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CELL BIOLOGY

Retrograde signaling from autophagy modulates stress responses

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Running Title: Retrograde signaling from autophagy

Keywords: autophagy, ATG5, ATG7, ATG9, Beclin 1, p62/SQSTM1, DNA damage response, p53, $p21^{CDKN1A}$, sestrins, Nrf2, Keap1, MiT/TFE, TFEB, NFκB/IKK, FOXO.

Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog 1; AMPK, AMP-dependent protein kinase; ATGs, autophagy-related proteins (human genes *ATG;* mouse genes *Atg)*; ATM, ataxia telangiectasia mutated; Beclin 1, BECN1, Bcl-2 interacting coiled-coil protein 1; Chk1, Chk2, checkpoint protein kinases 1, or 2; DDR, DNA damage response; DRAM, damage-regulated autophagy modulator; FIP200, focal adhesion kinase family-interacting protein of 200 kD; FOXO, forkhead box O; H2AX, histone 2AX; IKK, IκB kinase; Keap1, Kelch-like ECH-associated protein 1; LKB1, Liver B1 kinase; MEF, mouse embryonic fibroblasts; MiT/TFE, microphthalmia/ transcription factor E; mTORC1, mechanistic target of rapamycin complex 1; NFκB, nuclear factor kappa-light chain enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2; PARP1, ADP-ribose polymerase 1; p62, sequestosome 1 protein (gene *SQSTM1*); PI3K/vps34, class 3 phosphatidylinositol-3 kinase or vacuolar protein sorting protein 34; PINK1, PTEN-induced kinase 1; Rheb, *Ras* homolog enriched in brain; ROS, reactive oxygen species; TAK, TGF-β activated kinase; TIGAR, TP53-induced glycolysis and apoptosis regulator; TFEB, transcription factor EB; TSC1/2, tuberous sclerosis complex proteins TSC1 and TSC2; ULK 1/2, unc-51 like autophagy-activating kinases 1 and 2.

Macroautophagy is a general term for a cellular facility for the degradation within lysosomes of cytoplasmic components including whole organelles. As a basal process, it is essential for homeostasis and is functional in most cells all the time, but it is also an option for implementing appropriate responses to stress. The purpose of this review is to draw attention to findings which show that autophagy proteins can act to modulate and amplify the activities of transcription factors involved in stress response such as the p53, FOXO, MiT/TFE, Nrf2 and NFκB/Rel families. Thus, transcription factors not only amplify stress responses and autophagy, but are themselves subject to retrograde regulation by autophagy-related proteins. Physical interactions with autophagy-related proteins, competition for activating intermediates, and "signalphagy", which is the role autophagy plays in the degradation of specific signaling proteins, together provide powerful tools for implementing negative feedback or positive feed forward loops on transcription factors which regulate autophagy. Here we present examples illustrating how this network works together to regulate metabolic and physiologic responses.

Introduction

Macroautophagy (hereafter designated simply autophagy) is a catabolic process that sequesters cytoplasmic elements in double membrane vacuoles for degradation in lysosomes. Targets of autophagy can be particular, specific proteins, unspecific protein aggregates, or whole organelles, e.g. dysfunctional mitochondria. Basal autophagy is constitutive in most cell types (*1-3*) and it is estimated that basal autophagy is responsible for the catabolism of 1 - 1.5% of cellular protein per hour even under nutrient-rich conditions (*4*). However, since cell survival is constantly threatened by variations in both internal and external conditions, coherent autophagy programs must be able to respond appropriately to stress. Stresses, such as starvation, whether for nutrients or for appropriate growth factors, metabolic imbalance, hypoxia, oxidative stress, genotoxic stress, or unfolded-protein stress all impact on autophagy and induce adjustments, initially through post-translational modifications among the autophagy related (ATG) proteins, but extending then to transcription factors, e.g. those of the p53, Forkhead box O (FOXO), Microphthalmia/Transcription Factor E (MiT/TFE), nuclear factor erythroid-derived 2-related factor 2 (Nrf2) and Nuclear Factor kappa-light chain enhancer of activated B cells (NFκB)/ reticuloendotheliosis (Rel) families (*2,5-9*).

