ISAS-INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

Exploring the transcriptional regulation of autophagic signaling

Thesis submitted for the Degree of Philosophiae Doctor

Candidate:
Tamara Copetti

Supervisor
Prof. Claudio Schneider

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TRIESTE

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SYNOPSIS of PhD Project

Multicellular organisms have evolved a number of highly regulated barriers to oncogenic and stressful stimuli, including apoptosis, autophagy and senescence. The tumor suppressor p53 plays a pivotal and pervasive role in setting and executing such failsafe programs. During the last years three negative regulators of p53 functions, which are often deregulated in cancer cells have been identified in Claudio Schneider laboratory at LNCIB, Trieste. These include: calpain, hGTSE-1 (Monte et al., 2003; Monte et al., 2004) (Bublik in preparation) and MAGE-A tumor antigens, (Monte et al., 2006). In particular calpain was shown to inhibit susceptibility to p53-dependent apoptosis (Benetti et al., 2001), to regulate the transcription competent β-catenin pool (Benetti et al., 2005; manuscript attached), to activate specific NF-kB survival pathways (Demarchi et al., 2005), and more recently to be involved in the regulation of the autophagy-apoptosis switch (Demarchi et al., 2006; Demarchi et al., 2007; manuscripts attached).

The research project described in this thesis stems from these previous findings and it is part of a broader project aimed to dissect the calpain/calpastatin network interference in autophagic signalling. The ultimate scope of the project is to understand the molecular basis by which these pathways can enhance cancer cell survival through the analysis of novel targets involved in the regulation of the specific failsafe programs.

In particular, in this work we focused on the transcriptional regulation of autophagic signalling. To this aim, using bioinformatic tools, we performed an in silico analysis of a set of autophagic genes promoters and identified beclin 1 as a novel NF-kB transcriptional target. Notably, three NF-kB binding sites with different scores emerged from the in silico analysis of a region located around 1100 bp upstream of the ATG sequence of beclin 1. By means of luciferase reporter assays, we demonstrated that p65 overexpression enhances beclin 1 promoter activity, while its depletion leads to a decrease of transcriptional activity.

Upon induction of autophagy by ceramide and tamoxifen, previously reported to induce beclin 1 transcription, p65 depletion didn't show any significant effect on beclin 1 promoter driven transcription, possibly due to the interference of other transcription factors.

Indeed, a 380bp 3' region of beclin 1 promoter encompassing the NF-kB putative binding site with highest stringency, was responsive both to ceramide and tamoxifen treatment.

A significant decrease of beclin 1 mRNA levels was observed after p65 depletion by means of qPCR experiments, clearly demonstrating that p65 is involved in the regulation of beclin 1 transcription. Moreover, data obtained by semi-quantitative RT PCR experiments, highlighted the existence of alternatively spliced forms of beclin 1 and suggest that a complex and finely tuned regulation of beclin 1 transcription might occur and lead to different autophagic outcomes (survival/death).

Furthermore, induction of p65 expression led to increased beclin 1 protein steady state levels in an inducible p65 cell line system. Consistently, a net decrease of beclin 1 protein levels was observed both in an inducible IkBα cell line and in an adeno-associated virus (AAV) encoding IkBSR infected cell system.

Accordingly, we found that p65 is required for autophagy induction both in basal conditions and following ceramide and tamoxifen treatment. Indeed, p65 depletion or IkBSR (super repressor) overexpression, are coupled to a clear reduction of LC3I to LC3II processing and autophagosome formation.

In the light of the data presented in this thesis, it is likely that NF-kB transcription factor activity is required for the proper regulation and for determining the final outcome of the autophagic process. Indeed, preliminary ChIP data suggest p65 binding to beclin 1 promoter. Other NF-kB target genes have been demonstrated to regulate autophagy showing both negative (e.g. Bcl-2, Bcl-X_L and IL-13) and positive (e.g. IL-2, HIF-1, BNIP3, PTEN and IFNγ) autophagy induction abilities. Given that NF-kB transcription factor activation is a complex event that triggers waves of transcription, we suggest that it could be required for proper autophagy regulation in different cellular contexts and following different autophagic stimuli, in such a way to maintain both cellular homeostasis and to regulate cell stress responses.

In the light of the obtained evidences, proper understanding of the relationship between NF-kB and autopahgy is thought to be fundamental to clarify the basic molecular mechanisms that determine cell death and survival during tumor progression.

INTRODUCTION TO NF-kB FAMILY OF TRANSCRIPTION FACTORS

Nuclear factor-kappa B (NF-kB) transcription factor family consists of a series of proteins conserved from the phylum Cnidaria to humans and absent in yeast and in Caenorhabditis elegans in which NF-kB genes have been probably lost. The Rel/NF-kB family is comprised of the vertebrate NF-kB1 (p50/p100), NF-kB2 (p52/p100), Rel A (p65), c-Rel and Rel B, the viral oncoprotein v-Rel, Xenopus X-Rel1 and the Drosophila Dorsal, Dif and Relish factors. Usually, the diction NF-kB indicates homo- and heterodimers formed by the five members of this family; the ability of these members to form a variety of homo- and heterodimers is responsible for the subsequent differential control of gene expression. Along with NF-kB other regulatory proteins namely IKK (IkB kinase) and IkB are fundamental for the proper activation and termination of NF-kB signaling.

NF-kB transcription factors and the pathways controlling NF-kB activation are best characterized in immune and inflammatory response (Hayden et al., 2006), but they also represent the nuclear effectors of an intricated cellular pathway activated in response to a large number of extracellular stimuli in many cells; indeed, it plays important roles in a myriad of physiological and pathological scenarios in the organisms and at the cellular level regulating the expression of hundreds of human genes that are involved in diverse and key cellular and organismal processes including cell proliferation, cell survival, the cellular stress response, inflammation, innate and adaptive immune responses, secondary lymphoid organ development, osteoclastogenesis and oncogenesis.

Targeted genes responsive to the activated form of NF-kB in immune response can be grouped into those encoding cytokines, cell surface receptors, acute phase proteins and viral genomes including HIV-1. Genes involved in cell cycle adjustment, apoptotic response and different kinases are also linked with NF-kB activation. Because of the ability of NF-kB to regulate a diverse set of genes, specific gene expression pattern induced by NF-kB should be expected to have a profound effect in determining different cellular phenotypes, such as differentiation or transformation and cell death or survival.

Structural domains of the NF-kB family of transcritpion factors and of their regulatory proteins IKK and IkB

NF-kB family of transcription factors

NF-kB family of transcription factors is composed of two subfamilies that differ for their structural organization at the C-terminal domain: the 'Rel' and the 'NF-kB' subfamilies that can either homo- or hetero-dimerize for determining gene expression specificity.

NF-kB transcripion factors subfamilies dimers recognize 9-10 base pair DNA sites called 'kB sites' which are characterized of great variability (5'-GGGRNWYYCC-3'; R, denotes a purine; N, denotes any base; W, denotes an adenine or thymine and Y denotes a pyrimidine base) and both these subfamilies share a highly conserved 300-amino acid N-terminal domain called Rel Homology Domain (RHD) (Baldwin, 1996; Huxford et al., 1998), responsible for dimerization, interaction with the IkB family of NF-kB inhibitors, nuclear translocation and DNA binding. The C-terminal end of the RHD each protein bears a nuclear localization signal (NLS) (Latimer et al., 1998). Crystallographic determination of RHD structure of several NF-kB family members shed light on the Ig-like structure of 100 amino acids located in the C-terminal of the RHD, and demonstrated that this subdomain is fully responsible for dimerization; for this reason it is referred to as DimD (Huang et al., 2001). In the formation of most of the dimers, each monomer contributes symmetrical β-strands that pack against each other to form a β-sheet dimer interface. The amino acid residues constituting the dimer interface are highly conserved across the whole NF-kB family. Nonetheless, the interactions occurring at the dimer interface are not the only contributors for dimerization selectivity in that amino-acid sequences located outside of the dimer interface are also thought to be crucial, especially when considering RelB that is unable to homodimerize and in vivo gives rise to a swapped, interwined domain (Huang and Ghosh, 2005).

The N-terminal subdomain (NTD) of the RHD is resulted to be crucial for NF-kB contacting the DNA. Just as the DimD, it folds into an Ig-like domain making both specific and aspecific contacts with DNA, while DimD domain can give rise only to sequence non-specific interactions; the linker peptide connecting the two subdomains, that is the DimD and the NTD, also makes both specific and non specific contacts with DNA (Hoffmann et al., 2006).

The Rel subfamily includes RelA (p65), RelB and c-Rel proteins which contain, besides the N-terminal RHD, a C-terminal transactivation domain that doesn't seem to be conserved across the species at the sequence level but that retains the ability of activating transcription in many species including yeast.

The NF-kB subfamily consists of NF-kB1 (p105) and NF-kB2 (p100) genes, which contain the N-terminal RHD and are distinguished by long C-terminal domains that contain multiple copies of ankyrin repeats (ARD/ANK) and that can function like IkB proteins to sequester NF-kB dimers in an inactive state in the cytoplasm. NF-kB proteins are synthesized as long precursors, and their C-terminal processing corresponds to the activation of NF-kB pathway, in order to generate two shorter active DNA-binding subunits, p50 and p52 respectively, that are finally able to regulate gene expression by homo- or heterodimerizing with members of Rel subfamily (Perkins, 2007).

Processing of p100 and p105 can occur through several machanisms depending on the upstream activation of the pathway; p105 infact, is activated both in the absence of cell stimulation and subsequently to the activation of the so called 'canonical pathway' (see below): in these settings, p50 generation depends on a co-translational mechanism as a consequence of proteolysis of the p105 nascent polypeptide by the 26S proteasome (Cohen et al., 2004). Finally, p105 calpain–mediated processing has been demonstrated to occur in response to ceramide treatment (Demarchi et al., 2005). Instead, p100 processing depends on the 'non canonical pathway' activation (see below), where p52 formation is the result of phosphorylation-induced, ubiquitin-dependent processing of p100 by the 26S proteasome (Bonizzi et al., 2004).

IkB Kinases: the IKK complex

Most of the diverse NF-kB activation signalling pathways converge on serine/threonine IkB specific kinases (IKK) which are essential in signal transmission since they are able to phosphorylate IkB inhibitory proteins and target them to proteasomal degradation.

Two major types of pathways leading to NF-kB activation have been described: the canonical pathway and the non canonical one, both of which require IKKs activity for NF-kB activation.

In the canonical pathway, the major player in IkB phosphorylation is a large 700-900 kDa complex with high specificity for serine residues in the destruction box of IkBα (Chen et al., 1996) and requiring ubiquitination for its full activation.

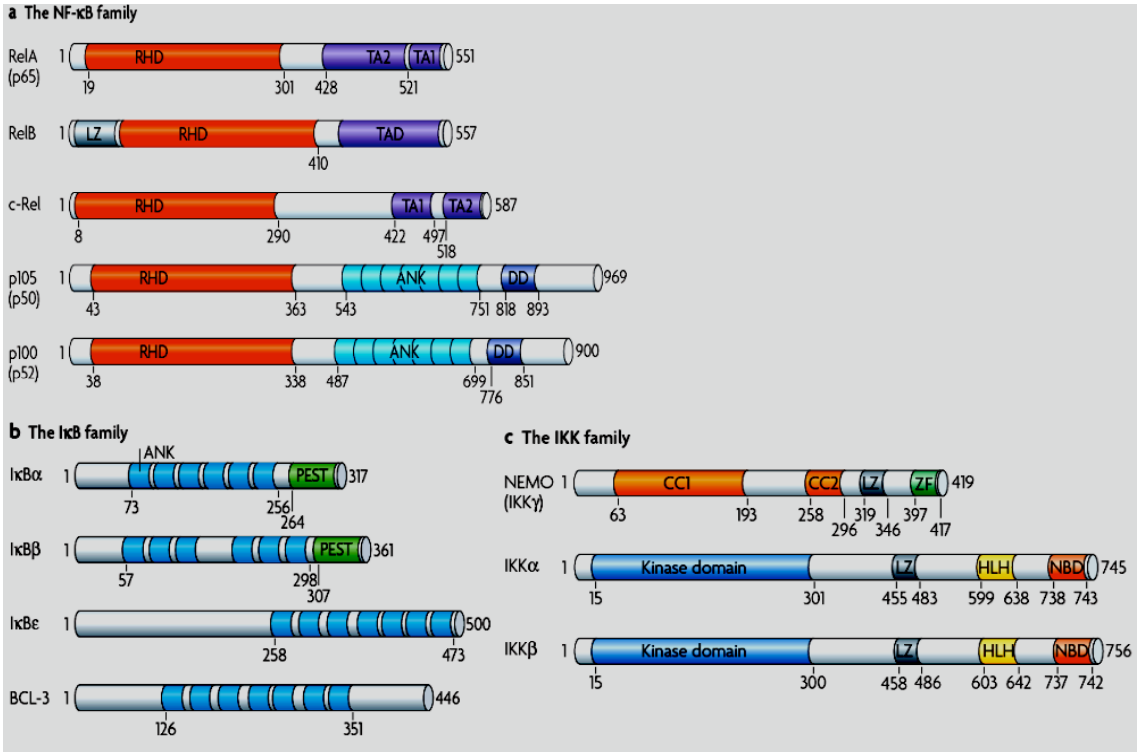

Figure 1: Mammalian members of the NF-kB, IkB and IKK families: members of each family and domains involved in their regulation are shown. (Perkins N.D., 2007; Nature)

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The basic components of this complex are IKKα, IKKβ active kinases and IKKγ/ NEMO (NF-kB essential modifier) regulatory subunit (DiDonato et al., 1997; Mercurio et al., 1997; Woronicz et al., 1997).

Both IKKα, and IKKβ contain an N-terminal kinase domain (KD) characterized by similar, but functionally different activation loops (T-loop): IKKβ is the primary target for proinflammatory stimuli and shows a higher catalytic activity towards IkBα than IKKα in the canonical pathway; in turn, IKKα is a more proficient kinase for p100 and plays a pivotal role in the activation of the non canonical pathway.

Of note, only IKKα KD bears a functional NLS (nuclear localization signal, aa 233-240) (Sil et al., 2004), while a ULD (Ubiquitin Like Domain, aa 307-384) is present within the IKKβ KD and it has been demonstrated to be crucial for its functional activity (May et al., 2004).

Other structural components of IKKα and IKKβ are the more C-terminal leucine zipper (LZ) and putative helix-loop-helix (HLH) motifs and the NEMO binding domain (NBD). LZ domain mediates dimerization of the kinases that is required for their activity (Karin, 1999). HLH domain intramolecular association with KD switches on IKK kinase activity and seems to be important in the postinductive downregulation of

the kinase itself (Hayden and Ghosh, 2004); finally, both IKKα and IKKβ bind to NEMO regulatory subunit via a short conserved sequence referred to as NEMO binding domain (NBD) and located at distal C-terminal.

The third core component of the IKK complex is the non-catalytic regulatory protein NEMO (IKKγ, IKKAP1, or Fip3). NEMO protein is highly conserved and represents an absolute requirement for IKKα and IKKβ activity: cells lacking NEMO cannot be activated by any of the classical NF-kB stimuli (TNFα, IL-1 or LPS). At the structural level, NEMO contains three coiled-coil regions (CC1-3), a leucine zipper (LZ) motif, a ubiquitin binding domain (UBD) located within the minimal oligomerization domain (MOD), and a C-terminal zinc finger (ZF) required for NEMO activation in response to genotoxic stress (Huang et al., 2002). NEMO oligomerization is necessary for IKK complex activation, in that once in an oligomeric state, NEMO can act as a scaffold allowing the cross-phosphorylation of the kinase dimers. N-terminal specific sequences of NEMO are then important for its interaction with IKKα and IKKβ (aa 1-196 and aa 65-196 for IKKα and IKKβ binding, respectively) and represent NEMO's kinase binding domain (KBD) (Tegethoff et al, 2003).

IkB proteins

IkB family members are characterized by the presence of five to seven ankyrin repeats (ARD/ANK) that assemble into elongated cylinders that bind the NF-kBs dimerization domain, such that their nuclear localization signal (NLS) is masked leading to cytoplasmic retention of the complex (Perkins, 2007). ARD domains seem to mediate also IkBs nuclear localization (Turpin et al., 1999). C-terminal and N-terminal regions of IkBs also contain leucine-rich nuclear export sequences (NES) (C-term aa 265–277; N-term aa 42-54) that are recognized by CRM1 receprtor and promote IkB nuclear export (Henderson and Eleftheriou, 2000).

There are seven IkB family members: IkBα, IkBβ, IkBε, IkBγ and BCL-3; p100 and p105 (p52 and p50 precursors respectively) also belong to this family, in that they present ARD/ANK domain in their C-terminal that inhibits NF-kB signalling. Conversely, BCL-3 IkB family member is distinguishable for its ability to interact with p50 and p52 and function as a nuclear co-activator rather than as a repressor of NF-kB signalling (Zhang et al., 2007).

IkBα, IkBβ, IkBε contain a conserved motif at the N-terminus consisting of two serine residues susceptible to phosphorylation by IKKβ, and one or two inducibly ubiquitinated lysine residues; both the phosphorylation and ubiquitination events are crucial for IkB targeting to proteasomal degradation.

Besides the ARD/ANK domain and the N-terminal motif previously described, IkBα and IkBβ proteins present in their C-terminal a motif associated with protein degradation termed PEST (domain rich in Proline); serine and threonine residues within this domain are phosphorylated by Casein Kinase II (CK2) that is activated by atypical NF-kB pathways and this event seems to trigger calpain mediated proteolysis of IkBα (Romieu-Mourez et al., 2002). Furthermore, it has been reported that following serum starvation and consequent increase of reactive oxygen species (ROS), an IkB long-lived pool underwent to selective lysosomal proteolysis. This degradation event seemed to require lgp96 (lysosomal glycoprotein-96 protein) and binding of hsc-73 (heat shock protein-73) to a KFERQ motif of IkB. This alternative IkB degradation mechanism is phosphorylation and ubiquitination independent, suggesting that, during cell starvation, chaperone mediated autophagy rather than the proteasome, might trigger IkB degradation (Cuervo et al., 1998).

Signaling Pathways to NF-kB: the 'canonical', the 'non-canonical' and the 'atypical' pathways

As discussed above, IKK ser/thr kinases act as a bottleneck for all the pathways that converge on NF-kB. IKK kinases phosphorylate and trigger the degradation of IkB family members, then enhancing NF-kB transactivation of target genes. Nonetheless these pathways are characterized by significant differences for the great variability among the selectivity and kinetics of IkB degradation, the different IKK and adaptor proteins and NF-kB dimers involved, leading to consequent different persistences of NF-kB activation. Indeed, all these aspects confer to individual NF-kB responses the ability to generate waves of activation and inactivation of various NF-kB family members that can influence the nature of the transcriptional response to a given stimulus (Hayden and Ghosh, 2004).

Further complexity to NF-kB signalling has emerged recently, with the characterization of atypical pathways involving IKK-independent mechanisms of activation as well as different utilization of IKK activity.

The canonical NF-kB pathway

The canonical or classical NF-kB pathway is the most frequently observed, it occurs ubiquitously and is induced in response to a variety of stimuli such as TNFα (Tumor necrosis Factor alpha), IL-1 (Interleukin 1), engagement of the T-cell receptor (TCR) and of B-cell receptor (BCR), bacterial infection, stimulation with lipopolysaccaride (LPS) etc.

Consequently to the engagement of the receptor, the signal is transduced through adaptor molecules to the IKK complex that phosphorylates IkBα, targeting it for proteasomal degradation. The whole process occurs within minutes and is finely regulated at each level.

TNFR engagement represents the more explicative model of activation of NF-kB classical pathway and results in NF-kB, p38, JNK and caspase activation.

Consequently to trimeric TNFα binding, TNFR oligomerizes, dissociates from SODD (Silencer of death Domain) endogenous inhibitor of TNFR, and becomes active. This receptor family lacks intrinsic enzymatic activity and needs to recruit adaptor molecules for transducing the signal through its cytoplasmic TNFR-associated death domain (TRADD) (Perkins and Gilmore, 2006). TRADD, consequently, directly recruits downstream TNF-receptor associated factor (TRAF) family of adapter molecules. In particular, TRAF2 and TRAF5 seem to be required specifically for NF-kB signalling via TNFR, in that TRAF2/5 double knockout cells show impaired TNF-induced IKK activation (Tada et al., 2001). Following TNFα stimulation, TRAF2 directly recruits IKKα and IKKβ by binding to the relative LZ domains (Devin et al., 2001). TRAF2 is also responsible for Receptor Interacting Protein 1 (RIP1) kinase recruitment that in this setting works as a scaffold molecule becoming K-63 ubiquitinated due to the E3-ligase actitvity of TRAF. Consequently RIP1 can nucleate the assembly of a signaling complex that leads to IKK activation. Notably, TRAFs E3 ligase activity is also fundamental for K-63 ubiquitination of NEMO; polyubiquitin binding domains (UBDs) of these proteins mediate their recruitment in a signaling complex where activation of IKK by trans-auto-phosphrylation can occur (Hayden and Gosh 2004; Scheidereit, 2006) thereby activating IKKβ. Activated IKKβ then phosphorylates IkBα at serines 32 and 36, which causes IkBα to become a substrate for the Skp1, Cdc53/Cullin1, F-box protein $^{\beta\text{transducin repeat containing protein}}$ ($SCF^{\beta TrCP}$) E3 ligase complex, and to be targeted to proteasomal destruction. This event causes the release of p50-, p65- and c-Rel

containing heterodimers and allows them to relocate to the nucleus. Once in the nucleus p50/p65, and p50/c-Rel heterodimers can bind and transactivate antiapoptotic genes among which Bcl-X_L, cIAP-1 and 2, FHC (Ferritin Heavy chain), MnSOD (Manganese Superoxide Dismutase), IkBα and many others.

The canonical NF-kB pathway can be terminated in several ways, the more striking of which occurs through a negative feedback: being the induction of IkBα transcription activated by NF-kB, newly synthesized IkBα can enter the nucleus, bind NF-kB heterodimers and relocalize them into the cytoplasm. Two other IkB family members that are not regulated by NF-kB, IkBβ and IkBε can inhibit this signaling. Other negative feedback loops also exist, in that genes encoding de-ubiquitinating enzymes such as Cylindromatosis protein (CYLD) and A20, which can degrade ubiquitinated K63 chains of TRAF and NEMO and counteract NF-kB activation process, are both RelA/c-Rel/p50 transcriptional targets. Conversely, positive feedback loops can also occur: c-Rel and Rel-B genes, together with the precursor proteins NF-kB1 (p105/p50) and NF-kB2 (p100/p52), contain kB elements in their promoter and this implies that the composition of NF-kB complex can change over time resulting in temporal changes in the spectrum of genes that will be induced and repressed (Saccani et al., 2003). Another positive feedback loop leading to fine regulation of NF-kB signalling, has been demonstrated to occur consequently to serum starvation: in this setting, an increase in ROS intracellular levels leads to NF-kB activation; contemporarily, a long-lived IkB pool undergoes a selective pathway of lysosomal proteolysis. This long-lived pool of IkB might allow cells to increase NF-kB transactivation potential more slowly than the proteasome pathway regulating in this manner long-lasting NF-kB signalling (Cuervo et al., 1998).

The non canonical NF-kB pathway

Non canonical or alternative NF-kB pathway is activated in response to a limited number of stimuli such as stimulation of CD40, lymphotoxin-β receptor (LTβ), B-cell activating factor of the TNF pathway (BAFF), and some viruses Human T-cell Leukaemia virus (HTLV-1), latent membrane protein-1 (LMP-1) of Epstein-Barr virus (EBV), Tax Kaposi's sarcoma-associated Herpes Virus (KSHV), viral FADD-like interleukin-1-β-converting enzyme (FLICE/ caspase8)-inhibitory protein (vFLIP) and specific engagement of members of TNF receptor associated factor (TRAF)-binding receptors of TNF receptor superfamily (Hauer, et al, 2005).

Of note, activation of non canonical pathway does not require the formation of IKKα, IKKβ and IKKγ complex, rather proceeds through IKKα homodimer formation and activation. Actually there is no direct evidence supporting the involvement of a separate IKK complex composed exclusively by IKKα homodimers in the non canonical pathway. Nevertheless a major role for IKKα emerges from the analysis of IKKβ and IKKγ knockout cells in which these two subunits seem to be dispensable for this alternative pathway activation (Bonizzi and Karin, 2004).

Activation of the non canonical pathway proceeds via NF-kB inducing kinase (NIK) which phosphorylates the T-loop serines of IKKα, leading to its activation and dimerization. NIK activated IKKα dimer, in turn, phosphorylates p100 within its C-terminal domain at Ser-866 and Ser-870 (Senftleben et al., 2001, Perkins, 2006). These phosphorylations create an $SCF^{\beta TrCP}$ binding site on p100 and trigger K48-linked ubiquitination at K-856 of p100, then inducing its processing to p52. In most of the circumstances the final outcome of this process is p52 association with RelB (Bonizzi and Karin, 2004). p52/RelB heterodimer then shuttles into the nucleus, and binds with higher affinity to distinct kB elements, resulting in the regulation of a specific subset of genes (Hoffman et al., 2006).

Interestingly, activation of non canonical pathway by viral Tax and vFLIP proteins again occurs through a physical recruitment of IKKα to p100, but does not require NIK for p100 processing (Xiao et al., 2001).

A feature of the alternative pathway is the slow kinetic of the onset of p100 to p52 conversion, that requires several hours compared to the fast IkBα processing that occurs within minutes. The alternative pathway also results in long-lasting NF-kB activation and *de novo synthesis* is required, while in the canonical pathway it is not. It is very well estabilished that non canonical signaling induced by both CD40 or BAFF receptor induces TRAF3 degradation and concomitant enhancement of NIK expression and this event requires *de novo synthesis*. In this context, TRAF3 acts as an inhibitor of the pathway, interacting directly with NIK and targeting it for proteasomal degradation (Liao et al., 2004).

Interestingly, recent data indicate that autophagic pathway may represent an alternative to proteasomal TRAF3 targeted NIK degradation. Infact, inhibition of Hsp90 by Geldanamycin treatment induces NIK degradation through an autophagic process and in

this settings, it may suppress NIK induced p100 processing, typical of the NF-kB alternative pathway (Qing et al., 2007).

Atypical pathways of NF-kB activation

Although the canonical and alternative pathways account for most of the physiological inducers of NF-kB, there is growing evidence that different physiological and not-physiological stimuli trigger NF-kB nuclear localization and DNA binding. These alternative mechanisms of NF-kB activation can be both IKK-dependent or -independent.

Concerning IKK-independent mechanisms, it has been demonstarted that short wavelength ultraviolet (UV-C) light or expression of Her2/ erbB-2/Neu oncogene can lead to casein kinase II (CK2) dependent phosphorylation and to subsequent degradation of IkBα (Kato et al, 2003; Ramieu–Mourez et al., 2002). In these circumstances, IkBα phosphorylation by CK2 occurs at serine residues within its C-terminal PEST domain instead of at classical N-terminal serines 32/36. UV-C light CK2 dependent phosphorylation of IkBα also depends on p38 MAP kinase pathway (Kato

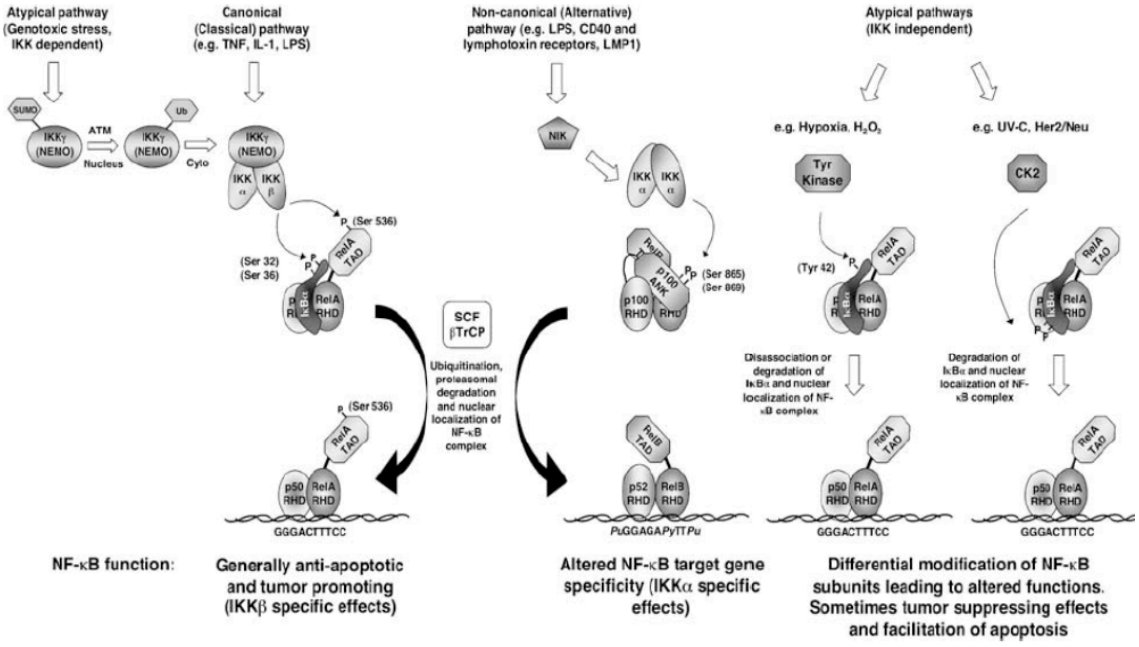


Fig 2: Pathways leading to NF-kB activation: canonical, non-canonical and atypical pathways (ND Pekins and TD Gilmore, 2006; Cell Death Diff.)

et al., 2003), while with Her2/erbB-2/Neu, IkBα degradation results from calpain activity rather than from the proteasome.

Other IKK-independent mechanism of NF-kB activation have been proposed to be activated in response to UV-C, hypoxia/reoxigenation, hydrogen peroxide or pervanadate treatment and nerve growth factor (NGF) stimulation. In these circumstances, IkBα phosphorylation occurs at tyrosine-42 and probably requires C-terminal PEST sequence for IkBα degradation to take place (Schoonbroodt et al., 2000; Perkins, 2007). However, in other settings, thyrosine-42 phosphorylation of IkBα does not target it for degradation and rather enhances Ikbα dissociation from NF-kB complexes (Perkins, 2007). Interestingly, as activated IKKα and IKKβ also phosphorylate RelA, these IKK-independent pathways have the potential to induce differentially modified forms of NF-kB subunits with distinct functions.

Some atypical mechanisms of NF-kB induction can be IKK dependent, but exhibit functional differences to classical and alternative IKK-dependent activation pathways. Genotoxic stimuli such as ionizing radiations or chemotherapeutic drugs (e.g. camptothecin and etoposide), lead to an NF-kB induction that requires IKKγ-dependent IKKβ activation. Here, NEMO translocates into the nucleus where it is first sumoylated on Lys-277and Lys 309 and then phosphorylated by the ataxia telangiectasia mutated checkpoint kinase (ATM) on Ser-85; sumoylation on NEMO is then replaced by mono-ubiquitination that causes NEMO to leave the nucleus as a complex with ATM (Perkins, 2007). The NEMO-ATM complex, once in the cytoplasm, can activate IKKβ through a process requiring IKK-associated protein ELKS (a protein rich in E, L, K and S residues) (Wu, et al., 2006). However, it remains to be determined whether this atypical pathway is engaged by all genotoxic stimuli and in all cell types.

The existence of these alternative NF-kB pathways underlines the heterogeneity of the mechanisms leading to NF-kB activation; indeed some stimuli such as UV-C, are able to activate both IKK-dependent and -independent alternative pathways.

Post-translational modifications regulating NF-kB activity and function

The enormous complexity that characterizes NF-kB, IkB and IKK post-translational modifications has been elucidated in recent years and has shed light on their contribution in enabling the fine control of NF-kB function. These regulatory modifications which include phosphorylation, ubiquitination, acetylation, sumoylation

and nitrosylation, determine the specificity of NF-kB regulated gene expression and its functionality as a regulator of critical cellular pathways such as cell growth, cytokine and chemokine secretion and apoptosis.

IKK complex

Induction of NF-kB by many stimuli requires activation of IKK through different postranslational modifications.

The first step in IKK complex activation involves IKKγ modification through K-63 linked ubiquitination. In the case of NEMO, different sites of ubiquitination and different E3 ubiquitin ligases are involved in its K-63 ubiquitination, depending on the activating upstream stimulus: TCR activation resullts in Bcl-10/MALT1-mediated and TRAF-6 dependent K63 ubiquitination at lysine 399, while TNFα and IL-1 require TRAF2 and TRAF5. By contrast, bacterial infection seems to require nucleotide binding oligomerization domain (NOD2/CARD15) and receptor interacting protein 2 (RIP-2) for K-63 ubiquitination of NEMO at lysine 285. In the classical pathway K-63 linked ubiquitinated IKKγ behaves as a K-63 linked polyubiquitin binding protein and binds polybiquitinated RIP-1. Ubiquitination removal is due to CYLD or A20 activation and is required for terminating NF-kB response (Perkins, 2006).

This cascade of modifications, therefore, serves to oligomerize and activate the pathway. Polyubiquitination of adapter and signalling proteins allows, for example, TAK1 K-63 ubiqutinated kinase targeting to IKK complex in response to TNFα, IL-1, TLR and TCR mediated activation of NF-kB. TAK, in turn, phosphorylates IKKβ within the serine loop at Ser-177 and 181 and renders it competent for IkBα phosphorylation response (Perkins, 2006).

Other kinases involved in IKKβ activation have been described, such as PDK1, SGK and MEKK3 (Perkins, 2006; Tanaka et al., 2005), although the mechanism by which these kinases are targeted to IKK complex is not known. Moreover, c-Src-dependent phosphorylation at Tyr-188 and Tyr 199 in response to TPA (12-O-tetradecanoyilphorbol-13-acetate) and TNFα has also been reported. In addition to all these kinases, autophosphorylation of IKKβ is catalysed by oligomerization of the whole complex and requires IKKβ mono-ubiquitination at Lys-163 (Carter et al., 2005).

IKKβ is also subjectected to S-nitrosylation at Cys-179 within the activation loop, and this modification inhibits IKKβ activity (Reynaert et al., 2004); interestingly, the same nitrosylation is required for some natural product-mediated inhibition of IKKβ (Chen et al., 2006; Perkins, 2006).

A further regulatory post-translational event is represented by IKKβ-mediated IKKγ phosphorylation at Ser-31, Ser-43 and Ser-376, and might serve to potentiate IKK complex activation (Perkins, 2006).

Different IKKγ modifications are then induced by genotoxic stress. As already reported in these circumstances IKKγ is first sumoylated on Lys-277 and Lys-309, then phosphorylated by ATM on Ser-85 and finally initial sumoylation is replaced by mono-ubiquitination (See Atypical Pathways).

Alternative IKKα modifications take place in response to activation of NF-kB non canonical pathway. Here, NIK-mediated activation of IKKα occurs through phosphorylation at IKKα Ser-176 and Ser-180 and in some cell types Akt/Protein Kinase B dependent phosphorylation of IKKα at Thr-23 has been reported (Hacker and Karin 2006).

Modifications of IkB proteins

IKKβ principal role in activating NF-kB signalling is phosphorylation of IkBα (Hayden and Gosh, 2004) that represents the major NF-kB inhibitor. IkBα, β or ε proteins, contain N-terminal IKKβ target serine residues that, once phosphorylated, trigger their own SCF-βTrCP E3 mediated K-48-linked ubiquitination and proteasomal degradation with different kinetics. Other kinases than IKKβ can phosphorylate IkBα: as already discussed, CK2 engagement following UV-C light exposure or overexpression of Her2 oncogene, allows IkBα phorsphorylation at Ser-283, Ser-289 and Ser-293 or at Thr-291, and Thr-299 residues inside the PEST C-terminal domain, and these modifications target IkBα for degradation. Conversely, CK2-mediated IkBβ phosphorylations at Serines 313 and 315 can block its NF-kB inhibitory activity. IkBα has also been reported to be inhibited by p56-lck, Syk and c-Src tyrosine kinases following hypoxia/reoxygenation, hydrogen peroxide exposure and NGF treatment by direct phosphorylation on Tyr-42 residue.

Another post-translational modification affecting IkBα activity is sumoylation at Lys-21 by SUMO-1 (small ubiquitin-like modifier 1), which counteracts IkBα ubiquitination at

the same residue. This event renders IkBα refractory to proteasomal degradation. Interestingly, IkBα sumoylation seems to be inhibited by IkBα N-terminal phosphorylation (Perkins et al., 2006).

Bcl-3 IkB family member is also highly susceptible to phosphorylation, but little is known about the sites and the kinases involved, and only GSK3β kinase has been demonstrated to induce Bcl-3 degradation by phosphorylating it at Ser-394 and –398 in unstimulated cells. Furthermore, K-63-linked polyubiquitination of Bcl-3 has been demonstrated to regulate its nuclear localization (Viatour et al., 2004).

Modifications of NF-kB subunits

As already discussed, non canonical and canonical pathways activation occur through different post-translational modifications of NF-kB subunits. Processing of p105 to p50 in the canonical pathway takes constitutively place in a co-translational manner, and inducible degradation of p105 can also occur without p50 generation (Perkins, 2007). Ikkβ has been shown to phosphorylate p105 at Ser-927 and Ser-932, and this event targets p105 to $SCF^{-\beta TrCP}$-mediated ubiquitination and subsequent proteasomal complete degradation. Interestingly, IKKβ can also induce $SCF^{-\beta TrCP}$ independent processing of p105, that in this case leads to p50 generation (Perkins, 2006).

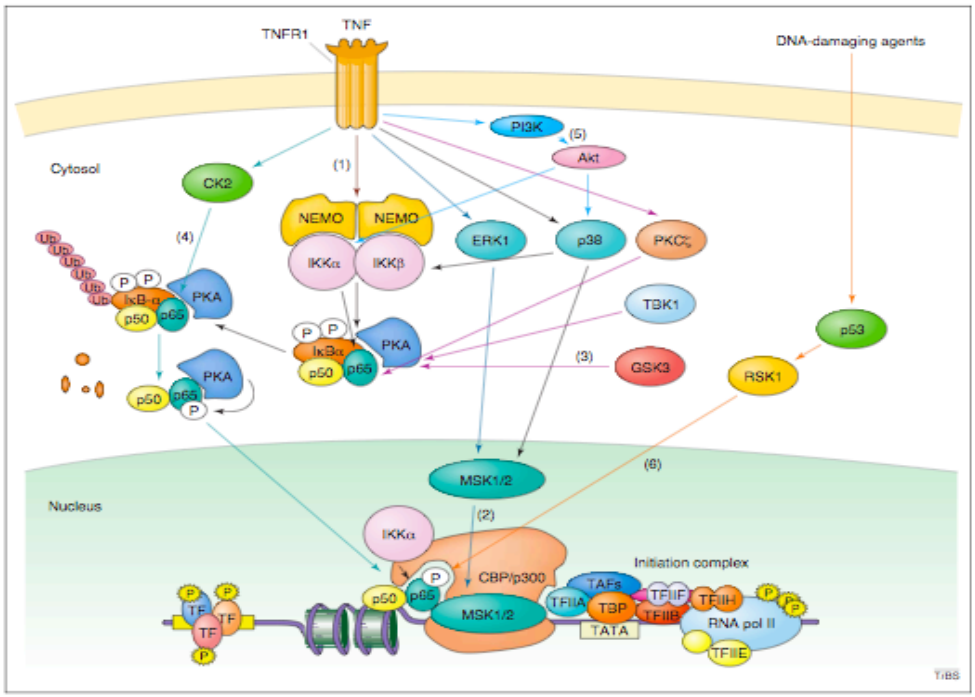


Fig.3: Modulation of the transcriptional activity of p65 phosphorylation (Viatour P., Merville M-P., Bours V. and Chariot A; TiBS, 2005)

In resting cells, p105 is phosphorylated by GSK3β at Ser-903 and Ser-907, causing p105 stabilization and p105 processing in response to TNFα stimulation (Demarchi et al., 2003). p105 processing has also a regulatory role, indeed it seems to be required for Tpl2/COT kinase release and MAP kinase pathway signalling. When physically associated with p105, Tpl2 kinase is unable to phosphorylate MEK1 substrate (Babu et al., 2006), but it retains the ability to phosphorylate p105, indicating that its kinase activity is not impaired by the association with p105.

Processing of p100 involves IKKα activity: phosphorylation occurs at Ser-866 and Ser-870 of p100 and results in $SCF^{-\beta TrCP}$-mediated K-48 linked polyubiquitination of p100 on Lys 855 (Amir et al., 2004). Following this initial phosphorylation events, IKKα further phosphorylates p100 at additional serine residues within the Rel Homology Domain, in order to ensure correct p100 to p52 processing or in order to regulate proper p52 dimerization or DNA binding (Perkins, 2006).

Rel members need also to undergo cytoplasmic modifications for efficient and specific signaling. RelA is phosphorylated by PKA (protin kinase A) at a highly conserved Ser-276; also p50 and c-Rel undergo PKA-mediated phosphorylation at equivalent sites of RelA Ser-276 (Guan et al., 2005). In the nucleus, the same Ser-276 residue of RelA (and the equivalent residue of c-Rel) undergoes MSK1 kinase mediated phosphorylation and this modification seems to disrupt intramolecular interactions between RelA N-and C-termini, allowing DNA binding and p300/CBP co-activators recruitment (Perkins, 2006). Thr-254 RelA phosphorylation by an unknown kinase is thought to be necessary for pThr-254Pro motif generation in RelA. This motif allows peptidyl-prolyl isomerase (Pin1) binding, favouring disruption of RelA/IkBs interactions and RelA translocation into the nucleus. Pin-1 also protects RelA from SOCS-1-mediated ubiquitination and degradation (Ryo et al., 2003). As Thr-254 is within RelA RHD Pin-1 could also be expected to affect RelA DNA binding and transcriptional activity.

Multiple RelA phosphorylation sites and candidate kinases have been reported to have a modulatory role on its transcriptional activity. Phosphorylation at Ser-536 and Ser-529 residues within the TA1 subdomain can stimulate RelA transactivation (Viatour et al., 2005) and have been detected in response to many inflammatory stimuli. Multiple Ser-536 kinases have been described: IKKβ, IKKα, IKKε, NF-kB activating kinase (NAK/TBK1/TANK-binding kinase-1), and RSK1 (Perkins, 2006). By contrast, only CK2 has been demonstrated to phosphorylate Ser-529. TA1 contains many other

phosphorylable residues, suggesting that other regulatory events might occur in this region. Other phosphorylation sites within the TA2 subdomain of RelA have been recently characterized. Ser-468 is a target for GSK3β, IKKε and IKKβ phosphorylation in response to TNF, IL-1β and T-cell stimulation. These modifications can both activate or inhibit NF-kB signalling and occur in different cellular compartments. Thr-505 phosphorylation within TA2 inhibits RelA transactivation by inducing increased association with HDAC1 and requires ARF mediated Chk1 checkpoint kinase activation (Rocha et al., 2003; Campbell et al., 2006). Phosphorylation on Thr-435 by an unknown kinase has also been reported to inhibit RelA activation (Perkins, 2006). It is noteworthy that these phosphorylations occur within evolutionarily conserved TA subdomains, suggesting that they regulate RelA association with co-activators and corepressors.

RelA is also inducibly acetylated at a number of sites, and these modifications have different effects on its activity. CBP and p300 co-activators acetylate RelA at Lys-218, -221 and-310 and enhance RelA DNA binding and transcriptional activity. Of note, RelA Ser-276 and –536 phosphorylation results in an increased p300 binding and therefore in enhanced RelA acetylation at Lys-310 (Perkins, 2006).

With regard to the other NF-kB subunits, PKCζ, NIK and IKKε inducible phosphorylation of c-Rel occurs at different serine residues and generally stimulates c-Rel activity. RelB Ser-368 (Ser-276 equivalent of RelA) phosphorylation regulates its dimerization with p52. p50 DNA binding activity is enhanced by Lys-431, -440 and –441 acetylation. p52 acetylation leads to different outcomes: it is, infact, thought to either induce p100 to p52 processing or to increase its DNA-binding activity (Deng et al., 2006).

Biological roles of NF-kB and IkB proteins

The signaling pathways that regulate life and death of a cell are the subject of intense biological studies, since mutations or misregulation of components of these pathways often give rise to defects in apoptosis and are implicated in a large number of diseases, such as neurodegeneration and cancer.

NF-kB family of transcription factors can be found in all cell types and has revealed a great diversity of functions and context in which it becomes activated. NF-kB proteins, infact, are regulators of the immune, inflammatory, stress, proliferative and apoptotic responses of a cell to a very large number of stimuli. Moreover, many reports suggest

apparently opposing or contradictory functions for NF-kB. Furthermore, NF-kB response is integrated with important pathways in the cell, such as p53 tumour suppressor pathway, JNK pathway and others (Dutta et al., 2006; Hoffman et al., 2006; Tergaonkar and Perkins, 2007).

NF-kB was first identified as a regulator of the expression of the kappa light chain gene in murine B-lymphocytes, and most of initial research had focused on understanding its function in the innate and adaptive immune responses. The importance of the NF-kB family members in the regulation of various aspects of immune system development, immune response and inflammation as well as in regulating apoptosis in the immune system, in the liver, nervous system, hair follicles and epidermal appendages, has been very well unveiled by studies on transgenic or knockout NF-kB mice (Tripathi and Agarwal, 2006).

Concerning the innate immune response an essential involvement of classical NF-kB pathway has been demonstrated, in that its activation is associated with increased transcription of a number of genes coding for chemokines, adhesion molecules and cytokines, all of which are required for migration of inflammatory and phagocytic cells to tissues and for pathogen destruction (Kucharczak et al., 2003). In these settings different Toll-like receptor (TLRs) isotypes are competent for specific pathogen pattern recognition, and activate NF-kB transactivation of a plethora of genes encoding inflammatory molecules and enzymes (IFNγ, IL-12 and iNOS). For example, TLR4 is the receptor for LPS component of Gram-negative bacteria and for some viruses; TLR2 recognizes a larger variety of microbial products (peptidoglycans and lipoproteins) and TLR5 recognizes bacterial falgellin; TLR3 and TLR9 recognize bacterial DNA or double-stranded RNA, respectively. Cellular types involved in innate immunity response are monocytes and macrophages, but also non-immune cells such as fibroblasts, endothelial and epithelial cells (Tripathi and Aggarwal, 2006).

Many of the events involved in innate immune response are also critical to subsequent adaptive immune response. In this context, survival of peripheral blood cells in response to antigens depends on BCR-mediated activation of NF-kB and induction of antiapoptotic target genes. c-Rel knockout primary B cells undergo apoptosis upon mitogenic stimulation; lack of p50 demonstrated that this member of NF-kB family is important for the survival of quiescent B-cells; similar impairments in B-cell proliferation and survival has emerged from IKK-β and IKK-γ knockouts. Furthermore, c-Rel and RelA double knockout caused B cell failure to mature IgM(lo) and IgD(hi).

Attenuation of apoptosis in response to BAFF-R engagement has been found to be necessary for B-cell development. All these phenotypes have shed light on a protective role of NF-kB in B-cell survival, due to the ability of these transcription factors to transactivate antiapoptotic genes such as Bcl-2, Bcl-XL and Bfl1/A1 (Tripathi and Aggarwal, 2006). B-cell activation and effector function of NF-kB has been clearly demonstrated by studies performed on mice deficient in NF-kB1, NF-kB2, RelA, c-Rel or BCL-3 genes and in all these models a compromised humoral response was observed due to defects in B-cell maturation and proliferation or to defective immunoglobulin class switching. NF-kB2, RelB and c-Rel have also important functions in hematopoietic and non-hematopoietic lineages and regulate second limphoyd organs development and architecture.

NF-kB family members are also directly involved in regulating T-cell functions, since an adaptive T-cell response is balanced by proliferation and expansion of antigen-specific T-cell during the initiation of the response and the loss of excess T-cells as the response resolves. In this case TCR engagement and co-stimulatory signalling by CD28 is required for full activation of naive T-cells. This event triggers NF-kB activation and subsequent cell survival via NF-kB mediated induction of antiapoptotic genes Bcl-2 and Bcl-XL (Zheng et al., 2003). Analysis of activated CD4+ or CD8+ T cells indicates that while NF-kB mediated induction of Bcl-XL and Bfl1/A1 is transient, Bcl-2 activation is delayed and IL-2 dependent (Verschelde et al., 2003). It has also been reported that E2F1 and p73 are involved in induction of apoptosis in mature T cells (Green, 2003), and in these settings a NF-kB dependent mechanism might antagonize cell death. Indeed, the identification of a kB site in the vicinity of p73 first exon suggested that NF-kB might interfere with E2F mediated transactivation of p73, counteracting, in this way, cell death (Kucharczak et al., 2003). All these evidences reveal a mechanism underlying the protective effects of inflammation and innate immunity towards T-cell apoptosis in the adaptive immune response (Gerondakis and Strasser, 2001). In contrast, NF-kB contribution to apoptosis has also been reported in mature T cells during activation-induced cell death (AICD) and it is imputable to NF-kB induction of FasL expression and subsequent activation Fas-death cascade (Zheng et al., 2001).

