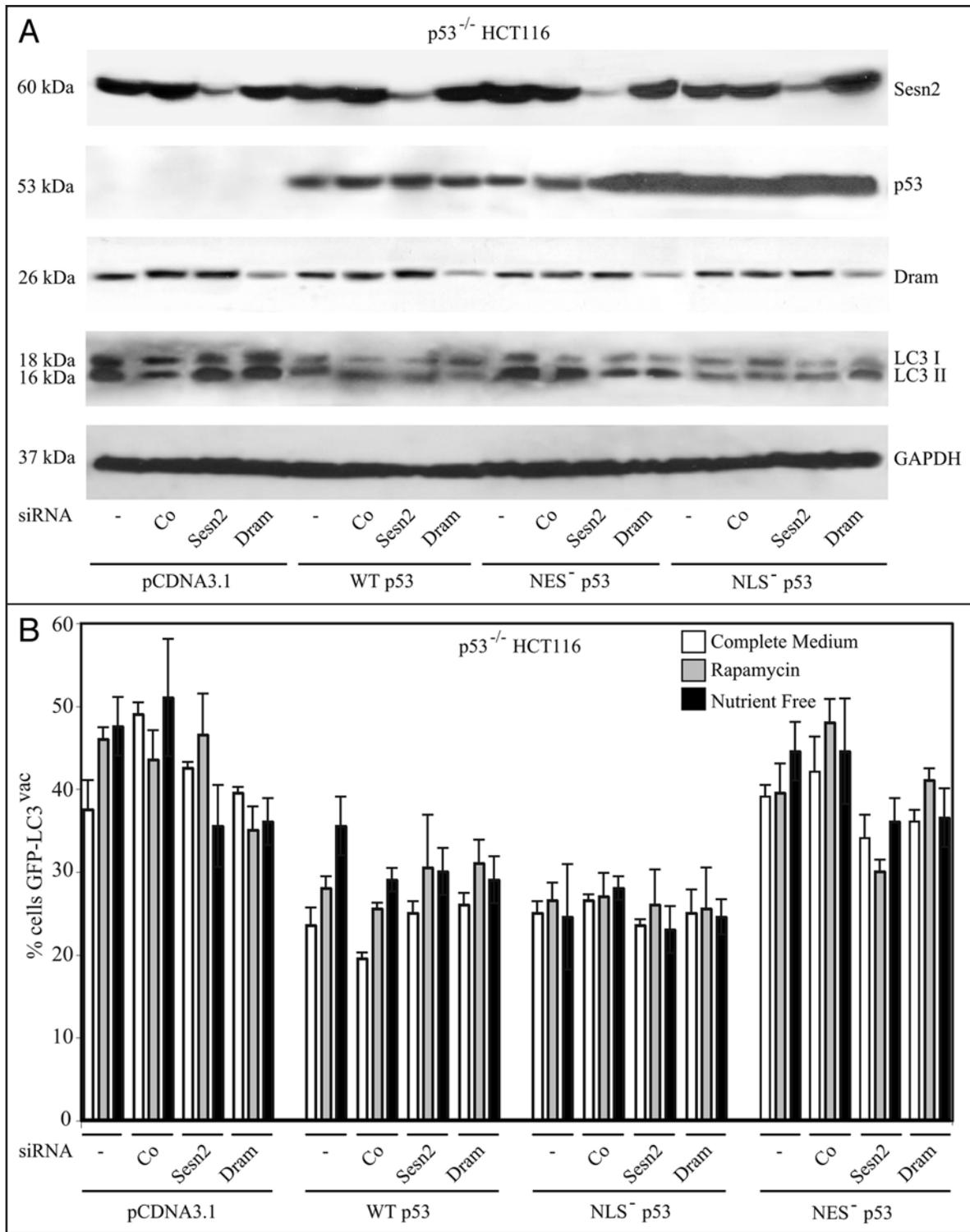


Figure 2. Failure of Dram and Sestrin2 to regulate autophagy in p53-negative cells. p53-deficient HCT116 cells were transfected with the indicated siRNAs, followed by transfection with different p53 constructs (wild type, NLS⁻, NES⁻), in the absence (A) or presence (B) of GFP-LC3 and immunoblot (A) or fluorescence microscopy (means ± SEM, n = 3) (B) and addition of autophagy inducers overnight.

and 10 mM Hepes at 37°C under 5% CO₂. HCT116 colon carcinoma cell lines (wild type or p53^{-/-}, generous gift from Dr. B. Vogelstein)²⁸ were cultured in McCoy's medium containing 10%