The identification of the *ATG* genes, first in yeast, then in mammals, provided the impetus for a molecular understanding of autophagy (*4,10*). Autophagy functions have been conserved in evolution from *Saccharomyces* to man; there are now more than 30 recognized *ATG* genes, some of which bear for historical reasons individual names, e.g. *Beclin 1*, *p62*/*SQSTM1*, *FIP200* (focal adhesion kinase family-interacting protein of 200 kD) whose products are required for autophagy. The canonical autophagic pathways (*10-13*), and also non-canonical pathways resembling or

overlapping with autophagy (*14-16*), have been characterized in excellent reviews; hence, they will not be elaborated here.

Autophagy is a part of a complex network bringing together the regulation of stress responses, metabolic oversight, and cell proliferation. This network includes interactions which connect many familiar, linear pathways (*6-9,11,12*). An integrated picture showing how the separate pathway fragments fit together in a whole network is surely an important goal for future research because there exists an evident clinical potential for targeting events in autophagy, especially for treatment of neurodegenerative and cardiovascular diseases or cancer. Various retrograde interactions between ATG proteins and transcription factors provide already hints about how this network may work and will be the subject of this review.

Stresses Trigger Autophagy

The central role of mTORC1 in the regulation of autophagy

Autophagy is a specific, modulated response after stresses such as nutrient starvation, growth/survival factor deprivation or the hyperproliferative stimulus induced by oncogenes, e.g. activated Ras (*17-20*). An ultimate target in all forms of stress is the multi-protein, mechanistic Target of Rapamycin Complex 1 (mTORC1), a master regulator of cell growth which integrates inputs from at least five pathways: growth factor stimulation, DNA damage, energy charge (ratio of ATP to AMP), sufficiency of amino acids and availability of oxygen (Fig. 1) (*21-23*). MTORC1 is made up of the Target of Rapamycin (TOR), a serine/threonine protein kinase, together with several regulatory subunits and the scaffold protein Raptor. MTORC1, localized in part on the

lysosome, is responsible for phosphorylating numerous targets including regulators of translation such as the ribosomal S6 kinase and the initiation factor eIF4E binding protein, 4E-BP1, to promote protein synthesis, but at the same time it negatively regulates autophagy by direct phosphorylation and inactivation of the unc-51-like kinases 1 and 2 (ULK1/2) and ATG13 of the phagophore initiation complex (*22*). Raptor is responsible for mTORC1 binding to the small GTPase Rag proteins associated with the Ragulator complex, and to Rheb (*Ras* homolog enriched in brain), which is also a small GTPase (*22*). All of these small GTPases are located at the lysosome which thus serves as a critical platform for regulation of mTORC1 activity (*24-26*). MTORC1 is active while tethered to the lysosome in association with the Rag and Rheb small GTPases, but since Rag GTPases bind to mTORC1 based on information about intra-lysosomal concentrations of amino acids communicated by the proton pump channel v-ATPase (*27*), mTORC1 becomes inactivated immediately under conditions of insufficient amino acids (*25,27*). Rag GTPases then release mTORC1, which is displaced from the lysosome and inactivated (*25*).

As with Rag-dependent monitoring of amino acid starvation, other inputs on mTORC1 are regulated by the Rag-dependent recruitment of the Tuberous Sclerosis Complex (TSC) containing TSC1 and TSC2, to the lysosome (*25*). The TSC1/2 complex is a GTPase activating protein (GAP); its association with the Rag GTPases brings it into proximity with Rheb (*25*). The GTP-bound form of Rheb is essential for mTORC1 activity, but is converted by an active TSC1/2 GAP to the GDP-bound form, followed by mTORC1 inactivation (*24,25*). Since however, TSC1/2 is under the subtle and varied control of the growth factor receptor (e.g. insulin) - PI3K - AKT pathway its activity is inhibited by AKT phorphorylation (*22,23*). On the other hand, other signals acting on the AMP-dependent protein kinase (AMPK) such as low oxygen tension, low

energy charge or increased levels of reactive oxygen species (ROS), provide a strong TSC1/2 activation (Fig. 1), thus provoking conversion of Rheb to the GDP-bound form with concomitant mTORC1 inactivation, displacement from the lysosome, and the initiation of autophagy in consequence (*22,25*).