As illustrated, NF-kB represents a key player of the inflammatory response for its ability to increase the expression of many cytokines, enzymes and adhesion molecules, and its deregulation has been found to be involved in many inflammatory chronic diseases such as asthma, rheumatoid arthritis, psoriasis, inflammatory bowel disease and

allergic diseases. All chronic inflammatory pathologies show an increased production of IL-1β, TNF-α, IL-5, IL-6, GM-CSF and iNOS enzyme and many therapeutic intervention is then oriented in trying to suppress NF-kB activation. NF-kB is, indeed, the target of many anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and immunosuppressant drugs, and several natural products (Gilmore and Herscovitch, 2006).

NF-kB, as previously discussed, represents a key player in the development and selection of T- and B-lymphocytes and it is critical for maintainance of immunological homeostasis through its ability to regulate apoptosis.

Other in vivo studies on $RelA^{-/-}$ mice provided clear evidence for NF-kB role in preventing apoptosis in different contexts. In fact, RelA deficient mice die as embryos manifesting massive liver apoptosis as a consequence of circulating TNF stimulation (Beg and Baltimore, 1996; Alcamo et al., 2001), a phenotype observed also in IKKβ deficient mice. Further studies demonstrated that RelA cytoprotective function in response to TNFα and other pro-inflammatory molecules was due to its ability to induce several survival genes, such as TRAF-1, TRAF-2, c-IAP1, c-IAP2, IEX-IL, Bcl-X_L and BfI-1/A1. On the other hand, kB binding sites have been found also in the promoters of IL-1β converting enzyme protease, c-myc and TNF-α genes, which are commonly involved in signal-induced programmed cell death. Studies in hepatocytes treated with TGF-β (transforming growth factor-β) unveiled again a protective role for NF-kB, as TGF-β induces apoptosis by stabilizing IkBα and inactivating NF-kB (Kucharczak et al., 2003).

NF-kB has been demonstrated to have an essential role in the development, survival and homeostasis of hair follicles and epidermal appendages (skin, teeth and eyes) and this has been very well illustrated by mice expressing a degradation-deficient IkBα and in heterozygous NEMO females, both of which manifest epidermal diseases resembling human anhydrotic ectodermal dysplasia (HED) and incontinentia pigmenti (IP), respectively (Fusco et al., 2004; Kucharczak et al., 2003).

Although most studies link NF-kB with prosurvival functions and prevention of apoptosis, several studies have clearly demonstrated a pro-apoptotic role of NF-kB because, along with AP-1, it can induce FasL expression and in some circumstances such as long term treatment of chronic inflammatory diseases, its continuous suppression could manifest itself in squamous cell carcinoma (Lind et al., 2004).

Notably, in the nervous system NF-kB represents a double-edged sword, inducing both anti- and pro-apoptotic processes. All cell types of the nervous system express functional p50/p65 heterodimer that can be induced by several stimuli such as membrane depolarization, nerve growth factor (NGF), activity-dependent neurotrophic factor (ADNF), opioids, the secreted form of β-amyloid protein, TNF, adhesion molecules and glutamate (Mattson and Meffert, 2006). Several mechanisms of activation and inhibition of NF-kB are known to affect neuronal survival in the context of developmental cell death and neurodegenerative disorders. Environmental insults such as oxidative and metabolic stress, neurotoxins and physical trauma trigger NF-kB activation whereas intracellular signals such as calcium, ROS and ceramide mediate stress- induced modulation of NF-kB activity. These intracellular signals are linked to specific receptors at the plasma membrane and use several pathways to activate NF-kB, including calcium/calmodulin-dependent kinase II, Akt and PKC. Target genes of NF-kB that promote neuron survival encode BDNF, Bcl-2, Bcl-XL, N-methyl-D-aspartate receptor subunits, MnSOD and IAPs. NF-kB has been implicated in NGF-mediated protection of developing sensory neurons to cytokines (Mattson and Meffert, 2006); in neurons, NF-kB becomes activated by binding of NGF to either $p75^{NTR}$ or TrkA receptors. TRAF6 mediates signaling downstream of $p75^{NTR}$ leading to activation of both NF-kB and JNK, and is linked to both pro-survival and pro-death functions. TrkA-linked p62 scaffold protein binds to atypical PkC and TRAF6 and acts as a platform for crosstalk between $p75^{NTR}$ and TrkA. Selective stimulation of these receptors and consequent NF-kB activation results in induction of sets of genes with significant overlap, suggesting that temporarily different waves of response can occur consequently to receptors stimulation by neurotrophins.

$RelA^{-/-}$ neurons show decreased survival compared to the wild type counterpart (Hamanoue et al., 1999). Moreover, NF-kB-mediated protective role against neurodegeneration in cortical neurons exposed to the amyloid β-peptide associated with Alzheimer's disease has also been reported. In this context, increased levels of IkBα and decreased NF-kB prosurvival activity lead to neurodegeneration (Bales et al., 1998). Surprisingly, an increased NF-kB activity has been observed in Parkinson's and ALS (amyotrophic lateral sclerosis) neurodegenerative diseaseses. However, in these settings NF-kB activity might activate an early defense mechanism against oxidative stress and mytochondrial disfunction (Mattson and Meffert, 2006). By contrast, proapoptotic effects for NF-kB have also been described in some models of neuronal cell injury, and

most of them seem to converge on p53 tumor suppressor. p53 is a detrimental factor for neuronal viability in response to ischemia/reperfusion and excitotoxic stress. For example, NMDA receptor activation in rat striatum activates NF-kB and leads to a concomitant increase of p53 and c-myc expression, inducing cell death (Kucharczak et al., 2001). Although it is well documented that in certain settings NF-kB promotes the expression of proapoptotic genes, an alternative mechanism is represented by the cellular milieu of the central nervous system: NF-kB-mediated production of proinflammatory cytokines, ROS, excitotoxins and induction of nitric oxide synthase in glial cells might indirectly promote cell death, resulting in glia-mediated neurotoxicity. A unifying hypotesis concerning the role of NF-kB in neurons is that activation of NF-kB in neurons could promote cell survival, while its activation in glial cells may induce the production of neurotoxins (Mattson and Meffert, 2006).

Another important context in which NF-kB activation leads to contradictory biological outcomes is viral infection. For many viruses, activation of NF-kB has been linked to their transforming activity. The first identified member of the Rel/NF-kB family has been v-Rel, the oncogene from avian Rev-T virus, derived from the cellular gene c-Rel that induces fatal lymphoma/leukemia in young birds (Gilmore, 1999). Oncogenic potential of v-Rel and of the other members of Rel/NF-kB family arises from their ability to suppress apoptosis from a variety of signals by upregulating antiapoptotic proteins. Many other viruses have utilized multifunctional proteins to hijack and stimulate NF-kB pathway. Tax oncoprotein of human T-cell leukaemiua virus type-1 (HLTV-1) that causes adult T-cell leukaemia (ATL) immortalizes T-cells by inducing NF-kB mediated upregulation of antiapoptotic genes. LMP-1 protein of human herpesvirus Epstein-Barr virus implicated in Burkitt's lymphoma also induces NF-kB signalling and increases expression of Bfl1/A1. Interestingly, this increase confers protection against apoptosis induced by growth factor deprivation in EBV-positive cell line (D'Souza et al., 2000). v-FLIP protein of human herpesvirus 8 (that causes Kaposi's sarcoma) also suppresses apoptosis triggered by growth factor deprivation by constitutive inducing IKK activity and Bcl-2 expression (Liu et al., 2000). Consistent with these evidences, many other viral proteins such as Tat, Vpr, Nef, X4 and gp120 of HIV virus (Choe et al., 2001; Fakruddin et al., 2004), A224L protein of African swine fever virus (ASFV) etc (Hiscott et al., 2006), affect NF-kB signalling to counteract apoptosis and to enhance viral replication and pathogenesis.

HIV infection leads to the progressive loss of CD4+ T cells and the almost complete destruction of the immune system in the majority of infected individuals. The earlier step in this viral infection is mediated by envelope glycoproteins, which bind to CD4 and to the coreceptors CCR5 or CXCR4. Interestingly, binding of X4 Env expressed on cells to CXCR4, besides triggering apoptosis of uninfected CD4+ T, can also induce autophagy and accumulation of Beclin 1 via CXCR4 (Espert et al., 2006; Espert et al., 2007).

Some viral proteins exploit NF-kB signaling to induce cell death. This is the case of Dengue, Sindbis and reovirus that promote cell death in infected cells; another example is E1A human adenovirus protein that has been reported to block IKK activity (Kucharczak et al., 2001).

Interestingly, when considering Sindbis virus, NF-kB seems to behave in a contradictory manner. In Sindbis virus infection, Bcl-2 induction decreases Sindbis virus replication in mouse brains, resulting in protection against lethal encephalitis. In the same context, the Bcl-2 interacting protein Beclin 1 has also been reported to increase mice survival. The mechanisms by which Beclin 1, in cooperation with Bcl-2-like proteins, functions to inhibit Sindbis virus replication and Sindbis virus-induced neuronal death are unknown, but it has been suggested that binding to Beclin somehow might protect Bcl-2 (or Bcl-X_L) from cleavage by caspases, thereby preventing Sindbis virus-induced cell death (Liang et al., 1998). This is, probably, another example in which the contradictory role of NF-kB in regulating both apoptosis and cell survival is determined by the death stimulus, by the cellular context and by the timing of modulating NF-kB activity relative to the death stimulus.

Overproduction of NF-kB antiapoptotic target genes has confirmed an undisputed pro-survival and pro-oncogenic role of this transcription factor; moreover, through its antiapoptotic activity NF-kB can also reduce the effectiveness of many common target therapies, which themselves activate NF-kB (Luo et al., 2005).

Nevertheless, NF-kB in some settings seems to behave as a tumor suppressor. For example, RelA has been shown to oppose epidermal proliferation driven by TNFR1 and JNK, and keratinocytes from mice lacking RelA are hyperproliferative in vitro. (Zhang et al., 2004). Also in this context contradictory NF-kB behaviour has been observed. Concerning with another example of NF-kB tumor suppressor function, some studies have shed in light that p53 tumor suppressor can activate NF-kB via MEK1 and $pp90^{rsk}$ mediated Ser-536 phosphorylation, and that NF-kB plays a crucial role in p53 mediated

apoptosis (Ryan et al., 2000). Moreover, it has been reported that loss of p65 can cause resistance to different agents that signal death through p53; this loss is able to enhance tumorigenesis induced by E1a and Ras (Ryan et al., 2004). In addition to p53, ARF can regulate also RelA activity through activation of ATM-and Rad3-related (ATR)/Chk1 checkpoint kinases, but in this case ARF signalling is thought to lead to Thr-505 phosphorylation of RelA and to repress the induction of Bcl-XL sensitizing cells to TNF-induced apoptosis (Basseres and Baldwin, 2006).

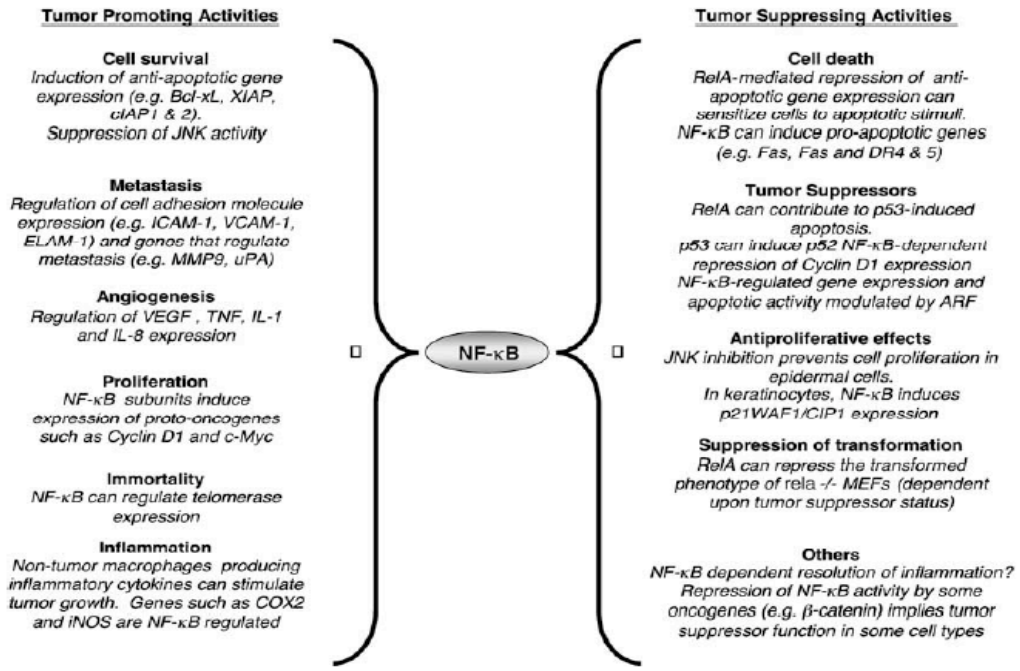


Fig.4: Different abilities of NF-kb in either promoting or suppressing tumor formation. (Perkins N.D. and Gilmore T.D., 2006; Cell Death Diff.)

It is then well established that, by regulating gene expression, NF-kB can promote a plethora of oncogenic processes: tumor cell proliferation through its ability to induce proto-oncogens such as cyclin D1 and c-myc; metastasis through its mediated induction of cellular adhesion molecules and metalloproteinases; angiogenesis through regulation of vascular endothelial growth factor and cell immortality through regulating telomerase (Luo et al., 2005). Furthermore, other pathways can influence NF-kB transcriptional activity adding a major level of regulation to NF-kB pro-oncogenic potential. Notably, NF-kB can further contribute to the tumorigenic process through its effect on infiltrating and non-cancerous macrophages; indeed, the secretion of NF-kB-regulated cytokines

and growth factors from these cells, helps to drive tumor cell proliferation and size (Greten et al., 2004).

With regard to the links of NF-kB to other pathways, a crosstalk between p53 tumor suppressor pathway and NF-kB has been demonstrated. In contrast to what discussed previously, NF-kB can also contribute to suppression of p53 activity through the induction of hdm2 expression, an inhibitor of p53 (Basseres and Baldwin, 2006). Finally, direct proapoptotic function of NF-kB rely on its ability to directly transactivate apoptosis-inducing genes, such as Fas, Fas-ligand and death receptors 4 and 5, TNFα, Bcl-XS, p53 and death receptor (DR) ligand TRAIL (Kucharczak et al., 2003). Interestingly, one study demonstrated that c-Rel-induced expression of TRAIL receptors DR4 and DR5 along with Bcl-XS was able to sensitize cells to TRAIL-induced apoptosis (Chen et al., 2003). It has to be noted that in a model of lumen formation in mammary gland acini, TRAIL induction has been demonstrated to be required both for apoptosis and autophagic process to occur (Mills et al., 2004).

Another important basis for NF-kB tumor activity is its ability to prevent reactive oxygen species (ROS) induced apoptosis and in this context NF-kB crosstalk with JNK pathway is thought to be fundamental. Several mechanisms have evidenced that NF-kB activation results in suppression of JNK activity. These include induction of Gadd45β expression, which functions as an inhibitor of the upstream MKK7 kinase and induction of XIAP, which, in addition to inhibit caspase-3 and –7 activities, can inhibit JNK. As discussed above, RelA-/- mice die for massive liver TNF-α induced apoptosis. TNF-α stimulation leads to increased ROS intracellular levels and, in this circumstance, JNK pathway and caspase activation trigger cell death. NF-kB activation directly counteracts both caspases and JNK effects, leading to cell survival (Bubici et al., 2006). Another interesting feature of NF-kB is that it can be directly activated by ROS and counteract ROS signalling through the transactivation of several genes such as ferritin heavy chain (FHC), manganese superoxide dismutase, metallothionein and glutathione S-transferase. In various tumors, NF-kB and ROS/JNK signaling appear therefore to have opposing effects. Infact, inducers of JNK cascade (MKK4, BRCA-1, JunB and JunC) have been implicated in tumor suppression, and both ROS and JNK can mediate tumor cell killing inflicted by radiation and some anticancer drugs. Conversely, NF-kB activation promotes cell viability during oncogenic transformation and survival of late-stage tumors (Bubici et al., 2006).

Recent work suggested a novel antiapoptotic function of NF-kB activation. NF-kB has been proposed to negatively regulate autophagy consequently to TNFα treatment using Ewing sarcoma stably expressing IkBα and in MCF-7 cells (a breast cancer cell line haploinsufficient for Beclin 1 one of the key players proteins of the autophagic process). It has been observed that consequently to TNFα treatment, an increase in ROS was implicated in autophagic vacuole formation. In these settings NF-kB has been proposed either to counteract ROS formation or to engage mTOR kinase, indeed reducing the overall autophagic activity of the cells (Djavaheri-Mergny et al., 2006). This work agrees with previous data linking ROS to autophagy and describing a death-related pathway, referred to as type II PCD, which is induced by high levels of ROS (Yu et al., 2004; Kiffin et al., 2006). Interestingly, a more recent work demonstrates that under starvation conditions, a consequent increase of ROS levels takes place and is both local and reversible (Shertz-Shouval et al., 2007). Data presented by the authors argue for a biphasic role of ROS in autophagy induction: an initial rapid increase in ROS levels occurs consequently to starvation, requires both PI3K class III kinase and Beclin 1 and favours early steps of the autophagic process; after this initial steps, the need of ROS buffering is required for the cells to complete the autophagic response, since a prolonged oxidizing environment inhibits the activity of Atg4 redox-sensitive enzyme that is necessary during the late steps of the autophagic process. Altogether, data presented in this work suggest that an unrestrained increase in ROS levels inhibits autophagy. Moreover, this study argued for an involvement of ROS in starvation-induced autophagy as signaling molecules of a survival pathway rather than of a death one.

INTRODUCTION TO THE AUTOPHAGIC PROCESS

Normal cellular development and growth require a well regulated balance between protein synthesis and degradation. Eukaryotic cells have two major avenues for degradation, the proteasome and autophagy. Autophagy, which literally means “self eating”, is a major mechanism for degrading long-lived cytosolic proteins and cellular organelles, such as peroxisomes, mitochondria and endoplasmic reticulum and, unlike the proteasome, terminates at the lysosome/vacuole, whereas the ubiquitin-proteasome system degrades specific short-lived proteins (Yorimitsu et al., 2005).

The autophagic process is evolutionarily conserved from yeast to mammals, and it was initially characterized in yeast as a mechanism used to promote survival under metabolic stress. In fact, consequently to nutrient starvation high levels of autophagy are induced in order to allow unneeded proteins to be degraded by the lysosome and the amino acids to be recycled for the synthesis of proteins essential for survival. Akin to yeast, in higher eukaryotes autophagy is induced in response to nutrient depletion that occurs in animals at birth after severing of the trans-placental food-supply, as well as in nutrient starved cultured cells and tissues (Kuma et al., 2004). Besides nutrient starvation, autophagy can be activated by many other forms of stress, including energy starvation (Lum et al., 2005), oxidative stress (Droge et al., 2007), mitochondrial dysfunction (Jin S., 2007), endoplasmic reticulum stress (Yorimitsu and Klionsky, 2007) and infections (Espert et al., 2007), playing an essential role in cell survival, in many phisiological cellular processes such as lifespan extention (Massey et al., 2006; Cuervo et al., 2005), and during development and differentiation (Levine and Klionsky, 2004). Due to the large spectrum of functions in which it is involved, an impairment of autophagy is thought to be detrimental in many pathological conditions, among which cancer, muscolar disorders and neurodegenerative diseases (Levine and Klionsky, 2004; Cuervo, 2006; Martinez-Vincente and Cuervo, 2007). In addition, autophagy has a very well estabilished role in the intracellular resistance to pathogens, contributing to endogenous MHC class II antigen processing and presentation of viral, bacterial, self- and tumor antigens (Münz, 2006; Menéndez-Benito and Neefjes, 2007). Conversely, there is evidence that autophagy is involved in type II programmed cell death (PCD) and might contribute to the pathology of some diseases (Bursch, 2004).

Three different types of autophagy have been described in mammalian cells: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Shintani et al., 2004; Cuervo, 2004). These three pathways differ in the procedures of delivering substrates to lysosomes, in the nature of these substrates and in the mechanisms involved in their regulation. Both micro- and macroautopahgy involve dynamic membrane rearrangements: microautophagy consists in the direct engulfment of cytosolic regions by the lysosome membrane (Reggiori and Klionsky, 2002), while macroautophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vescicle, termed autophagosome, before delivering to lysosomes. CMA process allows degradation of specific cytosolic proteins that contain a penta-peptide

consensus motif, indeed being more selective and having a limited degradative capacity (Cuervo et al., 2005; Massey et al., 2006).

Autophagy was morphologically identified at first in the 1960s in mammalian cells; however, the molecular mechanism for this process has only recently begun to be elucidated. One breakthrough for studying the molecular basis of autophagy was achieved in S.cerevisiae strain defective in proteinases A and B and in carboxipeptidase Y (CPY). Infact, shifting of this yeast strain from vegetative to nutrient-deficient conditions induced the formation of vescicles that were called autophagic bodies. Electron microscopy analysis of the autophagic bodies revealed that their content appeared morphologically indistinguishable from cytoplasm. Subsequent examination of yeast strains that failed to accumulate autophagic bodies led to the identification of several genes involved in this process (Tsukada and Ohsumi, 1993; Thumm et al., 1994), which were termed autophagy-related genes (ATG) (Klionsky et al., 2003). Besides micro- and macro-autophagy in yeast, an additional lysosomal pathway has been characterized, which involves most of ATG genes products and is referred to as 'cytoplasm-to-vacuole' (Cvt) pathway. This additional lysosomal pathway differs from autophagy for it has a biosynthetic function, leading to the transport of specific resident vacuolar hydrolases (aminopeptidase I [ApeI] and α-mannosidase [Asm1]) into the vacuole (Kim and Klionsky, 2000). The study of both autophagy and Cvt pathways allowed the identification of the ATG genes. Currently, more than 25 ATG genes have been identified in yeast, and the relative protein products seem to be specific to the autophagic process. Additionally, orthologs of the ATG genes have been recently identified and functionally characterized in higher eukaryotes such as mammals, insects, worms and plants; they all have been shown to play essential roles in autophagic cellular functions (Kim and Klionsky, 2000). These findings have revealed that the molecular machineries of autophagy found in yeast are also commonly utilized in several others eukaryotic cells, underlying the high conservation of this process also at the molecular level.

Induction and regulation of autophagy

Autophagy occurs at basal levels in normal growing conditions to perform homeostatic functions. Nonetheless, it is rapidly upregulated in response to some environmental stress and developmental transitions. In yeast, one of the major players in the autophagic regulation is the evolutionarily conserved target-of-rapamycin protein

(TOR) that specifically responds to nitrogen levels and behaves as a negative regulator of autophagy. In mammalian cells, the nutritional status and other cues such as temperature, oxigen concentrations and hormonal factors, are fundamental in the control of autophagy and such conditions tightly regulate mTOR activity.

Downstream of TOR kinase, several genes essential for autophagy and yeast related pathways have been characterized, although the nutrient sensor upstream of TOR remains unknown (Noda and Ohsumi, 1998). Under nutrient-rich conditions, active Tor kinase switches on protein translation and synthesis and inhibits autophagy whereas, in the absence of an adequate supply of nutrients, Tor becomes inactive and autophagic catabolism provides amino-acids and other components necessary for cell survival. The mammalian Tor protein (mTOR) also senses environmental changes in order to modulate autophagy, but the mechanism of regulation may be more complicated than in yeast. In yeast TOR controls the phophorylation state of Atg13, one of the proteins required for autophagy. In nutrient-rich conditions, mTOR mediated phosphorylation of Atg13 confers lower affinity for Atg1, a kinase involved in autophagic induction, and in this condition autophagy is repressed. In contrast, under starvation conditions or following treatment with the Tor inhibitor rapamycin or rapalogs, Atg13 is rapidly and partially dephosphorylated and interacts with Atg1 with higher affinity (Matsuura et al., 1997); the net result of this interaction is an increase of autophagic activity. Atg1 is the sole serine/threonine protein kinase identified among the Atg proteins, and it is essential both for the Cvt pathway and for autophagy (Harding et al., 1996). The role of Atg1 activity still remains arguable. However, the Atg1 kinase complex might be crucial for the induction and regulation of the autophagic process, possibly by swithcing between the Cvt pathway and autophagy in response to environmental changes.

The Atg1-Atg13 complex also interacts with several proteins, such as Vac8, Atg11 and Atg17. Atg11 and Vac8 are also phosphorylated independently of the Atg1-Atg13 complex, although the role of such phosphorylation is currently unknown (Scott et al., 2000). Atg17 is required for autophagy under starvation conditions, while it is not needed for the Cvt pathway under nutrient-rich conditions. The interaction of the Atg1-Atg13 complex with Atg17 is thought to have a major role in the regulation of autophagosome biogenesis in that Atg17 mutants that are not able to interact with Atg1-Atg13 complex show reduced autophagic capacity. These results suggest that the Atg1-Atg13-Atg17 complex might play a role in autophagy which is likely to be different

from induction, and might be involved in autophagosome formation, by specifically determining the ultimate size of vescicle. Although Atg1 is conserved in mammals, it remains to be estabilished how the Atg1 hortholog acts in mammalian autophagic system. In contrast, Atg13 and Atg17 horthologs have not been identified in mammals yet.

In yeast there are two TOR genes, TOR1 and TOR2 that give rise to two different complexes which share several functions such as regulation of translation, ribosome biogenesis, amino-acid transport and autophagy; unlike TOR1, TOR2 has additional functions on cytoskeleton regulation that cannot be inhibited by rapamycin (Loewith et al., 2002; Jacinto et al., 2004).

In mammals, a unique TOR gene exists that gives rise to two TOR complexes: mTOR Complex1 (containing mTOR, GβL, and raptor) which is rapamycin sensitive and mTOR Complex2 (containing mTOR, GβL, and rictor) that is rapamycin insensitive.

Early studies in mammalian cells established that the regulatory cascade upstream of mTor includes a class I phosphatidylinositol (PtdIns) 3-kinase (PI3K class I), PDK1 and Akt/PKB, while the phophatase PTEN acts antagonistically to the PI3K class I to induce autophagy. Amino acids are the most powerful inhibitors of autophagic sequestration activating the mTOR and S6K signalling cascade through a mechanism that is still unknown (Oldham et al., 2003). Besides amino acids, also hormones have a role in modulating autophagy; in fact, in mammals, hormones such as insulin or glucagon act antagonistically in modulating autophagy in the liver, but only at intermediate amino acid concentrations (Cuervo et al., 2005). Insulin alone, in fact, is not able to induce S6K phosphorylation and activation and actually, high levels of amino acids do not require the presence of insulin; on the other hand, glucagon inhibits S6K and stimulates proteolysis at intermediate levels of amino acids (Bugnet et al., 2003). In any condition, a linear relationship has been observed between the percentage of inhibition of autophagic proteolysis and the phosphorylation status of S6K, indicating the importance of this event in the regulation of autophagy (Blommaart et al., 1995). Also the inhibition of mTOR kinase with rapamycin reduces the phosphorylation of S6K, consequently inducing the autophagic pathway independently of aminoacids. Rapamycin not only induces protein degradation, but also partially reduces protein synthesis, indicating a coordinate control of opposing processes by the same signaling pathway aimed to the mantainance of cellular homeostasis (Blommaart et al., 1995).

The kinase mTOR seems to be the main regulator of the autophagic pathway where signals derived from both energetic and nutritional status, hormones and cytokines convey. Earlier studies demonstrated that growth factors and hormones, such as insulin, regulate mTOR Complex 1/S6K1 activation via calss 1 PI3K. These kinase resides near to the plasma membrane and its products are the lipids phosphatidylinositol-3,4-biphosphate ($PI(3,4)P_2$) and phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) that have been demonstrated to have an inhibitory effect on autophagy (Petiot et al., 2000; Criollo et al., 2007). Insulin and growth factors receptors engagement lead to the recruitment of adaptor molecules that bring class I-PI3K lipid kinase closer to the membrane and favour the transient production of ($PI(3,4,5)P_3$). Indeed, ($PI(3,4,5)P_3$) binds to PKB/Akt, recruiting it to the membrane where it is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) (Stokoe et al., 1997) and mTOR complex2 (Sarbassov et al., 2005). Activated Akt/PKB, in turn, phosphorylates TSC2 (Tuberous sclerosis complex 2) inactivates its GAP activity towards Rheb (Ras homolog enriched in brain) and targets it for degradation, subsequently leading to mTor activation (Corradetti and Guan, 2006), elevated protein synthesis, increased cell growth and inhibiton of autophagy. Downregulation of both class I-PI3K and mTor leads to autophagy activation as well as overexpression of the phosphatase PTEN (phosphatase and tensin homolog deleted from chromosome ten), which removes the phosphate from the 3-position of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ avoiding downstream signalling (Arico et al., 2001).

At present it is not clear whether aminoacids regulate mTOR directly or through the TSC1/2 complex, or simply reducing Rheb GTPase activity. It has been recently suggested that aminoacids can inhibit autophagy independently of mTor based on the observation that in myotubes rapamycin was unable to abolish the stimulation of proteolysis by leucine starvation (Mordier et al., 2000). Interestingly recent studies established a link between human vacuolar protein sorting 34 (hVps34, a class3 PI3K that uses phosphoinositide as a substrate to produce PtdIns(3)P, and is required for autophagic induction), and mTOR Complex1. A mechanism by which nutrients and glucose could lead to its activation was recently shown. In fact, it was demonstrated that in the absence of TSC1/TSC2, although mTOR Complex1/S6K1 activation was elevated and refractive to stimulation by insulin, it was still regulated by amino acids (Nobukuni et al., 2005). It was also demonstrated that overexpression of hVps34 drives S6K1 activation in the presence, but not in the absence of amino acids (Byfield et al.,

2005) and stimulation of cells with amino acids increases hVps34 activity. Of note, hVps34 is the only PI3K found in yeast. Indeed this suggests that the rise of systemic hormonal regulation during evolution has led to the insulin PI3K class I signalling pathway in order to control the growth and development of organisms (Nobukuni et al., 2007). The suggestion that hVps34 is a positive mediator in both autophagy and mTOR Complex1/S6K1 signalling appears contradictory and the mechanism by PtdIns(3)P signals to mTOR/Complex1/S6K is unknown. Nonetheless, given the role of PtdIns(3)P in recruiting proteins containing either FYVE or PX domains, it is possible that hVps34 is involved in forming a signalling complex that includes mTOR Complex1 and S6K. Moreover, glucose depletion has been reported to inhibit hVps34 activity, suggesting that amino acids and glucose are converging through a common mechanism to activate this signalling. Finally, it has recently been found that mTOR co-immunoprecipitates with hVps34 (Nobukuni et al., 2007).

Another possible explanation for the inability of rapamycin to reverse aminoacid-induced inhibition of autophagy in some cell types is that mTOR is present in the two distinct complexes mTOR Complex1 that is rapamycin sensitive and mTOR Complex2 that is not inhibited by this drug (Sarbassov et al., 2005). Raptor of mTOR Complex1 phosphorylates S6K1 and is involved in controlling cell growth, whereas rictor in the mTOR Complex2 is required for Ser473 phosphorylation of PKB and controls cell proliferation and survival. Indeed, the relative amount of mTOR sequestered in each of these two complexes might determine whether or not autophagy can be accelerated by rapamycin (Codogno and Meijer, 2005).

Other growth regulatory pathways can affect mTOR and TSC1/2 and mTOR signalling: Rsk1 and Erk can activate mTOR Complex1 in response to growth stimuli such as phorbol esthers and EGF by phosphorylating and inhibiting TSC2 (Corradetti and Guan, 2006) even if in some cellular contexts Erk has been shown to activate autophagy (Meijer and Codogno, 2004). Osmotic stress (hypertonic stress induced by sorbitol and mannitol) has been shown to reduce mTOR activity and S6K phosphorylation, independently of TSC2 and Rheb (Corradetti and Guan, 2006).

Along with the mainteinance of osmotic neutrality, translation and ribosome biogenesis are energetically expensive processes and low nutrient conditions strongly reduce mTOR activity, and couple to an increase in AMP concentration enhancing the autophagic process. Small decreases in ATP content are transmitted to mTOR by AMP-activated protein kinase (AMPK) which then phosphorylates and inactivates mTor

signalling and leads to the inhibition of protein synthesis (Corradetti and Guan, 2006). Interestingly, in murine myotubes, amino acid deprivation or AMPK activation lead to mTOR Thr^{2446} phosphorylation, while Ser^{2448} of mTOR is phosphorylated in response to amino acids and growth factors. These two phosphorylation sites are mutually exclusive because phosphorylation of one site inhibits phosphorylation of the other and vice versa. They may be viewed as switches, which integrate the counteracting signals of growth factors and nutrient deprivation (Cheng et al., 2004).

Autophagy is induced also in response to death inducing stimuli. For example, consequently to genotoxic stress, p53 is activated and triggers starvation-like responses by inhibiting mTOR. Thus a crosstalk between p53 and mTOR pathway was demonstrated and the means by which p53 is able to inhibit mTOR has been shown to require AMPK activation and functional TSC1/2 complex. In these ways etoposide treated cells switch from an anabolic to a catabolic state, and cell growth is halted; indeed, p53 induced activation of starvation response can represent a rapid damage-control mechanism that can temporarily prevent transmission of altered genetic material from mother to daughter cell (Zhaohui et al., 2005). Moreover, it has been found that PTEN expression can be driven by the p53 transcription factor, underlying again an important role for p53 in regulating mTOR pathway (Stambolic et al., 2001). Intriguingly, PTEN has been shown to be upregulated also after TNFα treatment via NF-kB in human leukemic cells; this effect seems to be cell context dependent (Lee et al., 2007; Kim et al., 2004).

Another exemple of autophagy induction in response to death inducing stimuli, is related to the expression of activated forms of death-associated protein kinase (DAPK) and death-associated related protein kinase-1 (DRP-1). These kinases are positive effectors of cell death in response to stimuli such as INFγ and TNFα, and trigger autophagy and cell death independently of caspase activity in carcinoma cells with non-functional p53 (Shohat et al., 2002; Inbal et al., 2002); moreover the expression of a dominant-negative form of DRP-1 blocked the induction of autophagy in MCF-7 in response to tamoxifen treatment (Codogno and Meijer 2005). Also TNF-related apoptosis-inducing factor (TRAIL) and Fas-associated death domain (FADD) have a role in promoting both apoptosis and autophagy (Mills et al., 2004; Thorburn et al., 2005). The ability of FADD to interact with the autophagic protein Atg5 has been shown to play a crucial role in death induction by INFγ, independently of caspase-8

activation (Pyo et al., 2005). Induction of autophagy has been observed in fibroblasts and monocytoid cells in response to the inhibition of caspase-8 (Yu et al., 2004). In this experimental setting the accumulation of autophagic vacuoles was dependent on receptor-interacting protein (RIP), a protein associated with the cytoplasmic domain of the death receptor, and on the activation of JNK and its upstream kinase MKK-7. RIP is a substrate of caspase-8, which cleaves and inactivates it, indicating that this caspase plays a role in processes other than apoptosis. Overexpression of hypoxia-inducible BH3-like domain containing protein (BNIP3) leads to autophagic type II cell death in cancer cell lines by opening of the mitochondrial permeability transition pore (Daido et al., 2004). Hypoxia inducible factor (HIF-1) expression has been demonstrated to be driven by mTOR, and HIF-1, in turn, is able to transactivate RTP801/Redd1 protein and inhibit mTOR (Corradetti and Guan, 2006). These data seem to be at odds with each other, but actually, hypoxia has been demonstrated to positively regulate the autophagic process in different contexts. Moreover, it is possible that hypoxia-mediated inhibition of mTOR by RTP801/Redd1 occurs at different time points. Of note, RTP801/Redd1 is also induced in response to energy stress, arsenic and hydrogen peroxide, glucocorticoid treatment and DNA damaging agents (Corradetti and Guan, 2006).

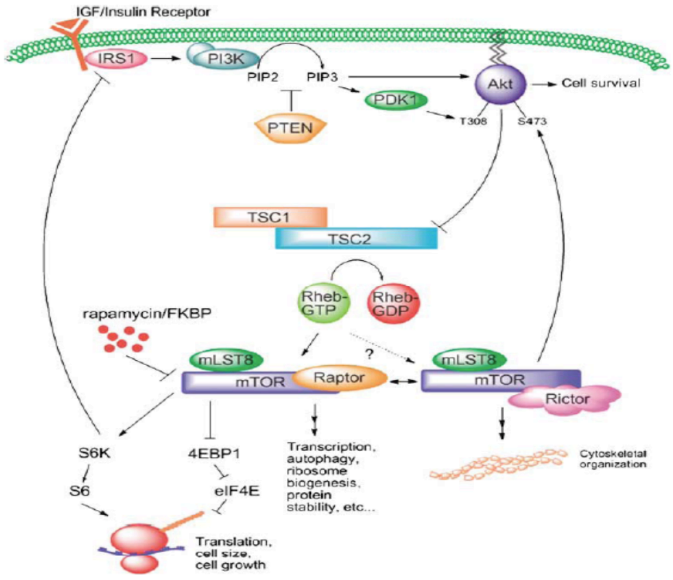


Figure 6: Tor kinase signalling in mammals (Corradetti and Guan 2006)

Also the accumulation of Atg proteins has been observed in mammalian cells undergoing autophagic cell death, and is dependent on the expression of Bcl-2 family members (Bcl-2 and Bcl-X_L) (Shimizu et al., 2004); moreover an accumulation of Beclin 1 has been described in ceramide and tamoxifen-induced autophagic death in

MCF-7 cells (Scarlatti et al., 2004). The upregulation of Atg proteins is probably important in determining the amplitude of the autophagic response. Indeed, the accumulation of Atg proteins is not a common trait during starvation-induced autophagy, the initiation of autophagy not requiring protein synthesis (Gozuacik et al. 2004). On the other hand, autophagic cell death requires protein synthesis (Codogno and Meijer 2005).

Molecular mechanism of autophagosome formation

Macroautophagy consists in the sequestration of portions of the cytoplasm into double membrane vescicles that fuse with the lysosomes, leading to the degradation of the inner membrane along with the content of the autophagosome. One of the first questions that arise is which is the origin of autophagosomes. It seems that in yeast there is a putative site called PAS (Pre-Autophagosomal-Structure) where these vesicles appear to be formed *de novo*, characterized by the presence of several Atg proteins, that constitute the machinery required for the formation of autophagosomes (Suzuki et al., 2001; Kim et al., 2002). In contrast to vesicles that generate by budding, an initial membrane must be formed in autophagy: this is the nucleation step of vesicle formation. The origin of this membrane is unknown. There is a general, although not universal, consensus that the endoplasmic reticulum (ER) is a source of the membrane for forming the vesicle. It has been reported that ER function and machinery of the early secretory pathway are required for autophagy and the Cvt pathway in yeast, although further studies are required for understanding this relationship (Ishihara et al., 2001; Hamasaki et al., 2003; Reggiori et al., 2004). The absence of conditional mutants in higher eukaryotes has prevented the analysis of the molecular machinery involved in autophagosome formation, and, as a consequence, all the studies have been performed mainly at cytological and morfological level. The existence of autophagosomes precursors in mammalian cells arose from some electron microscopy studies made in isolated rat hepatocytes where some thick, osmiophilic membrane multilayer was observed (Seglen, 1987; Fengsrud et al., 1995). These structures were termed phagophores or isolation membranes and their existence was confirmed by other studies of in vivo visualization of autophagosome formation using GFP-Atg5. These studies demonstrated that autophagosomes were generated by elongation of a small membrane structure, indeed from an autophagosome precursor. Formation of these structures didn't occur after tratment of cells with 3-MA or wortmannin, which are known inhibitors of PI3Ks that

are required at early steps of autophagosome formation (Mizushima et al., 2001). Furthermore, the same studies demonstrated that autophagosomes maturate from small cup-shaped cisternae, not simply derived from large pre-existing membranes, even if it was not clarified whether these small compartments were formed *de novo* or derived from some membrane source. These cisternae membranes, indeed, elongate, bend, and form spherical autophagosomes (Mizushima et al., 2001).

Elongation of the isolation membrane and biogenesis of autophagic vesicles involves two ubiquitin-like systems: Atg12–Atg5 and Atg8-phosphatidylethanolamine (Atg8-PE) protein conjugation systems (Ohsumi, 2001; Reggiori and Klionsky, 2002; Levine and Klionsky, 2004). The Atg12–Atg5 conjugation system, originally discovered in yeast, is widely conserved among eukaryotes. As in yeast cells, in mammalian cells Atg5 is mostly present as conjugated with Atg12. The initial step of the conjugation reaction results from the activity of Atg7 E1-like enzyme, which activates C-terminal glycine residue of mammalian Atg12. Such an activation results in the formation of an Atg12-Atg7 thioester intermediate (Tanida et al., 2004). Atg12 is subsequently transferred to mammalian Atg10 E2-like protein to form Atg12-Atg10 thioester intermediate (Mizushima et al., 2001). The last step consists in the attachment of C-terminal glycine of Atg12 to lysine 130 of Atg5 through an isopeptide bond (Mizushima et al., 1998). Mammalian Atg12-Atg5 conjugate is contained in large complex that contains also the Atg16L WD protein (Mitsushima et al., 1999). Atg16L, infact, can form homo-oligomers and mediates the formation of high multimeric structures of tetramers and octamers Atg12, Atg5, and Atg16L complexes. (Kuma et al., 2002; Mizushima et al., 2003). Although most of these complexes are cytosolic, small fractions localize on the isolation membrane during the elongation process being associated to its outer side. Interestingly Atg12-Atg5-Atg16L complexes dissociate from the membrane upon autophagosome completion, underlying their requirement to be fundamental during the elongation step. This requirement has been further confirmed by the expression of an Atg5 K130R mutant in Atg5 null embryonic stem (ES) cells: such a mutant is no longer conjugated to Atg12 but, all the same, it localizes at the autophagosome precursor with Atg16L. However, in this situation, no isolation membrane formation occurs (Mizushima et al., 2001).

The Atg12-Atg5 system is closely linked to the LC3 (MAP-LC3 microtubule-associated protein 1 light chain 3) ubiquitin-like system. LC3 is the mammalian orthologue of yeast Atg8; it is the first protein identified on the autophagosome membrane (Kabeya et

al., 2000) and requires several postranslational modifications before targeting to autophagosomes.

Similarly to yeast Atg8 conjugation, C-terminal region of LC3 is cleaved soon after its synthesis, and this processing is catalyzed by Atg4 cysteine protease (Mizushima et al., 1998). The resulting processed form, called LC3-I exposes then a glycine residue that becomes accessible to the E1-like Atg7, the same enzyme used in the Atg12-Atg5 conjugation system (Tanida et al., 2004). Activated LC3 is transferred to Atg3 E2-like enzyme which is then transferred to Aut1/Atg3 E2-like enzyme (Tanida et al., 2002) and is finally conjugated to phosphatidylethanolamine (PE). LC3 PE-conjugated form, called LC3-II, is tightly associated with the autophagosome on both the inner and the outer membrane bilayer. Unlike the Atg12-Atg5 conjugation, modification of LC3 with PE is a reversible event, indeed Atg4 can again cleave LC3 after the glycine residue to remove it from the lipid (Sherz-Shouval et al., 2007; Tanida et al., 2004). LC3-PE conjugation appears to be regulated in a complex manner in vivo because depletion of the Atg12-Atg5 conjugate causes instability of LC3-PE. Infact, Atg5 null cells overexpressing AtgK130R cannot target LC3 to the membrane. In this situation, autophagy is severely impaired and LC3 shows a diffuse cytosolic staining (Mizushima et al., 2001). LC3 autophagosomal localization is very well induced by different autophagic stimuli; for this reason, LC3 is considered one of the best markers for monitoring the autophagic process. Besides MAP1-LC3, other two Atg8 homologs have been found in mammalian cells: GATE-16 (Golgi-associate ATPase enhancer of 16 kDa), GABARAP (GABA receptor-associated protein) (Kabeya et al., 2000; Kabeya et al., 2004). All these proteins appear to be modified with lipids in the same manner and have been shown to localize to the autophagosome (Kabeya et al., 2004; Sherz-Shouval et al., 2007).

Indeed, during the membrane formation stage, the Atg12-Atg5-Atg16 complex drives the expansion and/or curvature of the membrane envelope and finally dissociates from the vesicle just before, or immediately after completion. On the other hand, LC3-PE is detected on both the forming intermediate vesicle and the completed autophagosome (Tanida et al., 2004; Ferraro and Cecconi, 2007).

Role of PI3-Kinase in autophagosome formation

Among the Atg components, PtdIns 3-kinase is class III has been proposed to play some role in membrane nucleation. Phosphatidylinositol 3-phosphate (PtdIns(3)P) is known to have an important role in various cellular functions. There is only one PtdIns 3-kinase, Vps34, identified in yeast. Vps34 is involved in forming two distinct PtdIns 3-kinase complexes (Kihara et al., 2001). Complex I is composed of Vps34, Vps15, Vps30/Atg6, and Atg14, while complex II contains the same proteins, except that Atg14 is replaced by Vps38. Notably, complex I disruption in yeast results in loss of autophagy and Cvt pathway.

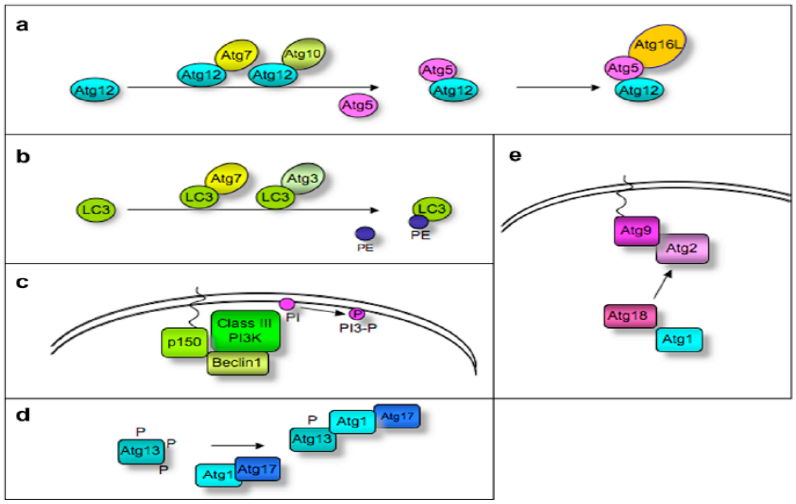


Figure 7: Two ubiquitin conjugation systems are involved in vescicle espansion during autophagosome formation. The result of these conjugations is the translocation of the structural component Atg8 (LC3 in mammals) to autophagosomal membrane. (Ferraro and Cecconi, 2007)

On the other hand, complex II depletion allows normal progression of autophagy and the Cvt pathway. PtdIns(3)P is known to be bound by proteins that have PtdIns(3)P-binding sites such as the PX and FYVE domains. Different yeast proteins, either containing or lacking PX or FYVE domains, have been found to interact with PtdIns(3)P and to be involved in either the autophagic process or in the Cvt pathway: Atg20 and Atg24, have PX domains, bind to PtdIns(3)P (Nice et al., 2002, Hettema et al., 2003) and seem to be involved only in the Cvt pathway, although they have been observed also at the PAS; Atg18, Atg21, and Atg27 also bind to PtdIns(3)P, even if none of them has known binding domains, and are then recruited to the PAS in a manner that is dependent on the PtdIns 3-kinase complex I (Guan et al., 2001; Wurmser

et al., 2002; Stromhaug et al., 2004). Atg18 is required for both the Cvt and autophagy pathways, whereas Atg21 and Atg27 are primarily required for the Cvt pathway.

During autophagy PtdIns(3)P might mediate the recruitment of some Atg components to the PAS to enable them to allow their involvement in the vesicle-forming machinery. Nonetheless further studies are required, because there is no direct evidence that PtdIns(3)P exists in the putative membrane of the PAS. In contrast to yeast, mammalian cells regulate autophagy via two PtdIns 3-kinase complexes, namely class I and class III (Codogno and Meijer., 2005). As discussed above, class I complex has a negative regulatory role on autophagy induction. This inhibitor effect is mainly due to mTOR hyperphosphorylation of the regulatory Atg13 protein. Atg13 forms a complex with Atg17 and Atg1 serine/threonine kinase proteins. Consequently to nutrient rich conditions, Atg13 m-TOR hyperphosphorylation inhibits its association with Atg1, and blocks autophagosome formation. On the contrary, nutrient starvation triggers Atg13 dephosphorylation and subsequent association with Agt1, favouring generation of the autophagosomes. Of note, Atg1 human homologues have been recently characterized, namely ULK1 and ULK2 (Yang YP et al., 2005; Ferraro and Cecconi, 2007). mTOR inhibition also enhances Atg8 and Atg14 gene expression. Infact, mTOR activation of Tap42 leads to PP2A inhibition and PP2A is required to activate some transcription factors that trigger the transcription of Atg8 and Atg14 genes (Ferraro and Cecconi, 2007).