FCS, 100 mg/l sodium pyruvate, 10 mM Hepes buffer, 100 units/ml penicillin G sodium and 100 µg/ml streptomycin sulfate at 37°C under 5% CO₂. All media and supplements for cell culture

were purchased from Gibco-Invitrogen (Carlsbad, USA). For serum and amino acid starvation, cells were cultured in serum-free Earle's Balanced Salt Solution medium (Sigma-Aldrich, St. Louis, USA), which we refer to as nutrient-free medium (NF). Three x 10⁴ cells were seeded in 24 well plates and grown for 24 h before treatment with lithium chloride (Lit, 10 mM; Sigma-Aldrich), rapamycin (Rapa, 1 μM; Tocris), pifithrin-α (PFT-α, 30 μM; Calbiochem) or thapsigargin (Thapsi, 3 μM; Calbiochem) for the indicated period (6 h, unless otherwise stated).

Plasmids, transfection and RNA interference. Cells at 80% confluence were transfected in 6-well plates with oligofectamine (Invitrogen) in the presence of 100 nM of siRNAs specific for human Sesn2 (Ambion, Applied Biosystems), Dram (Santa Cruz Biotechnology), a scrambled siRNA or a siRNA targeting the unrelated protein emerin.²⁹ Transient transfections were performed with Lipofectamine 2000 (Invitrogen), and cells were used 24 h after transfection, unless indicated otherwise. Cells were transfected with an empty pcDNA3.1 vector alone or together with plasmids encoding GFP-LC3,³⁰ in the presence or absence of NES-p53, NLS-p53 (gift from Dr. C.G. Maki) or WT p53.

Quantification of autophagy. Autophagy was quantified by counting the percentage of cells showing accumulation of GFP-LC3 in vacuoles (GFP-LC3^{vac}, of a minimum of 100 cells per preparation in three independent experiments).^{26,27} Cells presenting a mostly diffuse distribution of GFP-LC3 in the cytoplasm and nucleus were considered non-autophagic, whereas cells representing several intense punctate GFP-LC3 aggregates with no nuclear GFP-LC3 were classified as autophagic. Each GFP-LC3 staining was read by two independent investigators. Cells were fixed with paraformaldehyde (4% w/v) for GFP-LC3 and immunofluorescence assays. Cells were stained for the detection of p53 (Oncogene Sciences) revealed by goat anti-mouse immunoglobulin Alexa[®] fluor conjugates (Molecular Probes). Nuclei were labeled with Hoechst 33342 (10 μg/ml, Molecular Probes-Invitrogen). Fluorescence microscopy was analyzed with a Leica IRE2 microscope equipped with a DC300F camera.

Immunoblotting. Cells were lysed on ice in a buffer containing 1% NP40, 20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 1 mM dithiothreitol and 10 μg/ml aprotinin, leupeptin, pepstatin before centrifugation (20 min, 14,000 rpm, 4°C) and collection of supernatant. Thereafter, protein extracts were separated on precast polyacrylamide gels (gradient 4%–12% or 10% gels, from Invitrogen), and subjected to standard immunoblotting protocols. To this aim, the following specific primary antibodies were employed: anti-p53 (DO-1; Santa Cruz), anti-LC3 (Cell Signaling), anti-Sestrin2 (Abcam) or anti-DRAM (Abcam) revealed with the appropriate horseradish peroxidase-labeled secondary antibodies (Southern Biotech, Birmingham, USA) and the SuperSignal West Pico chemoluminescent substrate (Pierce Biotechnology, Rockford). Anti-GADPH (AbCys, Paris, France) antibody was used to control equal loading.

Statistical analysis. Student's t-distribution probability density function was used for calculation of p values (*p < 0.01).