AMPK a complex of 3 proteins and has a central responsibility for regulating energy charge by continuously monitoring the [AMP] / [ATP] ratio. As shown in Fig. 1, AMPK is also able to integrate information from various pathways. Glucose starvation, i.e. a rising [AMP] / [ATP] ratio, is an alarm signal, but also sestrin transactivation by p53 in response to stress activates AMPK (*28,29*), as does its phosphorylation by a p53-dependent activation of Liver B1 kinase (LKB1) (*30,31*) or by oxidized ataxia telangiectasia mutated (ATM) kinase dimers formed in the cytoplasm in the presence of reactive oxygen species (ROS) (*32*).

Autophagic responses are induced by many kinds of stress other than just starvation. However, since almost all stress responses overlap largely with the phenotype seen after starvation; the induction of autophagy is a central feature (*33,34*). This is because in practically all forms of stress a common denominator is the increased formation of ROS, which is released by the mitochondria as a consequence of almost any dysfunction or metabolic deficiency or imbalance, triggering autophagy (*34-36*).

The DNA damage response and p53

Fig. 1 also illustrates the DNA damage response (DDR) and indicates the triggers (red arrows) which are focused on the AMPK complex leading to initiation of autophagy, but potentially also to apoptosis. DDR is a prototype stress response made up of

several tasks: sensing DNA damage, its repair, and making physiological corrections like arresting proliferation and responding to increased metabolic demands with autophagy (*37*). Typical is the response to potentially lethal DNA double strand breaks caused by ionizing radiation and by some chemotherapy drugs. Briefly, such DNA strand breaks lead to the activation of the large (350 kD) serine/threonine protein kinase ATM which serves as master regulator and transducer (Fig. 1) for repair (*37*), but also acts in the stress response. ATM is activated by the formation of the Mre11-Rad50-NbsI (MRN) complex bound to the ends of DNA breaks and is essential for repair (*38*). Moreover, poly ADP-ribose polymerase 1 (PARP1) activation at DNA strand breaks produces chains of poly ADP-ribose (PAR) marking the site of injury. PARP1 activation is believed to be physiologically important owing to a bioenergetic collapse which can follow upon the massive ATP and NAD^+ consumption (*37,39*), and because PAR binds to and inhibits hexokinase (*40*), hence inhibiting the first step in glycolysis. Following PARP1 activation, a sharp rise in the [AMP] / [ATP] ratio and also increased mitochondrial ROS output, lead then to AMPK activation followed by mTORC1 inactivation and autophagy as in starvation.

A broad phosphorylation cascade is initiated by ATM in the nucleus following its activation, monomerization and autophosphorylation at several sites. The ATM kinase phosphorylates numerous targets (Fig. 1), e.g. locally, on the DNA such as on histone 2AX (H2AX), and on checkpoint 2 protein kinase (Chk2), which then, in turn is responsible for a rapid activating phosphorylation of p53 (*41,42*). Phosphorylation of human p53 at ser20 by Chk2 is believed to be important for its transactivation of $p21^{CDKN1A}$ and other stress response targets (*43*). However, concomitant with phosphorylation, p53 tetramerization is essential for its activation (*44,45*).

Noteworthy is the fact that following glucose starvation, human p53 is also subject to phosphorylation and activation from downstream by AMPK (see yellow arrow originating at AMPK; Fig. 1) (*46-48*). The tumor suppressor protein p53 is of particular importance in all forms of stress, partly because it is a transcription factor with broad responsibility for the oversight of cellular metabolism, autophagy and apoptosis (*49-52*) and partly because it has, as will be discussed below, important functions as an effector in the cytoplasm. After stress, transactivation of $p21^{CDKN1A}$ by p53 (Fig. 1) contributes to G_1 and G_2 cell cycle arrest, while other p53 targets, e.g. sestrins, promote autophagy and a conservative, catabolic metabolism by activating AMPK (*28,53*) and the lysosomal damage-regulated autophagy modulator (DRAM-1) protein (*54,55*), potentially contributes to either autophagy or apoptosis. Another p53 transcriptional target, TP53-induced glycolysis and apoptosis regulator (TIGAR), acts counter to autophagy, reducing ROS levels by increasing flux through the pentose phosphate pathway with concomitant NADPH production needed to scavenge ROS with reduced glutathione (*49*). Finally, among the many other genes transactivated by p53 under stress conditions, several, like *Puma, Noxa and Bax*, are pro-apoptotic (*2,35*).