In mammals, a complex similar to the yeast complex I has been described that involves the Apg6/Vps30 ortholog Beclin 1 and the Vps34 PI3K class III association and is required for autophagosome formation. Vps34 PI3K is localized on the trans-Golgi network and on endosomes and generates PtdIns(3)P; Beclin 1/Atg6 was observed on trans-Golgi network, complexed to Vps 34 (Kihara et al., 2001), and recently it was detected also on the endoplasmic reticulum and on mitochondria (Pattingre et al., 2005). Similarly to yeast, the production of PtdIns(3)P is required to allow the localization of other autophagic proteins to the pre-autophagosomal membrane (Kihara et al 2001). The yeast strains lacking APG6 gene are defective in autophagy induction upon nutrient starvation, and this function can be complemented by the ortholog Beclin 1 from higher eukaryotes, indicating the involvement of this gene in autophagy (Liu et al., 2005). Also in mammalian cells the requirement of Beclin 1 in autophagy induction has been assessed, indeed both beclin 1 deficient ES cells and Beclin 1 -/- mice showed reduced autophagic activity (Yue et al., 2003; Qu et al., 2003). Overexpression of Beclin1 in

MCF7 breast cancer cells increased autophagic vacuoles induction and protein degradation upon nutrients starvation in a 3-MA sensitive manner, further underlying the importance of a functional PI3K complex in this process (Liang et al., 1999). Recently, a new regulatory mechanism of autophagy has been proposed that involves modulation of Beclin 1/Vps34 complex activity by the anti-apoptotic protein Bcl-2. According to this mechanism, ER localized Bcl-2 sequesters Beclin 1 preventing the formation of Beclin 1/Vps 34 complex, and thus inhibiting its PI3K activity and autophagy induction. These studies showed that overexpression of Bcl-2 inhibited autophagic vacuole formation and protein degradation in breast carcinoma and colon carcinoma cell lines. Moreover the expression of Bcl-2 in Apg6-deficient yeast mutants prevented the rescue of autophagy induced by Beclin 1 under nutrient starvation condition. The inhibitory action of Bcl-2 towards autophagy has indeed been proposed to function as a rheostat that prevents autophagy to occur at elevated levels that could promote cell death; indeed, the relative amounts of Beclin1 and Bcl-2 complexed with each other within a cell might govern the threshold for transition from cell homeostasis to cell death (Pattingre et al., 2005). Interestingly, some recent reports showed that the ability of Bcl-2 to counteract autophagy induction is probably cell type and context dependent, indeed under ischemic condition, immortalized iBMK and MMEC beclin competent cells stably expressing Bcl-2 undergo autophagosome formation (Mathew et al., 2007; Karantza-Wadsworth et al., 2007). The same results were obtained in HeLa cells overexpressing Bcl-2 under ER stress stimuli (Criollo et al. 2007).

Vesicle formation

In yeast PI3K complex I might generate PtdIns(3)P at the PAS and facilitate the recruitment of certain Atg components, such as the vesicle-forming machinery. The Atg12-Atg5-Atg16 complex and Atg8-PE conjugate are included in this machinery, and their localization to the PAS also requires Atg9 integral membrane protein (Suzuki et al., 2001). The PtdIns 3- kinase complex I may function before Atg12-Atg5-Atg16 and Atg8-PE, since it is required for their proper localization at the PAS, while Atg9 might have a role prior the closure of the vesicle double layer (Ferraro and Cecconi, 2007). Atg9 also interacts with Atg2, and their association is required for autophagosome formation. Atg1 kinase and Atg 18 determine Atg2 proper localization on PAS. Mammalian orthologues of Atg9 and Atg2 have been already discovered in the human

genome, even if they have not been characterized yet (Ferraro and Cecconi, 2007). As already discusssed, Atg12-Atg5-Atg16 complex and Atg8-PE conjugate are proposed to be first located at the initial nucleation membrane and then to function in vesicle expansion.

Expansion of the vesicle presumably requires the delivery of lipids, possibly in the form of lipid bilayers present in transport vesicles; however, the origin of these lipids is not entirely known. Atg9 localizes at the PAS and mitochondria as multiple dots, and may cycle between these structures (Reggiori et al., 2004; Reggiori et al., 2005). It is not clear how Atg9 cycling functions. One hypothesis is that Atg9 marks the membrane source of the autophagosome. Atg9 may mediate the delivery of lipids/membrane by recycling from the donor (the mitochondria) to the acceptor membrane (autophagosome). This proposed lipid flow mediated by Atg9 cycling could be regulated via the Atg1-Atg13 complex and the PtdIns 3-kinase complex I in a manner that includes Atg2 and Atg18. The Atg9 protein is required for the recruitment of the Atg12-Atg5-Atg16 complex and Atg8-PE to the PAS (Suzuki et al., 2001) Atg9 cycling might then help the vesicle expansion in concert with the Atg12-Atg5-Atg16 complex and Atg8-PE. The presence of Atg5-Atg12 during the elongation process probably regulates the transport of additional membrane or lipids from somewhere to the isolation membrane, and recruits LC3 to the membrane. The mature autophagosome is therefore characterized only by the presence of LC3 as a specific marker. After Atg5 uncoating, presumed fusion machinery present on the vesicle would become exposed to drive the fusion of the autophagosome with the lysosome or vacuole. Thus, coating could act in part to prevent premature fusion of the forming intermediate vesicle with the vacuole.

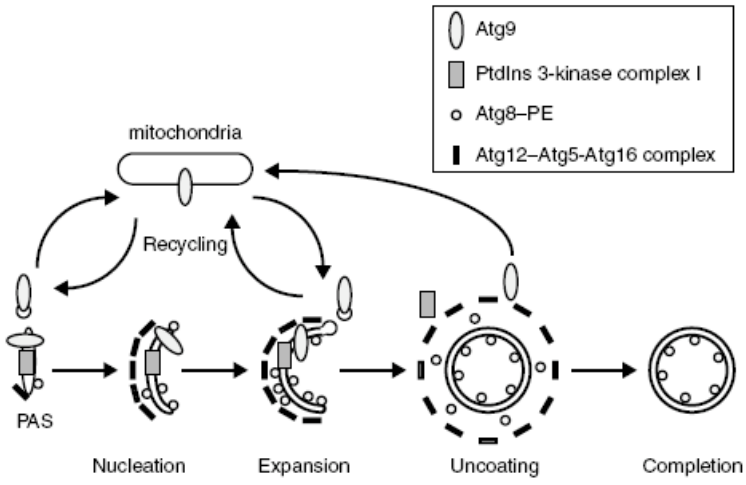


Fig. 8: Proposed mechanism of autophagosome formation (Yorimitsu and Klionsky, 2005)

In mammals, autophagosomes undergo a stepwise maturation process including fusion events with endosomal and/or lysosomal vescicles. Autophagosomes have been reported to fuse with early (Liou et al., 1997) and late endosomes (Berg et al., 1998), as well as lysosomes (Dunn et al., 1990; Gordon et al., 1992). The SKD1 AAA ATPase was recently shown to be necessary for autophagosome maturation (Nara et al., 2002). Early autophagosomes accumulated in cells expressing dominant negative forms of SKD1, indicating that fewer fusion events with endo/lysosomes took place. Hepatocytes derived from Vti1b SNARE protein deficient mice showed a delayed fusion between autophagosomes and multivescicular endosomes, suggesting that this protein participates to autophagosome fusion events both in yeast and in mammalian cells. Recently the small GTPase Rab7 has been identified on late autophagosomes (Jager et al., 2004). Rab7 is probably required for the final maturation of late autophagic vacuoles, and for the fusion with lysosomes Infact, it is needed for the formation of large perinuclear vescicular aggregates where the autophagosome marker LC3 colocalizes with the late endosomal and lysosomal markers Lysobiphosphatidic acid, a lipid present on these vescicles, and with Lamp1 (lysosome associated membrane protein 1), a membrane molecule associated with late endosomal/lysosomal compartment. Moreover, cells expressing Rab7 RNA interfering or a dominant negative form of Rab7 displayed improper accumulation of late autophagic vacuoles (Jager et al., 2004), suggesting a role for Rab7 in the final maturation of autophagic vescicles, most probably in the fusion with lysosomes. It appears that Rab7 plays a similar role in the late steps of both autophagosome and phagosome maturation, consequently it is possible for other molecules to be shared between the two pathways. RILP, Rab7 interacting ligand protein, is a molecule present on late endosome and lysosomes and it is known to mediate the movement of phagosomes from the periphery to the perinuclear region where they fuse with lysosomes. Therefore RILP requirement for autophagosome movements has been suggested. The late endosomal/lysosomal markers Lamp-1 and Lamp-2 (Lysosome assiciated membrane protein-1 and 2) have been detected on late autophagosomes (Eskelinen et al., 2002; Tanaka et al., 2000). Interestingly Lamp-2 deficient mice die at the age of 20-40 days (Tanaka et al., 2000), and show an accumulation of autophagic vacuoles in several tissues, while Lamp-1

deficient mice are normal (Andrejewski et al., 1999), suggesting that the lack of Lamp-1 is compensated by Lamp-2. The accumulation of autophagic vacuoles in Lamp-2 deficient mice correlates with an impared degradation of autophagosomes. Interestingly Lamp1 and Lamp2 accelerate the recruitment of Rab7 on autophagosomes, as Lamp1/Lamp2 double deficient cells show an accumulation of late autophagic vacuoles compared to wild type, while the early autophagosomes appear similar in both lines (Eskelinen et al., 2004).

Beclin 1 and autophagy

Beclin1 was originally identified in a yeast two-hybrid screen performed for the identification of Bcl2-interacting proteins. In this study Bcl2 interaction with Beclin 1 was demonstrated to be fundamental for the antiviral, antiapoptotic and survival promoting effects of beclin 1 on CNS Sindbis virus infection, suggesting that Beclin 1, via interactions with Bcl-2, can function in vivo in the CNS as an antiviral host defense mechanism (Liang et al., 1998). Human beclin 1 is a coiled-coil protein and shares structural similarities to the yeast autophagy gene Atg6/vps30. The beclin1 gene maps to a tumor susceptibility locus on human chromosome 17q21 and is monoallelically deleted in 40-75% of human breast, ovarian and prostate cancers (Aita et al., 1999).
Its involvement in the autophagic process protein was initially demonstrated by gene transfer sudies: human beclin when expressed in Apg6 null yeast strain was able to induce autophgy under nitrogen deprivation conditions. Moreover, when overexpressed in MFC7 cells (human breast carcinoma cells that present haploinsufficiency for Beclin 1 gene and display impaired ability to engage autophagic pathway) it increases basal levels of autophagy and nutrient deprivation-induced autophagy. In this context MCF7 enforced overexpression of Beclin 1 results in the loss of malignant morphological properties, decreased rates of proliferation, impaired clonogenity in vitro, and impaired ability to form tumors in nude mice (Liang et al., 1999). Beclin 1 was shown to localize at the trans-golgi network where it interacts with of PI3K class III presumably binding to C2 N-terminal domain of PI3K class III (Liang et al., 2007). Beclin 1-bound PI3K is functionally active and PtdIns(3)P might be essential for autophagosome formation due to their ability to recruit other Atg genes (Kihara et al., 2001). PI3K class III binding domain on Beclin 1 has been characterized and is located at aa 244-337 downstream the coiled-coil region and is referred to as ECD domain (Furuya et al., 2005). Interestingly Beclin 1 mutant lacking ECD shows impaired ability to activate autophagy, suggesting

that this mutant might act as a dominant negative protein. Beclin 1 has been found to localize also into mitochondria, ER and perinuclear membrane. A Beclin 1 NES (aa 180-189, downstream to Bcl2 binding site aa 88-150) has been characterized and enables Beclin 1 shuttling between nucleus and cytoplasm in a CRM1 dependent manner. Interestingly, nuclear localization of Beclin 1 impairs its ability to induce autophagy under nutrient starvation conditions (Liang et al., 2001). Beclin 1 null mice dye early in embryogenesis, demonstrating that autophagy is required during embryogenesis and for the mantainance of tissue homeostasis in vivo. Beclin 1+/- mutant mice develop wide spectra of tumors that seem to express decreased levels of Beclin 1 protein (Qu et al., 2003). Beclin 1 null ES cells show severely altered autophagic response, whereas their apoptotic response to serum starvation and UV treatment is identical to the wild type counterpart, establishing a role for autophagy and Beclin 1 in tumor suppression (Yue et all., 2003). Interestingly, Beclin 1 null ES cells failed to form embryoid bodies when cultured in the absence of LIF; this was attributable to a failure of autophagy beclin-dependent clearance of apoptotic cells during embryoid development that in the absence of Beclin1 failed to cavitate (Qu et al., 2007). Further evidences argue for a general involvement of Beclin 1 during embryonic development: in C.elegans, short interfering siRNA suppression of Beclin 1 inhibits autophagy and interferes with morphogenesis of the developmental stage known as the dauer diapause (Melendez et al., 2003). Beclin 1 expression and Atg genes upregulation are sporadically seen under nutrient starvation, while their protein levels are increased consequently to ceramide, tamoxifen, vitamin D analogues, radiations TNFα and other stimuli treatment (Scarlatti et al., 2004; Hoyer-Hansen et al., 2005). Bcl-2 binding to Beclin 1 has been demonstrated to negatively regulate Beclin 1 association with PI3K classIII and to inhibit Beclin 1-induced autophagy under starvation. Interestingly, Beclin 1 mutants lacking Bcl-2 binding domain enhance basal autophagosome formation and cell death in normal growth conditions (Pattingre et al., 2005). Nonetheless, in other studies performed in different cellular systems and under different autophagy stimulation conditions, Bcl-2 seems to be dispensable in Beclin 1-mediated autophagy regulation (Zeng et al., 2006; Karantza-Wadsworth et al., 2007; Matthew et al., 2007). For example, overexpression of Bcl-2 and Bcl-X_L in mouse wt embryonic fibroblasts (MEFs) treated with etoposide appears to stimulate, rather than inhibit, Beclin 1-dependent autophagic cell death (Shimizu et al., 2004). Besides Bcl-2, Beclin 1 also binds Bcl-X_L but not Bax (Liang et al., 1998; Furuya et al., 2005). Two recent

independent studies demonstrated that Beclin 1 binds both Bcl-2 and Bcl-X_L through a BH3-like amphipatic α-helix located inside the previously characterized Bcl-2 binding domain at aa107-135 (Oberstein et al., 2007; Maiuri et al., 2007), thus demonstrating that beclin1 is a new member of the BH3-only protein family. Interaction between Beclin 1 and Bcl-X_L is phisiologically relevant, as its inhibition by the BH3-only Bad protein can stimulate autophagy.

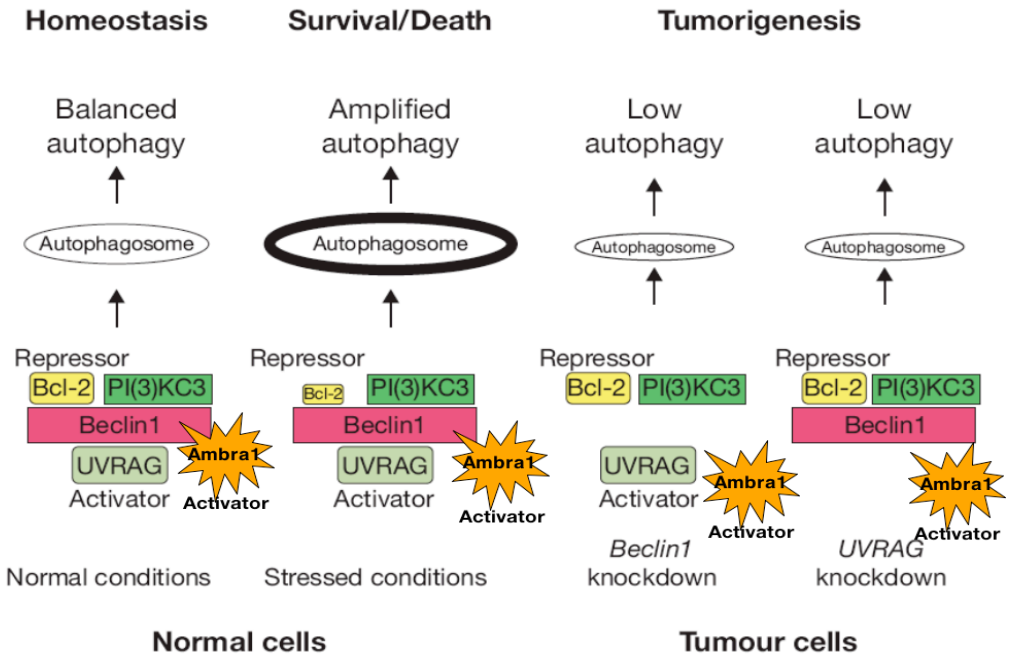


Fig. 9: Model for cross-regulatory mechanism of beclin 1 mediated modulation of autophagy ([modified figure] original: Liang et al., 2006, Nature Cell Biol.)

Moreover it suggests new modes of crosstalk between autophagic and apoptotic pathways. Recently, two novel Beclin 1 binding proteins, UVRAG and Ambra1, have been identified and have been demonstrated to positively regulate Beclin 1-mediated autophagy probably by facilitating its interaction with PI3K class III either in basal or in stress conditions (Fimia et al., 2007; Liang C. et al., 2007). Altogether, these data demonstrate that fine tuning of Beclin 1 interactions are determinant in the autophagic or apoptotic responses in different physiological contexts such as cell growth, tumorigenesis, host defense and development.

Biological outcome of autophagy induction

Besides its role in the turnover of proteins and organelles, autophagy has multiple physiological and pathological functions. When cells encounter environmental stressors such as nutrient starvation and pathogen infection, autophagy takes place, resulting in

either adaptation and survival, or death (Kondo et al., 2005). The observation of autophagic vacuoles in dying cells has suggested the existence of an alternative form of programmed cell death depending on autophagy induction and referred to as autophagic cell death or type II programmed cell death. Accumulating evidences are now indicating that in many conditions, in particular when cells have an intact apoptotic machinery, autophagy has a protective rather than a pro-death function (Levine and Yuan, 2005). It is therefore crucial to understand the mechanism regulating autophagy and in particular which molecular players differentiate the protective autophagy from autophagic death, in order to utilize them as a therapeutic strategy.

Autophagy as a cell death mechanism

The term "autophagic cell death" describes a form of programmed cell death morphologically distinct from apoptosis and presumed to result from excessive levels of cellular autophagy (Schweichel and Merker, 1973); in contrast to necrosis, both apoptotic and autophagic cell death are characterized by the lack of a tissue inflammatory response (Levine and Yuan, 2005). Large numbers of autophagic vacuoles have been observed in dying cells of animals of diverse taxa (Schweichel and Merker, 1973; Bursch, 2001; Clarke, 1990; Lockshin and Zakeri 2004). According to the consensus view, autophagic cell death occurs primarily when the developmental program (e.g., insect metamorphosis) or homeostatic processes in adulthood (e.g., mammary gland postlactational involution) require massive cell elimination. Recent studies have also described autophagic cell death in diseased mammalian tissue and in tumor cell lines treated with chemotherapeutic agents. In many of these cases, morphological features of autophagic and apoptotic cell death or of autophagic and necrotic cell death are observed in the same cell, making it difficult to understand the exact role of autophagy in cell demise.

In *Drosophila* autophagic cell death is observed during salivary gland regression, and is prevented by mutations in the ecdysone-regulated transcription factors BR-C and E74A (Lee and Baehrecke, 2001), but these mutations do not prevent autophagy in the same cells. In *Drosophila* the caspase inhibitor p35 blocks metamorphic cell death, but not autophagy, suggesting that cell death requires caspase-mediated apoptosis rather then autophagic cell death (Lee and Baehrecke, 2001). These studies suggest that autophagy is not required or sufficient to induce cell death in these experimental settings.

Characteristic	Autophagic cell death (type II PCD)	Apoptosis (type I PCD)
Morphological changes		
Cell membrane	Blebbing	Blebbing
Nucleus	Partial chromatin condensation, no DNA laddering	Chromatin condensation, DNA laddering, nuclear fragmentation
Cytoplasm	Increased number of autophagic vesicles, degradation of Golgi apparatus, polyribosomes and endoplasmic reticulum	Formation of apoptotic bodies, preservation of organelles
Biochemical features		
Cell-death pathway	Caspase independent	Caspase dependent
Detection methods		
Ultrastructure	Electron microscopy	Electron microscopy
Cell biological assays	Protein-degradation assay, LC3 levels	TUNEL staining, Annexin V staining, Hoechst nuclear staining, DNA fragmentation assay, sub G1 population

Figure 10: Comparison of autophagic cell death and apoptosis (Kondo et al., 2005).

However, there are also some experimental evidences where inhibitors of autophagy such as 3MA, inhibit cell death. This drug retards or partially prevents cell death in starved hepatocytes from carcinogen-treated rats (Schwarze and Seglen, 1985), in tamoxifen-treated mammary carcinoma cells (Bursch et al., 1996), in chloroquine-treated cortical neurons (Kaidi et al., 2001) in nerve growth factor (NGF)-deprived sympathetic neurons (Xue et al., 1999), in serum and potassium-deprived cerebellar granule cells (Canu et al., 2005), in serum deprived PC12 cells (Uchiyama, 2001), and in TNF-treated human T lymphoblastic leukemia cells (Jia et al., 1997). It must be noted that the use of 3MA pharmacological inhibitor can affect other pathways than autophagy, so as class III PI3K itself might be required for other survival pathways.

Yu et al. (Yu et al., 2004) published the first genetic evidence showing that autophagic cell death is dependent on ATG genes. The authors observed that inhibiting caspases with zVAD, a caspase inhibitor with broad specificity, or by knocking down caspase 8, surprisingly induced cell death in several cell lines. Morphologically, the cell death was characterized by accumulation of autophagic vacuoles, without the hallmarks of apoptosis. In these settings, 3MA and downregulation of beclin 1 and Atg7, inhibited both the accumulation of autophagic vacuoles and the cell death, indicating that classical autophagy induction was really needed for cell demise. Intriguingly, it was shown that the signaling pathway triggering the death process included the activation of RIP and JNK. These results, then, suggest that although the same autophagy genes are required both for autophagic cell death and starvation-induced autophagy, there might

be differences in the signal transduction pathways activating these two processes. Further, this study suggests that caspase 8 might act as a suppressor of autophagic cell death. It is possible that caspase 8 could act as a hub that regulates which death pathway, apoptotic or autophagic, the cell takes. Thus, apoptosis and autophagy may have complementary roles in cell death: if apoptosis fails, then cells have the option of dying via autophagy. This mechanism might be of importance for instance during viral infection, because many viruses have means to inhibit caspase activation (Eskelinen, 2005).

Another study demonstrating that autophagy genes are required for type II cell death was performed in embryonic MEFs double deficient for Bax and Bak, which are resistant to apoptotic death stimuli (Shimizu et al., 2004). When treated with apoptosis-inducing drugs like etoposide or staurosporine, double-deficient cells still underwent a cell death characterized by accumulation of autophagic vacuoles that could be inhibited by downregulation of Atg5, beclin 1, and by 3-MA. Of note, this etoposide-induced autophagic cell death was related to the deficiency of Bax and Bak, since it was not detected in cells deficient in Apaf-1 or caspase 9, or in wild-type fibroblasts treated with the caspase inhibitor zVAD. Interestingly, overexpression of Bcl-2 and Bcl-X_L in wild type cells triggered Atg5-mediated etoposide-induced autophagic death similar to that observed in the Bax/Bak double-deficient cells. Overexpression of Bcl-2 or Bcl-X_L in $Atg5^{-/-}$ cells did not trigger cell death, demonstrating the requirement of the autophagy protein Atg5 for cell death induction. Furthermore, silencing of Bcl-X_L with RNA interference in $Bax/Bak^{-/-}$ cells prevented the etoposide-induced accumulation of autophagic vacuoles and cell death, indicating Bcl-X_L was required for this death pathway. Since Bcl-2 and Bcl-X_L are known to interact with Beclin 1 (Liang et al., 1998) the authors suggested that Bcl-X_L might influence autophagosome formation at least partly via regulation of Beclin 1.

These studies showed that apoptotic stimuli can induce an autophagic cell death in cells where the apoptotic pathway is inhibited and that, under certain conditions, autophagic cell death can act as a substitute for apoptosis. Infact, autophagic cell death has not been observed in wild type cells treated with etoposide, it is not blocked by 3-MA and the main route for this cell demise is apoptosis. One explanation might be that apoptosis is faster than autophagic death; indeed the wild type cells are dying much more than the $Bax/Bak^{-/-}$. Another possible explanation is consistent with the theory of Lockshin and Zakeri claiming that cells preferentially die by apoptosis, but will die by any alternative

available route, including autophagy, if exposed to harsh enough stimuli (Lockshin and Zakeri, 2004). Interestingly, such a role for Bcl-X_L has not been demonstrated in starvation-induced autophagy. Infact, it was shown that different cell lines overexpressing a Beclin 1 lacking Bcl-2 binding domain died faster both in untreated and under nutrient starvation conditions (Pattingre et al 2005). Of note the recent finding that Beclin 1 is a BH3-only protein suggests that it can have some apoptotic functions by itself (Oberstein et al., 2007).

Other works demonstrated that downregulation of Atg5 expression suppressed both autophagic vacuole formation and cell death in HeLa cells treated with interferon-γ. On the other hand, overexpression of Atg5 or stimulation with interferon-γ induced 3-MA sensitive autophagic cell death (Pyo et al., 2005). Intriguingly, the caspase inhibitor zVAD inhibited cell death, but not the formation of autophagic vacuoles, suggesting that caspases might be involved in the signaling or execution of the cell death downstream of autophagic vacuole formation. Moreover, it was also shown that Atg5 interacted with the death domain of FADD (a component of the apoptosis-signaling cascade initiated by death receptors). The Atg5-mediated cell death, but not the accumulation of autophagic vacuoles, was blocked in FADD-deficient cells, suggesting that FADD is a downstream effector of Atg5. This result has two implications: first, it shows that the accumulation of autophagic vacuoles can be separated from cell death. Second, it suggests that an autophagy protein (Atg5) can mediate a death signal to a protein involved in apoptotic cell death (FADD). Of note, Yusefi et al. recently reported that Atg5 is a calpain (a non-lysosomal Ca^{++} dependent cysteine protease) substrate and that the resulting processed form triggers apoptotic cell death (Yousefi et al., 2006).

The results aforementioned suggest that autophagic vacuoles can act as the initiator of the death process, which then mediates the death signal to the apoptotic machinery that finalizes the cell demise However further studies are required to clarify the relationship between apoptosis and autophagic cell death and the role of Bcl2 family proteins in regulating auophagy and its crosstalk with apoptosis. Recent work performed in our laboratory further sustains these observations. We demonstrated that calpain is needed to trigger macroautophagy: cells lacking calpain activity show impaired autophagy and result more sensitive to apoptosis induced by different autophagic stimuli such as ceramide, etoposide and starvation (Demarchi et al., 2006). Accordingly, recent studies demonstrate that calcium mobilizing agents such as ionomycin and thapsigargin (that can also act as calpain inducers) are potent inducers of macroautophagy (Hoyer-Hansen

et al, 2007). Furthermore in this work it was demonstrated that calcium induces autophagy via a signaling pathway involving the Ca^{++} activate kinase CaMKKβ that is a direct activator of AMPK. As discussed in previous sections, AMPK inhibits mTOR activation and leads to autophagy induction. Another in vivo model substantiates an involvement of autophagy in the clearance and demise of specific cell types. In atherosclerotic plaques, macrophage infiltration is involved in destabilization of the plaque, whereas SMC (smooth muscle cells) contribute to plaque stability. Stent-based delivery of rapamycin in rabbits has been demonstrated to cause selective macrophage death while leaving SMC cells viable. Both cell lines showed equal mTOR inhibition under rapamycin; nevertheless, only macrophages died consequently to the treatment. This different behaviour has been attributed to the higher metabolic demand of macrophages in respect to SMC cells, suggesting that autophagy can lead to cell demise depending on the metabolic activity of specific cell lines (Verheye et al., 2007).

Autophagy as a cell survival mechanism

The pro-survival function of autophagic process is better documented than autophagic cell death and even though autophagy genes act as mediators of cell death in some experimental conditions, increasing evidence suggests that the accumulation of autophagosomes in dying cells or tissues commonly represents a failed rescue effort in response to external stress. Starvation induced autophagy is coupled to the survival of unicellular organism such as yeast; mutants in autophagic essential genes are in fact viable in normal growing medium, but die upon aminoacid withdrawal. In these conditions, the activation of autophagic degradation allows the cell to eliminate the structures and organelles in excess, and generates free fatty acids and amino acids necessary to fuel mitochondrial ATP production and maintain protein synthesis (Levine, 2005). Also, multicellular organisms including mammals activate autophagy in nutrient starvation conditions to sustain life. In *C. elegans*, silencing of the autophagy genes unc-51, bec-1, atg8 and atg18 impair survival during the dauer phase of development, indicating the importance of autophagy in recycling nutrients during starvation (Melendez et al., 2003). In newborn mice, autophagy activation is essential to allow survival during the severe starvation occurring after trans-placental nutrient supply is interrupted, and before nutrients are restored by milk feeding. Mice deficient in the essential autophagy gene Atg5 develop normally, but die within a day after birth, demonstrating the importance of the autophagic process to maintain survival in

starvation conditions (Kuma et al., 2004). Interestingly these Atg5$^{-/-}$ mice show signs of energy depletion, in particular the myocardial ATP production is reduced; moreover, they exibit low aminoacids concentration in plasma and tissues. Although death of individual cells has not been observed in these mice, it is possible that the function of tissues such as the heart and the diaphragm, which require high energy levels, is impaired due to the inability to induce autophagy. Also Atg7 conditional knockout mice show the same phenotype, moreover the cells of these mutants display multiple cellular abnormalities, such as the appearance of concentric membranous structures, deformed mitochondria and the accumulation of ubiquitin-positive aggregates, suggesting the importance of autophagy also in the quality control of proteins and organelles in quiescent cells (Komatsu et al., 2005).

Autophagy appears to be important also for maintaining cellular bioenergetics and survival when cells are unable to take up external nutrients, like for example during growth factor deprivation. In the absence of growth factors there is a decreased surface expression of nutrient transporters, decreased nutrient uptake and an intracellular deficiency of factors such as interleukin-3 (IL-3), cells undergo rapid apoptosis, but the inactivation of the apoptotic genes BAK and BAX prevents this death allowing the cells to survive (Lum et al., 2005). Immortalized interleukin-3 (IL-3) dependent cells derived from bone marrow of Bak-Bax double deficient mice survive in culture for weeks after IL-3 deprivation. The expression levels of the major glucose transporter GLUT-1 declined rapidly following IL-3 withdrawal; correspondingly, mitochondria membrane potential decreased and also ATP content fell, and these cells displayed the hallmarks of autophagy. Autophagy induction triggers progressive destruction of organelles in order to keep ATP at levels sufficiently high to allow cell survival, untill the occurrence of atrophy that finally degenerates into death. Notably readdition of IL-3 within six weeks recovers cell survival and proliferation. Treatment with 3-MA and Atg5 or Atg7 depletion increase cell death in IL-3 deprived cells, demonstrating autophagy requirement for the mantainance of the bioenergetic balance within the cell and survival (Boya et al., 2005). Furthermore, it was demonstrated that autophagy inhibition with chemical inhibitors (hydroxychloroquine, monensin, bafilomycin A1 and 3-MA) or by knocking-down autophagy essential genes such as ATG5, ATG12, ATG10 and BECLIN-1 in nutrient starved HeLa cells leads to apoptotic cell death, indicating that autophagy plays a pro-survival function during nutrient deprivation. Indeed, cell death triggered by autophagy inhibition is reduced by stabilization of mitochondrial

membranes with Bcl-2, and by caspase inhibitors. Interestingly, when fusion between autophagosomes and lysosomes is inhibited with lysosomotropic agents (hydroxychloroquine, monensin) or by LAMP-2 depletion, the nutrient starved cells undergo autophagic cell death. Also in this case, readdition of nutrients and removal of the drug rescues the normal viability of the cell, but if starvation is too long the cells reach the point-of-no-return, undergoing apoptosis. (Boya et al., 2005).

The pro-survival function of autophagy is not only restricted to nutrient starvation, but plays an important role also in cellular homeostasis in response to stressors, through the removal of damaged mitochondria and other organelles, in degrading intracellular pathogens, and in degrading protein aggregates too large to be removed by the ubiquitin-proteasome system (Levine and Yuan, 2005). It has been reported that autophagy protects mammary carcinoma, colon adenocarcinoma prostate carcinoma and malignant glioma cell line U373-MG from radiation induced cell death, and addition of inhibitors of the autophagic pathway restored sensitivity to radiation (Paglin et al., 2001; Ito et al., 2005). Another in vivo investigation performed in pigs demonstrated that autophagy is induced in chronically ischemic myocardium. Interestingly in this model, apoptosis is initially engaged (e.g. after few ischemic episodes), while the continuous exposure to the ischemic damage induces increased expression of autophagic genes such as beclin1, cathepsins, LC3 and others, contemporarily a decrease in apoptosis is observed. These data argue for a cytoprotective role of autophagy in chronically ischemic myocardium, indeed autophagy allows full functional recovery consequent to the removal of the ischemic injury (Yan et al., 2005).

Autophagy and diseases

Disease	Activation of autophagy	Inactivation of autophagy
Cancer		
Early stages	Blocks tumor growth	Favors tumor growth Makes cells unable to enter autophagic cell death after exposure to anticancer treatments
Late stages	Favors survival of cells in low-vascularized tumors Favors removal of damaged intracellular macromolecules after anticancer treatments	Prevents survival of cells in low-vascularized tumors Increases efficiency of anticancer treatments because damaged macromolecules cannot be eliminated
Vacuolar myopathies	Promotes elimination of the cytosolic autophagic vacuoles	Results in the accumulation of autophagic vacuoles that weaken skeletal and cardiac muscles
	If hyperactivated, could result in muscle waste	
Neurodegeneration		
Early stages	Favors removal of cytosolic protein aggregates	Increases accumulation of cytosolic protein aggregates
Late stages	Destroys irreversibly damaged neurons by autophagic cell death	
Axonal injury	Favors removal of neurotransmitter vesicles and damaged organelles	Prevents removal of damaged organelles and neurotransmitter vesicles. Cytosolic release of neurotransmitters induces apoptosis
	Provides energy and membranes for regeneration	Slows down regeneration
Infectious disease	Contributes to the elimination of bacterial and viral particles	Offers a survival environment for the bacteria that are able to inhibit autophagosome maturation Facilitates viral infection

Figure 11: Possible outcomes of autophagy activation or inactivation in different pathologies.

Autophagy and cancer

Cancer results from the misregulation of pathways that govern cell differentiation, cell proliferation, and cell survival. Autophagy may protect against cancer by sequestering damaged organelles, permitting cellular differentiation, increasing protein catabolism, and/or promoting autophagic death in order to mantain cellular homeostasis. Alternatively, autophagy may contribute to cancer by promoting the survival of nutrient starved cells. Recent data are most consistent with a model in which autophagy contributes to tumor suppression and defects in autophagy contribute to oncogenesis. Indeed, when baseline levels of autophagic degradation were compared, cancer cells showed reduced protein degradation than their normal counterpart (Gozuacik and Kimchi, 2004). The inhibition of a catabolic pathway could provide cancerous cell with some developmental advantages: first, early stage of tumor development require a higher level of protein synthesis than protein degradation and therefore inhibition of autophagy could maintain continuous tumor growth. Second, autophagy could decrease mutation rate and suppress oncogenesis by eliminating damaged organelles that could produce genotoxic stresses such as free radicals (Kondo et al., 2005). In accordance with this hypothesis, recent findings clearly demonstrate that autophagy limits metabolic stress to protect genome integrity, and defective autophagy increases DNA damage and genomic instability in different cell lines, facilitating cancer progression

(Mathew et al., 2007; Karantza-Wadsworth et al., 2007). Furthermore, the mammalian autophagy gene *beclin-1* has tumor suppressor activity in breast carcinoma cells (Liang et al., 1999), is commonly deleted in human breast ovarian and prostate cancer (Aita et al., 1999), and is a haploinsufficient tumor suppressor gene in mice (Qu et al 2003; Yue et al 2003). As seen with beclin1, UVRAG is monoallelically deleted in various human cancers. HCT116 cells bear a monoallelically frameshift mutation in UVRAG gene that generates a premature stop codon; indeed, overexpression of UVRAG in HCT116 cells has been demonstrated to decrease tumorigenesis of HCT116 in nude mice (Liang et al., 2006). Similarly, dowregulation of AMBRA1 expression, leads to increased proliferation rates (Fimia et al., 2007).

Several theories regarding the role of autophagy-dependent death and autophagy-dependent survival in cancer biology have been proposed (Shintani and Klionsky, 2004; Gozuacik and Kimchi, 2004). According to one theory, autophagy-dependent death is a mechanism of tumor suppression. Although there are no direct data to support this hypothesis, it is possible that autophagy is involved in the spontaneous or chemotherapy-induced death of existing tumor cells. The role of autophagy in cell death in apoptosis-competent cells is unclear; however, autophagy gene-dependent death in cells crippled in apoptosis (e.g., zVAD-treated cells; $bax^{-/-}$, $bak^{-/-}$ cells) may have relevance for cancer biology and therapy, since human tumor cells frequently contain mutations that render them resistant to apoptosis. One prediction is that such cells have an increased dependency on autophagy pathways for self-destruction, and that the impact of autophagy-dependent cell death on tumor progression may be greater in tumor cells that are resistant to apoptosis. Another prediction is that the enhanced autophagy-dependent death potential of apoptosis-resistant tumor cells might be exploited therapeutically by the administration of autophagy inducing agents. Indeed, there are examples of putative autophagic cell death in cancer cell lines treated with chemotherapeutic agents.

During tamoxifen-induced death of MCF7 cells (a cell type that contains a mutation in caspase-3), there is a marked upregulation of Beclin 1 expression (Scarlatti et al., 2004); moreover, temozolomide (a DNA alkylating agent) induces autophagy, but not apoptosis, in malignant glioma cells (Kanzawa et al., 2004). In both cases chemotherapy-induced autophagic cell death is inhibited by 3-MA (Scarlatti et al., 2004, Kanzawa et al., 2003) and also ceramide induces autophagic cell death in malignant glioma (Daido et al., 2004).

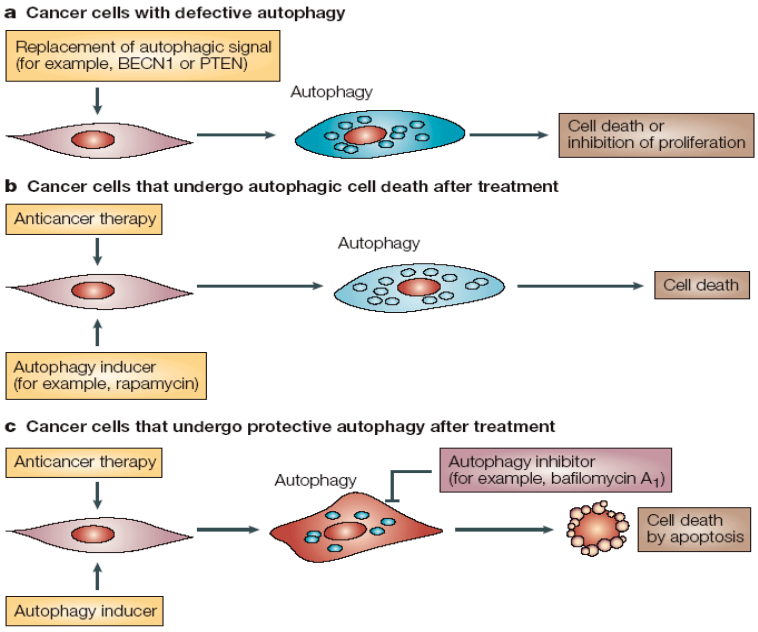


Figure 12: Potential strategies for treating cancer by manipulating the autophagic process (Kondo et al., 2005).

In addition, arsenic trioxide induces autophagy in malignant glioma (Kanzawa et al., 2003; Kanzawa et al., 2005), resveratrol induces autophagic cell death in ovarian cancer cells (Opipari et al., 2004), and soybean derived saponins induce autophagy in colon cancer cells (Ellington et al., 2005). However, direct evidence proving that autophagy is a bona fide death pathway in chemotherapy-treated cancer cells is lacking. In addition, rapamycin, an inhibitor of TOR kinase that has promising antitumor effects in human clinical trials (Huang and Houghton, 2003), is one of the most potent known inducers of autophagy, but is not known to induce autophagic cell death.

In contrast to potential pro-death effects, more clearly established pro-survival effects of autophagy during nutrient starvation might speed up tumor initiation and/or progression (Ogier-Denis and Codogno, 2003; Gozuacik and Kimchi 2004). As tumor cells grow beyond their blood supply, they are exposed to nutrient-limiting conditions, and it is possible that transformed cells use autophagy as a survival strategy in this setting. Indeed, tumor cell lines of diverse origins, including colon carcer, breast cancer, melanoma, hepatoma, and malignant glioma, switch on autophagy in response to nutrient starvation (Kondo et al., 2005), leading to hypothesize the existence of a positive selective pressure for the maintenance of autophagy in tumor progression.

Accordingly, recent studies revealed that autophagy enables cell survival in vitro and in vivo when apoptosis is inactivated (Degenhardt et al., 2006).

Autophagy and neurodegeneration

The accumulation of mutant or toxic proteins plays a major role in chronic neurodegenerative diseases (Martinez-Vincente and Cuervo, 2007). Neurodegenerative disordes are catalogued as protein conformational disorders, because they all originate consequently to protein misfolding events. Cells continously check the quality of proteins repairing or removing them when abnormalities are detected. Two major systems are involved in this quality control: the proteasome system and autophagy. An imbalance between efficiency of the quality control and the amount of altered proteins can lead to conformational disorders, in which intracellular accumulation of abnormal proteins occurs. For example deficits in the ubiquitine proteasome system are associated with various neurodegenerative diseases, since ubiqutin-positive inclusions frequently appear in neurons of animals or patients with neurodegenerative diseases. A direct involvement of autophagy in neurodegenerative disorders has been recently demonstrated: generation of neuron- specific autophagy Atg7 deficient mice showed behavioural defects and ubiquitinated-positive proteinaceous aggregates accumulation throughout the brain (Komatsu et al., 2006). Similar phenotypes were obtained in neural-cell-specific Atg5-/- mice (Hara et al., 2006) and in mouse embryos bearing Ambra1 functional deficiency (Fimia et al., 2007).

Morphologic evidence of autophagy has been reported in neurodegenerative diseases including Parkinson, Huntington, and Alzheimer diseases, and transmissible spongiform encephalopathies (Qin et al., 2003; Anglade et al., 1997; Yu et al., 2004; Liberski et al., 2004). Recent studies have provided insights regarding autophagy and neurodegenerative disease. α-synuclein, the major component of neuronal cytoplasmic inclusions that characterize Parkinson and other neurodegenerative diseases (Maries et al., 2003), is degraded both through the proteasome, and by chaperone-mediated autophagy pathways (Webb et al., 2003). Interestingly, pathogenic α-synuclein mutants associated with familial, autosomal-dominant forms of Parkinson disease (Polymeropoulos et al., 1997; Kruger et al., 1998) are inefficiently degraded by chaperone-mediated autophagy. Since the accumulation of wild-type α-synuclein in neuronal inclusions is common in adult-onset neurodegenerative diseases, these experiments suggest that defects in autophagy-related pathways may contribute to

multiple neurodegenerative diseases. Consistent with this hypothesis, autophagy has also been implicated in regulating the turnover of Huntingtin (Htt), the protein involved in Huntington disease, an autosomal-dominant neurodegenerative disorder caused by the expansion of a polyglutamine (polyQ) tract in Htt. The accumulation of expanded polyQ-containing proteins in insoluble aggregates in affected neurons is a hallmark feature of Huntington and other polyQ expansion diseases (DiFiglia et al., 1997). Although neuronal Htt proteins in inclusions are highly ubiquitinated, polyQ is a poor substrate for proteasomes (Venkatraman et al., 2004). Pharmacological evidences suggest a role for autophagy in the degradation of the N-terminus of Htt: 3-MA increases the aggregation of Htt with expanded polyQ in clonal striatal cells (Qin et al., 2003), while Rapamycin, an inducer of autophagy, reduces the aggregation of expanded polyQ in transfected cells (Ravikumar et al., 2002) and protects against neurodegeneration in a fly model of Huntington disease. In addition, it improves performance on behavioural tests and decreases aggregate formation in a mouse model of Huntington disease (Ravikumar et al., 2004). As the disease progresses, the presence of undigested materials accumulating inside autophagic vacuoles, the sustained activation of macroautophagy to sequester the aggregates and the removal of crucial cellular elements by autophagy along with the aggregates, could lead to a generalized cellular failure. In this case, the cell can use autophagy to undergo autophagic cell death (Cuervo 2004 et al.,). Other evidences implicating the involvement of autophagy in neurodegeneration are the observation of increased beclin1 levels found consequently to traumatic brain injury in mice near the injury site (Erlich et al., 2006). Moreover in traumatic brain injury rapamycin has a cytoprotective effect (Erlich et al., 2007), further confirming its fundamental role in the control of neurodegeneration.

RESULTS

In silico analysis of beclin 1 promoter

Many cell types, such as stem cells, must be able to adjust their proliferation rate in response to a wide variety of mitogenic stimuli. Checkpoint controls can cancel such cell cycle progression by activating the barrier-program unless prosurvival signalling raises the threshold required for entering such failsafe fate. Clear examples of such prosurvival mechanisms are the activation of NF-kB and PI3K/Tor signalling cascades. Autophagy is a program induced by damage/stress and nutrients/oxygen deprivation, while it is negatively regulated by the PI3k/Tor pathway: it can, in fact serve as a survival mechanism for cancer cells to avoid switching into apoptosis.

Recent work in our lab has focused on calpains, a family of sixteen intracellular nonlysosomal Ca^{2+}-regulated cystein proteases, mediating regulatory cleavage of specific substrates involved in a number of processes.

In particular, we have shown that calpain inhibits susceptibility to p53-dependent apoptosis (Benetti et al., 2001) and regulates the transcription competent β-catenin pool (Benetti et al., 2005). In addition, we have shown that calpain is involved in NF-kB activation and its relative prosurvival function in response to ceramide, where calpain deficiency strengthens the proapoptotic effect of ceramide (Demarchi et al., 2005). Most recently we further explored the involvement of calpain in the apoptotic switch and found that, in calpain deficient cells, autophagy is impaired, resulting in a dramatic increase in apoptotic cell death (Demarchi et al., 2006).

Many activators of NF-kB, such as TNFα, TRAIL, reactive oxygen species (ROS) and ceramide, work also as potent macroautophagy inducers. However, the possible crosstalk between these two signalling pathways is largely unknown.

The aim of this work was to investigate whether there was any relation between NF-kB or other transcription factors and regulation of autophagy.

As a first approach, we performed *in silico* analyis using CONFAC (Karanam and Moreno, 2004) in order to verify whether any transcription factor binding site (TFBS) was conserved in the promoters of autophagic genes both in humans and mouse. An input consisting of a list of autophagic genes was therefore submitted.

The CONFAC analysis of regions including 500bp upstream of the Trancription Start Sites (TSS) and including up to 15kbp of the first intron revealed that no TFBS was conserved between human and mouse.

However, considering the single outputs relative to each gene, beclin 1 was reported to bear 3 putative overlapping NF-kB binding sites inside the first intron.

Subsequently, a set of NF-kB target genes (together with beclin 1 (Figure 1A)) was analyzed with CONFAC in order to verify the results obtained during the previous analysis. Finally, a Mann-Whitney test was performed between the set of NF-kB target genes and a set of 250 randomly selected genes from RefSeq (Figure 1B). Two matrices, each containing the number of occurrences of every TFBS found by CONFAC in the two datasets were genrated and compared to verify the statistical significance of CONFAC results about NF-kB regulated genes.

The results corroborated the reliability of this analysis, confirming the significance of NF-kB binding sites occurrence in the promoter regions investigated.

These *in silico* analysis, therefore, suggested that beclin 1 might indeed represent a new NF-kB target gene.