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SUMMARY

Macro-autophagy is a conserved catabolic pathway that culminates in the degradation of old/damaged organelles, long-lived/aggregated proteins and portions of the cytosol for metabolic recycling to maintain cellular homeostasis. The absence of autophagy is frequently observed in many pathologies including cancers and neurodegenerative diseases. Beclin 1, a *bona fide* tumour suppressor, is the key autophagy regulatory protein that participates in autophagosome nucleation. In fact, Beclin 1 is a BH3-only protein that can interact with the BH3 receptor domain contained within Bcl-2 and its homologues. This interaction functions as an inhibitory check on autophagy. Some pharmacological agents such as ABT737, referred to as ‘BH3 mimetics’, occupy the BH3-binding grooves to competitively disrupt the inhibitory interaction between Beclin 1 and Bcl-2/Bcl-X_L allowing Beclin 1 to maintain the class III phosphatidylinositol-3-kinase activity of Vps34 for the phagophore formation. Autophagy is a complex process that is regulated by multiple protein complexes beyond that organized around Beclin 1. The energy sensors including AMP-dependent kinase (AMPK), mammalian target of rapamycin (mTOR), Sirtuin1 (SIRT1) as well as stress-integrating pathways such as those involving the inhibitor of NF- κ B (I κ B) kinases (IKK) and the tumour suppressor protein p53, all have a major impact on the regulation of autophagy. In many paradigms of autophagic stimulation, they all seem to act upstream of the dissociation of Beclin 1-Bcl-2. Our results reveal that ABT737 stimulate multiple pro-autophagic pathways to be optimally efficient. These results place SIRT1, AMPK/mTOR, HDM2 and IKK downstream of the dissociation of the Beclin 1-Bcl-2 complex. This study advocates that BH3 mimetics trigger multiple autophagy-stimulatory pathways maybe due to the high degree of connectivity that exists among autophagy-regulatory protein complexes meaning that a specific effect on the Beclin 1-interactome might affect other nodes in the autophagy-controlling network. These pathways cannot follow a linear hierarchy and rather must be interconnected in a complex circuitry, in which stimulation of autophagy by physiological triggers (such as starvation or organelle stress) induce an ensemble of intimately linked changes that are coupled to each other in positive feed forward loops constituting an indissociable ensemble that composes the “autophagic switch”.

RÉSUMÉ

La macro-autophagie est une voie catabolique conservée dans l'évolution permettant la dégradation des organites endommagés ou vieux, des protéines à longue durée de vie ou agrégées et des portions du cytosol pour le recyclage métabolique afin de maintenir l'homéostasie cellulaire. L'absence d'autophagie est fréquemment observée dans de nombreuses pathologies incluant les cancers et les maladies neurodégénératives. Beclin 1, un suppresseur de tumeur, est une protéine clé dans la régulation de l'autophagie et participe à la nucléation de l'autophagosome. Beclin 1 est une protéine “*BH3-only*” pouvant interagir avec le site de fixation au domaine BH3 présent dans la protéine Bcl-2 et ses homologues. Cette interaction inhibe l'autophagie. Certains agents pharmacologiques tels qu'ABT737, appelés «*BH3 mimetics*», occupent le site de fixation du domaine BH3 de façon compétitive pour perturber l'interaction inhibitrice entre Beclin 1 et Bcl-2/Bcl-X_L. Ceci permet à Beclin 1 de maintenir l'activité classe III phosphatidylinositol-3-kinase de Vps34 pour la formation du phagophore. L'autophagie est un processus finement régulé par de nombreux complexes protéiques. Les senseurs de la charge énergétique comme l'AMP-dépendant kinase (AMPK), la cible mammalienne de la rapamycine (mTOR), la Sirtuin1 (SIRT1) ou les voies d'intégration du stress telles que celles impliquant l'inhibiteur des kinases NF- κ B (I κ B α) (IKK) et le suppresseur de tumeur p53, ont tous un impact majeur dans la régulation de l'autophagie. Dans de nombreux paradigmes de stimulation autophagique, ils semblent tous agir en amont de la dissociation Beclin 1-Bcl-2. Nos résultats révèlent qu'ABT737 stimule plusieurs voies pro-autophagiques pour obtenir une efficacité optimale. Ces résultats placent la SIRT1, AMPK / mTOR, HDM2 et IKK en aval de la dissociation du complexe Beclin 1-Bcl-2. Cette étude démontre que les *BH3-mimetics* activent des voies multiples de stimulation de l'autophagie, peut-être en raison du degré élevé de connectivité qui existe entre les complexes protéiques de régulation de l'autophagie. Cela signifie qu'un effet spécifique sur l'interactome de Beclin 1 peut affecter d'autres voies dans le réseau du contrôle autophagique. Ces voies ne semblent pas suivre une hiérarchie linéaire, mais doivent être plutôt interconnectées dans un circuit complexe dans lequel la stimulation de l'autophagie par des déclencheurs physiologiques (tels que la carence en nutriments ou le stress des organites) induit un ensemble de changements intimement liés et impliqués dans une boucle de régulation positive qui constituerait un ensemble indissociable composant l'«*autophagy switch*».

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