After stress, phosphorylated, activated p53 can also be found in the cytoplasm (Fig. 1) where it acts, not as a transcription factor, but as a positive effector for autophagy, complexing with and activating LKB1 and triggering its phosphorylation of AMPK (*30,31*). Furthermore, under stress, such activated p53 can also take on a direct apoptogenic role in mitochondria (*56-58*).

Interestingly, however, in unstressed conditions, cytoplasmic p53 plays an entirely different role as an inhibitory effector of autophagy (Fig. 1) (*59-61*), at least partly owing to its binding to FIP200, an essential component of the ULK1/2 protein

kinase complex responsible for autophagy initiation (*61,62*). In stress-free conditions cytoplasmic p53 protein has a tonic, inhibitory influence on basal autophagy. Thus, experimental p53 depletion or treatment of unstressed cells with pifithrin-α, a p53 transcriptional inhibitor, causes an increase in basal autophagy (*59,60*). One speaks of a dual role for p53 (*59*).

Autophagy-Related Proteins Act Retrograde on Transcription Factors

ATG7 is an essential accessory protein for p53 transactivation of p21

If one takes the view that transcription factors act upstream of the proteins whose expression they control, then retrograde signaling means that these expressed products can act on and influence transcription factor function. Basal autophagy is believed to be maximal in the G_1/S phase of cell cycle, being inhibited in G_2/M, because "autophagic catabolism and growth associated anabolism are probably mutually exclusive" (*63*). Thus, it was unsurprising that after glucose or amino acid starvation of wild-type mouse embryonic fibroblasts (MEF), Lee et al. found that the fraction of cells entering into S phase declined by 60% within 3 hours (*35*). However, the same experiment performed with *Atg7* knockout MEF cells showed a reduction of just 20%. In fact, these authors found that expression of the cyclin-dependent kinase inhibitor p21 was lacking in *Atg7* knockout MEF cells, although as expected, starvation provoked its expression in wild-type MEF. The levels of $p27^{kip}$, known to be regulated by the FOXO family of transcription factors, however, were comparable in the two cell types. With chromatin immunoprecipitation, Lee et al. showed that in wild-type MEF cells, endogenous Atg7 was present together with p53 on the $p21^{CDKN1A}$ gene promoter

(Fig. 2) (*35*). The same complex could be found in both the cytoplasm and nucleus, and its abundance increased after nutrient starvation as the cells entered growth arrest.

Investigation of other ATG proteins such as Beclin 1 or Atg5 failed to reveal any similar physical association with p53. However, a subset of p53-regulated pro-apoptotic genes, *Puma, Noxa, Bax,* showed after starvation enhanced RNA and protein levels in both *Atg7* knockout and *Atg5* knockout as compared to wild-type cells, suggesting that these ATGs normally have a negative influence on transactivation of these pro-apoptotic genes (*35*). The role of Atg7 in its interaction with p53 seems to be to promote p53 tetramer formation and is distinguishable from the E1 ligase-like enzymatic activity of Atg7 needed for autophagy. Atg7 mutants lacking the C-terminal cysteine active site and, thus, non-functional for conjugation reactions in autophagy, were still able to bind p53 and promote $p21^{CDKN1A}$ transactivation. Taken together, the findings of Lee et al. indicate that Atg7 acts as an accessory protein for p53 in the process of the $p21^{CDKN1A}$ gene transactivation (*35*), much like other known accessory proteins involved with other p53 targets (*64,65*).

Ectopic ATG5 expression initiates a stress response

The goal of most anti-cancer therapy protocols is to induce irreparable DNA damage in tumor cells. Such protocols inevitably produce a DDR with activation of ATM kinase, p53 and a full program of stress responses (*66,67*). Low, sub-lethal doses of the topoisomerase II inhibitor, etoposide, or the DNA cross-linking agent, cisplatin were shown recently to induce strong upregulation of both ATG5 and p53 expression, as well as activation/phosphorylation of the latter (*68*). In these experiments, apoptosis levels remained low, but cell cycle arrest for 48 – 72 hours was observed. A rapid increase in autophagic flux was also documented, but this autophagic response was

secondary to p53 and ATG5 upregulation since the latter occurred even when autophagy was blocked by treatment with the PI3K type 3 inhibitor, 3-methyladenine. Blocking autophagic flux with 3-methyladenine, however, elicited a high level of caspase-dependent apoptotic cell death (*68*), which suggests that autophagy can be a preliminary to apoptosis.