Recent work from Farnham P.J. et al., focused on the identification of E2F regulated promoters using ChIP experiments, led to the isolation of three genomic fragments, all of which representing strong E2F binding sites (Weinmann et al., 2001). One of these fragments, referred to as ChET4, was demonstrated to correspond to the promoter region of beclin 1.

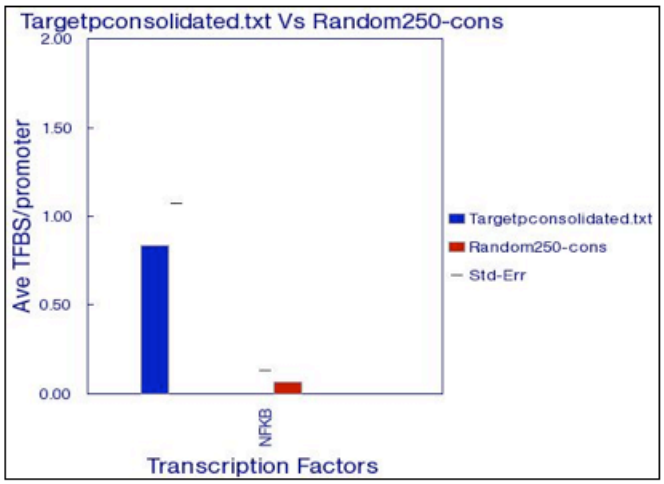
We therefore analyzed ChET4 sequence using Consite (Sandelin et al., 2004) tool. Results obtained confirmed that the ChET4 fragment contained the NF-kB binding site as expected (Figure 1C); moreover, the kB element was exactly positioned on ChET4 sequence. The kB site sequence on beclin 1 promoter, GGGACTTCCC, has been found to be positioned at around –100bp upstream the ATG of beclin 1.

Figure 1A

Fig 1: A) Gene list submitted to CONFAC analysis (including beclin 1 and a set of other NF-kb target genes). B) NF-kB binding site occurrence in the promoter regions investigated (blue) compared to its occurrence in the promoter regions of 250 randomly selected control genes (red). CONFAC output was submitted for subsequent statistical analysis using Mann-Whitney test. Parameters relative to the statistical analysis are reported in the right table of figure 1B. C) ConSite analysis of ChET4 sequence allowed exact kB site positioning on the first intron of beclin 1, at around –100 bp from beclin ATG. Genomic organization of beclin 1 whit respect to ChET4 sequence is shown in the box, and table in figure 1C shows the exact position of kB site on beclin 1 sequence.

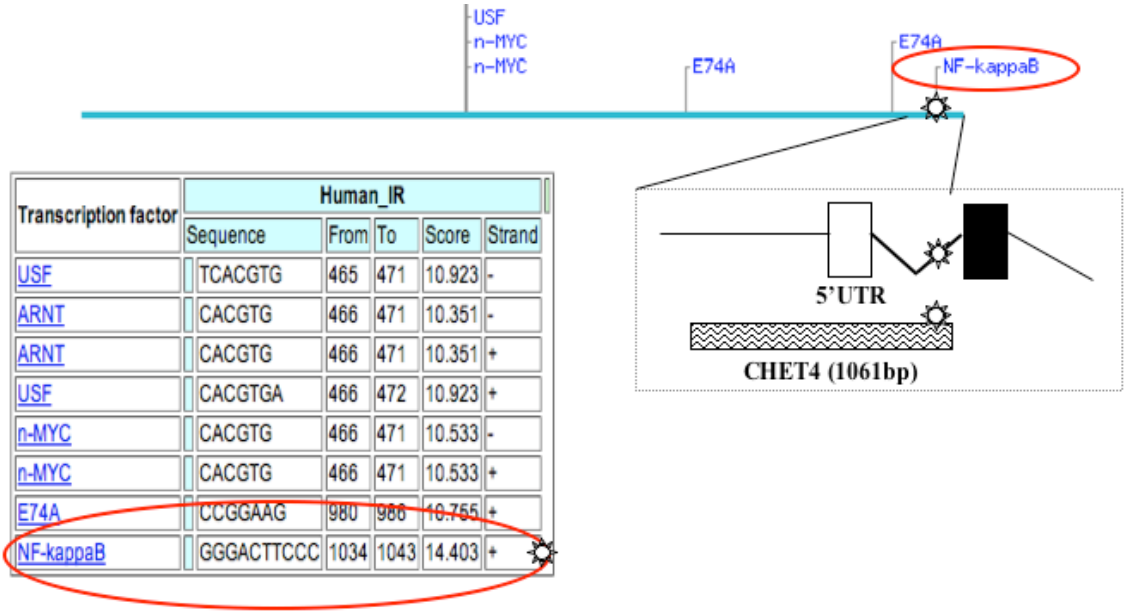
Figure 1B

Sample data : Targetpconsolidated p65 human target genes and beclin1
Control data: Random250-cons.confac selected
Cut-offs:Mean Difference: 0.50P-value: 0.05



Factor	NF-kB
Mean	0.833
SampleMean	0.0656
ControlMean Difference	0.768
P-value	8.3676e-06
Std-Dev - Sample	1.778
Std-Err - Sample	0.242
Std-Dev - Control	0.512
Std-Err - Control	0.0656

Figure 1C



Transcription factor	Human_IR				
	Sequence	From	To	Score	Strand
USF	TCACGTG	465	471	10.923	-
ARNT	CACGTG	466	471	10.351	-
ARNT	CACGTG	466	471	10.351	+
USF	CACGTGA	466	472	10.923	+
n-MYC	CACGTG	466	471	10.533	-
n-MYC	CACGTG	466	471	10.533	+
E74A	CCGGAAG	980	986	10.755	+
NF-kappaB	GGGACTTCCC	1034	1043	14.403	+

NF-kB subunit p65 enhances the transcriptional activity of beclin 1 promoter

The data obtained from the *in silico* analysis of beclin 1 promoter, prompted us to perform further investigations in order to clarify the role of NF-kB in the regulation of beclin 1 promoter, using transativation luciferase reporter assays.

Thus, pUC19 ChET4 clone was obtained from P.J. Farnham' laboratory. The region corresponding to the beclin 1 promoter was cloned into pGL3 luciferase reporter vector (Figure 2A) to perform transactivation luciferase reporter assays.

Figure 2A

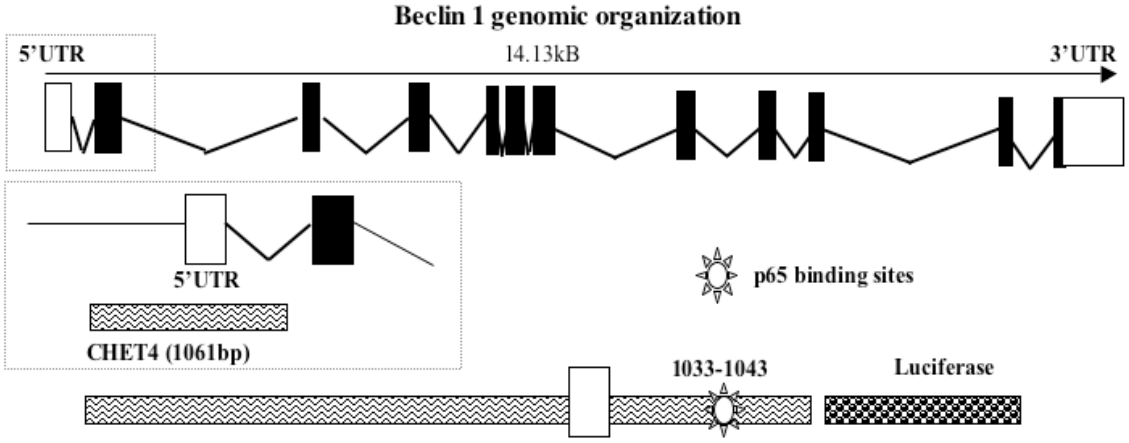


Figure 2 A) top of the figure represents beclin 1 genomic organization according to Ensemble ENSG00000126581: boxed region shows an enlarged view of beclin 1 5' untranslated region, first intron and second exon. In the middle box the same region is enlarged toghether with a piece of upstream promoter region, and exact localization of ChET4 is reported. Lower image shows KpnI/SacI insertion of ChET4 beclin 1 promoter region upstream to the luciferase gene.

pGL3ChET4 reporter was cotransfected into U2OS and HEK293T in combination with expression vectors for the following transcription factors E2F, p65, p50, p65 together with p50, with an empty vector as a negative control (Figures 2B and 2C). Data were normalized by cotransfection with CMV renilla TK vector. As shown in Figure 2B and 2C, overexpression of p65 alone efficiently enhanced beclin 1 promoter activity when compared to the empty vector. Notably, while both overexpressed E2F and p50 were unable to increase beclin1 promoter activity, synergism between p65 and p50 towards luciferase transactivation was observed, in particular in HEK293T (Figures 2B and 2C).

To address the role of constitutive p65 in the regulation of ChET4 beclin 1 promoter, a small interference RNA was used to selectively knock down endogenous p65. A scramble siRNA (SiCtrl) was used as a control. p65 expression was almost completely abolished 48h after transfection of the specific p65 siRNA. pGL3ChET4 was overexpressed in U2OS cells after p65 depletion or treatment with a scrambled siRNA as control (Figure 2D).

Figure 2B

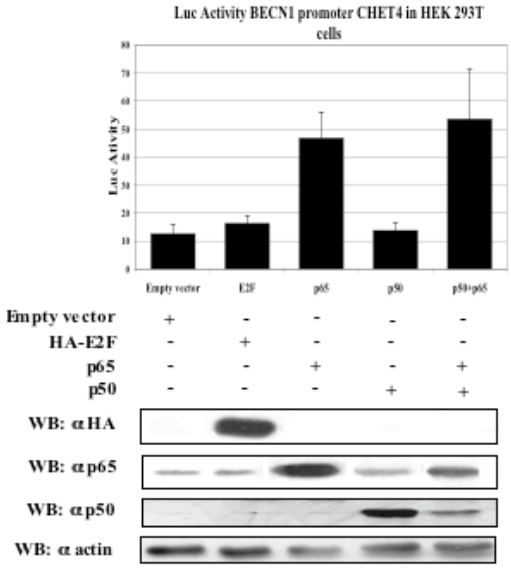


Figure 2C

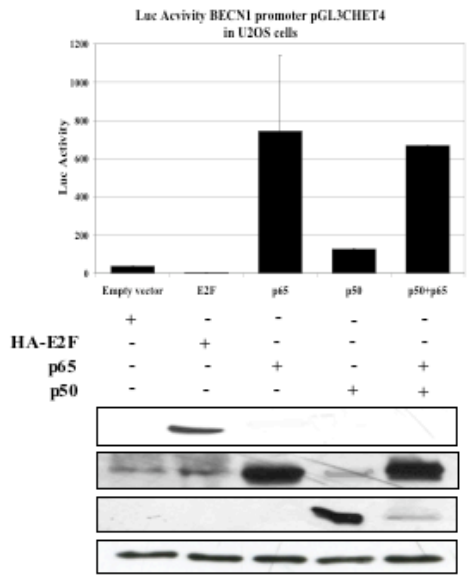


Figure 2B and 2C: HEK293T and U2OS cells were cotransfected with pGL3ChET4 and E2F, p65, p50, p65 and p50 or a control empty vector. Luciferase assays were performed 24h after transfection. Data represent the means of at least four independent experiments and error bars represent standard deviations; lower panels: Western blot analyses were carried out using the same lysates to monitor steady-state levels of each overexpressed protein.

Interestingly, depletion of endogenous p65 significantly decreased ChET4 transactivation activity (Figure 2D).

Ceramide and Tamoxifen are two well established autophagy inducers in several cell lines. Treatment with these drugs has been demonstrated to induce beclin 1 expression (Scarlatti et al., 2005). Moreover, ceramide can also trigger p65 activation (Demarchi et al., 2005; Boland and O'Neil, 1998). For these reasons, subsequently to p65 silencing and to an overnight pGL3ChET4 overexpression, U2OS cells were treated with 25μM

ceramide or with 10μM tamoxifen for 5 hours. As shown in Figure 2D, we couldn't see any increase of ChET4 transactivation after treatment of the cells with both drugs. Nonetheless, p65 depletion in all these experimental settings significantly decreases beclin 1 promoter activity.

Figure 2D

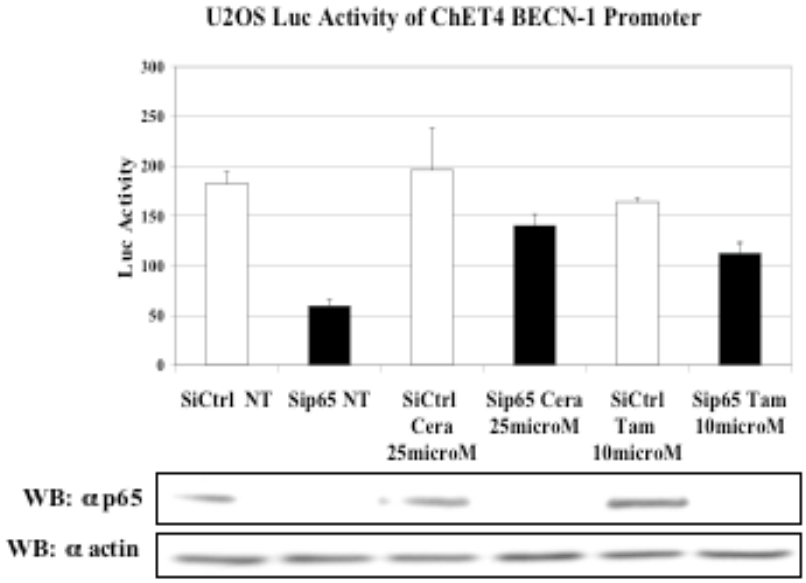


Figure 2D: U2OS cells. p65 expression was selectively knocked down after 48h. Subsequent overexpression of pGL3ChET4 was performed for 16h, followed by treatment with both 25μM ceramide or 10μM tamoxifen for 5h. Cell lysates were prepared immediately after treatment for the luciferase reporter assay. Data represent the means of at least three independent experiments and error bars represent standard deviations; lower panels: Western blot analyses were carried out on the same lysates to monitor steady-state levels of endogenous levels of p65.

Isolation of p65/RelA consensus on ChET4 beclin1 promoter

Preliminary *in silico* analysis of ChET4 beclin 1 promoter region with Consite tool, carried out at 85% cut off stringency evidenced the presence of two other putative NF-kB binding sites in a more upstream region, even if with a less significant score (Figure 3A).

In order to understand which of these three kB sites was actually required for the observed p65-mediated enhancement of beclin1 promoter activity, we decided to exclude the effect of the more upstream NF-kB binding sites. Analysis of ChET4 sequence showed that a unique site for SacI was present at 685bp. Therefore, SacI Hind

III region of CheET4 was excided and cloned it into pGL3 luciferase reporter vector. Thereafter we will refer to this region as p65 consensus region.

pGL3 p65 consensus region was cotransfected in combination with E2F, p50, p65

Figure 3A

Transcription factor	Human_IR				
	Sequence	From	To	Score	Strand
c-REL	GCGATTTTCC	21	30	8.445	+
p65	GCGATTTTCC	21	30	8.755	+
SP1	CCCCAGCCTC	33	42	7.002	-
c-REL	TGGGATTACA	52	61	6.647	+
SP1	ACCATGCCCA	72	81	9.172	-
c-REL	GGAATTACAA	184	193	6.539	-
SP1	ACCATGCCCG	203	212	8.507	-
SP1	ACCACGCCCG	385	394	9.305	-
SP1	GAGACGGGGT	417	426	7.424	+
c-REL	CGGGGTTTCA	421	430	8.509	+
p65	CGGGGTTTCA	421	430	8.140	+
SP1	ACCGCGCCCG	520	529	8.639	-
SP1	AGCCTCCCCA	557	566	7.198	-
SP1	TCCCAGCCCG	634	643	8.274	-
SP1	ACAGTGCCTC	812	821	7.198	-
SP1	TCCCTGACCC	939	948	6.115	-
c-REL	TGGGACTTCC	1033	1042	9.677	+
p65	TGGGACTTCC	1033	1042	9.110	+
c-REL	GGGACTTCCC	1034	1043	8.048	+
p65	GGGACTTCCC	1034	1043	8.783	-
p65	GGGACTTCCC	1034	1043	11.345	+
SP1	TCCCTCCCTT	1040	1049	6.400	-

Figure 3A Output of p65 Consite Analysis of ChET4 sequence performed at 85% stringency cut off: red circles indicate other two NF-kB binding sites on ChET4 beclin 1 promoter region. At the bottom of the table the third NF-kB binding site is reported.

expression vectors or a control empty vector both in HEK293T and U2OS cells.

As shown in Figures 3B and 3C, the effects of p65 on this shorter construct encompassing p65 consensus were similar, if not more pronounced, to the ones observed with ChET4 construct which covers a broader promoter region.

In light of these latest results it was likely that the kB site emerged with CONFAC analysis should be the only one responsible for the effects seen on beclin 1 promoter after p65 overexpression. To further confirming these data, again, we assessed the effect of knocking down endogenous p65 using specific p65 siRNA and scrambled siRNA as a control (SiCtrl). Also in this case, we tested the effect of both ceramide and tamoxifen on p65 consensus region of ChET4. Silencing and treatment conditions were identical to the ones previously used for monitoring the effect of specific p65 depletion on ChET4 promoter activity.

Notably, in these settings we could observe that both ceramide and tamoxifen treatments enhanced the transcription driven by the p65 consensus binding region.

These data can be explained considering that the effect of p65 binding on a restricted region is cleared from the possible interference due to the interaction of other transcription factors that may mask p65 binding. Although both drugs, previously shown to activate p65, clearly amplified the activity of the reporter driven by the p65 consensus binding region, this effect couldn't be observed using ChET4. A possible explanation for this discrepancy is that other endogenous transcription factors could bind and regulate ChET4 activity in response to the drugs.

Figure 3B

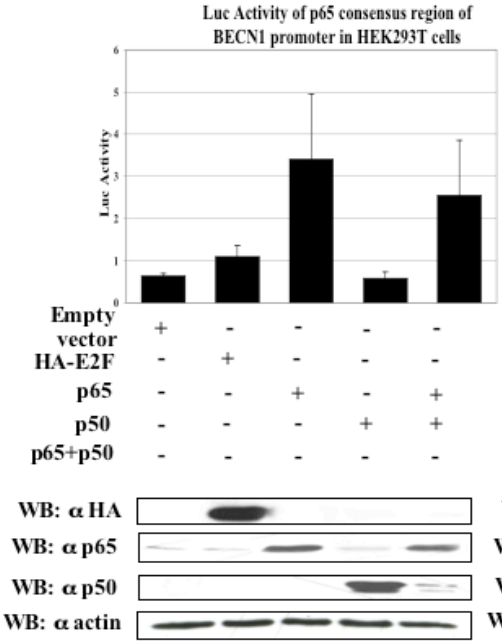


Figure 3C

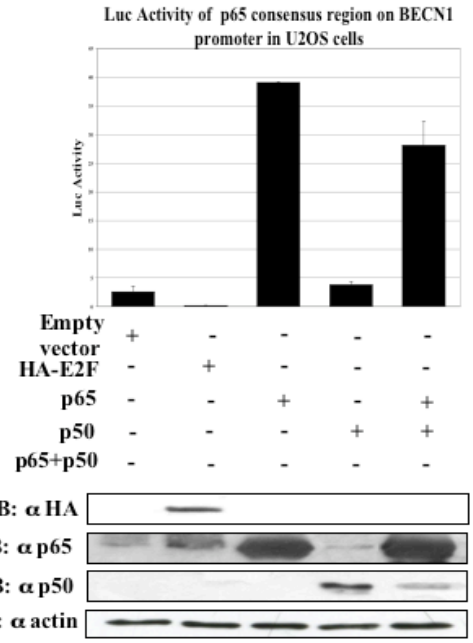


Figure 3D

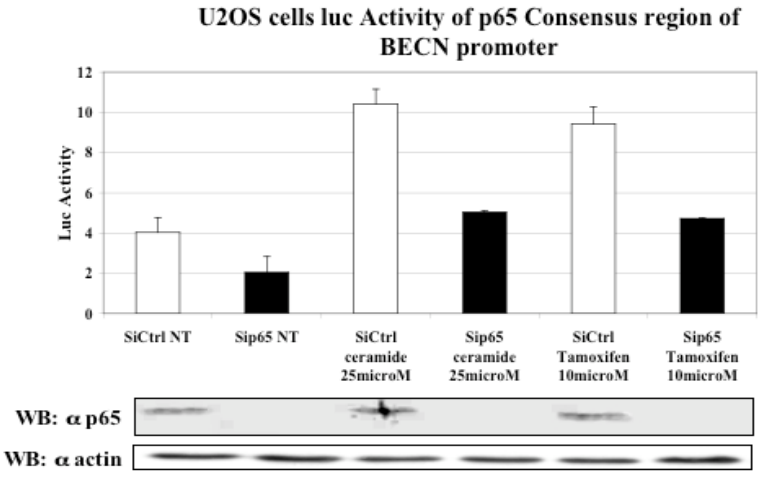


Figure 3: B) and **C)** HEK 293T cells and U2OS cells were cotransfected with pGL3ChET4 and E2F, p65, p50, p65 and p50 or a control empty vector, respectively. Luciferase assays were performed 24h after transfection. Data represent the means of at least five independent experiments and standard deviations are shown; lower panels: Western blot analyses were carried out on the same lysates to monitor steady-state levels of each overexpressed protein. **D)** U2OS cells. p65 expression was selectively knocked down for 48h. Subsequent overexpression of pGL3ChET4 was performed for 16h, followed by treatment with either 25μM ceramide or 10μM tamoxifen for 5h. Cell lysates were prepared immediately after the treatment for subsequent luciferase reporter assay. Data represent the means of at least three independent experiments; lower panels: Western blot analyses were carried out on the same lysates to monitor steady-state levels of endogenous levels of p65.

p65 regulation of beclin 1 transcription

Data obtained from the previous experiment argued for an involvement of either p65 or the p65/p50 dimer in the regulation of beclin 1 promoter. p50 has been reported to bind kB elements on the promoters of NF-kB target genes through its RHD, but lacks C-terminal transactivation domain, indeed cannot be responsible for any transactivation.

These pieces of evidence prompted us to investigate whether p65 could regulate beclin 1 mRNA levels.

HEK 293 cells were transfected with p65, E2F or with a control empty vector and analyzed by semi-quantitative RT-PCR. The primers used for the amplification step mapped at a central codifying region of beclin 1 and were previously demonstrated to work properly in semi-quantitative RT-PCR experiments (Scarlatti et al., 2005). We named these primers B5' and B3', B5' being the primer on exon 4 while B3' the one positioned on exon 8.

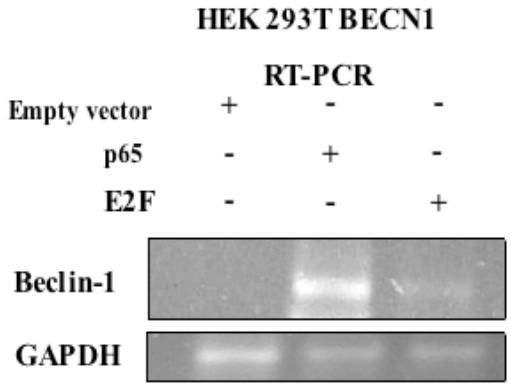


Figure 4A: RT-PCR analysis of beclin 1 and GAPDH mRNA expression in HEK 293T. Cells were transfected as indicated. RNAs were reverse-transcribed and amplified with specific primers for beclin 1

and GAPDH as described under "Experimental Procedures." The relative beclin 1 mRNA level was normalized by reference to the GAPDH. Upper panel: the band about 500bp expected to be amplified using B1 and B2 set of primers specific for beclin 1 region exon 4-8 (B1 and B2 primers) was significantly increased following p65 overexpression with respect to a control empty vector. RT-PCR analysis was repeated three times. A representative experiment is reported.

HEK 293T cells overexpressing p65 showed a significant enhancement of beclin 1 mRNA compared to transfection of either a control empty vector or of E2F transcription factor (Figure 4A).

Thus, we went on trying to better elucidate the effect of specific p65 siRNA on beclin 1 transcription using RT-real time PCR experiments.

At genomic level, beclin 1 gene transcript consists of 12 exons. This genomic organization suggests that alternative splicing of beclin 1 RNA is likely to occur. In fact, besides beclin 1 full lenght transcript, EBI database reports the existence of several clones coming from different libraries that seem to contain beclin 1 alternative spliced forms in both human and mouse species. Therefore, we decided to perform some preliminar analysis. Indeed, we divided beclin 1 region into three parts in order to monitor the effect of specific p65 silencing on each region by semi-quantitative RT-PCR. Three set of primers were designed: A5'/A3', B5'/B3' and C5'/C3' as indicated in Figure 4B.

As positive controls, two specific beclin1 siRNAs were used (indicated as SiB1 and SiB2 in Figure 4B [red]) while a scrambled control siRNA was used as a negative control (SiCtrl).

Thus, U2OS cells were transfected with p65 siRNA, SiB1, SiB2 and control siRNA (SiCtrl). 72 hours later total RNA was extracted and subjected to reverse transcription. PCR amplifications using the three different sets of primers were performed and the relative beclin 1 mRNA level was normalized by reference to the GAPDH.

As shown in Figure 4C, selective silencing of p65 significantly decreased amplification of beclin B5'-B3' and C3'-C5' fragments, with a more prominent effect on C3'-C5' fragment. SiB1 was more efficient than SiB2 in decreasing the amplification of beclin1 B5'-B3' and C3'-C5' fragments. Notably, we couldn't obtain any amplification using A5'/A3' set of primers (a 516bp amplificate was in fact expected). Other tests were performed using B5'/A3' and A5'/B3'sets of primers and demonstrated that A5' primer probably did not match any cDNA sequence (data not shown).

Figure 4B: beclin1 transcript according to ENSEMBL ENSG00000126581. Sets of primers used for RT-PCR analysis of beclin 1 transcript. Positions of both beclin1 siRNAs used as positive controls (SiB1 and SiB2) are reported in red color

Figure 4B

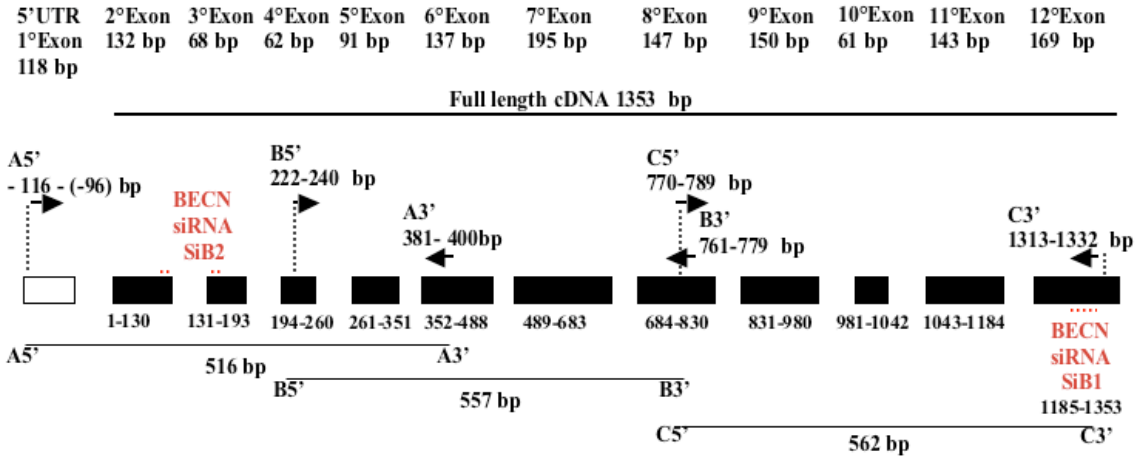


Figure 4C

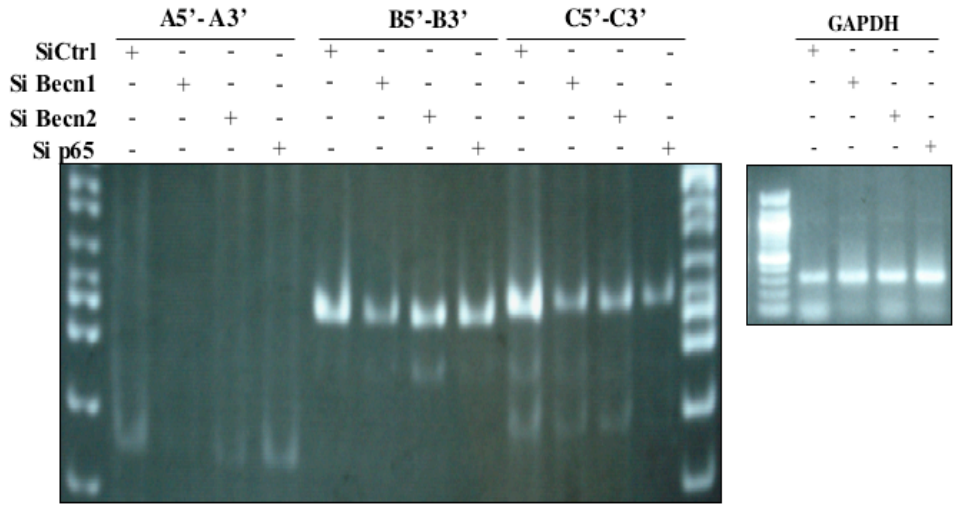


Figure 4C: RT-PCR analysis of beclin 1 mRNA fragments obtained using the three different sets of primers reported in figure 4B and GAPDH mRNA expression in U2OS cells. Cells were tranfected as indicated. RNAs were reverse-transcribed and amplified with the indicated set of primers for beclin 1 and with GAPDH primers as described under "Experimental Procedures." The relative beclin 1 mRNA level was normalized by reference to the GAPDH. Left pannel: the major bands of around 500bp expected to be amplified using B5'/B3' and C3'/C5'sets of primers were significantly downregulated following specific p65 siRNA, SiB1 or SiB2 tranfection with respect to a transfected scrambled control siRNA. Note that no specific A5'-A3' fragment has been amplified.

Analysis of beclin 1 transcripts using different databases underlined that beclin 1 first exon is poorly characterized and several annotated beclin1 transcripts were shown to lack this exon. Notably, other three amplificates, besides the expected ones and with lower molecular weight were amplified using B5'/B3' and C5'/C3' sets of primers, and they seemed to be differentially regulated by the specific beclin 1 siRNAs used. This suggests that actually other beclin1 transcripts, different from the full lenght one, might exist.

Data from these preliminary assays prompted us to design a beclin 1 Taqman probe and the relative primers on the twelfth beclin 1 exon for subsequent quantitative PCR analysis. In fact, C5'-C3' amplificate was efficiently silenced both with p65 siRNA and SiB1. Moreover, p65 silencing efficiently decreased also the lower molecular weight amplificates.

U2OS cells were then transfected with p65 siRNA, SiB1 and a scramble control siRNA (SiC-). After 72 hours of transfection, total RNA was extracted and reverse transcribed as explained in 'Experimental procedures' section. Real Time PCR reactions were performed using the aforementioned specific Beclin 1 Taqman probe and a GAPDH probe was used for subsequent data normalization. Calculation of the mRNA values was performed using ΔΔCt method. Specific p65 siRNA was demonstrated to decrease beclin1 mRNA levels in a significant manner with respect to both control siRNA and SiB1 in U2OS cells.

We could therefore conclude that constitutive p65 regulates beclin 1 transcription.

In attempt to further clarify whether, besides Beclin 1 mRNA, p65 could positively regulate also beclin 1 protein steady state levels, p65 was overexpressed alone or toghether with p50 in U2OS, HEK 293T or Hela cells and western blot analysis of beclin 1 protein levels was performed. Unfortunately, in these settings, we were unable to see any p65-mediated change in the regulation of beclin 1 steady state levels (data not shown), and since we did not know whether the lack of such expected p65- mediated effect on beclin 1 could be directly attributable to inefficient transfection or to postranslational beclin 1 regulation, we decided to investigate p65 role in beclin 1-mediated downstream effects.

Figure 4D

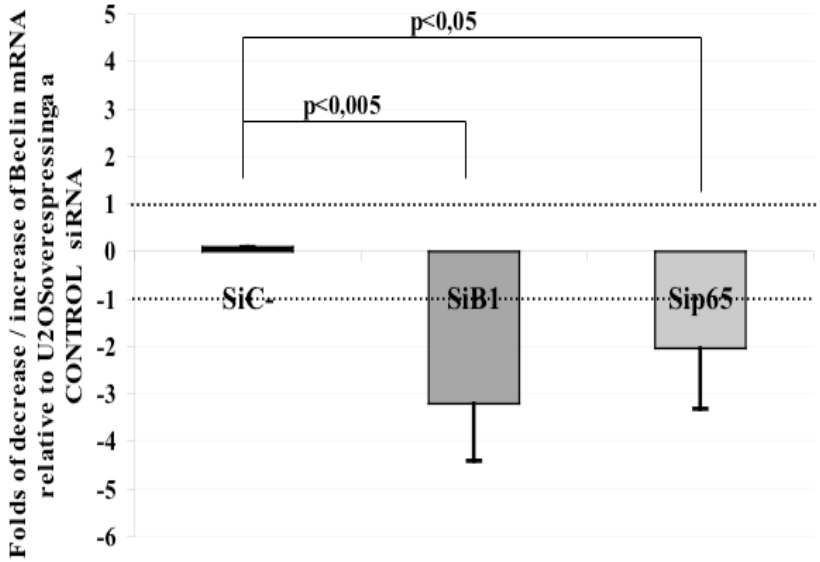


Figure 4D: qPCR analysis of beclin 1 mRNA in U2OS cells. Cells were transfected with siRNAs as indicated. Equal amounts of total RNAs were reverse-transcribed and amplified with specific Taqman beclin 1 FAM probe and with Taqman GAPDH VIC as described under "Experimental Procedures." Student's *t*-test was used to assess the statistical significance of the observed beclin 1 mRNA differences in qPCR experiments.

Production of anti-LC3 antibody

The clear effect of p65 on beclin 1 transactivation prompted us to investigate whether it could regulate beclin 1–mediated autophagy induction.

The data reported in the previous sections demonstrated that p65 could regulate beclin1 transcription. In order to clarify whether p65 could have any effect also on autophagy, we decided to produce an antibody against LC3, since commercially available antibodies didn't show appropriate immunoreactivity against LC3 protein. Indeed, in our previous work (Demarchi F. et al., 2006) we used an αLC3 antibody kindly provided by Yoshimori's lab.

The strategy used for αLC3 antibody production was very similar to the one used by Yoshimori (more specific information can be found in "experimental procedures" section). We decided to produce two differently tagged RatLC3 recombinant proteins in BL21 DE3 bacteria strain: a 6His GST tagged LC3 recombinant protein was used for rabbit immunization and a 6His tagged LC3 recombinant protein was used for subsequent rabbit serum purification. Proper induction of the expression of both recombinant proteins was assessed (Figure. 5A).

Figure 5A

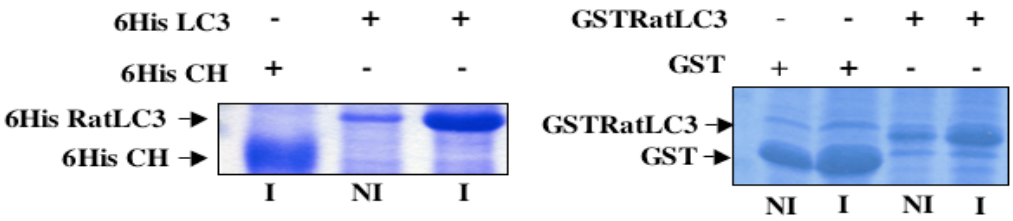


Figure 5A: expression of recombinant proteins was tested. Left panel: Coomassie gel loaded with lysates of BL21DE3 bacteria strain transformed with constructs codifying for 6His Rat LC3 or for another 6His protein previously produced are shown. Right panel: Coomassie gel loaded with lysates of BL21DE3 bacteria strain transformed with constructs codifying for GST alone or GST Rat LC3. NI: not induced; I: induced (0,5M IPTG, 37°C).

Recombinant proteins were further analyzed by western blot using specific αGST antibody and Yoshimori's αLC3 antibody. The recombinant proteins were efficiently expressed and were recognized by both antibodies (Figure 5B). Proteins were subsequently purified onto a nickel column. A rabbit was then immunized with the 6His GST tagged LC3 recombinant protein.

Figure 5B

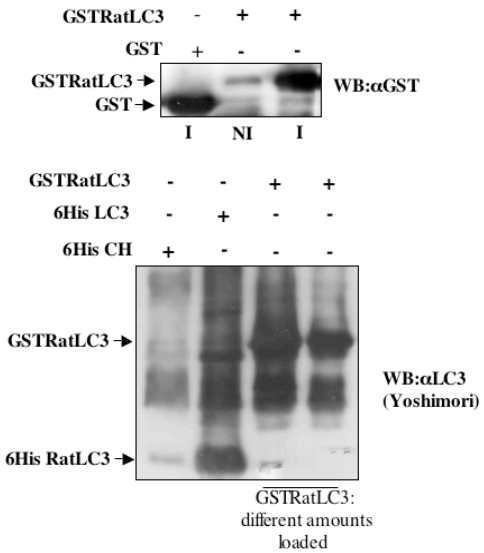


Figure 5B: BL21 DE3 bacteria were transformed with constructs codifying 6His GST Rat LC3, 6His Rat LC3 or for 6His CH and GST alone as controls. Upper panel: Western blot analysis of bacteria expressing 6His GST Rat LC3 using αGST antibody. Lower panel: Western blot analysis of bacteria expressing 6His GST Rat LC3 and 6His rat LC3 using a specific αLC3 antibody provided by Yoshimori's lab.

Sera from immunized rabbit were subsequently purified using a column onto which 6His LC3 had been covalently crosslinked. Purified αLC3 antibody was then tested in human cells overexpressing either human or rat LC3 proteins. In fact, high degree of homology among rat, human and murine LC3 primary amino acid sequences exists, indeed the antibody was expected to recognize also huaman and murine LC3.

As shown in Figure 5C, our purified αLC3 antibody efficiently recognized overexpressed human and rat LC3.

Next, we tested the immunoreactivity of our antibody against endogenous murine LC3 in wild type murine embryo fibroblast (MEF) cells in which autophagy was induced by ceramide and tamoxifen treatment. Figure 5D clearly shows that our αLC3 antibody recognized murine LC3. Moreover, it efficiently recognized both LC3I and LC3II forms. Thus, induction of autophagy can be easily followed using this antibody. We performed the same analysis also using human cell lines and the same result was obtained (data not shown, see below).

Figure 5C

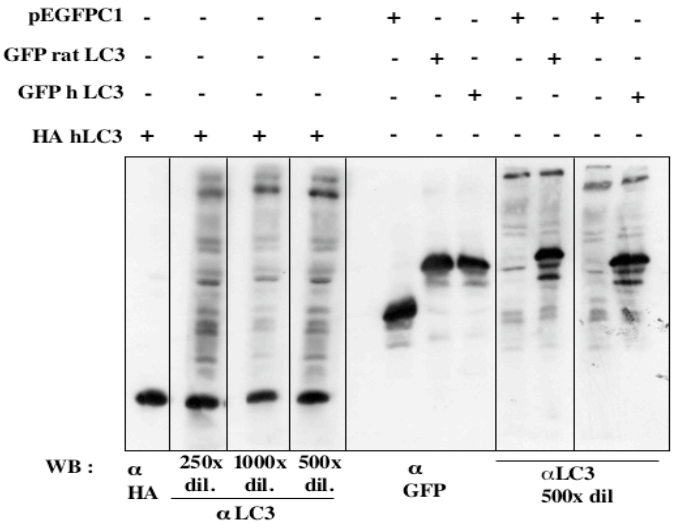


Figure 5C: Lysates from U2OS cells overexpressing HA tagged human LC3, GFP tagged human LC3, GFP tagged Rat LC3 or GFP alone were subjected to SDS-PAGE and subsequently analyzed by western blot using our purified αLC3 antibody. αLC3 antibody showed good immunoreactivity against both human and rat overexpressed LC3 proteins.

Figure 5D

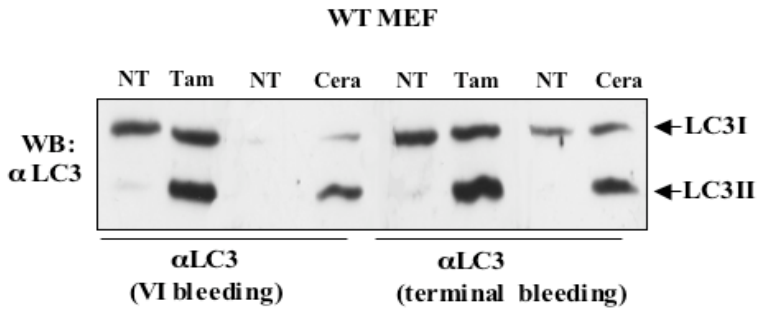


Figure 5D: wild type MEF cells were treated for 12h with 25μM ceramide or 10μM tamoxifen. Cell lysates were then subjected to SDS-PAGE and subsequently analyzed by western blot using αLC3 antibody purified from different bleedings. αLC3 antibody produced in our lab efficiently recognized both LC3I and LC3II forms in murine embryo fibroblast cell line. Autophagy induction is indicated by the increase of LC3II band observed after treatment with both drugs and due to LC3I processing.

p65 positively regulates autophagy in different human cell lines

In the previous sections we brought to light that p65 positively regulates beclin 1 transcription, probably by directly binding to its promoter and inducing its expression. These observations prompted us to perform further biochemical analysis in order to clarify whether p65 was involved in the induction of the autophagic process.

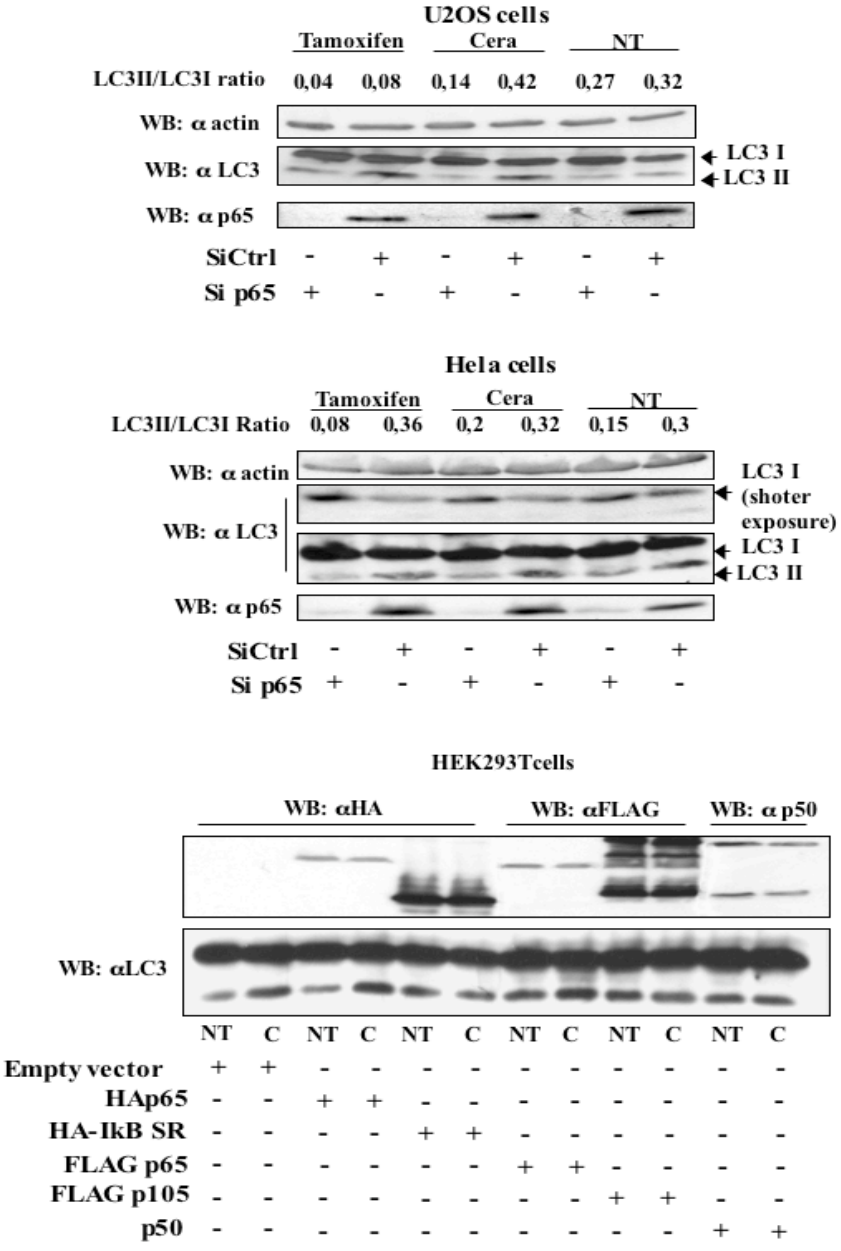
U2OS, HEK 293T and HeLa cell lines were analyzed for their ability to undergo the autophagic process consequently to ceramide or tamoxifen treatment and the effect of p65 knock down or of overexpression of either p65 or of p65 inhibitor proteins was monitored.

As shown in Figure 5A, p65 silencing was able to block LC3I to LC3II processing, indicating that p65 could mediate autophagy induction. Notably, p65 silencing exerted this kind of blockage also in not treated cells; indeed, it is likely that p65 might affect cellular constitutive autophagy. We also asked whether the disappearance of LC3II band following p65 silencing could be attributed to an induction of p65-mediated LC3II lysosomal processing; nonetheless, it is unlikely that such a process occurred, since accumulation LC3I was observed after p65 knockdown.

Effect of p65 and of p65 inhibitors (IkBSR [an IkB protein in which serine 32 and 36 have been substituted with alanine residues and therefore cannot be targeted to proteasomal degradation], p50 and p105) overexpression on LC3I processing were also assessed in HEK 293T cells treated or not with 25μM ceramide.

Figure 6A: Upper and middle panels: Silencing of p65 decreases LC3 processing. U2OS and Hela cells were transfected with specific p65 siRNA and with a scramble control RNA for 72h and subsequently treated for 12h with 25μM ceramide or 10μM tamoxifen. Effect of p65 knock down on LC3 processing (e.g. on autophagy) was evaluated. Cell lysates were subjected to SDS-PAGE and subsequently analyzed by western blot using our αLC3 antibody. Autophagy induction is indicated by the increase of LC3II band observed after tratment with both drugs. Quantification of LC3I and LC3II bands was performed using Image J program and values reported indicating LC3II/LC3I ratio are representative of the autophagic process activation. Lower panel: LC3I processing in HEK 293T overexpressing the indicated constructs was monitored.

Figure 6A



Of note, when overexpression of p65 inhibitor proteins was performed we couldn't see any increase of LC3I processing following ceramide treatment, while the same processing was not blocked following p65 overexpression. Unfortunately, HEK293T cell line LC3II levels seemed to be significantly higher with respect to LC3II levels observed in U2OS and Hela cell lines, rendering LC3I processing more difficult to detect.

Next, the effect of p65 depletion on autophagosome formation was monitored by means of immunofluorescence experiments using GFP-LC3 as a read-out. U2OS cells transfected either with specific p65 siRNA or with a scrambled control RNA for 72 hours; later small amounts of human GFP LC3 (about 100ng) were overexpressed in the same cells, and after 6 hours the cells were treated with tamoxifen for 12 hours.

In the absence of an autophagic stimulus, GFP LC3 staining appears diffused throughout the cytoplasm, while following autophagic induction, autophagosome formation is evidenced by a punctuate perinuclear staining. As shown in Figure 6B, p65 silencing significantly abolished autophagosome formation, this effect being more prominent in tamoxifen treated cells.

Figure 6B

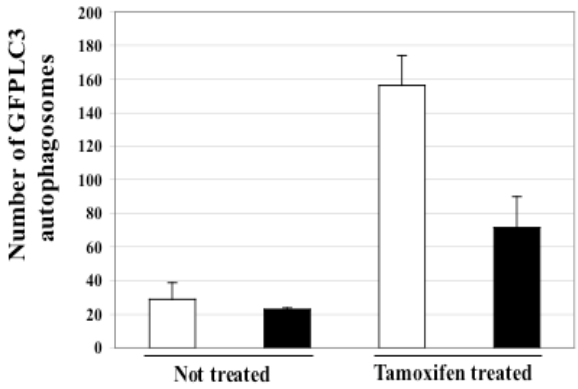
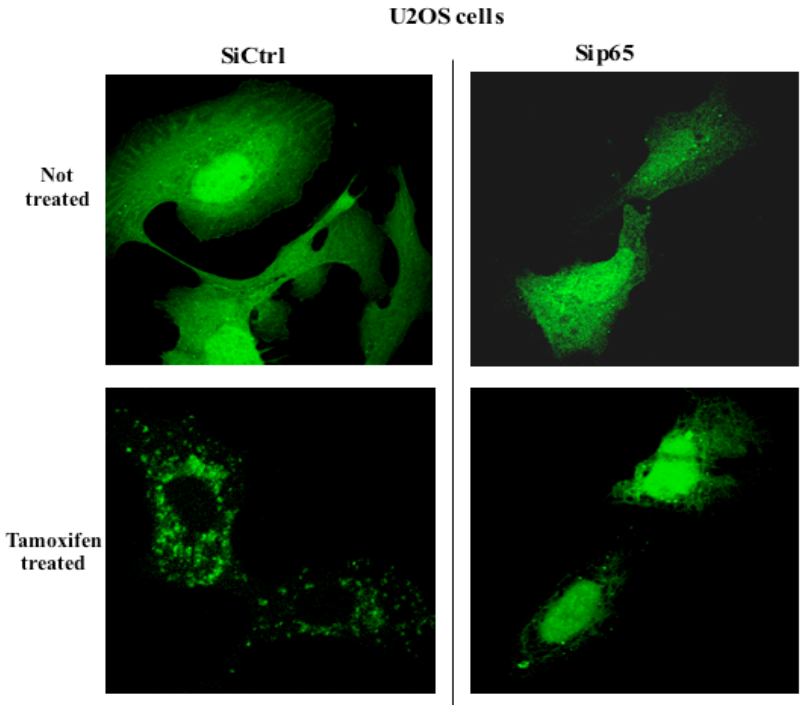


Figure 6B: U2OS cells were transfected with specific p65 siRNA and with a scramble control RNA for 72h; subsequent overexpression of 100ng of human GFP LC3 for 6h was performed. Finally cells were either treated or not with 10μM tamoxifen for 12h. Immunofluorescence was than performed and some representative images are shown in the lower panel. Upper panel: 200 cells were counted in each experiment, and the number of cell presenting a punctuate GFP LC3 staining are reported. Data represent the means of at least three independent experiments.



Beclin 1 steady state levels and autophagy regulation in U2OS derivative cells lines inducible for p65 or IkBα SR expression

The results reported in the previous section argue for an involvement of p65 in autophagy induction suggesting that this induction might result from the upstream p65-mediated enhancement of beclin 1 transcription. In spite of evidences demonstrating p65-mediated upregulation of beclin 1 transcription, we were not able to see any significant change of beclin1 protein steady state levels neither with the use of a specific p65 siRNA, nor by overexpression. Several can be the reasons beyond these discordant data. One explanation could rely in the low transfection efficiencies that can be reached when performing transfection of either siRNAs or cDNAs with the conventional protocols. In fact, using conventional transfection strategies usually the transfection efficiencies obtained range from 20 up to 50%; thus, the final effect of transfection could not be strong enough when considering specific processes. An other possibility that arises when working with transcription factors, is that transcription and translation of other target genes, beyond the one of interest, might interfere by temporal and spatial means in some processes (e.g. in translation or in the activation of proteolytic systems etc), rendering the detection of the final effect more difficult to see. Furthermore, it is necessary to bear in mind that p65 signalling occurs through tightly regulated waves of

activation; consequently it is fundamental to work at appropriate times after p65 activation.

In order to circumvent these problems, we decided to produce U2OS cell lines inducible for both p65 and IkBSR. Indeed, in this way, we could assure a 100% p65 and IkBSR expression efficiency and, more importantly, we could better regulate the timing of p65 or IkBSR induction/expression.

U2OS ponasterone A inducible cell lines for either p65 or IkBSR (Super Repressor) were obtained as described in the experimental procedures section.

Inducibility of two clones for each inducible cell line was tested. As shown in figure 7A

Figure 7A

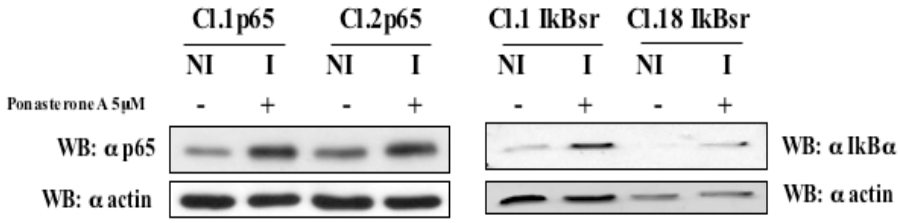
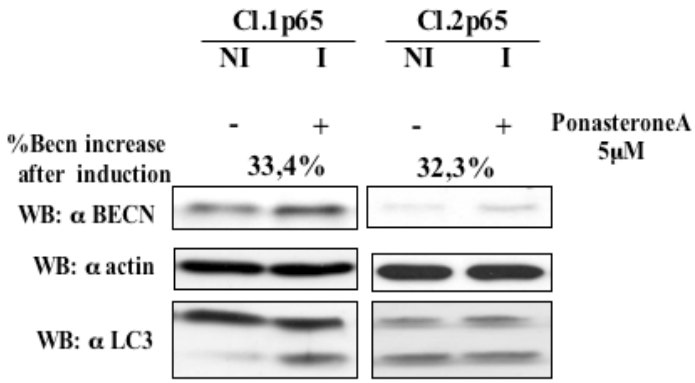


Figure 7A: generation of U2OS cell lines inducible for either p65 or IkBSR inducible cell lines were obtained as described in the 'experimental procedures' section. Two clones for each kind of inducible U2OS cells were tested. Treatment with 5μM ponasteron A for 16h significantly increased the expression of both p65 and IkB of the selected clones.

all the clones selected showed proper induction of p65 or IkBSR after ponasterone A treatment.

Figure 7B



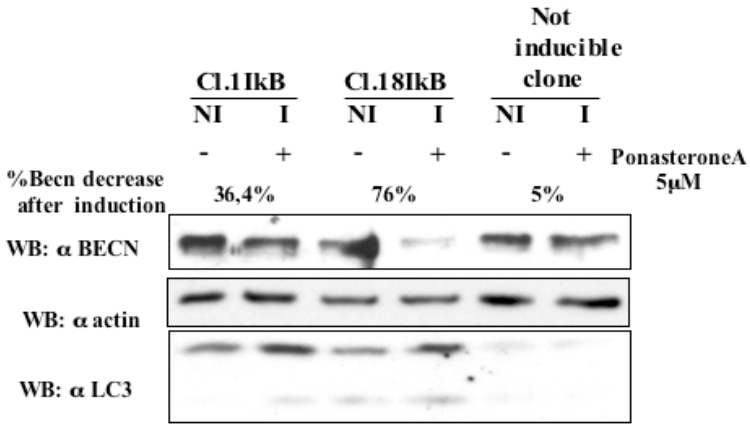


Figure 7B: p65 upregulates beclin 1 steady state levels and induces autophagy. Upper panel: clones 1and 2 inducible for p65 were treated with 5μM ponasterone A for 6 hours. Cell lysates were subjected to western blot analysis for monitoring beclin1 levels and LC3I processing. Lower panel: western blot analysis of clones 1 and 18 inducible for IkBSR was preformed; again, beclin1 levels and LC3I processing were monitored. (NI: not induced; I: induced). Quantification of the bands was performed using Image J tool.

Therefore, we went on monitoring beclin 1 protein levels and LC3I processing in all the clones selected after induction with ponasterone A. Figure 7B shows that p65 induction significantly enhanced beclin 1 levels; on the other hand, LC3I processing efficiently occurs in Cl.1p65, but not in Cl.2p65. One likely explanation for this contradictory behaviour might be provided when considering that p65 induction triggers the activation of several downstream effectors, mainly through its ability to transactivate a plethora of target genes. Among them, IkBα undergoes to p65-mediated transactivation after ponasterone A treatment (see Figure 7D lower panel) and this event can both halt or limit p65 signalling.

Analysis of beclin1 levels and LC3I processing in Cl1IkB and Cl.18IkB revealed a prominent downregulation of beclin1 steady state levels and a net decrease of LC3I processing.

Several experiments have been performed using these clones in order to verify p65 or IkBSR-mediated effects in autophagy induction. A common observation was that the aforementioned effects were more reproducible when using IkBSR clones. Explanation for these observations might again be the ones mentioned above: activation of p65 leads to downstream activation of several genes, the effect of which is hardly predictable.

Thus, IkBSR might represent a better tool for understanding p65-mediated effect in our experimental settings.

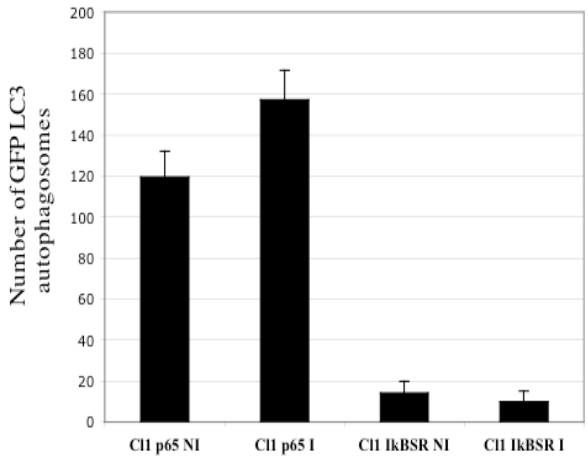
Finally, immunofluorescence assays were performed and each p65 or IkBSR clone was tested for its ability to induce autophagosome formation. All the clones were transfected with low amounts of human GFPLC3 for 6h and then induced with ponasterone A for 12 hours. Figure 7C shows representative images. Similarly to what was emerged by previous western blot analysis of the clones, in this settings p65 expression induction increases the number of GFPLC3 positive autophagosomes. Strikingly, in these settings, IkBSR was almost completely unable to induce autophagosome formation. Furthermore, endogenous LC3 staining and autophagosome formation was monitored by fluorescence microscopy and the ability of p65 to induce autophagosome formation was confirmed (Figure 7D).

Altogether, the data obtained with these derivative U2OS cell lines, stronlgy suggest that p65, in these settings, is a positive regulator of the autophagic process. Nonetheless, p65-mediated regulation of autophagy is likely to be finely tuned, since p65 induction caused waves of activation or inactivation of its signalling. For this reason, the use of a derivative U2OS cell line expressing IkBSR that overcomes p65 tuning and halts is signalling, better elucidated p65 role in the autophagic process.

Thus, the results presented led us to hypothize that p65-mediated autophagy induction should occur via p65 upstream ability to directly regulate beclin 1 expression at a transcriptional level.

Furthermore, since we demonstrated that IkBSR almost completely abolishes autopahgosome formation, it is likely that a canonical NF-kB is involved in the regulation of autophagy.

Figure 7C



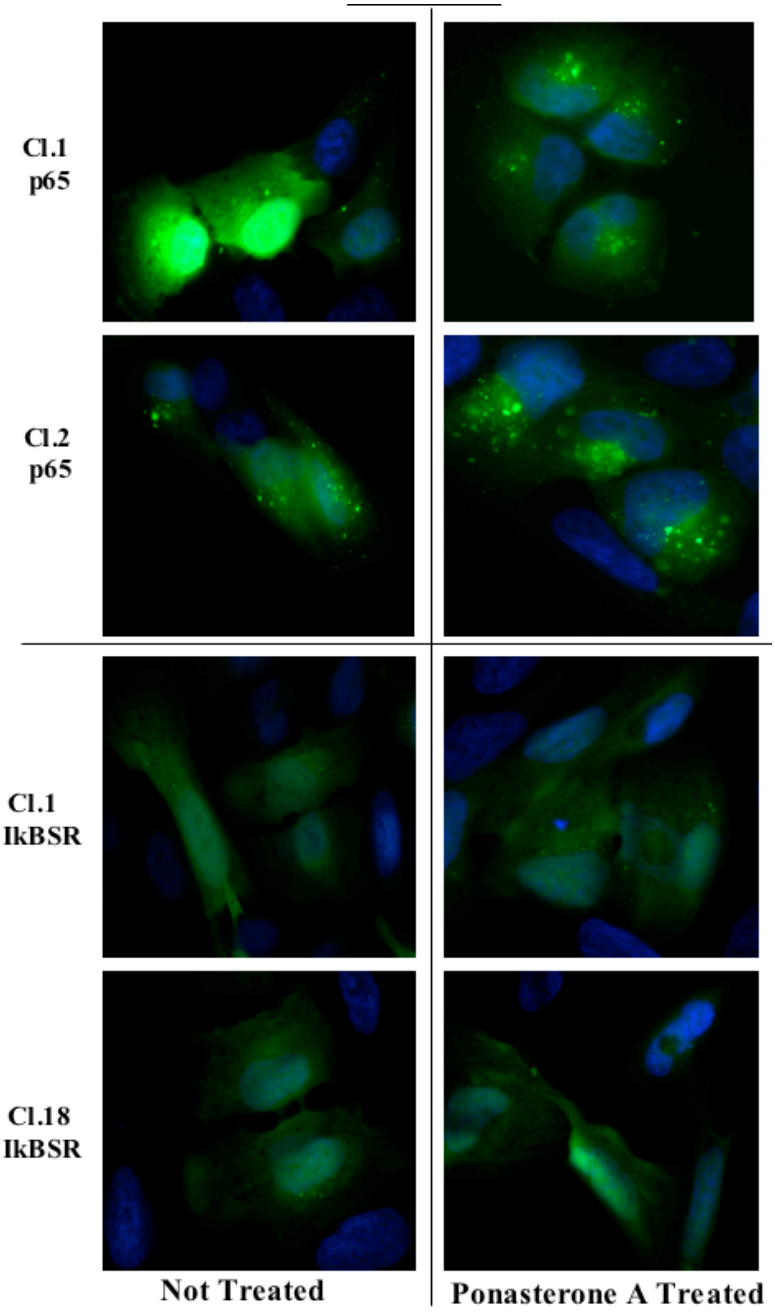


Figure 7C: Lower panels: U2OS derivative Cl1p65, Cl2p65, Cl1IkBSR and Cl8IkBSR cells were initally transfected with low amounts (100ng) of GFPh LC3 for 6h and then treated or not with ponasterone A for 12h; immunofluorescence was performed and some representative images are shown in the upper panel. Upper panel: 200 cells were counted in each experiment, and the number of cell presenting a punctuate GFP LC3 staining are reported. Data represent the means of at least three independent experiments.

Figure 7D

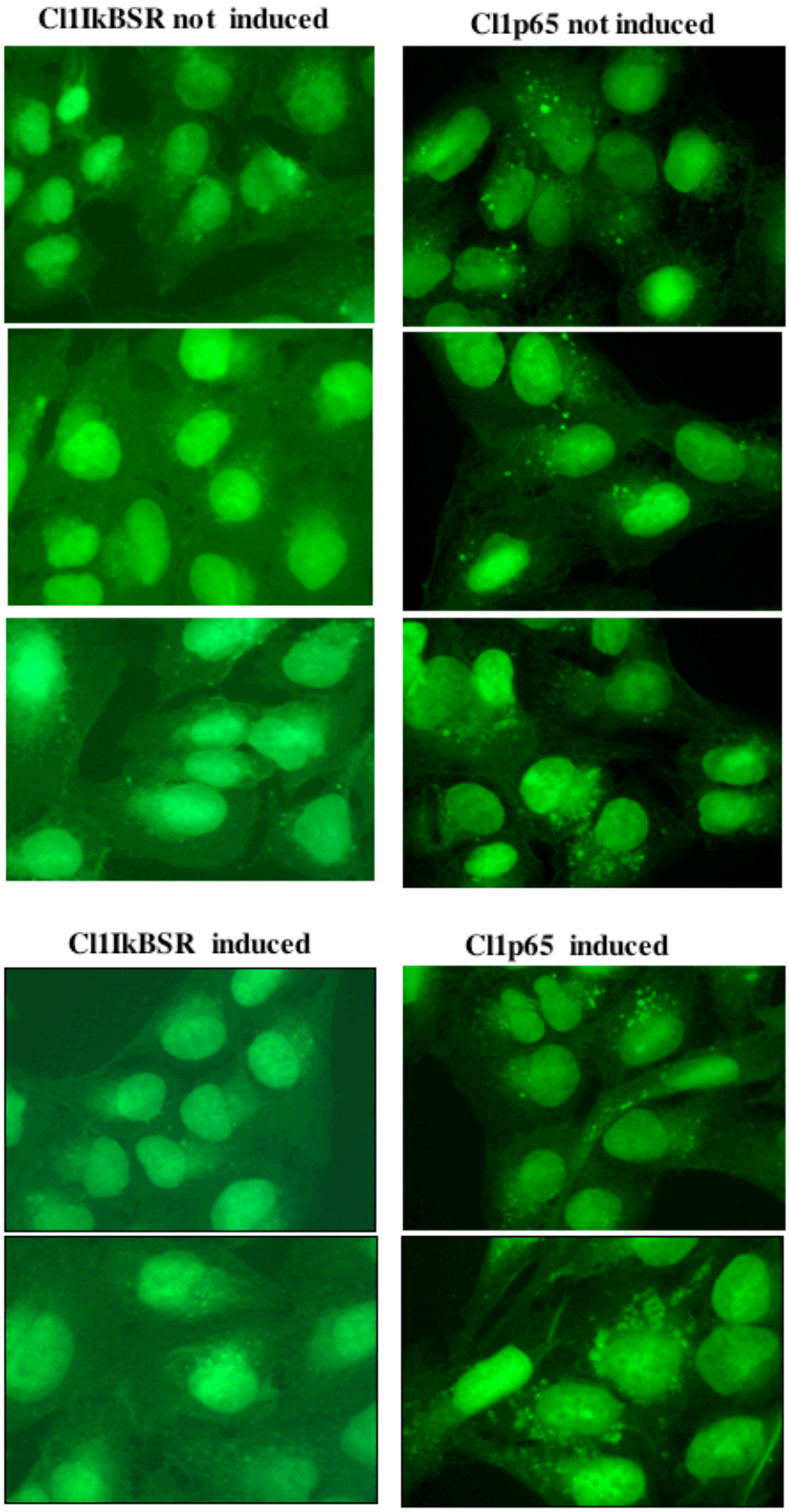
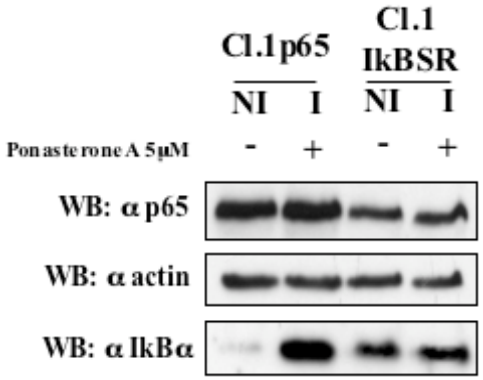


Figure 7D: p65 Upper panels: U2OS derivative Cl1p65 and Cl1IkBSR cells were treated or not with ponasterone A for 12h; afterwards the cells were fixed and analyzed by fluorescence microscopy; endogenous LC3 staining was monitored using anti-LC3 anibody. A punctuate staining indicates

autophagosome formation. Representative fields are shown. Lower panel: contemporaneously, the same clones were treated in the same way and subjected to western blot analysis.



Involvement of p65 mediated autophagy during K562 chronic myeloid leukemia differentiation towards megakaryocytic phenotype in response to PMA

In order to address the physiological significance of p65 mediated autophagy our investigations were focused on human K562 chronic myeloid leukemia cell line.

K562 cells have been frequently used as a model system for studying early steps in megakaryocytic and erythroid differentiation. In particular these cells can be induced by PMA to differentiate into cells with megakaryocytic characteristics, including cell growth arrest, changes in cell morphology and adhesive properties, and expression of markers associated with the megakaryocytes (Alitalo, 1990).

Previous studies demonstrated that PMA-induced differentiation of K562 cells was significantly downregulated by NF-kB inhibitors, whereas NF-kB subunit transfected cells were shown to be more sensitive to PMA-induced differentiation into megakaryocyte phenotypes than parental cells (Kang et al., 1996; Kang et al., 1998). Furthermore, M.I Colombo (Colombo and Fader, 2006) recently reported indicate that K562 GFP-LC3 expressing cells present a high basal level of GFP-LC3 bodies with respect to CHO cells suggesting a role for the autophagic pathway in the differentiation process of this cell type.

In light of these observations, we used this cellular system to explore whether NF-kB inhibition could prevent autophagy induction.

To this aim HA- tagged IkBαSR was subcloned into an adeno-associated virus (AAV) expression vector and its expression was tested in Hela cells.

As shown in Figure 8A, 2 days of infection with AAV-HA-IkBαSR were sufficient to induce proper HA-IkBα expression and to downregulate NF-kB-mediated transactivation of IkBα and COX-2 target genes when compared with AAV-MCS control infection in Hela cells.

Moreover AAV-HA-IkBα infection induced a clear downregulation of beclin 1 steady state levels and an impaired LC3I processing, confirming the previous results obtained by specific p65 knockdown on LC3I processing in the same cell line.

Figure 8A

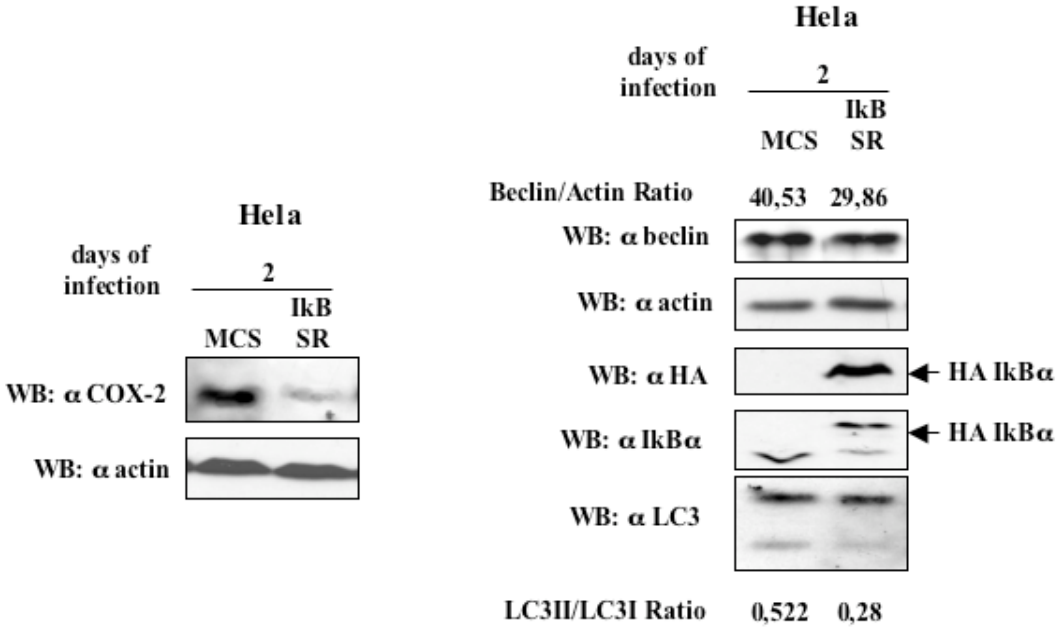


Figure 8A: HeLa cells were subjected to AAV-HA-IkBα and AAV-MCS infection for two days. Western blot analysis of COX-2 (left panel), beclin 1, actin, IkBα and LC3 (right panel) protein levels was performed. Quantification of beclin 1, actin and LC3I and LC3II was performed using Image J program.

Next we went on analyzing the effect of NF-kB inhibition in K562 cells. 6 days after infection with AAV-HA-IkBαSR, HA-IkBαSR expression was still detectable as well as its effect on downregulation of COX-2 NF-kB target gene (Figure 8B).

As expected PMA-mediated NF-kB induction resulted in a clear increase of COX-2 in AAV-MCS infected control cells, whereas AAV-HA-IkBαSR infection perfectly rescued the effect of PMA treatment.

Interestingly, PMA treatment resulted in the induction of autopahgic activity in K562 cell line, as demonstrated by the clear increase of LC3I to LC3II processing observed in

AAV-MCS infected cells following PMA treatement. Of note, in these settings, HA-IkBα clearly rescued PMA induced autophagy. Furthermore and coherently with the aforementioned effect on LC3I processing, HA-IkB expression downregulated beclin 1 protein steady state levels both in not treated and in PMA-treated cells. These results confirm that p65 is involved in autophagy induction also in K562 cellular system, possibly suggesting that p65-mediated autophagy induction might be required for K562 cells to undergo megakaryocyte differentiation.

Figure 8B

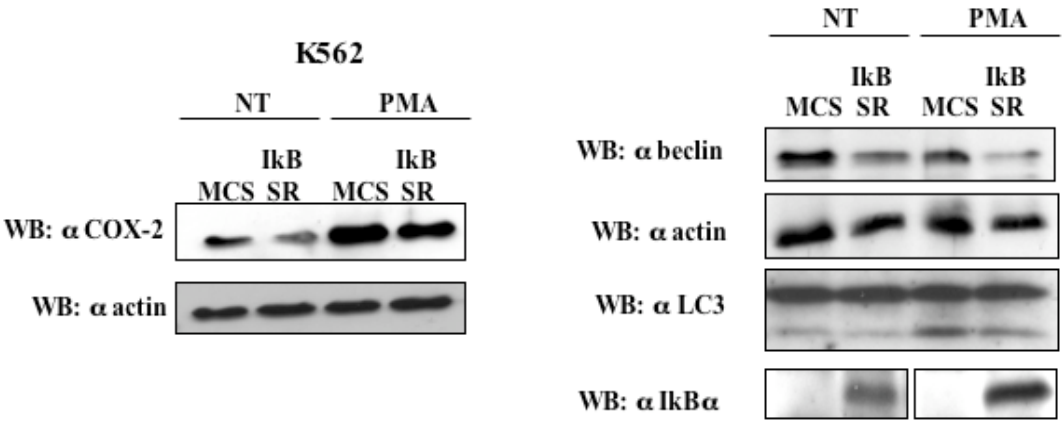


Figure 8B: K562 cells were infected for 6 days with AAV-HA-IkBαSR and AAV-MCS as a control, and treated or not with PMA (10ng/ml). Western blot analysis of cell lysates and steady state levels of COX-2 (left panel), beclin-1, LC3, IkBα and actin (right panel) was performed.

Preliminary evidences of p65 binding to beclin 1 promoter using chromatin immunoprecipitation (ChIP) assay

In light of the results presented in the paragraphs "p65 enhances transcritional activity of beclin 1 promoter" and " Isolation of p65/RelA consensus on ChET4 beclin 1 promoter", it was reasonable to hypothesize a p65 binding to beclin 1 promoter in the p65/RelA consensus region, specifically to the putative kB site identified with the previously reported *in silico* analysis.

In order to verify this hypothesis, preliminary chromatin immunoprecipitation (ChIP) assays of endogenous p65 were performed in Hela cells expressing or not HA-tagged IkBαSR. In order to reach 100% HA-IkBαSR expression, proper HA-tagged IkBαSR expression was reached after 2 days of infection with AAV-HA-IkBαSR.

Preliminary ChIP of endogenous p65 assays were thus performed in Hela cells infected with AAV-HA-IkBα or AAV-MCS as a control. In this settings we could observe a decreased ability of p65 to bind the identified putative kB site in the promoter of endogenous beclin 1: the ability of p65 to bind beclin 1 promoter in Hela cells expressing HA-tagged -IkBαSR was in fact almost abolished, with respect to Hela cells infected AAV-MCS (Figure 9).

Figure 9

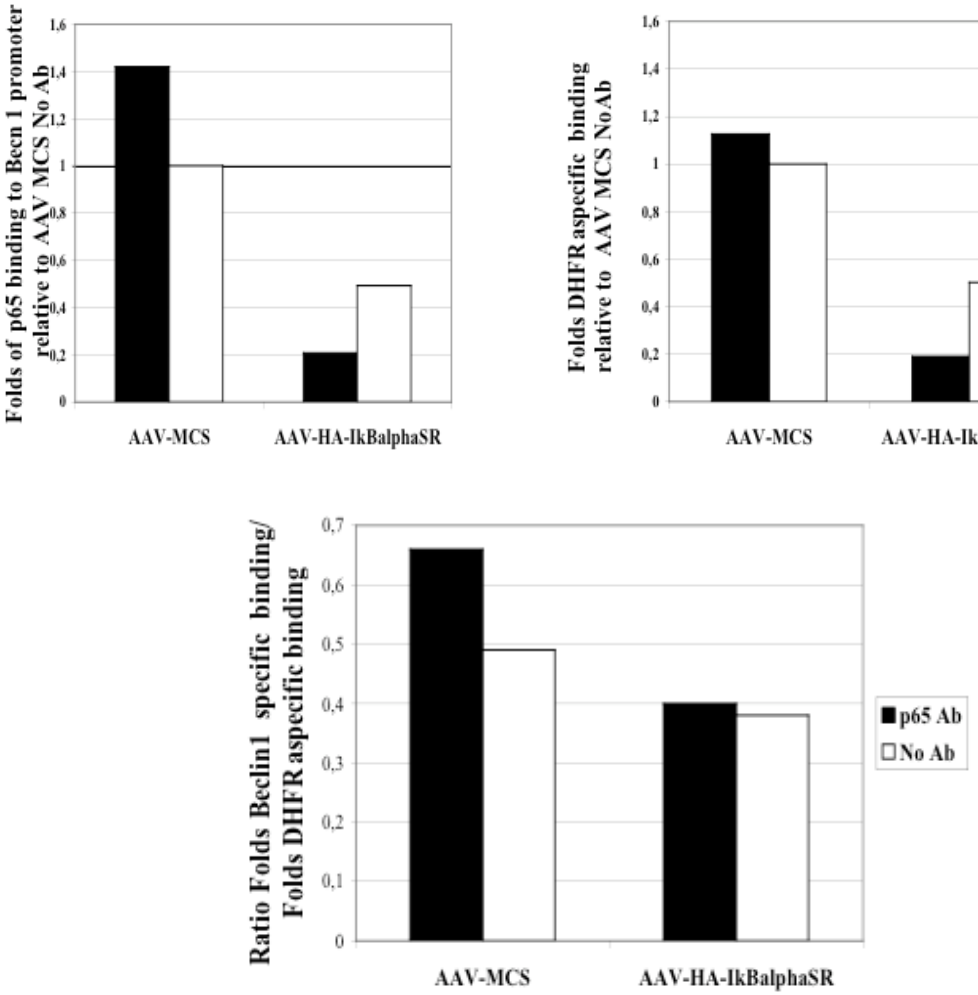


Figure 9: ChIP experiment was performed in Hela cell line infected for 2 days with AAV-MCS or AAV-IkBαSR. Black bars: IP with αp65 specific antibody; white bars: IP performed using an unrelated antibody. In the upper panel ChIP results relative to Beclin 1 specific bindind and to DHFR aspecific binding are shown. Lower panel represents the ratio of the folds of p65 specific binding to beclin 1 promoter normalized with DHFR.

DISCUSSION

NF-kB family of transcription factors can be found in all cell types and gives rise to a wide and often contradictory spectra of biological responses, depending on the cellular context and on the inducing stimulus. A further level of complexity is given by NF-kB activation pathway modalities, which show significant differences concerning the modes and the kinetics if IkB degradation, the requirement of different IKK, adaptor proteins and NF-kB dimers. All of these aspects account for NF-kB ability to generate waves of activation and inactivation in response to a given stimulus, leading to finely tuned persistence of its signaling and transcriptional response (Tiana et al., 2007).

Recent work performed in our lab focused on calpain involvement in the modulation of alternative pathways of cell death has shown that calpain is involved in NF-kB activation and in its relative prosurvival function in response to ceramide (Demarchi et al., 2005). In this work we demonstrated that p105 and p50 are calpain targets and that calpain expression knock down resulted in the stabilization of endogenous p105 and p50. Ceramide treatment of cells deficient for calpain activity impaired p105 processing and NF-kB signaling and triggers apoptosis. Altogether these data suggest that ceramide engages an NF-kB-dependent survival pathway that counteracts ceramide-induced cell death and that calpain activity is required for NF-kB prosurvival function in response to ceramide treatment. Furthermore, previous works demonstrated that regulation of NF-kB transcriptional activity partially relies on calpains through degradation of IkBα (Shumway et al.1999; Schoonbroodt et al., 2000), suggesting that calpains act in parallel to the proteasome in regulating NF-kB. Several works demonstrated that ceramide, besides triggering apoptosis, also induces alternative cell death pathways among which necrosis and autophagy. The involvement of ceramide in autophagy induction was further strenghtened by Scarlatti and Lavieu, who investigated the mechanisms leading to autophagy induction after ceramide treatment (Lavieu G. et al., 2006; Scarlatti F. et al., 2004).

More recently we investigated the role of calpain in the modulation of cell death, addressing its role in autophagy. We demonstrated that calpain depletion impairs the formation of bona fide autophagosomes both in human and mouse cell lines following amino acid or serum starvation and treatment with ceramide, rapamycin and etoposide. (Demarchi F. et al., 2006) In the same work we also highlighted the importance of calpain in inducing cytoprotective autophagy: knock down of both calpain and Atg5

increased apoptosis following aminoacid starvation. In these experimental settings, the effect of calpain depletion resulted to be more severe than Atg5 depletion, indicating that additional and probably upstream prosurvival mechanisms, such as NF-kB prosurvival activation, are lost in the absence of calpain.

Altogether these data strongly argued for an involvement of NF-kB and possibly other transcription factors in the autophagic induction and prompted us to further explore their involvement in this process.

In order to identify whether a common transcriptional regulation mechanism for the autophagic genes might exist, we screened the promoter region of a list of autophagic genes conserved both in mouse and human genome using CONFAC tool. Even if not any common transcription factor was shared among the selected promoters, NF-kB consensus binding sites were found both on mouse and human beclin 1 promoter.

Beclin 1 gene involvement in the autophagic process has been previously well documented and it has been defined a tumor suppressor gene since it is frequently monoallelically deleted in a high percentage of human breast, prostate and ovarian cancers (Aita et al., 1999). Enforced expression of beclin 1 gene has been demonstrated to induce autophagy in different contexts, but the final outcome of this autophagic process seems to be cell type specific and stimulus dependent. Indeed, autophagy has been proposed to represent on one hand a pathway of cellular demise (PCD II), especially in cells with impaired ability to undergo apoptosis and, on the other hand, to function as a prosurvival mechanism allowing survival during starvation and in response to stressing agents by assuring proper removal of damaged organelles or unfolded proteins. Independently of the stimulus used, beclin 1 represents an absolute requirement for autophagy to be induced.

Interestingly many stimuli known to induce autophagy have also been reported to activate NF-kB (e.g. ROS (Bubici et al., 2006), ceramide (Demarchi et al., 2005), TNFα, TRAIL (Perkins, 2007), but also viral (Hiscott et al., 2006) and bacterial infections (Hayden et al., 2006).

We then wished to better analyze beclin 1 promoter and its regulation by p65 transcription factor. To this aim a region corresponding to beclin 1 promoter localized immediately upstream to beclin 1 ATG-sequence, and previously isolated in Dr. Farnham's lab was cloned in a luciferase reporter vector and the ability of different transcription factors to transactivate luciferase gene was analyzed. We found that p65 alone and together with p50 strongly upregulates beclin 1 promoter activity. To further

characterize beclin 1 promoter and given that different kB sites with different scores were predicted to be present in beclin 1 promoter by the bioinformatic tools used, isolation of the most 3' region of the promoter encompassing the kB site with the highest score, was performed and its regulation by p65 was investigated. We found that this beclin 1 promoter region is highly responsive to p65 transcription factor in U2OS and 293T cell lines, and shows a clear increase of the reporter activity that is further enhanced by p65/p50 heterodimer (Figures 2B, 2C, 3B and 3C).

In order to validate beclin 1 promoter responsiveness to autophagic stimuli, its transactivation potential was evalueted following ceramide or tamoxifen treatment, two previously characterized autophagic stimuli. Intriguingly, we found that different susceptibility arose following treatment with these stimuli and was dependent on the promoter region considered. In fact, when considering a beclin 1 promoter region encompassing ~1100 bp upstream of ATG, the activity following both ceramide and tamoxifen treatment was almost identical to the one observed in not treated cells (Figure 2C). In contrast, when a restricted 5' region encompassing the most probable kB site was considered (p65 consensus region), a significant transactivation of the luciferase gene was observed following ceramide and tamoxifen treatment (Figure 3D).

These data argue for a complex regulation of beclin 1 promoter, in which several transcription factors could regulate beclin 1 transcription depending on their abundance in different cellular contexts, or on their availability in specific phases of cell cycle. For example, we observed a net decrease of the transcriptional activity following E2F binding to beclin 1 promoter when perfoming luciferase reporter assays (Figures 2B, 2C, 3B and 3C).

Of note, recent works demonstrate the importance of beclin 1 and of autophagy in preventing genomic instability following hypoxic stimuli in cells in which the apoptotic pathway was impaired due to Bcl-2 overexpression, suggesting that several cell cycle regulated transcription factors are likely to be involved in beclin 1 and thus in autophagy regulation (Karantza-Wadsworth et al., 2007; Mathew et al., 2007).

More importantly beclin 1 promoter transcriptional activity following depletion of endogenous p65 was significantly downregulated independently of the promoter region used and also in not treated cells (Figures 2D and 3D). These pieces of evidence suggest that p65 regulation of beclin 1 transcription could be constitutive and might contribute to cellular homeostasis. In fact, besides being activated following different stress

signals, autophagy is constitutively required as a step of 'quality control' for organelles and proteins, in order to maintain cellular homeostasis (Massey et al., 2006).

Data obtained from semi-quantitative RT-PCR in 293T cells (Figure 4A) and from qPCR experiments further enforce the aforementioned hypothesis: we demonstrated that in not treated U2OS cells, p65 depletion is sufficient to decrease beclin 1 mRNA transcription and the same data were obtained using HeLa cell line (Figure 4D and data not shown).

Despite the clear effect observed on beclin 1 mRNA levels following p65 depletion, in the same conditions we were unable to see any change in beclin 1 protein steady state levels. This problem was circumvented only by the production of a U2OS derivative cell line inducible for p65 expression. Induction of p65 led to an increase of beclin 1 protein levels (Figure 7B). Intriguingly we observed that beclin 1 protein upregulation was dependent on the time of p65 induction, suggesting that a kinetic of beclin 1 protein regulation exists. In fact, we confirmed that p65 induction triggers IkBα trascription and upregulation (data not shown); this event could engage a negative feedback mechanism that might halt or limit beclin 1 transactivation thereby influencing the timing of beclin 1 expression. One of the most important characteristics of p65 activation is the ability to generate waves of transcription that are tightly regulated depending on the persistence of the p65 activating stimulus. When considering the autophagic process this modality of p65 activation could be fundamental, since inappropriate induction of autophagy could be detrimental for cell fate and lead to cellular demise.

Another explanation for the observed results relies in Beclin 1 postranslational modifications that might tightly regulate its stability and turnover. Preliminary in vitro proteolysis assays performed in our lab have demonstrated that Beclin 1 protein is a calpain substrate and it is more susceptible to degradation than p53. Interestingly, following ceramide treatment, both calpain and NF-kB are activated, suggesting that complex beclin 1 postranslational modifications might occur.

Of note, when an inducible IkBSR derivative U2OS cell line was used, clear downregulation of beclin 1 protein was observed (Figure 7B). In this experimental settings, IkBSR induction downregulated Beclin 1 protein levels independently of the duration of IkBSR induction.

These results prompted us to better characterize the role of p65 in autophagy induction. LC3 processing and its conjugation with PE represents a reliable and widely used marker of autophagy induction. Autophagy induction can be examined either by

immunofluorescence assays to follow the appearance of punctuate fluorescent LC3-containing structures, or by western blot analysis to detect the appearance of the phosphatidylethanolammine-conjugeted form of LC3 (LC3II), which is found associated to the autophagosomal membranes. Alternatively, a GFP-LC3 expression plasmid can be transfected in the cells of interest, and the formation of punctuate fluorescent autophagosomal structures can be analyzed by microscopy.

In order to monitor autophagy induction we produced an antibody that showed good immunoreactivity towards both processed and unprocessed human, murine and rat LC3 protein. We used this reagent for further dissecting p65 role in autopahgy induction.

We observed impaired formation of LC3II form in both U2OS and HeLa cell lines following p65 depletion, suggesting that a blockage in autophagosome formation occurs and that p65 is required for autophagy induction (Figure 6A). This blockage already occurs at basal level in not treated cells and is maintained after ceramide or tamoxifen treatment, implying a p65 requirement both in cellular homeostasis control and for the proficient induction of autophagy that is observed following ceramide and tamoxifen treatment (Figure 6A). The evidence of an impairment in autophagosome formation was enforced by immunofluorescence assays in which diffuse GFP-LC3 staining was observed following p65 depletion, thereby suggesting that autophagosome formation is halted. A more striking impairement in auophagosome formation was observed using U2OS derivative cell lines inducible for p65 or IkBSR expression. In these experimental settings, while GFP-LC3 punctuate staining clearly appeared in p65 inducible cell line, it does not appear in IkBαSR cell line, where no GFP-LC3 structures are formed (Figure 7C).

Similar results were obtained in 293T cell line where LC3I processing has been shown to be impaired following transient overexpression of NF-kB inhibitors, such as IkBαSR or p105 (Figure 6A). In the same cell line we further observed that a blockage in autophagy induction again occurs following p50 overexpression (Figure 6A). Of note, p50 can, in some circumstances, act as a p65 inhibitor at the transcriptional level, since it is able to bind the same kB elements but it is not compentent for subsequent gene transactivation. This competition mechanism was already evident in the luciferase assays presented in this work, in which no significant activation of beclin 1 promoter has been observed following p50 overexpression (Figures 2B, 2C, 3B and 3C). These data further suggest that p65 is likely to be required at a transcriptional level, since it

regulates beclin 1 transcription as demonstrated by the semi-quantitative RT real time PCR and by qPCR experiments performed in this work.

Another interesting result emerged from semi-quantitative RT-PCR analysis of beclin1 mRNA performed in U2OS cell line (Figure 4C): the data obtained in these experiments strongly suggest the existence of alternative spliced forms of beclin1 gene. Some important evidences arose from these analysis and we confirmed that beclin 1 5'UTR is poorly characterized. We reached this conclusion using two different primers: one of them (A5') matched the most 5' region of 5'UTR and together with A3' primer was not able to amplify any 5' region as shown in figure 4C. Another primer was then designed and matched a more internal region of beclin 1 5'UTR, but similarly to A5', it was not able to amplify the region expected when considering Ensemble beclin 1 genomic organization (ENSG00000126581) (data not shown). Several annotated cDNA sequences in EBI and ENSEMBL databases further confirmed this observation.

Concerning other alternative spliced forms of beclin 1, we showed that the use of a set of primers expected to amplify a cDNA fragment corresponding to a region spanning from exon 4 to exon 8 led to the amplification of both this expected region of 557 bp and of a lower molecular weight fragment around 300bp. A similar result was obtained when amplification was performed with another set of primers expected to amplify a cDNA fragment spannig form exon 8 to exon 12. In this case two other cDNA fragments with lower molecular weights around 280 and 150 bp respectively were amplified. These pieces of evidences suggest that a further level of beclin 1 transcriptional regulation might exist that might confer further complexity to the regulation of the autophagic process and to the ability of specific transcription factors to transactivate different beclin 1 splice variants in a cell type and stimulus specific manner.

Beclin 1 has been demonstrated to contain different domains that enable its interaction with some regulatory proteins. A Bcl-2 binding domain (aa 86-158 corresponding to exons 5 and 6) has been characterized; recently resolution of the Bcl-XL beclin 1 peptide structure unveiled the existence of a BH3 domain inside Bcl-2 binding region (aa107-135). Interestingly, the coding sequence for Bcl-2 binding region of beclin 1 has been found to be deleted in some human infant brain cDNA clones of the Merck EST database, suggesting that beclin 1 alternative transcript lacking this region might exists (Liang et al., 1998). Moreover norther blot experiments also evidenced that besides the mRNA codifying all 12 beclin 1 exons of 1.7kbp, another transcript of 1.4kbp also

exists in severel human and mouse tissues (Liang et al., 1998). In line with these previous reported observations, our semi-quantitative analysis of beclin 1 region spanning form exon 4 to exon 8 further confirmed the existence of an alternative amplified cDNA in this region. Of note, Beclin 1 CCD also maps to this region and is required for Beclin 1 tethering of UVRAG (Liang et al., 2006).

Similarly, amplifications performed with primers designed on exon 8 an exon 12 showed that other cDNAs, besides the expected one of 562bp, are likely to exist. Noteworthy, this region comprises the so called ECD domain, which is needed for beclin 1 interaction with Vps34. These evidences, once proved, should be carefully taken in consideration when characterizing beclin 1 functions in autophagy, but also in other cellular processes such as apoptosis. Indeed, additional work is required in order to unveil and confirm the existence of beclin 1 alternative spliced forms.

Concerning p65, we demonstrated that its ability to positively regulate autophagy, in the cellular systems considered, is probably the result of beclin 1 transcriptional regulation. Interestingly, both Bcl2 and Bcl-XL p65 transcriptional targets have been demonstrated to block the autophagic process in some experimental settings. These data seem to be in contrast with our findings on a positive p65-mediated regulation of autophagy. Nonetheless other evidences suggest that in many cellular contexts Bcl-2 overexpression is unable to block autophagy (Shimizu et al., 2004; Mathew et al., 2007). Moreover, the recent identification of beclin1 BH3 domain, that is both necessary and sufficient for Bcl-XL binding, implies that beclin 1 may be also able to regulate Bcl-2 in some circumstances, rather than, or in addition to the recently reported ability of Bcl-2 to regulate Beclin 1 (Pattingre et al., 2005). Intriguingly, with respect to Bcl-2 family, BH3 domain could mediate novel and unknown beclin 1 functions, serving as an adaptor for loading of mitochondria into autophagosomes (Kundu and Thompson, 2005; Edinger and Thompson, 2003). It is noteworthy that different Bcl-2 pools have been suggested to regulate autophagy: considering the aforementioned suggestions, one could speculate that a mitochondrial Bcl-2 pool might positively regulate rather than inhibit autophagy. On the other hand, ER targeted Bcl-2 pools that have been shown to actually inhibit autophagy (Pattingre et al., 2005; Kroemer et al., 2007), might serve to halt, limit or regulate the proper mambrane sorting that occur at the early steps of autophagosome formation.

A serie of cell lines expressing mutant beclin 1 deficient in binding Bcl-2, all display increased levels of type II cell death that can be suppressed by Atg5 specific RNA

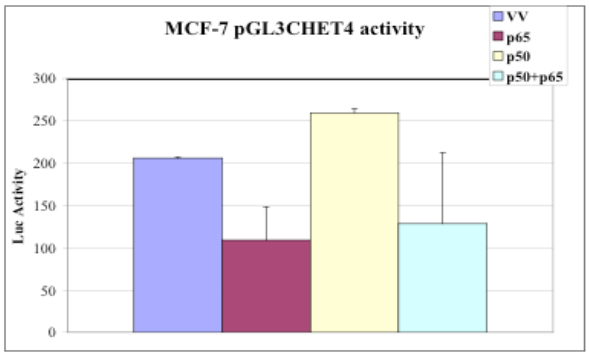
interference (Pattingre et al., 2005). In similar settings, p65-mediated Bcl-2 proper expression could counteract an autophagic activation that could be detrimental for the cell, switching on an autophagic prosurvival program through its ability to finely tune the expression of genes that present, at least at a first glance, quite opposite functions, such as beclin 1 and Bcl-2.

In contrast to our findings, recent work performed by Djavaheri-Mergny et al., reported that NF-kB activation represses autophagy induced by TNFα (Djavaheri-Mergny et al., 2006). This discrepancy could be explained when considering that autophagy is context and stimulus dependent. In our experimental conditions, in fact, we used different autophagic stimuli to induce autophagy, such as ceramide and tamoxifen. Our choice was dictated by our previously published results: as reported above, we clearly demonstrated that ceramide induces NF-kB activation (Demarchi et al., 2005) and that ceramide induced autophagy requires calpain (Demarchi et al., 2006). The different stimulus used might then account for the discrepancy of our data. Indeed, in accordance with this observation the same authors reported that autophagy can be induced in the same manner in either NF-kB-competent or in NF-kB incompetent cell lines following nutrient starvation, further confirming that different p65 regulation of autophagy may be required depending on the stimulus employed.

Since it has been very well documented that NF-kB activation triggers waves of responses in order to finely regulate expression of its target genes, we used inducible cellular systems in which NF-kB activation or repression can be stimulated in a controlled manner. Thus, the use of these different cellular systems might at least partially explain the alternative NF-kB role observed in this work. Interestingly, in line with our RT-real time beclin mRNA analysis, Djavaheri-Mergny et al. demonstrated that inhibition of p65 in a cell system stably expressing MAD1 (the murine IkB ortholog) does not lead to increased beclin 1 transcription both in not treated conditions and following TNFα treatment. Nonetheless, in the same experimental settings they observed an increase of Beclin 1 protein steady state levels, suggesting that postranslational regulation of Beclin 1 protein is likely to occur and might be responsible for beclin 1 upregulation and for autophagy induction (Djavaheri-Mergny et al., 2006).