A surprising observation, however, was that simply expressing ATG5 ectopically in cells without drug treatment, also stimulated p53 upregulation, its phosphorylation and activation followed by *p21*^{CDKN1A} transactivation (*68,69*). Thus, ectopically expressed ATG5 induces not just autophagy, but also an authentic stress response (Fig. 2), implying that ATG5 may impact on AMPK, since an upstream effect of activated AMPK on p53 has been documented (*47*). Exactly how this happens is unclear, but a feed-forward loop between p53 transactivation of sestrin, followed by AMPK potentiation and additional p53 activation, is proposed in Fig. 2 as a possible mechanism consistent with known pathways (*46-48*). Ectopically expressed Beclin 1, while able to trigger autophagy in Jurkat cells, failed to induce p53 activation or growth arrest (*68*).

Interestingly, enforced ATG5 expression, besides causing autophagy and p53 upregulation and activation, culminates in mitotic catastrophe (Fig. 2) (*68*). Within 72 hours after ATG5 gene transfer, most cells exhibited evidence of mitotic catastrophe, i.e. were either multi-nucleate or exhibited enlarged, abnormal nuclei. An important insight into the mechanisms responsible emerged from cell fractionation studies using Jurkat cells (*68,70*). Unexpectedly, it was found that ATG5 localizes to a significant degree in the nucleus (Fig. 2). Beclin 1, had been previously reported to possess a homologous leucine-rich region acting as a nuclear export signal (*71*) and a homologous sequence was also detected in ATG5 (*68*). Both Beclin 1 (*72*) and ATG5

(*68*) have been found to physically associate with survivin/BIRC5 (Fig. 2). Since survivin/BIRC5 is an essential component of the chromosome passenger complex, competition between nuclear ATG5 and Aurora B for BIRC5 was shown to have consequences for recruitment of the chromosome passenger complex to centromeres during mitosis (*68,70*). In cells ectopically-expressing ATG5, Aurora B was present at centromeres only in reduced amounts at prometaphase, whereas survivin/BIRC5 was present there in normal amounts, but afterward was retained at the centromeres at anaphase, unlike normal cells, which by that stage exhibited survivin/BIRC5 mainly at the central spindle (*68*). Any such disturbance in cytokinesis must lead to genetically abnormal cells, mitotic catastrophe, polyploidy, aneuploidy, imbalance in gene dosage, and eventually, in some fraction of the cells, to tumorigenesis (*73*).

A feedback regulation by ATG7 on FOXO$_1$

FOXO transcription factors transactivate many genes involved in autophagy which represent survival responses to stress (*ULK1*, *LC3*, *Gabarapl1*, *ATG5*, *ATG8*, *ATG12*, *ATG14*, *Beclin 1* and *Bnip3*) (*74-76*).

Interestingly, an interaction of ATG7 with FOXO$_1$ was reported recently. ATG7 is able to bind to acetylated FOXO$_1$ in the cytoplasm (*77-79*), forming ATG7/FOXO$_1$ complexes which stimulate autophagy (Fig. 3). The accumulation of acetylated FOXO$_1$ in the cytoplasm takes place owing to a prior disassociation of Sirt2 deacetylase from FOXO$_1$ under the influence of oxidative stress. In this role, FOXO$_1$ acts as a cytoplasmic effector, not a transcription factor, since Zhao et al. could show that FOXO$_1$ mutants defective for transcription factor activity retain the ability to bind ATG7 and initiate autophagy (*78*). The induction of autophagy results presumably from the

activation of ATG7 in the ubiquitination-like conjugation reactions leading to formation of ATG5-ATG12 and LC3-phosphatidylethanolamine which are fundamental for the formation and closure of the autophagosome. At the same time, being complexed to ATG7, $FOXO_1$ is thus retained in the cytoplasm transcriptionally inactive (Fig. 3). Hence, this phenomenon represents a kind of negative feedback on $FOXO_1$ acting as a transcription factor.