Djavaheri-Mergny et al., showed that p65 depletion in MCF-7 cells is sufficient for autophagy induction. Notably, we observed that p65 overexpression could not activate beclin 1 promoter in some luciferase assays performed in the same cell line (Figure 1

Discussion). One explanation for this behaviour is that p65 response is cell type dependent. In fact it has been previously demonstrated that in this ER positive and tamoxifen senstive breast cancer cell line p50, rather than p65, is likely to bind DNA (Zhou et al., 2007; Zhou et al., 2005). Moreover, the same reports also demonstrate that not any change in the activity of NF-kB driven promoter can be observed following several SERMs (selective estrogen receptor modulators among which tamoxifen) treatment, even if they have been demonstrated to activate p65 in other ER positive breast cancer cell lines (Biswas et al., 2005). We also observed similar results following tamoxifen, ceramide and TNFα treatment (data not shown). Interestingly, it was suggested that NF-kB signalling in these cell lines could be largely attributed to p50 DNA-binding and coimmunoprecipitation experiments further demonstrated that, since p50 lacks a transactivation domain, it requires Bcl-3 co-activator binding for becoming transcriptionally competent (Zhou et al, 2007).



p65 doesn't enhance transcriptional activity of beclin 1 promoter ChET4 in MCF7 breast cancer cell line. MCF7 cells were cotransfected with pGL3ChET4 and E2F, p65, p50, p65 and p50 or a control empty vector. Luciferase assays were performed 24h after transfection. Data represent the means of at least four independent experiments and error bars represent standard deviations;

Therefore, it is likely that other pathways besides the canonical, requiring IkB degradation and probably IKK independent, might be involved in NF-kB signalling in MCF7 cell line.

Work performed by Djavaheri-Mergny et al., also demonstrates that ROS are involved in TNFα induced autophagy in cells lacking NF-kB activity and that antioxidant agents completely abolished autophagosome formation. Noteworthy, a recent report shows that following nutrient starvation a first wave of ROS production is required for autophagosome formation and that it is Beclin 1/Vps34-dependent. The same work also demonstrates that a persistence of ROS signaling at later stages is responsible for

autophagy impairment, since an oxidizing environment is able to cause Atg4-dependent LC3 delipidation inhibition and halts autophagy (Scherz-Shouval et al., 2007). These observations, strongly argue for a step-dependent role of ROS, underlining that a ROS buffering wave is required for autophagy to occur that follows a first wave of ROS production. Such a system seems to be more coherent with a tightly regulated mode of NF-kB activation, indeed several NF-kB target genes such as MnSOD, FHC etc., efficiently buffer ROS production. Interestingly, TNFα has been demonstrated to stimulate ROS production and to trigger strong NF-kB activation. Notably TNFα an TRAIL have been reported to stimulate autophagy in T-lymphobalstic (Jia et al., 1997; Jia et al., 2006; Mills et al., 2004) cells and in a model of lumen formation in mammary acini, suggesting that NF-kB activation might be differentially required for autophagy induction in different cellular contexts.

Interestingly, a work performed by Cuervo et al., demonstrated that serum removal caused a significant decrease of IkBα, while following NH_4Cl treatment (in order to inhibit lysosomal degradation) this IkBα levels remained unchanged. These evidences strongly argued that IkBα is degraded through a lysosomal route. Notably, under the same conditions, no changes were detected in the NF-kB degradation rates (Cuervo et al., 1998). When the same authors went on analyzing the relative kinetics of IkBα degradation in cells cultured in the presence and in the absence of serum, they observed that after a first fast wave of IkB degradation occurring within minutes and that could not be inhibited by NH4Cl in both the treatment conditions, a portion of IkBα was subsequently slowly degraded in cells supplemented with serum. The same portion of IkBα was also detected in cells deprived of serum, even if the degradation occurred in a faster way. Strikingly, following 2 days of starvation, a significant decrease in IkBα cytosolic level was observed and was paralleled by a concomitant significant increase in NF-kB (p50/p65) DNA binding activity. Another interesting evidence coming from this work concerned the modalities of NF-kB activation, which was demonstrated to occur in a cell type specific manner and to be subjected to rises and falls, further undrlying that NF-kB signalling modalities occur in a time dependent manner. In these experimental settings the use of PDTC antioxidant inhibits NF-kB signalling and IkBα degradattion independently of the presence or absence of serum. Hydrogen peroxide incubation under conditions of serum deprivation were shown to markedly increase

IkBα degradation, suggesting a partecipation of ROS in the targeting of IkBα to lysosomes in response to serum withdrawal (Cuervo et al., 1998).

Altogether, the evidences reported suggest that further investigations about NF-kB signalling activation and involvement in autophagy are required, and need to be carefully examined. In our experimental settings we demonstrated that: 1) NF-kB positively regulates beclin 1 at the transcriptional level; 2) NF-kB is required for constitutive autophagy to occur; 3) NF-kB is required for ceramide and tamoxifen autophagy induction; 4) NF-kB regulation of beclin 1 protein levels is likely to occur in a time dependent manner; 5) we suggest that NF-kB might regulate alternative beclin 1 transcripts. Moreover, our evidences indicate that NF-kB might trigger a costitutive autophagic prosurvival function, rather than a type II PCD program.

Other work is in progress to better characterize p65 binding on beclin 1 promoter kB site by ChIp experiments and EMSA assays. Recent data suggest that p65 binding to beclin1 promoter might require formation of a complex since luciferase reporter assays demonstrate a 2 fold reporter activation following p65/p50/p300 cotransfection with respect to the p65/p50 heterodimer transfected alone (data not shown). Another interesting point, that needs to be further explored, concerns beclin 1 splice variants identification and functional characterization. Recent evidences about beclin 1 structural oraganization, in fact, point for a beclin 1 involvement in processes other than autophagy (Maiuri et al., 2007).

Understanding these beclin 1 features and p65 regulation of the autophagic process needs to be investigated also in different cellular systems and might be instrumental for the future design of more efficient therapeutical strategies against specific malignancies.

EXPERIMENTAL PROCEDURES

Cell lines and treatments

U2OS, 293T and Hela cell lines were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). K562 cells were grown in suspension in RPMI medium supplemented with 10% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml).

C2Ceramide tamoxifen and PMA were purchased from Sigma and were used at the indicated doses for the indicated times.

The pVgRxR and pIND plasmids (ratio 1: 9) were co-transfected into U2OS cells by the calcium phosphate method.

U2OS derivative cell lines were obtained by cotransfection of pVgRXR and pIND p65 or pIND IkBαSR in the ratio 1:9 using calcium phosphate method. Selection was performed with Zeocin (0.5 mg/ml, Invitrogen) and G418 (0.5 mg/ml, Sigma). The positive inducible clones were identified by Western blot analysis. To induce p65 or IkBαSR expression, Ponasterone A, a synthetic analog of ecdysone (Invitrogen), was added to the culture medium at a final concentration of 5 μM for 24 h.

Transfection and vectors

DNA: Subconfluent cells were transfected using Calcium Phosphate method, Lipopectamine 2000 Reagent (Invitrogen) or FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. Unless stated otherwise, cells were analyzed 24 h after transfection.

pIND and pVgRXR were from Invitrogen. pGL3 vector was from Promega. pIND p65, pIND IKB SR, pGL3 ChET4, pGL3 p65 consensus, pcDNA3HAhp65 and pCMVtagFLAGp65 were generated by subcloning the relative PCR products into the vectors of interest. pRSVhRelA/p65 and pRSVNF-kB1/p50 were kindly provided by Professor N.Perkins; and pCMV4HA-IkBαSR was kindly provided by Professor Shao-Cong Sun; pcDNA3FLAG p105 was kindly provided by Professor C.Scheidereit; pEGFPhLC3 and pEGFP rat LC3 were a kind gift of Professor I.Tanida and T.Yoshimori respectively; pCMVHAE2F1 was kindly given by Dr. Del Sal G.

pAAV-MCS was from Stratagene Corporation; pAAV-HAIkBαSR was generated by HA-IkBαSR subcloning from pCMV4HA-IkBαSR into pAAV-MCS vector. Replication-deficient Adeno-associated viruses were obtained according to Stratagene Instruction Manual guidelines.

siRNA: Cells in mid-log growth phase were transfected with siRNA's (MWG) using Oligofectamine Reagent (Invitrogen) as recommended by the manufacturer. Cells transfected with siRNA were analyzed after 48 or 72 h. The control siRNA used was AACCUUUUUUUUUGGGGAAAA (siCONT/SiCtrl). The mRNA targeted sequences for beclin1 twelfth exon and p65 were the following: Beclin 1 (NCBI Ac No. AF077301) (GAUUGAAGACACAGGAGGCTT) (Boya et al. 2005); RelA/p65 (NCBI Ac No. M62399) (GCCCUAUCCCUUUACGUCATT).

Western Blot Analysis

Western blot analysis was performed according to the standard procedures using the following primary antibodies: affinity purified anti-ratLC3 polyclonal antibody, anti-Beclin 1 polyclonal antibody (Santa Cruz Biotechnology), anti-actin polyclonal antibody (Sigma), anti HA monoclonal antibody (Sigma), anti-p50 monoclonal antibody (Santa Cruz Biotechnology), anti-FLAG monoclonal antibody (Sigma), anti-IkBα (Cell Signaling Technology).

Immunofluorescence analysis

Cells were plated on glass coverslips in 3 cm culture dishes. After washing with PBS, cells were fixed in 3% paraformaldehyde in PBS, treated with 1% glycine in PBS, and permeabilized in 0.01% saponin in PBS. The staining was performed using specific antibodies incubated in 5% bovine serum albumin in PBS/0,01%saponin at 37°C followed by fluorescein isothiocyanate-conjugated secondary antibodies (Sigma).

Semi-quantitative RT-PCR of Beclin 1

RNA from $2x10^6$ cells was extracted using using TRIzol Reagent (Invitrogen). Then it was incubated with RQ1 Rnase free DNase (Promega) for 30 min at 37 °C. First strand cDNA synthesis was performed with 4 μg of RNA by using the SuperScript First Strand Synthesis SystemTM (Invitrogen). The following primers were used to amplify GAPDH cDNA (5"-cggagtcaacggatttggtcgtat-3"/5"-agccttctccatggtggtgaagac-3") while

for beclin 1 cDNA amplification the following set of primers have been used: A5'/A3 (5'-gggaagtcgctgaagacaga-3'/5'-gatccacatctgtctggccc-3'); B5'/B3' (5'-ccaggatggtgtctctcgca-3'/5'-ctgcgtctgggcataacgca-3') and C5'/C3' (5'-cccagacgcagctggataag-3'/5'-ggacacccaagcaagacccc-3'). The thermal cycle conditions were 1 cycle for 2 min at 94 °C, followed by 27 cycles of 1 min at 94 °C, 1 min at 63 °C, and 1 min at 72 °C and terminated by 1 cycle for 5 min at 72 °C. Under these conditions PCR products were amplified in the linear range. GAPDH primers were added to the amplification mixture after the beginning of the seventh cycle, during the denaturation phase. Aliquots of each PCR were subjected to electrophoresis at 100 mV on a 2% agarose gel in the presence of 0.5 μg/ml ethidium bromide. The products of the amplification were revealed under UV light and photographed on Kodak paper.

qPCR

Quantitative Real Time PCR was performed as follows: briefly, RNA 3⦸ dishes U2OS cells (previously silenced for 48 or 72h) was extracted using using TRIzol Reagent (Invitrogen). Subsequently it was incubated with RQ1 Rnase free DNase (Promega) for 30 min at 37 °C in a thermocycler. RQ1 DNase Stop Solution was added to terminate the reaction. First strand cDNA synthesis was performed with 0,5 μg of RNA using oligo dT by and the SuperScript II Rnase H Reverse Transcriptase (Gibco BRL), according to manufacturer instructions. Of note, the same reaction was performed on the same samples in the absence of SuperScript II Rnase H Reverse Transcriptase in order to check genomic DNA background during amplification (it always resulted undertermined, indicating no genomic DNA contamination). 50ng of each sample was used for mRNA quantitation. The Beclin 1 gene expression was detected by real time qPCR assay. Quantification of beclin 1 mRNA was achieved by means of the ABI-PRISM 7000 Sequence Detection System (PE Applied Byosystems) using TaqMan Universal Master Mix (Applied Byosystems). RT-PCR was based upon Taqman fluorogenic detection system Taqman (Applied Byosystems), using a fluorogenic oligonucleotide probe designed to hybridize the specific target sequence. The Taqman probes were labeled at 5' end with the fluorescence reporter dye FAM for beclin1 or VIC for GAPDH control, and at the 3' end with the quencher dye TAMRA. The sequences for gene-specific forward and reverse primers and the probes were designed using Primer Express 1.0 software (PE Applied Biosystems). The following sequences of primers and probe were used for RT-PCR of Beclin 1 mRNA: beclin-1 primers on

12° exons : 5'-gtcctccccgtatcataccattc-3'/5'-acgatggtaaagggagggaagt-3'; Taqman FAM probe: 5'-cagtggcggctcc-3'; pre-developed Human GAPDH probe was purchased by Applied Biosystem . RT-PCRs were performed in a MicroAmp optical 96-well plate. 25 microliters of reaction mixture were used, containing 50 ng of total RNA, 1x Taq-Man Master Mix buffer, 0.9 μM forward and reverse primers, 0.2 μM TaqMan probe. Amplification settings were performed as previously described (Kaeser et al. 2002), Triplicate RT-PCR reactions were prepared for each sample. The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (Ct) was determined for each sample, and the average Ct of triplicate samples was calculated for both Beclin1 and GAPDH. ΔΔCt method was used in order to calculate folds of increase ore decrease relative to the control sample. A one-tailed Student's *t*-test was used to assess the statistical significance of the observed mRNA differences in qPCR experiments as previously reported (Benetti et al., 2007). Microsoft excel was used for statistical calculations. Diferences were considered significant at $P<0,05$.

Production of anti-LC3 antibody

Briefly, a polyclonal anti-LC3 antibody against full lenght recombinant rat LC3 protein was produced in rabbit as follows: pETM30 rat LC3 and pETM11ratLC3 constructs were obtained by subcloning rat LC3 PCR product. BL21 DE3 bacteria strain was transformed with these constructs and the relative proteins (e.g. 6His GST rat LC3 and 6His rat LC3 respectively) were purified using NiNTA agarose resin. Proteins were eluted from the resin by means of a step imidazole gradient (Imidazole 100-250mM); 1mg of pure 6His GST rat LC3 protein was used for rabbit immunization and 4.5mg 6His rat LC3 were covalently coupled to Affi-Prep 10 matrix (BioRad) following manufacturer instructions. 6His rat LC3 coupled to Affi Prep 10 matrix was used for subsequent rabbit sera purification.

***In silico* analysis**

In silico analisys of the autophagic genes and NF-kB target genes datasets was performed using CONFAC (Karanam and Moreno, 2004). The length of sequence analyzed upstream of the TSS was 500bp. Transfac parameters were set as follows: Matrix Similarity and Core Similarity at 0,85 and at 0,95 respectively. The Vertebrate matrix was used and Repeatmasker was applied.

ConSite (Sandelin et al., 2004) was used to analyze ChET4 sequence using a TF score cutoff of 85% and 95%. “95%” TF score cutoff analysis confirmed the presence and the position of the kB site identified using CONFAC; “85%” TF score cutoff was used in order to verify the exact position of kB elements other than the one identified with CONFAC.

Chromatin Immunoprecipitation (ChIP)

Cells were crosslinked with 1% final formaldehyde using Fixing solution (11% formaldehyde, NaCl 100mM, EDTA 1mM, EGTA pH8 0,5mM, TrisHCl pH8.0 50mM) for 10 min, neutralized with 125 mM glycine in PBS, collected and washed in PBS. Nuclei were prepared by hypotonic lysis (EDTA 10mM, EGTA 0,5mM TrisHCl pH8.0 10mM, 0.25% Triton X-100), washed in B2 solution (EDTA 1mM, EGTA 0,5mM, NaCl 200mM, TrisHCl pH8.0 10mM) centrifugated, and resuspended in RIPA-50 buffer (20 mM Tris HCl, pH 7.5, 50 mM NaCl, phophatase inhibitors, NaF 10mM and Na3VO4 1mM, DFP 2mM and Proteinase Inhibitor Cocktail (Sigma)). Chromatin was sonicated with Bioruptor (Diagenode) to 500-1000 bp average fragment size and cleared by centrifugation. Detergents were added to the sonicated samples: NP-40 0,5% DOC 0,5% and SDS 0,1% final concentrations. IP was performed overnight at 4°C with either: rabbit anti-p65 sc-109 and goat anti-p65 sc-372 (both Santa Cruz Biotechnology); a negative control was performed in the presence of isotype-specific unrelated Ab. Real-time PCR was performed on an ABI PRISM 7000 cycler, using TaqMan Universal PCR Master Mix (Applied Biosystems) as described before. Folds of promoter occupancy were calculated using ABI-PRISM 7700 software method. Primer sequences: ChET4 primers 5’-gtcctccccgtatcataccattc-3’/5’-acgatggtaaagggagggaagt-3’; Taqman FAM probe: 5’-aacggcgcaggttg-3’; DHFR primers: 5’-tcgcctgcacaaatagggac-3’/5’-agaacgcgcggtcaagttt-3’; Taqman FAM probe: 5’-gggcggccacaatttcgcg-3’.

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The Calpain System Is Involved in the Constitutive Regulation of β-Catenin Signaling Functions*

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Roberta Benetti‡§, Tamara Copetti‡, Stefania Dell'Orso‡, Edon Melloni¶, Claudio Brancolini‖, Martin Monte‡, and Claudio Schneider‡‖**

From the ‡Laboratorio Nazionale del Consorzio Interuniversitario per le Biotecnologie, Area Science Park, Padriciano 99, 34012 Trieste, Italy, the ¶Department of Experimental Medicine-Biochemistry Section and Centre of Excellence for Biomedical Research, Università di Genova, Viale Benedetto XV, 16132 Genova, Italy, and the ‖Dipartimento di Scienze e Tecnologie Biomediche, Università di Udine, Piazzale Kolbe 4, 33100-Udine, Italy

β-Catenin is a multifunctional protein serving both as a structural element in cell adhesion and as a signaling component in the Wnt pathway, regulating embryogenesis and tumorigenesis. The signaling fraction of β-catenin is tightly controlled by the adenomatous polyposis coli-axin-glycogen synthase kinase 3β complex, which targets it for proteasomal degradation. It has been recently shown that Ca^{2+} release from internal stores results in nuclear export and calpain-mediated degradation of β-catenin in the cytoplasm. Here we have highlighted the critical relevance of constitutive calpain pathway in the control of β-catenin levels and functions, showing that small interference RNA knock down of endogenous calpain *per se* (*i.e.* in the absence of external stimuli) induces an increase in the free transcriptional competent pool of endogenous β-catenin. We further characterized the role of the known calpain inhibitors, Gas2 and Calpastatin, demonstrating that they can also control levels, function, and localization of β-catenin through endogenous calpain regulation. Finally we present Gas2 dominant negative (Gas2DN) as a new tool for regulating calpain activity, providing evidence that it counteracts the described effects of both Gas2 and Calpastatin on β-catenin and that it works via calpain independently of the classical glycogen synthase kinase 3β and proteasome pathway. Moreover, we provide *in vitro* biochemical evidence showing that Gas2DN can increase the activity of calpain and that *in vivo* it can induce degradation of stabilized/mutated β-catenin. In fact, in a context where the classical proteasome pathway is impaired, as in colon cancer cells, Gas2DN biological effects accounted for a significant reduction in proliferation and anchorage-independent growth of colon cancer.

β-Catenin plays a dual role both as a major structural element of cell-cell adherent junctions and as a pivotal signaling molecule in the Wnt pathway, transmitting transcriptional cues into the nucleus (1–3). In the absence of Wnt signaling, the cytoplasmic levels of β-catenin are kept low through interaction with a protein complex that can phosphorylate β-catenin and target it to ubiquitin-mediated proteasomal degradation (4). Activation of Wnt signaling leads to inactivation of glycogen synthase kinase 3β (GSK3β),[1] a kinase responsible for phosphorylation of β-catenin, resulting in accumulation of cytoplasmic β-catenin (5). Enhanced cytoplasmic levels of β-catenin allow its translocation to the nucleus where it cooperates with a member of the T cell factor (TCF)/lymphocyte-enhancer binding factor (LEF) family of transcription factors to activate expression of target genes (6). The three regulatory genes in this pathway most frequently mutated in human cancers are APC, β-catenin, and axin. Their mutations result in the accumulation of nonphosphorylated β-catenin, thereby constitutively activating gene transcription and promoting carcinogenesis (7). Wnt signaling operates via cell surface receptors to stimulate also the activation of Gq pathway and the subsequent increase in intracellular calcium (8). Li and Iyengar (9) highlighted the consequences of activation of Gq signaling pathway showing that calcium release from internal stores results in μ-calpain-dependent degradation of β-catenin.

Calpains are a large family of calcium-dependent intracellular proteases whose precise and limited cleavage of specific proteins, in concert with their endogenous inhibitor Calpastatin, is thought to play an integral regulatory aspect in various signaling pathways from cytoskeletal remodeling to cell cycle regulation and apoptosis (reviewed in Ref. 10). In addition to Calpastatin, we have recently characterized Gas2 as an important calpain inhibitor (11).

Gas2 is a component of the microfilament system and colocalizes with actin fibers (12); it binds the calmodulin-like region of m-calpain (domain III and IV of large subunit) with its amino-terminal domain and with its carboxyl-terminal region exerts an inhibitory function (11). The isolated amino-terminal region has been demonstrated to act as a dominant negative form of Gas2 (Gas2DN), being able to bind, but not to inhibit, calpain activity and to rescue *in vivo* the effects of Gas2 on calpain function.

Here we underline the contribution of the calpain system to β-catenin degradation, showing that m-calpain, as regulated by the endogenous inhibitors Calpastatin and Gas2, can modulate β-catenin levels and activity in the absence of external stimuli. More importantly, we focused our attention on the properties of

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** To whom correspondence should be addressed. Tel.: 39-040-398995; Fax: 39-040-398990; E-mail: schneide@lncib.it.

[1] The abbreviations used are: GSK3β, glycogen synthase kinase 3β; TCF, T cell factor; LEF, lymphocyte-enhancer binding factor; GFP, green fluorescent protein; siRNA, small interference RNA; BrdUrd, bromodeoxyuridine; HA, hemagglutinin; Gas2DN, Gas2 dominant negative; IVT, *in vitro* translated; E1, ubiquitin-activating enzyme; CH domain, Calponin homology domain; wt, wild type; m-calpain, milli-calpain.

Gas2DN, which can abrogate both Gas2 and Calpastatin effects, providing biochemical evidence that it can stimulate calpain activity. The Gas2DN-mediated calpain activation was utilized in colon cancer cells, where β-catenin degradation via proteasome is impaired. We show that in this cellular context, the calpain system controls deregulated β-catenin and that the biological effects of Gas2DN account for a significant reduction in the proliferation and anchorage-independent growth of colon cancer cells, therefore representing a potentially critical way to control deregulated β-catenin.

MATERIALS AND METHODS

Cell Lines, Transfections, and Reporter Assay—Cells were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C and 5% CO_2. Transfections were performed using FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. Luciferase assays were performed with the dual luciferase kit (DLR) from Promega.

U2OS are human osteosarcoma cells; HCT-116 are human colon carcinoma cells harboring β-catenin mutation on exon 3 of β-catenin with three base deletions at codon 45. LS-174 are human colon carcinoma cells harboring β-catenin mutation, whereas HT-29 are human colon carcinoma cells harboring APC deletion. MEF GSK3β−/− are murine embryo fibroblasts null for GSK3β, provided by Dr. J. Woodgett (Ontario Cancer Institute); H1299 are lung carcinoma cells. The TS-20 cell line is a temperature-sensitive mutant derived from the mouse cell line BALB/c 3T3 obtained from Dr. Gianni Del Sal (Laboratorio Nazionale del Consorzio Interuniversitario per le Biotecnologie, Trieste, Italy).

Plasmids—pEGFPC1-Gas2DN (12), pCMVHA-tagged β-catenin cloned from pMT2-VSV-tagged β-catenin (13), pGDSV7S Gas2wt (12), and the Calpastatin plasmid (PM194) have been described previously (14). TOP Luc plasmid was provided by H. Clever. His_6-tagged Gas2wt, His_6-tagged Gas2DN, and His_6-tagged Calpastatin were generated as described below under "Calpain Activity Assay."

Western Blot and Antibodies—Western blot analysis was performed according to standard procedures using the following primary antibodies: anti-β-catenin (C19220; Transduction Laboratories), anti-Gas2 antibody (12), anti-Calpastatin (monoclonal antibody-C-270), anti-HA (Roche Applied Science), and anti-m-calpain polyclonal antibody (C0728) and anti-actin polyclonal antibody (A2066) from Sigma. Anti-GFP (Invitrogen):polyclonal GFP antiserum (Invitrogen).

Stable Cell Lines—To obtain GFP-Gas2DN/HCT116 and GFP/HCT-116 cells, selection was performed for 2 weeks in the presence of 1 mg/ml G418. G418-resistant colonies were clonally expanded, and expression of Gas2DN and GFP was analyzed by Western blot.

Calpain Cleavage Assay in Vitro and in Vivo—In *vitro* cleavage assay was performed as previously described (11). The *in vivo* protein assays were performed on HCT-116. Cell lysates were prepared freshly at 4 °C in ice-cold Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.4% Nonidet P-40, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 1 mM EGTA) and rotated for 30 min at 4 °C. Cell debris was pelleted by centrifugation (15 min, 13,000 RPM, 4 °C), and the supernatant was used in β-catenin cleavage assay. 2 mM $CaCl_2$ was added for endogenous m-calpain activation, and different aliquots of the samples were collected at the indicated time points. 8 mM EGTA was added to one of the samples to completely inhibit calcium-dependent calpain reaction.

Inhibition of m-calpain Expression by RNAi—Small interfering RNA duplexes, si-m-calpain, were purchased from Dharmacon: siCONT 5′-AACCUUUUUUUUUGGGGAAAA-3′, si-m-calpain 5′-GUACCUCAACCAGGACUAC-3′, and siGENOME SMARTpool Upgrade MU-009979–00-0020 human CAPNS1 for calpain small subunit. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggestions.

Immunofluorescence and BrdUrd Incorporation Assay—After transfection with the various plasmids, cells were fixed with paraformaldehyde (3% in phosphate-buffered saline) and then processed for immunofluorescence with 12CA5 anti-HA antibody (Roche Applied Science). For BrdUrd incorporation assay, 24 h after transfection cells were pulsed with 50 μM BrdUrd for 4 h and fixed with paraformaldehyde. To reveal incorporated BrdUrd, coverslips were treated with 50 mM NaOH for 30 s and immediately washed with phosphate-buffered saline. BrdUrd was revealed by anti-BrdUrd monoclonal antibody (GH Healthcare) followed by incubation with rhodamine isothiocyanate (RITC)-conjugated anti-mouse IgG2a antibody (Southern Biotechnology). Nuclei were counterstained with Hoechst 33342.

Soft Agar Assay—For each cell pool, 30,000 cells were suspended in 2 ml of Dulbecco's modified Eagle's medium + 10% fetal bovine serum and warmed to 37 °C. 200 μl of prewarmed (52 °C) 5% agarose/phosphate-buffered saline solution was mixed with the cell suspension and then layered into 60-mm dishes that were previously coated with 2 ml of 1% agarose in Dulbecco's modified Eagle's medium. The agar was allowed to solidify at room temperature for 20 min before the addition of 2 ml of growth medium to each well. After 14 days, the colonies were counted.

Subfractionation Experiments—Subfractionation experiments were performed as indicated by the manufacturer (ProteoExtract subcellular proteome extraction kit 539790; Calbiochem).

Calpain Activity Assay—Recombinant His_6-tagged Gas2 (pQE11Gas2wt), His_6-tagged Gas2DN (pETM11Gas2DN), and His_6-tagged Calpastatin (pETM11Calpastatin) were used to evaluate calpain activity in biochemical studies. To generate His_6-tagged Gas2wt protein, full-length Gas2wt was subcloned from pGDSV7SGas2wt (12) into pQE11 vector using BamHI and HindIII sites. For the construction of His_6-tagged Gas2DN and Calpastatin fusion proteins, specific oligonucleotides upstream and downstream containing, respectively, NcoI and BamHI or NcoI and XhoI sites were used to generate polymerase chain reaction fragments of Gas2DN or Calpastatin that were cloned in pETM11 vector. One unit of calpain activity was defined as the amount of the enzyme causing the production of 1 μmol acid-soluble NH_2 revealed with fluorescamine and using acid-denatured globin as substrate (15). Rat skeletal muscle m-calpain activity was monitored after incubating for 3 min with His_6-tagged recombinant proteins Gas2, Gas2DN, and Calpastatin in a calpain assay buffer containing 50 mM sodium borate buffer, pH 7.5, 50 μM $CaCl_2$, and rat skeletal muscle m-calpain. Activity was measured 10 min after the addition of 1 mM calcium and 2 mg/ml denatured globin as previously described (15). Gas2 and Calpastatin, known calpain inhibitors, were used as control of calpain activity in the same assay.

RESULTS

Constitutive Activity of Calpain in Vivo Regulates β-Catenin—To address the role of constitutive activity of calpain toward β-catenin, we designed small interference RNA (siRNA) to selectively knock down calpain levels. Because the two ubiquitous calpain isoforms, m- and μ-calpain, are functional only when associated with the common regulatory small subunit, we decided to design siRNA to selectively knock down the small regulatory subunit to block both calpain isoforms and a siRNA to specifically knock down m-calpain to address the relevance of this isoform in this context. The ability of the specific siRNAs (siSmall or si m-calpain) to down-regulate calpain protein levels was tested in U2OS cells, where expression of endogenous small subunit of calpain or of endogenous m-calpain was, respectively, almost completely abolished already at 24 h after specific silencing transfection (Fig. 1*A*). A scrambled siRNA (*siControl*) was used as control. To evaluate the effects of calpain siRNAs toward the activity of β-catenin, U2OS cells were transfected as indicated in Fig. 1*B*. The TOPflash construct is a β-catenin-sensitive promoter widely used as an indicator for binding of β-catenin to hTCF-4. The resulting complex is responsible for activating transcription of the TOPflash reporter (16). Data were normalized by cotransfection with a pCMV-*Renilla* luciferase vector, which controls for the variability in transfection efficiency. Luciferase assays were performed 48 h after transfection, and Western blot analyses were carried out using the same lysates to monitor steady-state levels of endogenous or overexpressed β-catenin. As shown in Fig. 1*B*, down-regulation of calpain levels as induced either by siSmall or si m-calpain resulted in the stabilization and activation of both endogenous and overexpressed β-catenin. These results strengthen the argument for a role of the m-calpain isoform for the observed effects. As a negative control for the transcriptional competence of β-catenin, we used FOPflash reporter, which contains mutant Tcf-4 binding site, and this was not

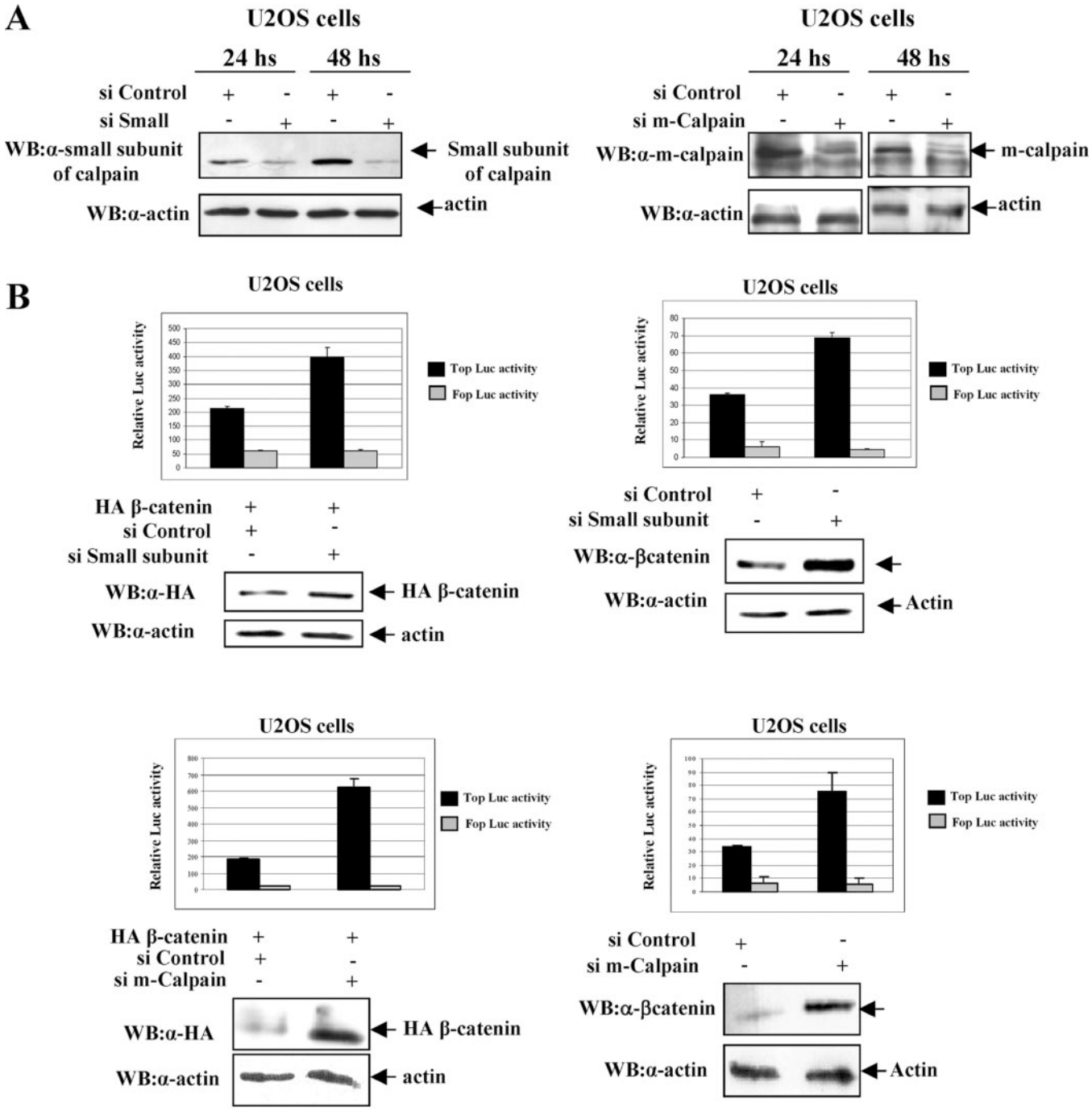

FIG. 1. **Constitutive activity of calpain toward β-catenin.** *A*, U2OS cells were transfected with siControl and siSmall (*left panel*) or siControl and si m-calpain (*right panel*). 24 and 48 h later, lysates were analyzed by Western blot using anti-small subunit of calpain (*upper panel*) and anti-actin (*lower panel*) antibodies in the first case and anti-m-calpain (*upper panel*) and anti-actin (*lower panel*) in the second case. *B*, U2OS cells were transfected as indicated. 24 h later, Western blot analysis was performed using anti-HA antibody (*left panels*) and an anti-β-catenin (*right panels*). Loading control was tested using anti-actin antibody (*lower panels*). Luc activity was determined in the same lysates by calculations of Top-Luc and Fop-Luc (as negative control). The data represent means ± S.D. from three independent experiments. *C*, U2OS cells were transfected with siControl and si-m-calpain. 36 h later cell fractionation was performed and cytoplasmic (*C*), membrane (*M*), and nuclear (*N*) fractions were analyzed. Western blot was performed using anti-β-catenin antibody or anti-c-Fos and anti-actin to check for fractionation efficiency. *D*, the same experiments as in *panel C* were performed in H1299 cells. *E*, confocal microscope image of U2OS cells transfected with HA-tagged β-catenin together with siControl (*a*) or si-m-calpain (*b*) and indicating change of β-catenin localization. Panels show two different corresponding fields of cells stained for β-catenin (*red*).

affected by β-catenin and siControl or siSmall/si m-calpain (Fig. 1*B*).

These observations suggest that, by regulating calpain and particularly the m-calpain isoform, the free cytoplasmic levels of β-catenin could be altered, inducing its translocation into the nucleus where it can function as a transcription factor. To confirm our hypothesis, we transfected U2OS cells with siControl and si m-calpain. 36 h after siRNA transfection, cell fractionation was performed and cytoplasmic (*C*), membrane (*M*), and nuclear (*N*) fractions were separately analyzed. Western blot analysis indicated that the increased levels of β-catenin after m-calpain down-regulation are significantly accumulated

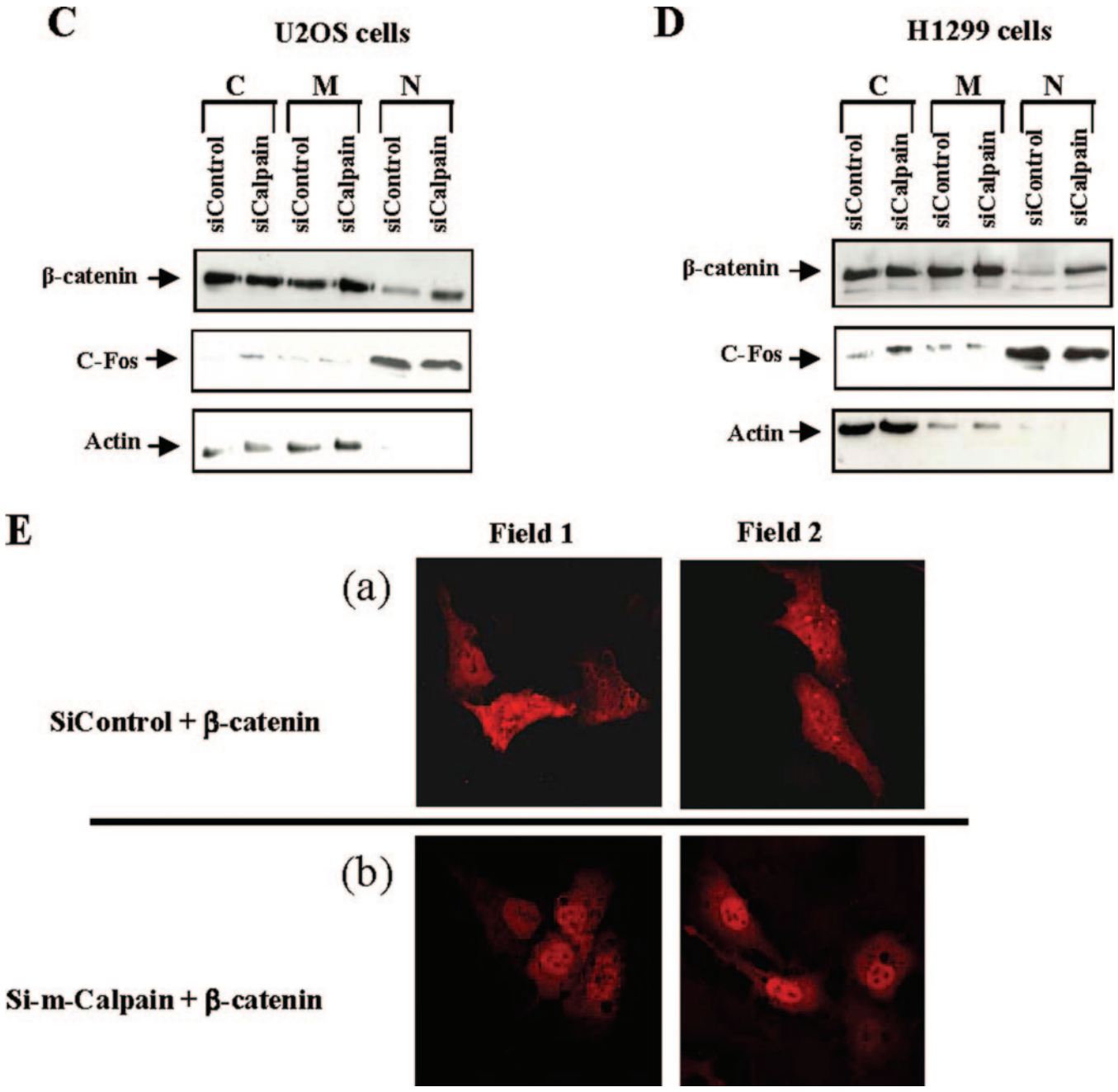

FIG. 1—*continued*

in the nucleus (Fig. 1*C*). The same results were obtained in the H1299 cell line (Fig. 1*D*) and by using siSmall (data not shown). Cellular localization of overexpressed β-catenin after si m-Calpain treatment was also tested by immunocytochemistry, and a supportive change in the nuclear localization was observed by knocking down calpain expression (Fig. 1*E*). Altogether, these results demonstrate that the calpain proteolytic system can alter the transcriptionally active fraction of β-catenin, thus representing a potential mechanism to regulate β-catenin in parallel to the proteasome.

Gas2 and Calpastatin, the Two Endogenous Inhibitors of Calpain, Regulate the Levels and Function of β-Catenin—To better characterize the constitutive m-calpain function toward β-catenin, we decided to analyze the effects of both its endogenous inhibitors, Gas2 (11) and Calpastatin (10). As a first approach, we tested whether overexpression of Gas2 and Calpastatin might enhance the steady-state levels of β-catenin *in vivo*. Cotransfection of HA-tagged β-catenin with Gas2 (Fig. 2*A*, *left panel*) and Calpastatin (*right panel*) caused a substantial accumulation of β-catenin, indicating that calpain regulation by its inhibitors could function as a constitutive system to regulate β-catenin.

As a next step we evaluated the transcriptional competence of stabilized β-catenin by determining the effect of Gas2 and Calpastatin overexpression on the ability of β-catenin to stimulate transcription from TCF-responsive elements. The relative luciferase activity (normalized by cotransfection with pCMV-*Renilla* luciferase) was significantly stimulated by overexpression of Gas2. A similar response was obtained with transfection of Calpastatin (Fig. 2*B*), suggesting that both Gas2 and Calpastatin overexpression can render β-catenin transcriptionally competent. As a control, we looked for the ability of Gas2 and Calpastatin to cause β-catenin translocation into the nucleus (Fig. 2*C*) where it can function as a transcription

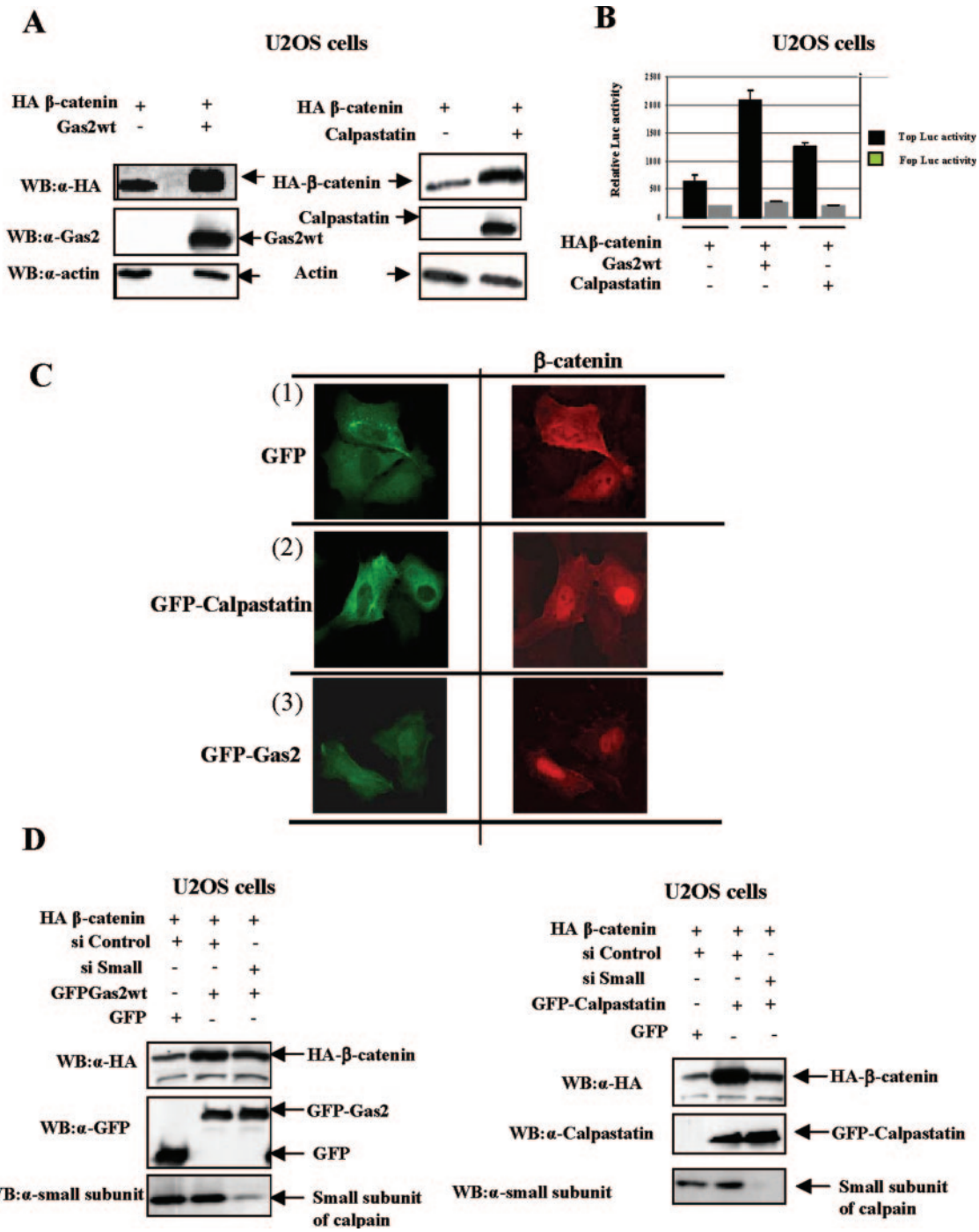

FIG. 2. **Gas2 and Calpastatin regulate levels and function of β-catenin.** *A*, U2OS cells were transiently transfected as indicated. Western blot analysis was performed using anti-HA (*upper panel*), anti-Gas2 or anti-Calpastatin (*middle panel*), and anti-actin (*lower panel*) antibodies. *B*, U2OS cells were cotransfected with indicated plasmids. Luc activity was evaluated 48 h later. Data represent means ± S.D. from three independent experiments. *C*, confocal microscope images of U2OS cells transfected with HA-tagged β-catenin together with GFP (*1*), GFP-Calpastatin (*2*), and GFP-Gas2 (*3*). Panels show corresponding fields of cells stained for β-catenin (*red*) and expressing GFP-tagged proteins (*green*). *D*, U2OS cells were transfected with HA-β-catenin together with a combination of vectors and siRNA oligos as indicated. Cells were harvested 48 h after transfection and analyzed by Western blot analysis using the indicated antibodies. The nonspecific band with *arrows* was considered as a loading control.

factor. We therefore transfected β-catenin into U2OS together with GFP-tagged Gas2, GFP-tagged Calpastatin, and GFP protein alone as negative control. 24 h after transfection, immunofluorescence analyses were performed to visualize β-catenin localization. As can be seen in Fig. 2*C*, the presence of both GFP-tagged Gas2 and GFP-tagged Calpastatin was able to relocalize the cytoplasmic β-catenin into the nucleus. Finally, to confirm that endogenous calpain is critical for the effect of Gas2 and Calpastatin on β-catenin stability, HA-tagged β-catenin levels were monitored after transfection in U2OS cells together with Gas2 or Calpastatin and SiControl or siSmall. Calpain silencing abrogated both Gas2 and Calpastatin effects on β-catenin stabilization, thus indicating the critical role for calpain in the Gas2 and Calpastatin regulation of β-catenin levels (Fig. 2*D*). Altogether, these data suggest that Gas2 and Calpastatin, as endogenous calpain inhibitors, play important roles in controlling β-catenin levels, localization, and transcriptional activity.

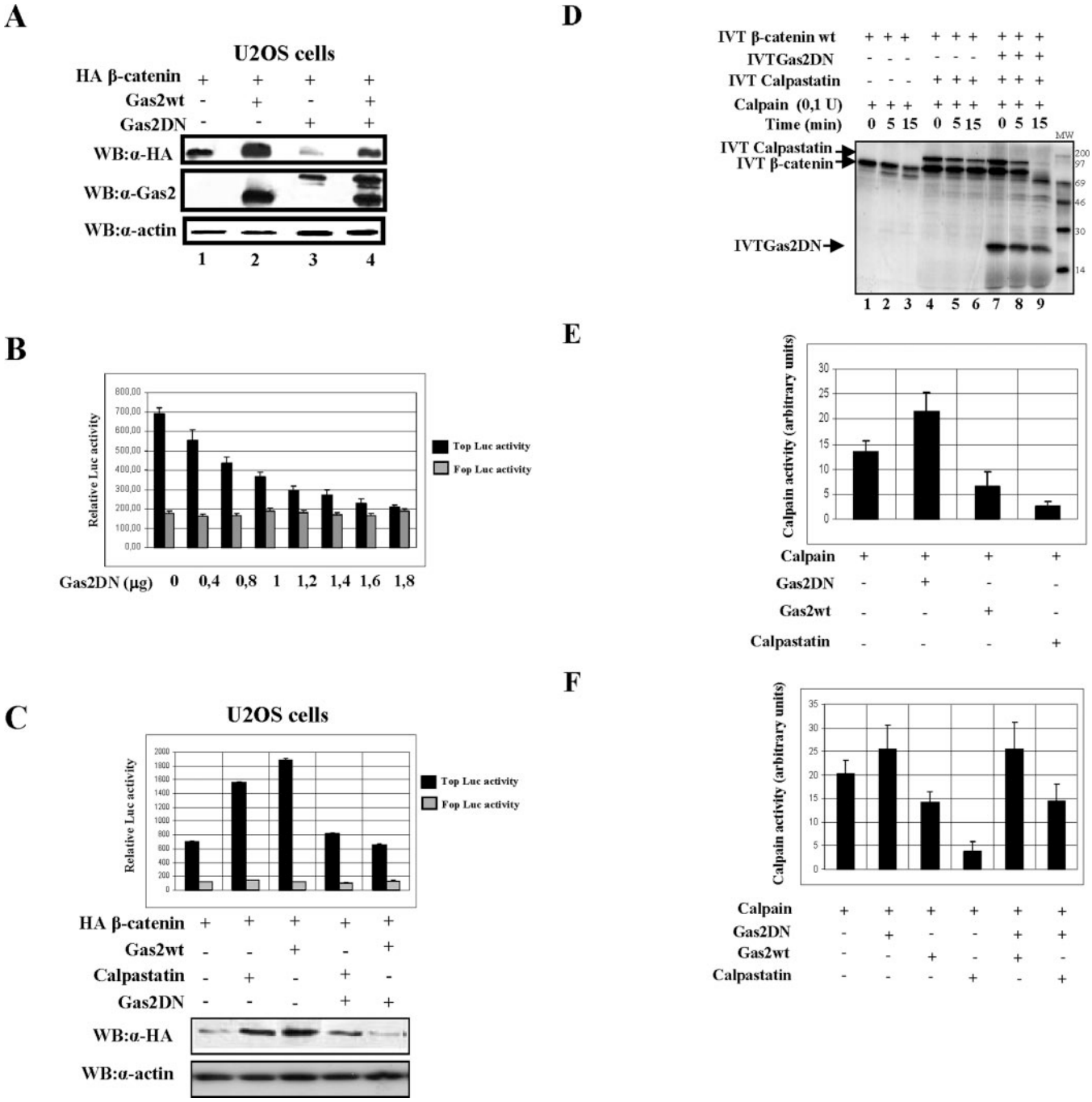

FIG. 3. **Gas2DN can counteract both Gas2 and Calpastatin effects.** *A*, U2OS cells were transfected as indicated. Protein levels were monitored by using anti-HA (*upper panel*), anti-Gas2 (*middle panel*), and anti-actin (*lower panel*) as loading control antibodies. *B*, U2OS cells were transfected with Top-Luc reporter together with increasing amounts of Gas2DN. Luc activity was evaluated 48 h later. Data represent means ± S.D. from at least three independent experiments. *C*, U2OS cells were transiently transfected with the combination of plasmids as indicated. Luciferase and Western blot analysis were performed with the same lysates. *D*, IVT β-catenin was incubated with m-calpain (*lanes 1-3*), m-calpain and IVT Calpastatin (*lanes 4-6*), or m-calpain, IVT Calpastatin, and IVT Gas2DN (*lanes 7-9*). Samples were collected at the indicated time points, analyzed by SDS-PAGE, and evaluated by autoradiography. *Arrows* indicate full-length proteins. Being itself a calpain substrate, the Calpastatin band is also degraded during the time although it is fully functional as a calpain inhibitor after its cleavage (40, 41). *E*, biochemical assay to test the effect of recombinant Gas2, Calpastatin, and Gas2DN on m-calpain activity. Assay was performed as described under "Materials and Methods." *F*, biochemical effect of Gas2DN on Gas2 and Calpastatin ability to inhibit calpain activity. Assay was performed as described under "Materials and Methods."

Gas2DN Blocks Both Calpastatin and Gas2 Regulatory Arms on Calpain Activity—We have previously characterized Gas2DN as a dominant negative form of Gas2, demonstrating that it can still bind m-calpain without showing any inhibitory function (11). We therefore decided to analyze the effects of Gas2DN on the levels and activity of transfected β-catenin.

Gas2DN was transfected into U2OS cells together with β-catenin alone or β-catenin and Gas2. As can be seen in Fig. 3*A*, its overexpression reduced the levels of β-catenin and of the Gas2-dependent β-catenin stabilization.

As a next step we evaluated the transcriptional competence of endogenous β-catenin by determining the effects of Gas2DN overexpression on the ability of β-catenin to stimulate transcription from TCF-responsive elements. We observed that Gas2DN significantly decreased β-catenin transcriptional activity in a dose-dependent manner (Fig. 3*B*). Altogether, the

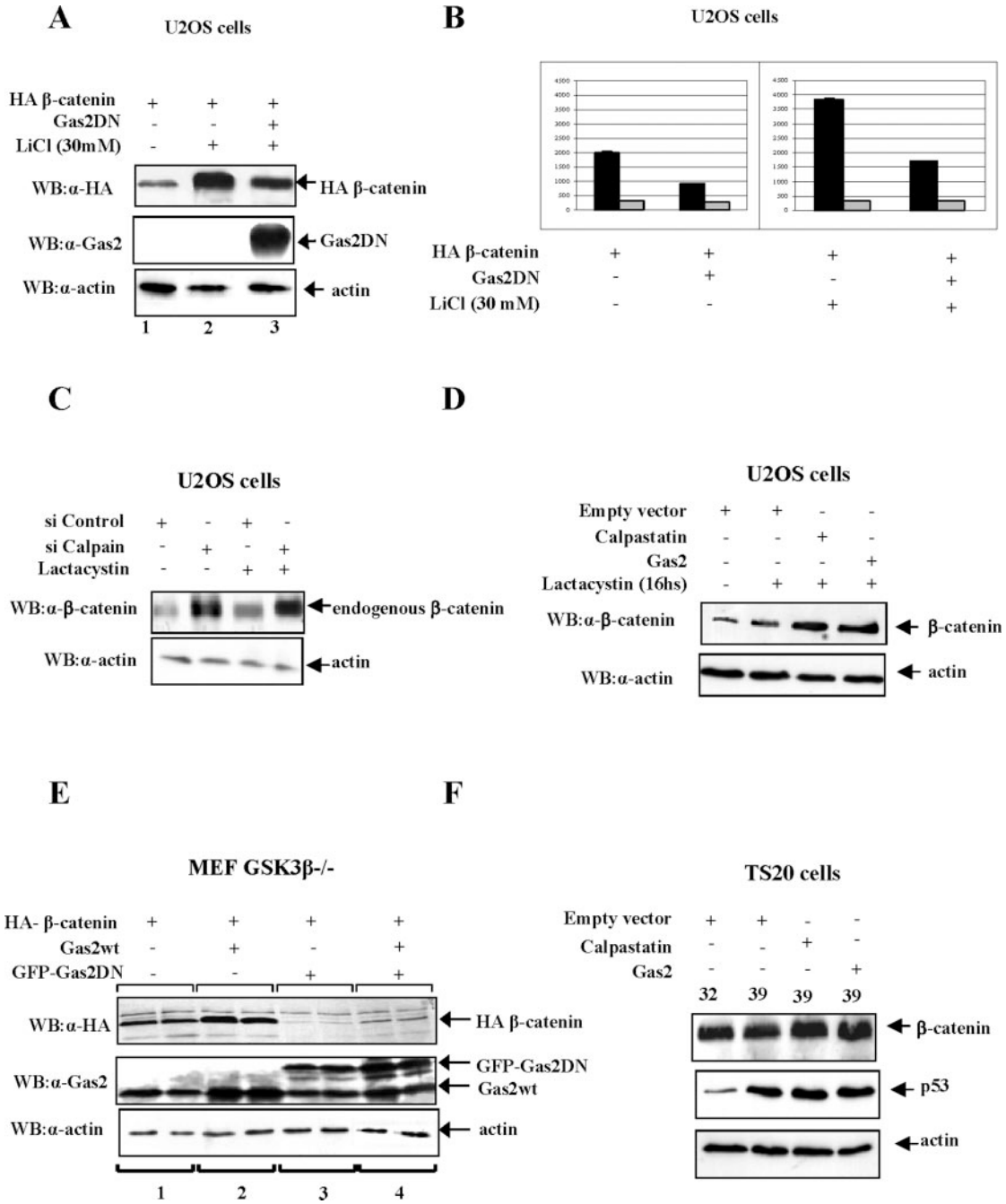

FIG. 4. **Gas2DN induces β-catenin degradation independently of GSK3β, and calpain regulates β-catenin functions independently of the proteasome.** *A*, U2OS cells were transfected with indicated plasmids and then treated with an aqueous vehicle or LiCl 30 mM for 18 h as indicated. Cells lysates were analyzed by Western blot using anti-HA (*upper panel*), anti-Gas2 (*middle panel*), and anti-actin (*lower panel*) antibodies. *B*, U2OS cells were transfected with the indicated plasmids and then treated with an aqueous vehicle or LiCl 30 mM as indicated. Luc activity was evaluated 48 h later. Data represent mean ± S.D. from three independent experiments. *C*, U2OS cells were transfected with siControl or si m-calpain and treated or not with lactacystin for the indicated times. Lysates of the cells were then analyzed using anti-β-catenin antibody and anti-actin as a loading control. *D*, U2OS cells were transfected with the indicated plasmids and treated with lactacystin for 16 h. Lysates of the cells were then analyzed as in *panel C*. *E*, GSK3β−/− mouse embryo fibroblasts were transfected as indicated. Protein levels were monitored by using anti-HA (*upper panel*), anti-Gas2 (*middle panel*), and anti-actin as loading control (*lower panel*) antibodies. *F*, TS20 cells were transfected as indicated. Cells were then grown at non-permissive temperature for 12 h. Cell lysates were subsequently analyzed by Western blot, and the endogenous levels of β-catenin, p53, and actin as loading control are indicated.

data indicating the relevance of m-calpain pathway in β-catenin regulation, together with the evidence that Gas2DN is able to down-regulate β-catenin levels and function (Fig. 3, *A* and *B*), prompted us to investigate whether Gas2DN interference with m-calpain activity could counteract not only Gas2 but also Calpastatin function, thus serving as a general reagent to control calpain pathway. As shown in Fig. 3*C*, in U2OS cells HA-β-catenin transcriptional activity as stimulated by Gas2 or Calpastatin coexpression was repressed in both cases by Gas2DN, and as expected HA-β-catenin levels, due to the presence of Gas2 or Calpastatin, were clearly reduced when Gas2DN was cotransfected. We therefore conclude that Gas2DN can also rescue Calpastatin effects on β-catenin stabilization. We decided to perform an *in vitro* assay to analyze whether the ability of Calpastatin to protect calpain-dependent β-catenin degradation could be abrogated by Gas2DN. As

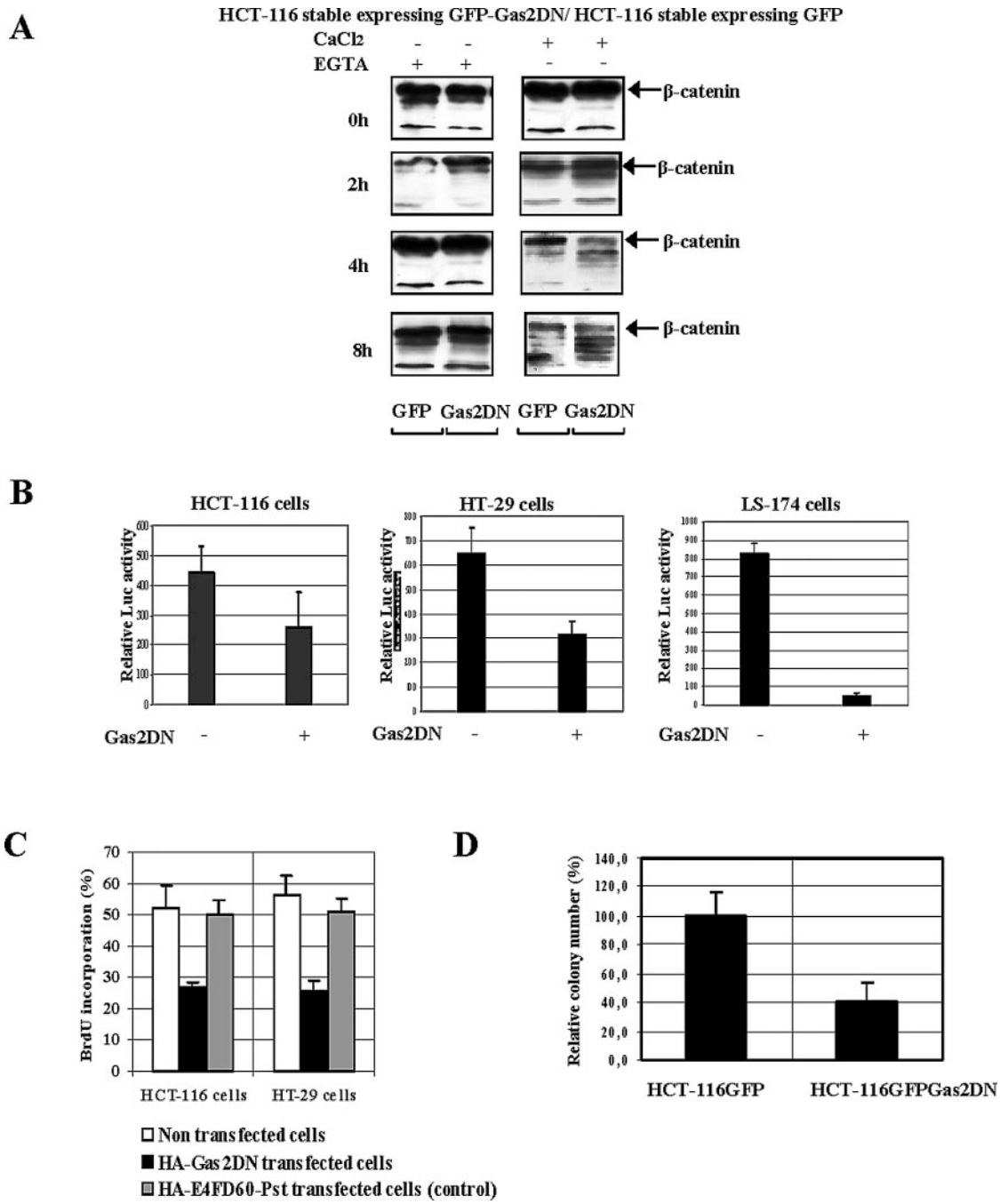

FIG. 5. **Gas2DN affects β-catenin activity and growth rate in colon carcinoma cells.** *A*, proteolytic cleavage of endogenous β-catenin by endogenous m-calpain was followed in GFP/HCT-116 or GFP-Gas2DN/HCT-116 cells. Aliquots of the samples were collected at the indicated time points in the presence of $CaCl_2$ (2 mM) or EGTA (8 mM). β-Catenin proteolysis in each sample was monitored by Western blot. *B*, HCT-116, HT-29, and LS-174 were transfected as indicated. Luciferase activity was measured 48 h after transfections. Graphs are the mean of at least three different experiments. *C*, HCT-116 and HT-29 cells were transfected with equal amounts of HA-Gas2DN or a control protein (HA-E4DΔ60-Pst). 24 h after transfection cells were pulsed with 50 μM BrdUrd for 4 h and then fixed and stained with anti-BrdUrd and anti-HA antibodies. Results are the mean of at least four independent experiments and were obtained by scoring more than 500 cells for each transfected construct in every cell line. Percentage of S-phase inhibition = [(percentage of cells in S-phase in the background − percentage of cells in S-phase in the transfected cells)/(percentage of cells in S-phase in the background)] × 100. *D*, 3×10^4 GFP/HCT-116 or GFP-Gas2DN/HCT-116 cells were plated as described under "Material and Methods." The colony number obtained with GFP stable expressing cells was set as 100%. Experiments were repeated five times with consistent and repeatable results.

shown in Fig. 3*D*, whereas Calpastatin can efficiently block β-catenin degradation (compare *lanes 4-6* with *lanes 1-3*), Gas2DN can effectively recover the inhibitory effect of Calpastatin on m-calpain (*lanes 7-9*). The same amount of *in vitro* translated (IVT) glyceraldehyde-3-phosphate dehydrogenase failed to show a similar result (data not shown). To provide final evidence of the calpain activator properties of Gas2DN, its effect on calpain activity was tested in a biochemical *in vitro* assay measuring calpain activity. Rat skeletal muscle m-calpain activity was measured as described under "Materials and Methods" and was monitored in the presence of both the recombinant inhibitors Gas2wt and Calpastatin and in the presence of Gas2DN. Whereas the function of calpain, as can be observed in Fig. 3*E*, was efficiently blocked by its inhibitors Gas2 and Calpastatin, the presence of recombinant Gas2DN induced an increase in calpain activity, suggesting a potential

activator role for this protein. With the same biochemical approach we confirmed *in vitro* the ability of Gas2DN to counteract the inhibitory effects of both Gas2 and Calpastatin (Fig. 3*F*). We therefore conclude that Gas2DN can disengage endogenous m-calpain from both its inhibitors, Gas2 and Calpastatin, possibly because both inhibitors share the same binding region on the calmodulin-like domain of m-calpain, as already described (11, 17).

Calpain Regulates β-Catenin Functions Independently of GSK3β Activity and of the Proteasome—After showing Gas2/Calpastatin/Gas2DN as tools to regulate β-catenin levels and function via calpain, we investigated whether β-catenin could be regulated in the absence of GSK3β, a critical component of the multimolecular complex responsible for targeting β-catenin to the proteasome (18). As a first approach, the Gas2DN effect as a general calpain activator was analyzed on stabilized β-catenin in U2OS cells treated with LiCl, a known inhibitor of GSK3β activity (19). Under these conditions, Gas2DN overexpression was still able to reduce β-catenin levels (Fig. 4*A*) as well as to decrease its transcriptional competence (Fig. 4*B*), indicating that its effect on β-catenin is a GSK3β-independent process. However, because β-catenin can also be degraded in a proteasome-dependent, but GSK3β-independent, mechanism (20, 21), we investigated whether calpain is truly acting in a proteasome-independent manner by using lactacystin as a proteasome-specific inhibitor. To set the conditions we followed the well characterized p53 protein stability as an independent marker of proteasome inhibition. In U2OS cells we found that p53 reaches its highest levels 8 h after lactacystin treatment and maintains such levels for 24 h (data not shown), suggesting that during this time the proteasome system can be considered significantly blocked. In the same context, transfections of U2OS cells with siControl or si m-calpain confirmed that m-calpain knock down causes the increased levels of β-catenin even when the proteasome is blocked (Fig. 4*C*). The same effects were observed when lactacystin-treated cells were transfected with Gas2 and Calpastatin (Fig. 4*D*). Altogether, these data indicate that calpain acts in a GSK3β- and proteasome-independent manner to regulate β-catenin levels and functions. To confirm this hypothesis, we chose two alternative cell lines, respectively knocked out for GSK3β (mouse embryo fibroblast GSK3β−/−) and containing a thermolabile ubiquitin-activating enzyme (E1) that is inactivated at 39 °C, thus preventing ubiquitination (TS20 cells). In GSK3β−/− mouse embryo fibroblasts, Gas2wt was still able to stabilize β-catenin (Fig. 4*E*), and, more importantly, Gas2DN maintained its ability to reduce β-catenin levels either alone or when cotransfected with Gas2. Finally, as shown in Fig. 4*F*, transfection of TS20 cells at the non-permissive temperature (39 °C) with Gas2 or Calpastatin resulted in the accumulation of endogenous β-catenin, confirming that ubiquitination of β-catenin does not seem to be required for its regulation by calpain system. We can therefore conclude that calpain modulates β-catenin levels and activity independently of both GSK3β and proteasome function, thus indicating that the calpain pathway could play an alternative role when canonical proteasome-mediated β-catenin degradation is impaired.

Gas2DN Modulates the Activity of β-Catenin in Colon Carcinoma Cells, Reducing Their Proliferation and Anchorage-independent Growth—Colon cancer cells represent an interesting model to study the relevance of calpain pathway because in this context canonical proteasome-mediated β-catenin degradation is strictly impaired. Therefore we decided to test whether Gas2DN is able to induce endogenous β-catenin degradation in HCT-116 colon carcinoma cells, which harbor mutations in β-catenin that prevent proteasome-dependent turnover. We generated a cell line stably expressing GFP-Gas2DN or GFP (GFP-Gas2DN/HCT-116 and GFP/HCT-116). Lysates of these cells were then separately incubated with $CaCl_2$ to activate m-calpain or EGTA to block its activity. Aliquots were taken at defined time points (0, 4, and 8 h) to follow the kinetics of β-catenin degradation. As can be observed in Fig. 5*A*, *lanes 3* and *4*, addition of calcium, but not EGTA, to the cell lysates promoted β-catenin degradation. Notably, GFP-Gas2DN/HCT-116 cells show degradation of β-catenin starting earlier and becoming significantly more pronounced compared with GFP/HCT-116 (Fig. 5*A*, *lanes 3* and *4*). In all cases β-catenin cleavage was inhibited by EGTA (*lanes 1* and *2*).

These results indicate that endogenous m-calpain can degrade endogenous β-catenin in cells that are defective in regulating its levels through the classical proteasomal pathway. Even more interesting, it suggests the possibility that Gas2DN can stimulate such degradation.

We then tested the effect of Gas2DN in various colon carcinoma cell lines, where proteasome degradation is blocked because of mutations of β-catenin itself (HCT-116 and LS-174) or of its regulator APC (HT-29). As shown in Fig. 5*B*, overexpression of Gas2DN induced a significant reduction in β-catenin activity in all tested cells. These results suggest that overexpression of Gas2DN can regulate the activity of a form of β-catenin that is not under the control of proteasome-dependent degradation.

Because accumulated evidence demonstrates that β-catenin alters cell cycle progression (22), we tested whether the negative effect of Gas2DN on β-catenin was also manifested in the proliferation rate of colorectal cancer cells. Transient overexpression of Gas2DN in HCT-116 and HT-29 cells showed a significant inhibition of S-phase entry as assessed by BrdUrd incorporation assay (Fig. 5*C*).

Finally, because a characteristic feature of transformed cells is their ability to grow in an anchorage-independent way and β-catenin is involved in this process, we compared the ability of GFP-Gas2DN/HCT-116 or GFP/HCT-116 cells to form colonies in semisolid medium. Significant reduction in the ability to form colonies for Gas2DN-expressing cells was observed with respect to GFP-expressing cells (Fig. 5*D*). Altogether, these results demonstrate that Gas2DN modulates the function of deregulated β-catenin and reduces both proliferation and anchorage-independent growth in a context of deregulated β-catenin.

DISCUSSION

The intracellular signaling pathway of Wnt is most conserved during evolution and regulates cellular proliferation, morphology, motility, cell fate, and organ development. Aberrant Wnt signaling pathway is an early event in 90% of colorectal cancers that results in β-catenin accumulation. It can occur through inefficient phosphorylation of β-catenin by GSK3β or because of mutations on APC, β-catenin, and/or axin. As a result of a defective degradation via proteasome, accumulated β-catenin translocates into the nucleus and becomes transcriptionally active, enhancing expression of genes regulating cell proliferation and survival. Our results indicate a constitutive calpain-dependent mechanism regulating β-catenin stability and function and suggest Gas2DN as a reagent to activate calpain pathway, emerging as an effective way to reverse oncogenic β-catenin/LEF/TCF signals that occur when β-catenin degradation via the proteasome is defective (Fig. 6).

In this study, we have shown the physiological and constitutive involvement of calpain in the regulation of signaling β-catenin, which can translocate into the nucleus where it functions as a transcription factor. The physiological involvement of calpain in the regulation of β-catenin was demonstrated by

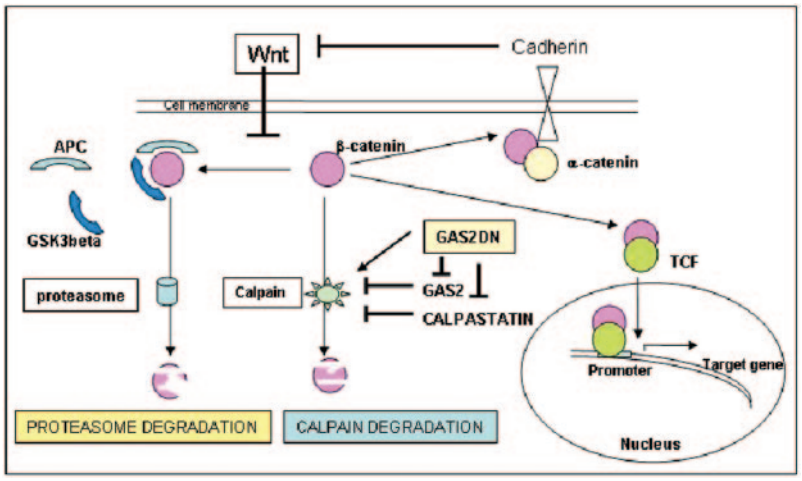

FIG. 6. **A working model for the regulatory network involving Gas2, Calpastatin, Gas2DN, calpain, and β-catenin.** Control over calpain activity by overexpression of its inhibitors Gas2 and Calpastatin is crucial to up-regulate β-catenin levels, to relocate β-catenin to the nucleus, and therefore to increase its signaling properties through a pathway that does not require the proteasome function. Gas2DN, disengaging calpain from both its inhibitors, stimulates endogenous calpain activity *versus* β-catenin and therefore can be used to down-regulate β-catenin signaling potential in the context of colon cancer, where accumulated β-catenin cannot be degraded through the classical proteasome pathway.

siRNA-mediated knock down of calpain that resulted in a significant increase of both the levels and transcriptional activity of β-catenin and in its clear translocation into the nucleus (Fig. 1). Control over calpain activity through the overexpression of Gas2 and Calpastatin was shown to be crucial for β-catenin regulation (Fig. 2). To better characterize the effects of both inhibitors, we studied the effects of Gas2DN, which was initially defined as Gas2wt-dominant negative form, being able to still bind calpain but not to inhibit its activity. We provided evidence that Gas2DN can be considered a novel and specific molecule to modulate calpain activity because it can efficiently counteract the inhibitory effect of both Gas2 and Calpastatin *in vivo*. The recombinant Gas2DN protein can also be used to stimulate endogenous calpain activity in a biochemical *in vitro* assay (Fig. 3). These results suggest that Gas2DN can disengage endogenous calpain from both its inhibitors, Gas2 and Calpastatin, possibly because Gas2 and Calpastatin share the binding region on the calmodulin-like domain of calpain. In fact, we have previously demonstrated that the amino-terminal region of Gas2 (which comprises Gas2DN) binds subdomains III and IV of m-calpain and that the Gas2 CT region is required to exert the inhibitory function (11). The inhibitory domains of Calpastatin are defined by three highly conserved regions, A, B, and C. Whereas subdomain B is responsible for inhibition, regions A and C are responsible for binding calpain in a strictly Ca^{2+}-dependent manner to subdomains III and IV, where Gas2 also binds (23, 24). Binding of regions A and C of Calpastatin shifts the conformational equilibrium of calpain toward the active form, but activation of calpain is not manifested because its active site is blocked by subdomain B. Regions A and C, on the other hand, lack this inhibitory potential and have been suggested as potential activators for m-calpain (24). Similarly, Gas2DN, lacking the carboxyl domain of Gas2 that is responsible for the calpain inhibitory function, could be involved in calpain activation.

In all tested cases, regulation of calpain proteolytic activity through overexpression of Gas2DN was shown to be crucial to regulate both β-catenin protein levels and its transcriptional activity in the absence of GSK3β or under conditions where GSK3β is blocked through the addition of LiCl. The use of LiCl has been reported to similarly block GSK3α (25), thus excluding any role for both GSK3 isoforms (Fig. 4).

These results indicate that the calpain pathway works independently of the β-catenin phosphorylation state as regulated by GSK3 activity. Moreover, because it has been demonstrated that β-catenin can also be degraded in a GSK3β-independent but proteasome-dependent manner (20, 21), we performed a set of experiments to exclude any involvement of the proteasome in the calpain-mediated degradation of β-catenin. We provide evidence that when the proteasome pathway is impaired by using a specific proteasome inhibitor the alternative calpain-dependent β-catenin degradation is still active. The same conclusions were obtained using a mutant cell line with a thermolabile E1 ubiquitin-activating enzyme, demonstrating that calpain function toward β-catenin can occur in the absence of a functional proteasome pathway. Altogether, these observations suggest that under physiological conditions the calpain system can cooperate with the proteasomal pathways to regulate β-catenin degradation and could therefore represent a mechanism to recapture non-phosphorylated and accumulated cytoplasmic β-catenin, targeting it to an alternative degradation when the most established proteasome pathway becomes altered during tumorigenesis, such as in a colon carcinoma context. Our findings are in apparent contrast with a prior study on prostate tumor cells, in which it has been reported that calpain cleavage, by removing the NH_2-terminal regulatory domain of the β-catenin protein, leads to the accumulation of a stable 75-kDa deletion able to increase TCF/LEF-dependent transcription (26). However, in the context of colon cancer cells we observed that calpain-dependent cleavage of endogenous β-catenin causes a complex pattern of smaller degradation products (Fig. 5*A* and data not shown), suggesting either that this unique 75-kDa proteolytic cleavage product is not formed in colon cancer or that it is unstable in these cells. The different degradation patterns observed in colon cancer cells and prostate cells could be due to the different complexes formed by β-catenin in the two cell types, limiting further degradation of the 75-kDa product in the case of prostate cells by calpains, or to amino acid difference of β-catenin at the cleavage sites. The molecular basis of this difference represents an interesting subject for future investigations.

Considering the relevance of β-catenin function in colon cancer cells, we showed that colon cancer cells stably expressing Gas2DN are suppressed both in proliferation and anchorage-independent growth (Fig. 5). These results underline the relevance of calpain activity toward β-catenin regulation. It is worth noting the recent evidence obtained with microarray analysis on colon carcinoma cells associating increased expression of Calpastatin mRNA (27) with increased metastatic potential. This could represent an interesting system to definitely link β-catenin and the calpain system, possibly suggesting a potential mechanism to alternatively regulate β-catenin degradation along with the metastatic phenotype.

In conclusion, we demonstrated that accumulated β-catenin that cannot be degraded through the proteasome can be targeted to the alternative calpain-dependent degradation, thus

blocking its oncogenic activity. In this respect, Gas2DN emerged as a tool to modulate the protease activity. Interestingly Gas2DN (amino acids 1–171) contains one Calponin homology domain (CH domain) (amino acids 37–153). This domain has been found in a variety of proteins ranging from actin cross-linking to signaling elements and has been proposed to function with a quite striking variability (28). In general, proteins containing a single CH domain act as regulatory proteins (29). It will be interesting to clarify whether and how the single CH domain could be linked to modulation of calpain activity. In this view, it can be hypothesized that the CH domain could function in the recruitment of calpain to a specific subcellular compartment for localized activation, therefore providing in such a microenvironment the requested calpain conformational changes necessary to reduce the calcium requirement for the activation. Our findings reveal a mode of control over calpain that is extensively implicated in diverse cellular and developmental contexts and open interesting avenues on the mechanisms regulating its activation, thus complementing the described requirement for Ca^{2+} signaling (30–32). Further biochemical *in vitro* and *in vivo* studies on the involvement of the CH domain contained in Gas2 with respect to calpain activity could finally provide new tools for therapeutic intervention in the control of the Wnt/β-catenin signaling pathway and, specifically, for the treatment of colon cancer.

In addition to its function in the conventional Wnt signaling pathway, β-catenin also binds tightly to the cytoplasmic domain of type I cadherins and plays an essential role in structural organization and function of cadherins by linking cadherins through α-catenin to the actin cytoskeleton (33, 34). Progress in understanding β-catenin signaling, as well as cadherin-mediated cell adhesion, generally followed separate lines of investigation. However, it is clear that there are many connections between these pathways; alteration in cell fate, adhesion, and migration are, for example, characteristics of cancer in which cells ignore normal regulatory cues from their environment. Unchecked Wnt signaling (4) and/or the loss of cell-cell adhesion (35–37) are involved in cancer induction and progression. Moreover, recent studies have shown that both Wnt signaling and cadherin-mediated cell-cell adhesion are important in the organization and maintenance of stem cells (38). The critical component of these pathways is β-catenin, and the key events are the regulation of β-catenin stability and availability. In this view, calpain could really represent an important bridge to switch the signaling of nuclear β-catenin that affects gene expression to a signaling toward the cytoskeleton and cell polarity in tissues. In fact, whereas inhibition of calpain activity through its endogenous inhibitors causes β-catenin translocation to the nucleus where it becomes transcriptionally competent (Fig. 1), stimulation of calpain activity induces β-catenin exit from the nucleus (9) (Fig. 3), where it lacks its transcriptional ability (Fig. 3) and could be strictly involved in cytoplasmic signaling. Cell migration requires dynamic interaction among a cell, its substratum, and the actin cytoskeleton. It is worth noting that calpain is an attractive candidate to be a regulator of cell migration and membrane protrusion through its capacity to regulate focal adhesion dynamics and rear retraction (39, 40). In this context it will be very interesting to connect the regulation of β-catenin by the calpain system to the described role of calpain activity with respect to E-cadherin degradation (41, 42) to contribute to the integration of the complex and still puzzling interplay of cell motility, cytoskeleton rearrangement, and cell polarity required during tissue morphogenesis. In this perspective tight and localized control on calpain activity could represent a critical link for the different β-catenin actions both during development and in a cancer context.

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Calpain is required for macroautophagy in mammalian cells

Francesca Demarchi,[1] Cosetta Bertoli,[1] Tamara Copetti,[1] Isei Tanida,[2] Claudio Brancolini,[3] Eeva-Liisa Eskelinen,[4] and Claudio Schneider[1,3]

[1]Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, 34012 Trieste, Italy
[2]Department of Biochemistry, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-8421, Japan
[3]Dipartimento di Scienze e Tecnologie Biomediche, Universitá degli Studi di Udine, 33100 Udine, Italy
[4]Department of Biological and Environmental Sciences, Division of Biochemistry, 00014 University of Helsinki, Helsinki, Finland

Ubiquitously expressed micro- and millicalpain, which both require the calpain small 1 (CAPNS1) regulatory subunit for function, play important roles in numerous biological and pathological phenomena. We have previously shown that the product of GAS2, a gene specifically induced at growth arrest, is an inhibitor of millicalpain and that its overexpression sensitizes cells to apoptosis in a p53-dependent manner (Benetti, R., G. Del Sal, M. Monte, G. Paroni, C. Brancolini, and C. Schneider. 2001. *EMBO J.* 20:2702–2714). More recently, we have shown that calpain is also involved in nuclear factor κB activation and its relative prosurvival function in response to ceramide, in which calpain deficiency strengthens the proapoptotic effect of ceramide (Demarchi, F., C. Bertoli, P.A. Greer, and C. Schneider. 2005. *Cell Death Differ.* 12:512–522). Here, we further explore the involvement of calpain in the apoptotic switch and find that in calpain-deficient cells, autophagy is impaired with a resulting dramatic increase in apoptotic cell death. Immunostaining of the endogenous autophagosome marker LC3 and electron microscopy experiments demonstrate that autophagy is impaired in CAPNS1-deficient cells. Accordingly, the enhancement of lysosomal activity and long-lived protein degradation, which normally occur upon starvation, is also reduced. In CAPNS1-depleted cells, ectopic LC3 accumulates in early endosome-like vesicles that may represent a salvage pathway for protein degradation when autophagy is defective.

Introduction

Macroautophagy, which is usually referred to as autophagy, is responsible for degradation of the majority of intracellular proteins in mammalian cells, particularly during starvation-induced proteolysis. Cytosolic proteins, organelles, and selected regions of the nucleus can be cleared by autophagy to ensure cellular homeostasis and remove damaged or unwanted products (Klionsky, 2005). Cytoplasmic constituents are first enclosed by a double-membrane autophagosome; next, the outer membrane of the autophagosome fuses with the lysosome, with the consequent destruction of the cargo and the inner membrane of the autophagosome by hydrolytic enzymes.

The autophagic pathway has been dissected at the molecular level in yeast, in which 27 genes, referred to as *ATG*, have been found to be required for autophagosome formation (Klionsky and Ohsumi, 1999; Yorimitsu and Klionsky, 2005). Two ubiquitin-like conjugation systems are involved in the process: one mediates the covalent attachment of Atg12 to Atg5, and the other mediates the conjugation of Atg8 to phosphatidylethanolamine (Ichimura et al., 2000). The resulting products, Atg12–Atg5 and Atg8-phosphatidylethanolamine, are essential for proper autophagosome formation. Only a small fraction of the Atg12–Atg5 complex localizes at the autophagic isolation membrane throughout elongation, and the complex dissociates when the phagosome is complete. On the other hand, Atg8 remains on the mature autophagosome constituting the recognized consensus marker of this structure. These two conjugation systems are highly conserved in mammalian cells. *ATG5*-deficient stem cells have been instrumental for demonstrating that the Atg12–Atg5 complex localizes at autophagosome precursors and plays an essential role in autophagosome formation (Mizushima et al., 2001). The production of *ATG5*-deficient mice has revealed that autophagy is required for neonatal

Correspondence to Claudio Schneider: schneide@lncib.it

Abbreviations used in this paper: 3MA, 3-methyladenine; AV, autophagic vacuole; CAPNS, calpain small; EBSS, Earle's balanced salt solution; MEF, mouse embryonic fibroblast; PI, propidium iodide.

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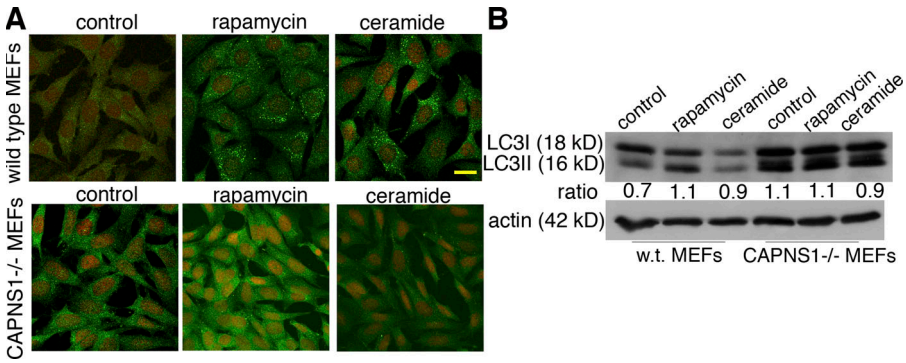

Figure 1. **CAPNS1$^{-/-}$ MEFs lacking calpain activity are defective in autophagosome formation.** (A and B) Wild-type and CAPNS1$^{-/-}$ MEFs were stimulated for 3 h with rapamycin or ceramide and analyzed by immunofluorescence using an anti-LC3–specific antibody and PI to stain nuclei (A) or were lysed and used for Western blot analysis with the same antibody to detect both cytoplasmic LC3I and membrane-associated LC3II (B). Actin was used as a loading control. (B) The ratio between the intensities of LC3II and LC3I bands is indicated below each lane. Bar, 20 μm.

survival before maternal feeding (Kuma et al., 2004). The analysis of endogenous LC3, which is one of the mammalian homologues of Atg8, and the production of transgenic mice expressing a fluorescent autophagosomal LC3 have shown that the regulation of autophagy is organ dependent and that the role of autophagy is not restricted to the starvation response (Mizushima et al., 2004).

A pivotal role for autophagy in growth control has emerged from studies on Beclin1, the mammalian homologue of *ATG6*. The Beclin1 gene is deleted in several types of breast cancer that are unable to activate autophagy (Liang et al., 1999) and functions as a haploinsufficient tumor suppressor gene (Yue et al., 2003). In addition to involvement in growth control, programmed autophagy plays a role in differentiation (Ghidoni et al., 1996; Lee et al., 2002) and tissue remodeling (Rusten et al., 2004). Moreover, autophagy may be artificially induced by radiation and treatment with certain drugs, including rapamycin (Noda and Ohsumi, 1998), ceramide (Scarlatti et al., 2004), arsenic trioxide (Kanzawa et al., 2003), and tamoxifen (Bursch et al., 2000); the final outcome of such induction is still a matter of debate (Kroemer and Jaattela, 2005).

The calpain family of cysteine proteases is composed of both ubiquitous and tissue-specific isoforms that share homology in their protease domain and are calcium dependent (Goll et al., 2003). The best-characterized ubiquitous calpains are the isoforms μ- and m-calpain, which are also known as calpain 1 and calpain 2, respectively. Both contain an 80-kD catalytic subunit and share a common 28-kD regulatory subunit commonly known as calpain 4 that is required for proper activity. Hereafter, calpain 4 will be referred to as calpain small 1 (CAPNS1) in accordance with the nomenclature reported at the calpain website (http://ag.arizona.edu/calpains). A homologue to CAPNS1, CAPNS2, has been described previously (Schad et al., 2002). This protein appears to be a functional equivalent to CAPNS1 in vitro; however, its in vivo function and tissue distribution are still controversial. Interestingly, ubiquitous calpains are associated with the endoplasmic reticulum and Golgi, a likely reservoir for autophagosome membranes (Hood et al., 2004), where the essential autophagy regulatory complex PI3K–Beclin also localizes (Kihara et al., 2001).

Calpain is required for normal embryonic development. Indeed, the targeted disruption of CAPNS1 is embryonic lethal at days 10 and 11 as a result of severe defects in vascular development (Arthur et al., 2000). Calpain is a regulator of adhesive complex dynamics in adherent cells (Bhatt et al., 2002); it plays an important role in osteoclastic bone resorption (Hayashi et al., 2005) and is required for phagocytosis in human neutrophils (Dewitt and Hallett, 2002).

We have become interested in calpain function because of our observation that the product of GAS2, a gene specifically induced at growth arrest, is in fact an inhibitor of millicalpain and that its overexpression sensitizes cells to apoptosis in a p53-dependent manner (Benetti et al., 2001). More recently, we have shown that calpain is also involved in NF-κB activation and in its relative prosurvival function in response to ceramide, in which calpain deficiency strengthens the proapoptotic effect of ceramide (Demarchi et al., 2005). In this study, we further explore the involvement of calpain in the apoptotic switch and find that in calpain-deficient cells, autophagy is impaired with a resulting dramatic increase in apoptotic cell death.

Results

The lack of calpain activity is coupled to a block in autophagy

We have previously reported that calpain plays a crucial role in cell death regulation in response to ceramide. Indeed, we demonstrated that the lack of calpain activity results in a considerable

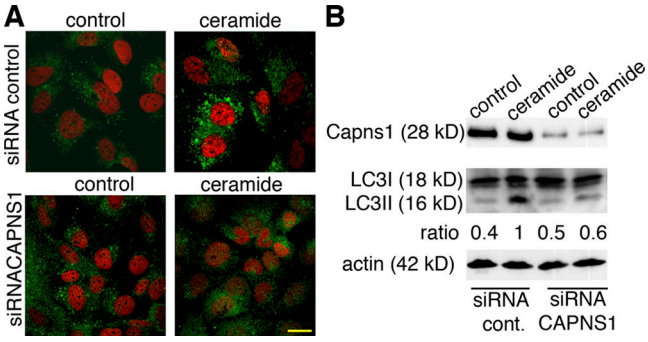

Figure 2. **CAPNS1 depletion is coupled to a block in autophagosome formation in human cells.** (A and B) U2OS cells were silenced with control siRNA or CAPNS1 siRNA and, 72 h later, were stimulated with ceramide for 3 h to trigger macroautophagy, as indicated in the figure. Afterward, the cells were fixed and decorated with anti-LC3 and PI (A) or were lysed to perform Western analysis against endogenous LC3 (B). Actin was used as a loading control; anti-CAPNS1 antibody was used to check silencing efficiency. (B) The ratio between the intensities of LC3II and LC3I bands is indicated below each lane. Bar, 20 μm.

increase in apoptotic cell death (Demarchi et al., 2005). To further investigate the involvement of calpain in the modulation of alternative types of cell death, in this study, we address the role of calpain in autophagy. LC3 is the only available specific marker to detect autophagosomes in mammalian cells (Kabeya et al., 2000).

To directly tackle the question of whether calpain is required in autophagy, we analyzed endogenous LC3 in response to ceramide and rapamycin by means of immunofluorescence in wild-type and CAPNS1$^{-/-}$ mouse embryonic fibroblasts (MEFs). CAPNS1$^{-/-}$ MEFs, which are also known as calpain 4$^{-/-}$ MEFs, were derived from CAPNS1 (calpain 4) knockout mice and were shown to lack calpain activity (Arthur et al., 2000). Wild-type and CAPNS1$^{-/-}$ MEFs were induced with each stimulus for 3 h or were left untreated. Afterward, the cells were fixed and subjected to immunofluorescence analysis with an anti-LC3 antibody. As shown in Fig. 1 A (top), wild-type MEFs are faintly labeled by anti-LC3 antibody and show a clear induction of autophagosome formation after rapamycin or ceramide treatment. On the other hand, CAPNS1$^{-/-}$ MEFs show a higher diffuse background that does not substantially vary after stimulation with rapamycin or ceramide (Fig. 1 A, bottom).

In a parallel experiment, rapamycin and ceramide were used to stimulate wild-type and CAPNS1$^{-/-}$ MEFs, and the lysates were analyzed by Western blotting to detect both cytoplasmic LC3-I and its proteolytic derivative LC3-II that preferentially associates with autophagosomal membranes (Kabeya et al., 2004). In agreement with the immunofluorescence data, both rapamycin and ceramide trigger an increase of LC3II with respect to LC3I in wild-type cells, whereas the ratio between the two LC3 forms is not considerably altered by both stimuli in CAPNS1$^{-/-}$ MEFs (Fig. 1 B).

The fate of endogenous LC3 upon autophagy induction was also addressed in human U2OS cells. U2OS cells were transfected with a scrambled siRNA (siRNA control) or with a CAPNS1-specific siRNA. 72 h later, autophagy was induced by a 3-h incubation with ceramide, and the cells were either fixed and analyzed by immunofluorescence or lysed and subjected to Western blot analysis. As shown in Fig. 2 A, CAPNS1 depletion prevents the formation of bona-fide autophagosomes upon induction with ceramide. Accordingly, the increase in the endogenous membrane-bound LC3II after ceramide treatment is clearly defective after CAPNS1 silencing (Fig. 2 B).

Calpain-deficient cells show a defect in lysosomal activation and long-lived protein degradation

To further assess the requirement of calpain for the autophagic process, we followed the increase of lysosomal activity occurring

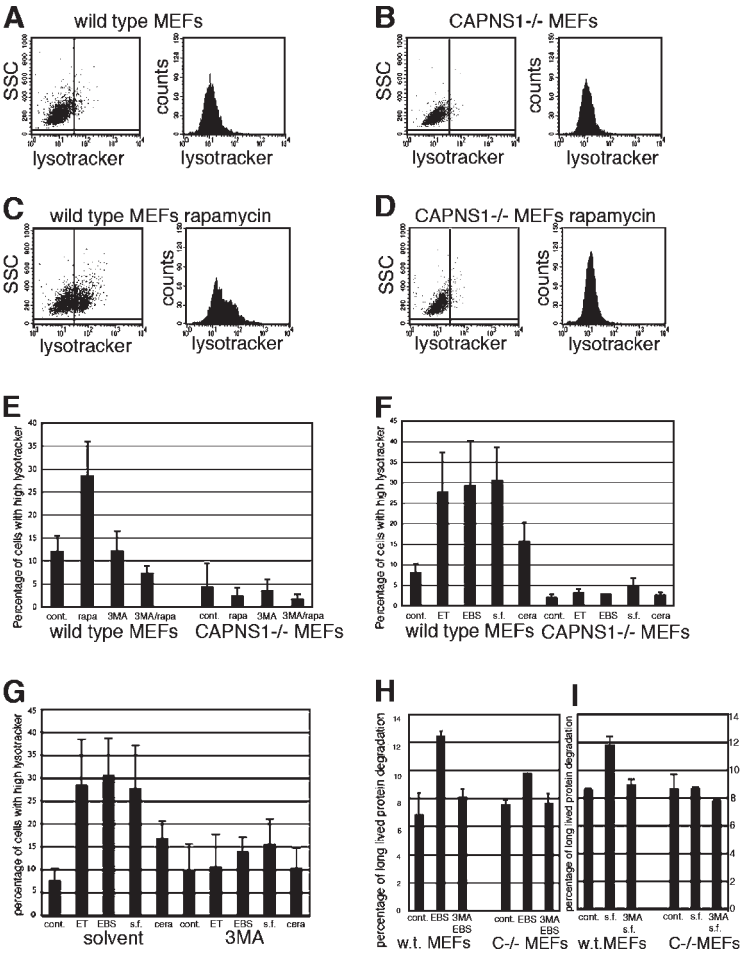

Figure 3. **Lysosomal induction and long-lived protein degradation are hampered in CAPNS1-deficient MEFs.** Wild-type and CAPNS1 MEFs were incubated with the stimuli indicated, namely rapamycin (rapa), etoposide (ET), EBSS (EBS), serum-free medium (sf), and ceramide (cera), in the presence or absence of 3MA and were trypsinized, stained with LysoTracker green, and analyzed by FACS. (A–D) Representative FACS profiles obtained after LysoTracker staining of wild-type and CAPNS1$^{-/-}$ MEFs before and after rapamycin treatment. The gate set between 10^1 and 10^2 separates the cells with high fluorescence from the background population. (E–G) The histograms report the percentage of cells with high LysoTracker staining and represent the mean values obtained in four independent experiments. (H and I) Wild-type and CAPNS1$^{-/-}$ MEFs were grown for 4 h in amino acid–free medium (H) or in serum-free medium (I) in the presence or absence of the autophagy inhibitor 3MA, and, subsequently, long-lived protein degradation was scored as the percentage of TCA-soluble counts on total radioactivity in a standard protein degradation assay. The histograms report the mean values obtained in four independent experiments. Error bars represent SD. SSC, side scatter.

after autophagy induction by means of LysoTracker staining and FACS analysis. Wild-type and CAPNS1$^{-/-}$ MEFs were incubated with rapamycin for 24 h, trypsinized, stained with LysoTracker green, and analyzed by FACS. The results obtained in a typical experiment are reported in Fig. 3 (A–D). A net increase in the percentage of cells that are highly labeled with LysoTracker occurs after rapamycin treatment of wild-type MEFs (Fig. 3 C) as compared with control untreated cells (Fig. 3 A), clearly showing that rapamycin results in an increase in lysosomal activity. On the contrary, in CAPNS1$^{-/-}$ MEFs, rapamycin is ineffective in inducing an increase in LysoTracker fluorescence intensity (Fig. 3, compare B with D). The mean values of the percentage of cells highly labeled by LysoTracker obtained in four independent experiments are reported in Fig. 3 E. To determine whether the increase in lysosomal activity was dependent on macroautophagy induction, 3-methyladenine (3MA) was added just before stimulation with rapamycin. 3MA clearly inhibits the increase in LysoTracker staining, indicating the specificity of the induction (Fig. 3 E). Next, other autophagic stimuli, namely etoposide, starvation in Earle's balanced salt solution (EBSS) or serum-free medium, and ceramide, were used to induce autophagy in wild-type and CAPNS1$^{-/-}$ MEFs using the same type of assay. As shown in Fig. 3 F, all four stimuli induce autophagy-dependent lysosomal activation after 3 h in wild-type MEFs but not in CAPNS1$^{-/-}$ MEFs; moreover, the increase in LysoTracker labeling is dramatically reduced upon the addition of 3MA just before induction (Fig. 3 G).

To further investigate the requirement of calpain in autophagy, we monitored long-lived protein degradation upon amino acid starvation both in wild-type and CAPNS1$^{-/-}$ MEFs according to standard protocols (Pattingre et al., 2003). Amino acid starvation induces an increase in bulk protein degradation in both cell lines. However, such an increase is considerably lower in calpain-deficient cells as compared with wild-type cells (Fig. 3 H). A reduction in protein degradation occurs in the presence of the inhibitor 3MA in both cell lines, indicating that although considerably reduced, autophagy may still occur in CAPNS1$^{-/-}$ MEFs as a consequence of amino acid starvation. Alternatively, this result might be caused by an effect of 3MA on alternative degradation pathways, as a similar effect is also observed in autophagy-defective *ATG5*$^{-/-}$ cells (Mizushima et al., 2001). Similar results were obtained by studying long-lived protein degradation upon serum starvation (Fig. 3 I), confirming the importance of calpain in activating protein degradation through the autophagic process.

Calpain is required for autophagosome formation in response to rapamycin

Electron microscopy was used to directly analyze and quantify autophagosomes in wild-type and CAPNS1$^{-/-}$ MEFs grown in control serum-containing medium and after autophagy induction with rapamycin or amino acid–free medium (EBSS). The morphology of early autophagic vacuole (AV [AVi]) and late AV (AVd) profiles of wild-type MEFs are shown in Fig. 4 (A and B, respectively). The quantitative results obtained are represented in Fig. 4 C.

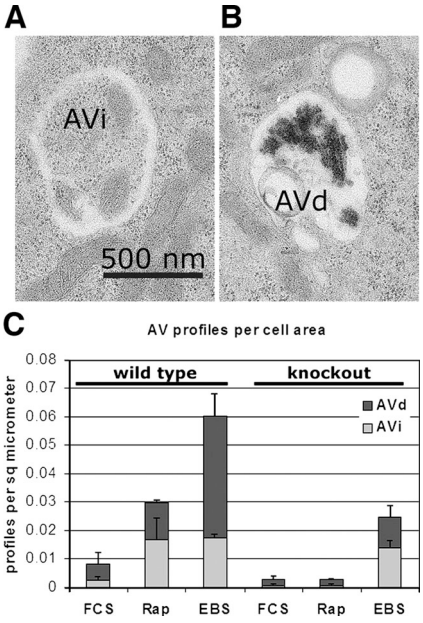

Figure 4. **Autophagosome formation is hampered in CAPNS1$^{-/-}$ MEFs.** (A and B) The electron micrographs show the ultrastructure of early (A) and late autophagosomes (B) in wild-type MEFs. The cell area on the grid squares was estimated by point counting using negatives taken at 600×. (C) Quantification of autophagosomes in wild-type and CAPNS1$^{-/-}$ MEFs (knockout) grown in control serum-containing medium (FCS) or induced for 3 h with EBSS (EBS) or rapamycin (Rap). Error bars indicate the SEM. The number of AVi and AVd profiles was counted under the microscope at 12,000× from four to five grid squares for each sample.

These electron microscopy experiments demonstrate that in CAPNS1$^{-/-}$ MEFs, autophagosome formation is completely abolished in response to rapamycin. In the case of EBSS-induced autophagy, CAPNS1$^{-/-}$ MEFs do show autophagosome formation but at a substantially reduced level compared with wild-type MEFs. In this last case, as shown in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200601024/DC1), the morphology of the autophagosomes is not altered in CAPNS1$^{-/-}$ MEFs with respect to wild-type MEFs. This led us to conclude that the mechanics of autophagosome formation are still functional in CAPNS1$^{-/-}$ MEFs, however severely impaired in their efficiency. The diminished accumulation of autophagosomes in CAPNS1$^{-/-}$ MEFs maintained in EBSS medium may therefore suggest that the dramatic reprogramming of cell functions occurring upon amino acid deprivation may also involve the activation of some autophagic pathways not strictly requiring calpain function. Alternatively, because EBSS possibly represents a stronger and broader stimulus in respect to rapamycin, residual calpain activity caused by some other calpain isoforms may be sufficient to trigger autophagy, albeit at a reduced level.

This evidence prompted us to monitor Tor activity in both cell lines before and after rapamycin treatment using S6 kinase phosphorylation as readout. The results shown in Fig. S3 (available at http://www.jcb.org/cgi/content/full/jcb.200601024/DC1) clearly indicate that Tor is active in both cell lines, where it is inhibited with similar efficiency upon rapamycin addition.

Further studies are required to define the key regulatory elements targeted by calpain for the efficient induction of autophagosome formation.

Ectopic LC3 constitutively forms discrete bodies in calpain-deficient MEFs

A widely used method to study autophagy is monitoring the redistribution of the overexpressed autophagosome marker LC3 from a mostly diffused localization to a punctuated pattern upon the addition of an autophagic stimulus. Therefore, in parallel to the studies on endogenous LC3, we monitored autophagosome formation after overexpressed GFP-LC3 localization in wild-type and CAPNS1$^{-/-}$ MEFs. 16 h after transfection, the cells were preincubated with the macroautophagy inhibitor 3MA or solvent alone, and ceramide was added for 3 h to induce autophagy (Scarlatti et al., 2004). The cells were subsequently fixed to analyze overexpressed LC3 by fluorescence microscopy. As shown in Fig. 5 A, in wild-type MEFs, LC3 produces a mostly diffuse staining in the absence of stimulus and after 3MA treatment. After induction with ceramide, LC3 accumulates in the autophagosomes, whose formation is prevented by 3MA pretreatment, indicating the specificity of autophagy induction. However, in CAPNS1$^{-/-}$ MEFs, LC3 accumulates in specific bodies even under basal conditions; incubation with the autophagy inhibitor 3MA in this case does not prevent the formation of LC3 spots. In addition, the number of cells with intensely stained bodies does not substantially increase after the addition of ceramide (Fig. 5 B). Each experiment was repeated at least four times, obtaining reproducible results. For each experiment, the percentage of cells with LC3 bodies was counted, and the mean value ± SD is reported in the figure. At least 200 cells were analyzed for each independent experiment.

To further monitor autophagy induction, wild-type and CAPNS1$^{-/-}$ MEFs overexpressing GFP-LC3 were challenged with several stimuli that were previously used to induce autophagy, namely rapamycin (Noda and Ohsumi, 1998), amino acid–free medium (Neely et al., 1977), serum starvation (Pfeifer, 1973), and etoposide (Shimizu et al., 2004). All of the treatments triggered autophagosome formation in wild-type MEFs (Fig. 5 C) while leaving unchanged the LC3 staining pattern in CAPNS1$^{-/-}$ MEFs (not depicted). Altogether, these results further suggest that autophagy is impaired in cells lacking calpain activity, where overexpressed LC3 constitutively accumulates into specific bodies.

Calpain silencing is coupled to the accumulation of overexpressed LC3 in human cells

To verify whether the constitutive accumulation of ectopic LC3 into the observed specific bodies in CAPNS1$^{-/-}$ MEFs was a peculiarity of the cell line used or a consequence of calpain inactivation, we monitored the effect of calpain silencing by siRNA on autophagosome formation in the human osteosarcoma cell line U2OS. An *ATG5*-specific siRNA that has been previously described (Boya et al., 2005) was used as a tool to prevent autophagy, allowing the distinction between true autophagosomes

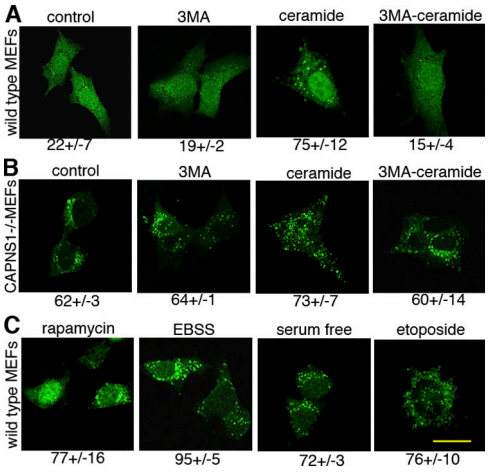

Figure 5. **Ectopic LC3 constitutively accumulates in vesicles in CAPNS1$^{-/-}$ MEFs.** (A–C) Wild-type (A and C) and CAPNS1$^{-/-}$ MEFs (B) were transfected with GFP-rLC3, and, 16 h later, the cells were treated for 3 h with the stimuli indicated. Afterward, the cells were fixed and analyzed by fluorescence microscopy. Representative fields are shown. The different patterns of GFP-LC3 distribution were scored in 250 transfected cells for at least three independent experiments. The mean of the percentage of cells with intensely stained bodies and relative SD are indicated beneath each image. Bar, 20 μm.

and other undefined structures containing ectopic LC3. U2OS cells were transfected with a scrambled siRNA (siRNA control) or with a CAPNS1-specific siRNA in combination with *ATG5* siRNA or an ineffective siRNA as a control.

48 h later, the cells were transfected with GFP-LC3, and, after an additional 16 h, autophagy was induced by a 3-h incubation with ceramide. The fixed cells were finally analyzed by fluorescence microscopy. As shown in Fig. 6 A, LC3 appears diffuse in control U2OS cells and, after induction with ceramide, becomes associated with autophagosomes, which are not formed by specifically inhibiting autophagy through *ATG5* siRNA. On the other hand, CAPNS1-silenced U2OS cells show LC3-positive bodies in the absence of stimulation, and the distribution of GFP-LC3 is not altered by *ATG5* silencing. In addition, the LC3 staining pattern is not considerably affected by ceramide (Fig. 6 B).

To mark and analyze the LC3 pattern specifically in the cells where CAPNS1 is silenced, we developed an siCAPNS1-GFP p-superior plasmid (siCAPNS1-GFP) allowing the simultaneous expression of CAPNS1 siRNA and GFP. In parallel, we produced a U2OS-derivative cell line expressing HcRed-LC3 (U2OS-LC3) as a tool to study autophagy induction. U2OS-LC3 cells were transfected with a control vector (sicontrol-GFP) or with siCAPNS1-GFP plasmid; after 48 h, they were stimulated or left unstimulated with ceramide for 3 h and were fixed and analyzed by fluorescence microscopy.

As shown in Fig. 6 C, in the cells transfected with the control plasmid, HcRed-LC3 appears diffuse before induction and forms autophagosomes after ceramide treatment as expected. However, siCAPNS1-GFP–transfected cells show a punctuated LC3 pattern both in the presence and absence of ceramide stimulation. Collectively, the data obtained in U2OS cells are in agreement with the data obtained comparing wild-type

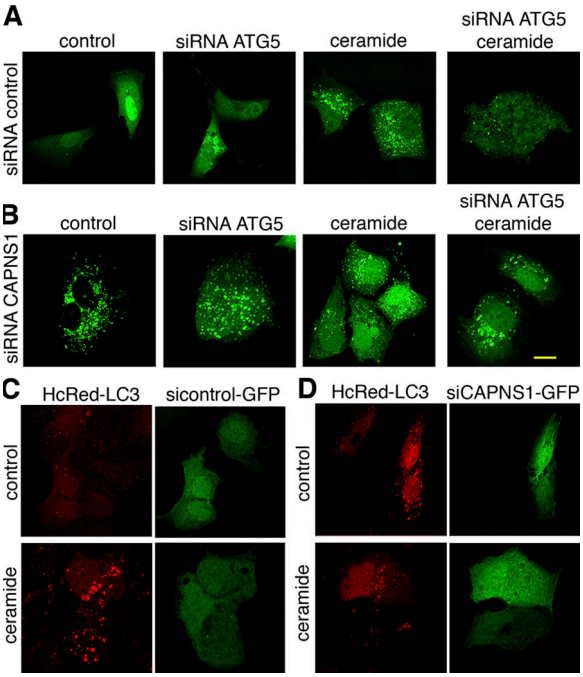

Figure 6. **Ectopic LC3 forms specific bodies in CAPNS1-depleted human cells.** (A and B) U2OS cells were silenced with a control siRNA (A) or with a CAPNS1 siRNA (B) in combination with a control siRNA or *ATG5* siRNA as indicated. After 48 h, the cells were transfected with GFP-hLC3 and, 16 h later, were incubated for 3 h with ceramide or solvent alone as a negative control. They were subsequently fixed and analyzed by fluorescence microscopy. Representative fields are shown. (C and D) A U2OS-derivative cell line stably expressing HcRed-hLC3 (U2OS-LC3) was established. U2OS-LC3 cells were transfected with si-control-GFP (C) or siCAPNS1-GFP (D) expression vectors and, 48 h later, were either treated with ceramide or with solvent alone for 3 h as indicated. Finally, the cells were fixed and analyzed by fluorescence microscopy. The different patterns of GFP-LC3 distribution were scored in 250 transfected cells for at least three independent experiments. Bar, 20 μm.

with CAPNS1$^{-/-}$ MEFs, demonstrating that in CAPNS1-depleted cells, overexpressed LC3 accumulates in specific bodies independently of autophagy induction.

Ectopic LC3 bodies constitutively present in CAPNS1-depleted cells are enriched in endosome markers

To investigate the identity of LC3-containing structures in CAPNS1-depleted cells, we analyzed whether overexpressed LC3 would eventually colocalize with specific endosomal markers both in control and in CAPNS1-silenced U2OS-LC3 cells. 48 h after silencing with control or CAPNS1 siRNA, autophagy was induced by ceramide together with pepstatin to inhibit lysosomal activity and freeze any eventual transient colocalization event. Thereafter, the cells were fixed and subjected to immunofluorescence against the markers LAMP-2, EEA1, and transferrin receptor and were analyzed by a confocal microscope. Representative images are shown in Fig. 7. The colocalization of LC3 with the endosomal markers was quantified using ImageJ software (colocalizer plug-in). The Pearson correlation coefficient (R) is reported near each merged image. Altogether, the data indicate that overexpressed LC3 bodies are enriched in endosome markers specifically in calpain-depleted cells.

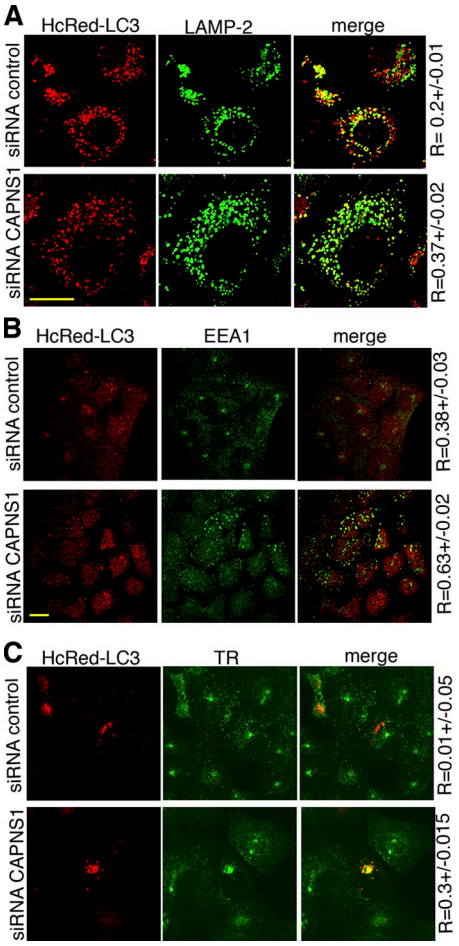

Figure 7. **Ectopic LC3 bodies are enriched with endosome markers in CAPNS1-depleted cells.** (A–C) U2OS-LC3 cells were silenced with a control siRNA or a CAPNS1 siRNA as indicated and, 48 h later, were stimulated for 3 h with ceramide to induce macroautophagy. Pepstatin A was added to inhibit lysosomal activity and freeze any colocalization event between LC3 vesicles and lysosomes. Subsequently, the cells were fixed, decorated with anti–LAMP-2 (A), anti-EEA1 (B), and antitransferrin receptor (TR; C), and analyzed by confocal microscopy. Images of representative fields were taken. The Pearson's correlation coefficient, R, is reported for each merge image. Bars, 20 μm.

To precisely define the identity of the LC3-positive bodies that accumulate in calpain-deficient cells, immunoelectron microcopy studies were performed. U2OS cells were transfected with GFP-LC3 and siRNA specific for CAPNS1 as described in the first section of Results and were fixed in PFA. Double labeling with GFP and LAMP-2 antibodies allowed us to detect GFP-LC3 bodies and also mark endosomes in the same experiment. As expected, GFP-LC3 partly accumulates in specific bodies, as shown in Fig. 8 (A and B). The gold particles indicating the presence of GFP-LC3 bodies (Fig. 8, large gold dots) were associated with endosome-like structures, some of which were also positive for LAMP-2 (Fig. 8; small gold dots are indicated by arrowheads). The morphology and LAMP-2 labeling pattern of GFP-LC3 structures corresponded to that of early endosome-like vesicles, clearly confirming that GFP-LC3 bodies accumulating in CAPNS1-depleted cells are not autophagosomes.

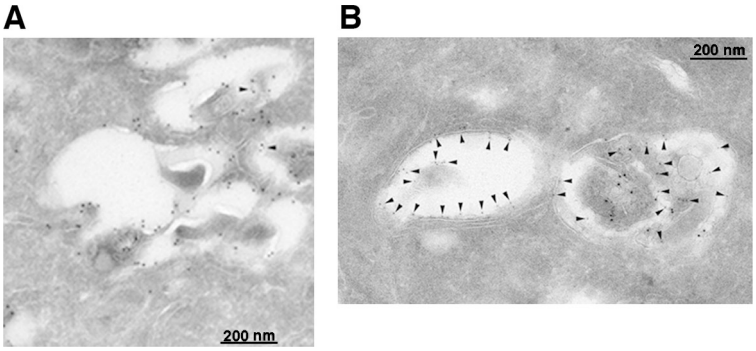

Figure 8. **GFP-LC3 bodies in CAPNS1-depleted cells are endosome-like structures.** Representative immunoelectron microscopy microphotographs showing the ultrastructure of GFP-LC3 bodies in CAPNS1-depleted U2OS cells. Large gold dots mark GFP-positive structures, whereas small gold dots (indicated by arrowheads) are labeled with anti–LAMP-2.

The calpain-dependent impairment of autophagy sensitizes cells to apoptosis

We have previously reported that ceramide triggers apoptosis and induces an NF-κB–dependent prosurvival pathway through calpain. Accordingly, in calpain-deficient cells, apoptosis is enhanced (Demarchi et al., 2005). In this study, we have demonstrated that calpain is required to induce autophagy in response to ceramide, etoposide, and amino acid or serum starvation. Recent studies indicate the existence of a negative cross-regulation between autophagy and apoptosis (Yu et al., 2004; Boya et al., 2005; Takacs-Vellai et al., 2005). However, there are examples of a mutual requirement between the two pathways (Camougrand et al., 2003; Martin and Baehrecke, 2004). Therefore, we decided to define the effect of the calpain-related autophagy block on apoptosis induction in the cellular systems used for this study.

Wild-type and CAPNS1$^{-/-}$ MEFs were incubated with the autophagy inducers etoposide, ceramide, serum-free medium, EBSS, or vinblastine for 20 h. Afterward, the cells were double labeled with annexin V and propidium iodide (PI) and were analyzed by FACS. The results obtained in a typical experiment of EBSS induction are reported in Fig. 9 (A–D). The histogram presented in Fig. 9 E summarizes the results of four independent experiments for each specific stimulus. A dramatic increase in apoptosis can be seen in calpain-deficient cells after induction with all of the stimuli, with the exception of vinblastine. Vinblastine is a microtubule-depolymerizing agent, and, although it can trigger an autophagic response, it has inhibitory functions on late autophagic events (Punnonen and Reunanen, 1990). Interestingly, we have previously reported that the toxicity of taxol, an indirect inhibitor of autophagy by virtue of its block of microtubule depolymerization, is almost comparable in wild-type and CAPNS1$^{-/-}$ MEFS (Demarchi et al., 2005). Collectively, the aforementioned data highlight the cytoprotective potential of autophagy in MEFs and strongly argue for a strict correlation between the impairment of autophagy in calpain-deficient cells and induction of the apoptotic switch.

To further confirm this hypothesis, a similar approach was followed using human U2OS cells silenced either with *ATG5*, CAPNS1-specific siRNA, or a combination of the two. 48 h after silencing, the cells were shifted to amino acid–free medium and incubated for a further 20 h to trigger autophagy. Cell death was then quantified by means of PI and annexin staining followed by FACS analysis. Fig. 10 (A–F) presents the results of a representative experiment and indicates that both *ATG5* and CAPNS1 silencing sensitize U2OS cells to apoptosis induced by amino acid starvation. The mean values obtained in four independent experiments are reported in Fig. 10 G. The effect of CAPNS1 silencing is the most severe, suggesting that CAPNS1 depletion in addition to the block of autophagy also prevents other antiapoptotic mechanisms such as NF-κB activation (Demarchi et al., 2005).

Discussion

In this study, we demonstrate that calpain regulates macroautophagy in two cellular systems lacking calpain activity: MEFs derived from CAPNS1 knockout mice and CAPNS1-silenced

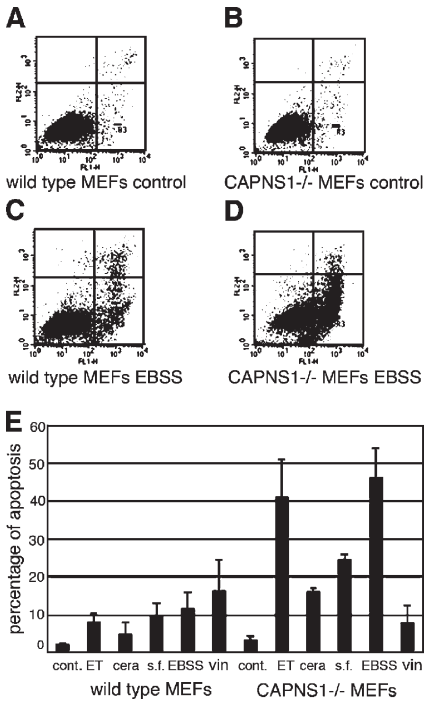

Figure 9. **CAPNS1$^{-/-}$ MEFs are more sensitive to apoptosis.** (A–D) Wild-type and CAPNS1$^{-/-}$ MEFs were treated with etoposide (ET), ceramide (cera), EBSS, serum-free medium (sf), and vinblastine (vin) or left in control medium (cont) for 20 h and stained with PI and annexin V–FITC. A set of representative experiments with EBSS is shown. The cells in the bottom right quadrant were scored as apoptotic. (E) The mean values obtained in four independent experiments with all of the stimuli. Error bars represent SD.

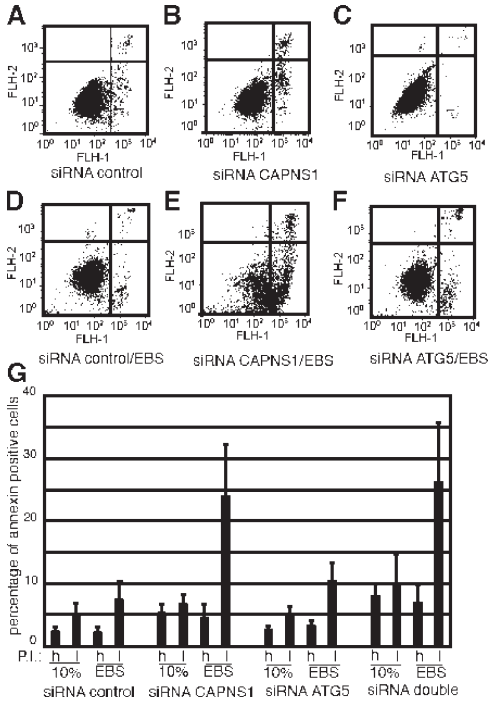

Figure 10. **Depletion of CAPNS1 results in increased apoptosis in human cells.** U2OS cells were transfected with a siRNA specific for CAPNS1, *ATG5*, or a combination of the two. An ineffective siRNA was used as a negative control. 48 h later, the cells were shifted to amino acid–free medium for an additional 20 h, stained with PI and annexin-FITC, and analyzed by FACS. (A–F) Results of a set of representative experiments. (G) The mean of the values obtained in four independent experiments. Bars indicate the percentage of annexin-positive cells with high (h) PI staining (top right quadrant in A–F) and low (l) PI staining (bottom right quadrant in A–F). Error bars represent SEM.

human cells. In addition, we shed some light on the relationship between apoptosis and autophagy.

Calpains are activated by several stimuli that trigger macroautophagy, including starvation (Gomez-Vicente et al., 2005), ceramide (Tanabe et al., 1998; Demarchi et al., 2005), etoposide (Varghese et al., 2001), and arsenic trioxide (Karlsson et al., 2004). Milli- and microcalpain, which both require CAPNS1 for activity, are localized at the endoplasmic reticulum and Golgi, where the autophagic machinery works (Kihara et al., 2001; Pattingre et al., 2005).

In both calpain-null systems used in this study, deficient autophagy was shown by four different approaches: analysis of exogenous and endogenous LC3 by immunofluorescence and Western blotting, quantification of lysosomal induction, long-lived protein degradation assays, and electron microscopy. Altogether, the data presented indicate that in CAPNS1-deficient cells, the autophagic program is not efficiently activated. As a result of this defect, CAPNS1-deficient cells are more sensitive to apoptosis induced by several autophagic stimuli, including ceramide, etoposide, and starvation.

In light of our findings connecting calpain and autophagy, it will be interesting to investigate this relationship in vivo under physiological and pathological conditions as well as in response to chemotherapy. Previously reported data lend support to such a possibility. The dual role of autophagy has been seen during cancer progression, where, at the initial stages, autophagy prevents tumor growth (Liang et al., 1999), whereas at advanced stages, it might favor tumor cell survival (Paglin et al., 2001). An example of this is calpain 9, which appears to be a tumor suppressor of gastric cancer, whereas calpain activation has been shown to be involved in cell transformation and invasion (Carragher and Frame, 2002; Xu and Deng, 2004).

Most interestingly, this parallel is maintained in other degenerative pathologies. Autophagy is protective during the initial stages of neurodegeneration (Webb et al., 2003; Ravikumar et al., 2004), whereas it becomes deleterious at later stages (Yue et al., 2002). A similar dual role could be played by calpain in neuronal diseases, where it may be protective at the initial stages of neurodegeneration (Kim et al., 2003) and hyperactivated and detrimental at advanced stages (Park and Ferreira, 2005). A detailed investigation of the molecular cross talk between these two systems in living organisms might be instrumental for performing appropriate strategies of molecular intervention.

The precise molecular network linking autophagy, apoptosis, and other types of cell death is still unresolved (Assuncao Guimaraes and Linden, 2004; Kroemer and Jaattela, 2005). Autophagy is protective against low radiation damage (Paglin et al., 2001) and against mutant huntingtin-induced cell death (Bjorkoy et al., 2005); it is essential for maintaining cell survival after growth factor withdrawal in $Bax^{-/-}Bak^{-/-}$ cells (Lum et al., 2005). Furthermore, the inhibition of macroautophagy triggers apoptosis (Boya et al., 2005; Gonzalez-Polo et al., 2005). On the other hand, autophagy is coupled to cell death in $Bax^{-/-}Bak^{-/-}$ MEFs (Shimizu et al., 2004) and in tamoxifen-treated MCF7 cells (Bursch et al., 2000). In addition, autophagy and apoptosis are positively interconnected in several systems, including *Drosophila* development (Baehrecke, 2003), rat retinal tissue development (Guimaraes et al., 2003), and peripheral nerves of adult rats after damage (Xue et al., 1999). Collectively, our data further support the argument that macroautophagy is a survival strategy activated both in response to serum and amino acid deprivation. In addition, we have shown that macroautophagy is activated and also plays a protective role upon treatment with specific drugs, such as etoposide and ceramide; thus, the knockdown of autophagy could be a strategy for improving chemotherapy protocols.

Ectopic LC3 is a powerful tool to study autophagy (Kabeya et al., 2000). In this study, we confirmed that in MEFs and U2OS human osteosarcoma cells, the formation of LC3 autophagosomes is efficiently inhibited by treatment with the autophagy inhibitor 3MA and by siRNA-mediated depletion of the essential autophagy gene *ATG5*, respectively. However, in $CAPNS1^{-/-}$ MEFs and siCAPNS1-depleted cells, we observed that ectopic LC3 constitutively accumulates in the absence of any specific autophagic stimulus into cytoplasmic endosome-like bodies enriched with endosomal markers. Therefore, we hypothesize that such LC3 bodies could represent a default or salvage lysosomal pathway for protein degradation with slower clearance kinetics, which becomes manifest in a calpain-deficient cellular context. A similar pathway may be predicted for the endogenous proteins that accumulate in diseases coupled to autophagy defects.

Further studies are required to analyze the detailed mechanisms involving calpains in the formation of autophagosomes and in lysosomal-mediated protein degradation pathways. Given the strategic localization of milli- and microcalpain at the endoplasmic reticulum, it would be tempting to speculate that calpain may play a role in the delivery of membranes to the autophagosome. Alternatively, calpain could modulate one or more key components of the signaling networks, ultimately leading to autophagosome formation. The complete block of autophagosome formation occurring in rapamycin-treated CAPNS1$^{-/-}$ MEFs favors such a possibility.

Materials and methods

Chemicals and reagents
C2 ceramide, bafilomycin, pepstatin A, 3MA, rapamycin, and etoposide were purchased from Sigma-Aldrich. LipofectAMINE reagent and Oligofectamine were purchased from Invitrogen. SMARTpool for CAPNS1 silencing was obtained from Dharmacon. Micro- and millicalpain siRNAs were purchased from Santa Cruz Biotechnology, Inc.

Plasmids and constructs
HcRed-hLC3 and pGFP-hLC3 were constructed by inserting human LC3 in HcRed and pGFP plasmid, respectively. The plasmid pGFP-rLC3 was a gift from T. Yoshimori (National Institute of Genetics, Shizuoka, Japan). The plasmid siCapns1-GFP was obtained by subcloning a double-stranded oligonucleotide containing a sequence complementary to CAPNS1 siRNA into commercial p-superior–GFP plasmid according to the manufacturer's instructions. All constructs were checked by sequence analysis.

Cell culture
Wild-type and CAPNS1$^{-/-}$ MEFs (Dourdin et al., 2001) were gifts from P.A. Greer (Queen's University, Kingston, Ontario, Canada). U2OS cells and the aforementioned mouse fibroblasts were grown in DME supplemented with 10% FCS.

Immunological procedures and quantifications
Standard protocols for immunoblotting and immunofluorescence were used. Rabbit serum raised against LC3 was provided by T. Yoshimori. Monoclonal antibody against LAMP-2 was purchased from BD Biosciences. LAMP-1–specific antibody was purchased from Santa Cruz Biotechnology, Inc., and monoclonal anti-CAPNS1 was purchased from Sigma-Aldrich. Monoclonal antitransferrin receptor (OKT9) was previously described (Sutherland et al., 1981). Band quantification from Western blots was performed using the Image 1.63.sea program (Scion). Secondary antibodies for immunofluorescence were FITC conjugated, and the cells were mounted using Mowiol medium. Analysis and acquisition were performed using a confocal laser-scanning microscope (Axiovert 100 M; Carl Zeiss MicroImaging, Inc.) with a 63× NA 1.25 or 100× NA 1.30 oil objective (Leica) at room temperature. Images were imported using LSM-510 software (Carl Zeiss MicroImaging, Inc.). ImageJ software (National Institutes of Health) was used to quantify the colocalization observed by immunofluorescence.

Transfection
Stable transfections of U2OS cells with HcRed-LC3 were performed by the calcium phosphate method using standard procedures. U2OS, CAPNS1$^{-/-}$, and control mouse fibroblasts at 60–80% confluency were transiently transfected or oligofected using FuGene (Roche) or Oligofectamine (Life Technologies) according to the manufacturer's instructions.

Statistical analysis
Results are expressed as means ± SD of at least three independent experiments performed in triplicate or quadruplicate unless indicated otherwise. Statistical analysis was performed using a *t* test, with the level of significance set at $P < 0.05$.

LysoTracker labeling and quantification
To label lysosomes, LysoTracker green (Invitrogen) was used at a final concentration of 75 nM. The cells induced for 3 or 24 h with the stimuli were trypsinized, resuspended in phenol-free DME, 10% FCS, and 75 nM LysoTracker green, incubated for 30 min at 37°C, and analyzed with the CellQuest program (BD Biosciences) by FacsCalibur (Becton Dickinson).

Protein degradation assay
Cells were labeled in complete medium in the presence of [^{14}C]valine for 18 h, washed, and incubated with cold medium for 1 h to allow the degradation of short-lived proteins. Then, after extensive washing, the medium was replaced with EBSS for 4 h in the presence or absence of 10 mM 3MA. Finally, the proteins were precipitated with TCA, and radioactivity was measured. Protein degradation was calculated as the percentage of TCA-soluble counts on total radioactivity.

Electron microscopy
Monolayers were fixed in 2% glutaraldehyde in 0.2 M Hepes, pH 7.4, for 2 h and were scraped off the dish during fixation. The pellets were embedded in Epon using routine procedures. Approximately 60-nm sections were cut and stained using uranyl acetate and lead citrate and were examined with an electron microscope (JEM 1200EXII; JEOL). The number of AVi and AVd profiles was counted under the microscope by systematically screening the sections at 12,000× using grid squares as sampling units, as previously described (Eskelinen et al., 2004).

All AVs occurring in the grid were recorded. The area of cells on each grid square was estimated by point counting using photographic negatives taken at 600×. Four to five grid squares were screened for each sample. The number of vacuoles per cell area was then counted for each grid square separately.

For immunogold electron microscopy, U2OS cells were fixed in 4% PFA in 0.2 M Hepes, pH 7.4, for 2 h at room temperature and were further processed as previously described (Eskelinen et al., 2002). The cell sections were analyzed by immunogold electron microscopy with rabbit anti-GFP and mouse anti–LAMP-2 antibodies followed by the secondary antibodies goat anti–rabbit conjugated to 10 nm gold and goat anti–mouse coupled to 5 nm gold.

Annexin V and PI staining and FACS analysis
Translocation of phosphatidylserine to the cell surface was monitored by using an annexin V–FITC apoptosis detection kit (Sigma-Aldrich). Cells were trypsinized, washed in PBS, and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Cell density was adjusted to 2–5 × 10^5 cells/ml. 1 μl of recombinant human annexin V–FITC/a and 2 μl PI were added to 100 μl of cell suspension; the mixture was briefly mixed and incubated for 10 min at room temperature in the dark. Afterward, 400 μl of binding buffer was added to the cells that were then analyzed by FACScan (Becton Dickinson). 15,000 events were collected in list mode fashion, stored, and analyzed by CellQuest software (BD Biosciences).

Online supplemental material
Fig. S1 shows that the depletion of microcalpain impairs macroautophagy. Fig. S2 shows that the fine ultrastructure of autophagosomes is not altered in CAPNS1$^{-/-}$ MEFs. Fig. S3 shows that rapamycin inhibits Tor activity in wild-type and CAPNS1$^{-/-}$ MEFs. Fig. S4 shows that LC3II is associated with membranes in CAPNS1$^{-/-}$ MEFs. Fig. S5 shows that the induction of LysoTracker-labeled bodies is reduced in CAPNS1$^{-/-}$ MEFs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200601024/DC1.

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Addendum

Calpain as a Novel Regulator of Autophagosome Formation

Francesca Demarchi[1,*]
Cosetta Bertoli[1]
Tamara Copetti[1]
Eeva-Liisa Eskelinen[2]
Claudio Schneider[1,3,*]

[1]Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie AREA Science Park; Padriciano, Italy

[2]Department of Biological and Environmental Sciences Division of Biochemistry; University of Helsinki; Helsinki, Finland

[3]Dipartimento di Scienze e Tecnologie Biomediche; Universita' degli Studi di Udine; Udine, Italy

*Correspondence to: Francesca Demarchi; Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie AREA Science Park; Padriciano 99, 34012 Trieste, Italy; Tel.: +39.040.398992; Fax +39.040.398990; Email: demarchi@lncib.it/ Claudio Scheider; Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie AREA Science Park; Padriciano 99, 34012 Trieste, Italy; Tel.: +39.040.398992; Fax +39.040.398990; Email: schneide@lncib.it

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KEY WORDS

calpain small 1, autophagosomes, endosome, GFP-LC3 bodies, apoptosis

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Addendum to:

Calpain is Required for Macroautophagy in Mammalian Cells

F. Demarchi, C. Bertoli, T. Copetti, I. Tanida, C. Brancolini, E.-L. Eskelinen and C. Schneider

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ABSTRACT

Ubiquitously expressed μ- and m-calpain proteases consist of 80-kDa catalytic subunits encoded by the *Capn1* and *Capn2* genes, respectively, and a common 28-kDa regulatory subunit encoded by the calpain small 1 (*Capns1*) gene.

The μ- and m-calpain proteases have been implicated in both pro- or anti-apoptotic functions. We have found that *Capns1* depletion is coupled to increased sensitivity to apoptosis triggered by a number of autophagy-inducing stimuli in mammalian cells. Therefore we investigated the involvement of calpains in autophagy using MEFs derived from *Capns1* knockout mice and *Capns1* depleted human cells as model systems.

We found that autophagy is impaired in *Capns1*-deficient cells by immunostaining of the endogenous autophagosome marker LC3 and electron microscopy experiments. Accordingly, the enhancement of lysosomal activity and long-lived proteins degradation, normally occurring upon starvation, are also reduced.

In *Capns1*-depleted cells ectopic LC3 accumulates in early endosome-like vesicles that might represent a salvage pathway for protein degradation when autophagy is defective.

BACKGROUND

Calpains constitute a family of sixteen intracellular nonlysosomal Ca^{2+}-regulated cysteine proteases, mediating regulatory cleavages of specific substrates involved in a number of processes during differentiation, life and death of the cell.[1] Their most studied involvement is in the regulation of the dynamic rearrangements of cytoskeletal architecture during cell migration[2]: depending on the cellular context, a range of stemming effects have been shown to be controlled by calpains such as synaptic function/remodelling in neurons[3] or angiogenesis in endothelial cells.[4]

μ-calpain and m-calpain, encoded by *Capn1* and *Capn2,* respectively, are ubiquitously expressed and require a common 28-kDa regulatory subunit encoded by the *Capns1* gene for stability and activity. Targeted disruption of *Capns1* is embryonic lethal at day 10–11, due to severe defects in vascular development.[5] Both μ-calpain and m-calpain are specifically inhibited by calpastatin, which binds them in a substrate competitive manner[1] and by Gas2,[6] a component of the microfilament network system, specifically induced at growth arrest.[7]

The calpain/calpastatin system associates with the membranes of the endoplasmic reticulum, Golgi apparatus, and plasma membrane, as well as lipid rafts, through hydrophobic interactions. Such specific compartmentalization of the calpain network seems to regulate the access to Ca(2+)-rich microdomains and to membrane-associated phosphoinositides[8] both involved in the activation of calpain enzymatic activity.

We have shown that inhibition of m-calpain throught Gas2 binding increases susceptibility to p53-dependent apoptosis[6] and the free transcriptional competent β-catenin pool.[9] In addition, calpain is required for NF-κB activation in response to ceramide.[10] Interference with calpain activity indeed strengthens the pro-apoptotic effect of ceramide. This finding led us to explore the involvement of calpain in autophagy. We discovered that in calpain-deficient cells autophagy is impaired, thus contributing to the apoptotic switch. The involvement of calpain in necrosis and apoptosis is well established.[1] Very recently Yousefi et al. reported that calpain could cleave the autophagy protein Atg5, determining a switch from autophagy to apoptosis.[11] Altogether these data could indicate that calpain is active at all subsequent stages of the stress response leading to divergent biological outcomes depending on the specific timing, the compartment where the enzyme is activated and the endogenous substrates with respect to the specialized functions of the responding cells.

PLEASE REFER TO FIG. 1 WITHIN THE TEXT

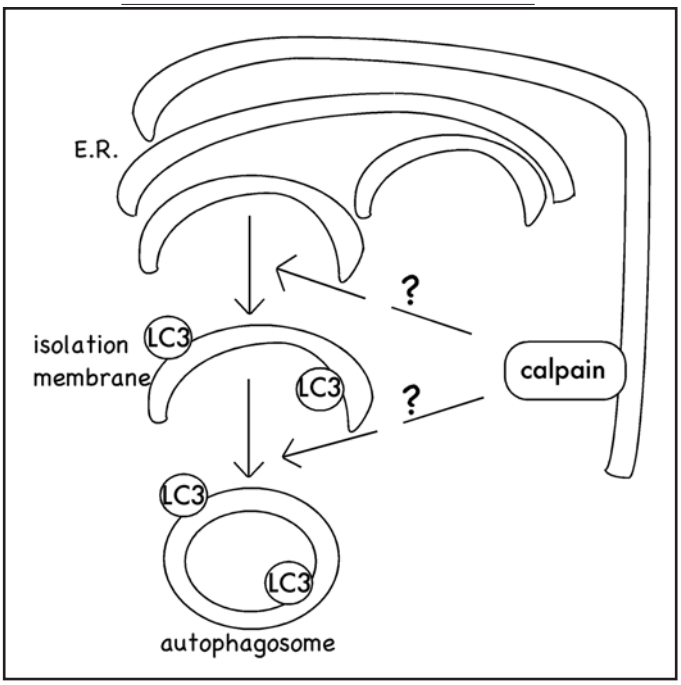

Figure 1. Calpain may act at two possible regulatory steps of autophagosome formation. Calpain resides in the endoplasmic reticulum and can orchestrate membrane delivery to the isolation membrane. Alternatively calpain may be required to remove some block regulating the formation of the autophagosome from the isolation membrane.

IS CALPAIN INVOLVED IN AUTOPHAGY?

To directly tackle the question of the role of calpain in autophagy we compared endogenous LC3 upon autophagy induction in wild type versus *Capns1*$^{-/-}$ MEFs and in human U2OS osteosarcoma cells versus calpain-depleted U2OS.

In both cellular systems autophagic stimuli trigger an increase of membrane-bound LC3II with respect to uncleaved LC3I, whereas the ratio between the two LC3 forms is not significantly altered upon autophagy induction in *Capns1*$^{-/-}$ MEFs and in *Capns1*-depleted U2OS.

The final result of the autophagic process is protein degradation in the lysosome. Therefore we monitored long-lived proteins degradation as well as induction of lysosomal activity upon amino acid starvation in wild type and *Capns1*$^{-/-}$ MEFs. Both readouts allowed us to further underscore the requirement of calpain for efficient autophagy.

Although immunocytochemistry and protein degradation assays are increasingly accepted as reliable tools to investigate autophagy, electron microscopy still remains the gold standard technique. We demonstrated that in *Capns1*$^{-/-}$ MEFs autophagosome formation, as followed by electron microscopy, is completely abolished in response to rapamycin. In the case of EBSS-induced autophagy, *Capns1*$^{-/-}$ MEFs do show autophagosome formation, however, at a significantly reduced level compared to wild type MEFs.

Further studies are required to analyze the detailed mechanisms involved in the specific interference of calpain in the formation of autophagosomes and the consequent effects in lysosomal-mediated protein degradation pathways.

HOW DOES CALPAIN MODULATE AUTOPHAGY?

Interestingly we have found that ectopic GFP-LC3 constitutively forms discrete bodies in calpain-deficient MEFs. Accordingly, *Capns1*-silenced U2OS cells show GFP-LC3 positive bodies in the absence of stimulation, and the distribution of GFP-LC3 is not altered by *Atg5* silencing. Ectopic LC3 bodies constitutively present in *Capns1*$^{-/-}$ MEFs and in *Capns1*-depleted U2OS cells have an endosome-like ultrastructure and are enriched in the endosome marker LAMP1.

Given the strategic localization of μ- and m-calpain at the endoplasmic reticulum, it would be tempting to speculate that calpain might play a role in the delivery of membranes to the phagophore. Interestingly Rim13, the calpain-like protease of yeast, interacts with Vid22, a plasma membrane protein involved in vacuole import and degradation. It also interacts with Snf7, a protein recruited from the cytoplasm to the endosomal membrane, which works in the sorting of transmembrane proteins into the multivesicular body pathway.[12]

Mammalian calpain could modulate one or more key components of the signalling and trafficking networks involved in autophagosome formation. The complete block of autophagosome formation occurring in rapamycin treated *Capns1*$^{-/-}$ MEFs favors such a possibility. In particular calpain could have a broad interfering effect on phosphoinositide signalling, eventually regulating phosphatases localized at specific membrane compartments. Indeed very recent studies demonstrated that calpain deficiency is coupled to alterations in Akt[13] and JNK signalling.[14]

A second, non exclusive, mechanism could be the requirement of calpain in the processing of cytoskeletal connections, possibly organized through the microtubule network, which prevent/block the maturation of the isolation membrane to an autophagosome. A candidate substrate could be spectrin that was shown to recruit the dynactin-dynein complex to membrane phospholipids, thus playing an essential role in the retrograde axonal transport of a number of vesicular cargoes.[15] Interestingly, a recent study showed that microtubules support production of starvation-induced autophagosomes and not their targeting and fusion with lysosomes.[16] The authors of these findings propose that microtubules serve to deliver only mature autophagosomes for degradation providing a spatial barrier between phagophores and lysosomes.

It is well established that calpain is essential for microfilament dynamics at the leading edge of a migrating cell.[2] We hypothesize that it could play a similar role in orchestrating the dynamic changes of the cytoskeleton coupled to autophagosome formation.

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