Furthermore, a tumor suppressive effect of this cytoplasmic $FOXO_1$ function was also demonstrated, being ascribed to ATG7/$FOXO_1$-induced autophagic cell death (*78,80*). Interestingly, Zhu et al. have reported that $FOXO_3$ maintains a transcriptional repression on $FOXO_1$ expression (Fig. 3) (*81*). These authors demonstrated that $FOXO_3$ knockdown is followed by a spontaneous $FOXO_1$-dependent upregulation of autophagy.

P62-dependent selective autophagy creates feedback loops for Nrf2 amplification in stress response pathways

Autophagy is vitally important for the genetic stability of the cell. Early reports by Mathew et al. demonstrated that loss of functional autophagy causes genetic instability owing to accumulation of toxic p62/SQSTM1-target protein aggregates together with dysfunctional mitochondria and attendant elevated ROS production (*82,83*). It must be remembered that p62/SQSTM1 (hereafter designated just p62) is not just an autophagy receptor/adaptor; it is also a substrate that must be removed and degraded with the cargo via autophagy. Enforced p62 over-expression and accumulation induced ROS production, DNA damage, aneuploidy and tumorigenesis (*83*). Furthermore, inhibition of autophagy shows up a fundamental defect in DNA repair:

loss of autophagy leads to reduced levels of Chk1, an essential factor for homologous recombination, and to a corresponding deficit in DNA repair of double strand breaks (*84*). This is explained by the fact that inhibition of autophagy causes elevated proteasomal activity with enhanced Chk1 degradation (*84*).

The idea that autophagy can be selective for particular protein targets is now well-established and is pivotal for explaining some important feedback pathways. Gao et al. ascribed to autophagy an essential role for the disposal of the Dishevelled (Dvl) protein under conditions of nutrient stress and demonstrated an inverse correlation between autophagic activity and levels of cytoplasmic Dvl, hence, also the intensity of Wnt signaling (*85*). The term “signalphagy” was recently coined by Belaid et al. for dedicated, selective autophagy which aims to regulate levels of particular, individual signaling proteins (*86*). These authors demonstrated that autophagy is responsible for the rapid and selective degradation of active RHOA-GTP (*87*). Interestingly, the inactive form of RHOA was subject to proteasomal degradation. The basis for this selective degradation is the specificity of p62 and related autophagy receptor/adaptor proteins for individual, ubiquitinated targets, mediating their association with lipidated LC3 at the developing autophagosome (*87*).

P62 is the most studied of the receptor/adaptor proteins selectively targeted for autophagic degradation. Its gene, *SQSTM1*, is a target for transactivation by the transcription factor Nrf2 (*5*). *SQSTM1* transactivation by Nrf2 is induced as a part of the overall anti-oxidant and detoxification response, seen, for example, under conditions of increasing intracellular ROS concentrations. Interestingly, p62 plays an essential retrograde role in controlling levels of Nrf2 which is vitally important for maintaining the redox balance in the cell (Fig. 4) (*5,88*). The transcription factor Nrf2 is subject to ubiquitination through the binding of kelch-like ECH-associated protein 1

(Keap1), a component of a Cullin-3-type ubiquitin ligase, and is retained in the cytoplasm and targeted for degradation in the 26 S proteasome (Fig. 4). Since p62 serves as a specific receptor for Keap1, however, regulating its elimination by autophagy (Fig. 4) (*88*), this mechanism provides “chronic oxidative stress signaling through a feed-forward loop” (*5*) which, however, is conditional on a functional autophagic flux.

This loop concept leaves us asking how the p62 function is regulated. One way is clearly its transcriptional upregulation as a target of Nrf2-dependent transcription. But what if p62 levels are low, and Nrf2 is entirely bound up by Keap1 and subject to degradation? Ichimura et al. have demonstrated that a specific mTORC1-dependent phosphorylation of p62 on Ser351 causes significantly increased affinity of p62 for Keap1 with concomitant degradation through autophagy (*89*). Furthermore, recent findings have established that sestrin expression also activates p62/Keap1 binding and autophagic degradation (*90*). Thus, p62 activation by phosphorylation and/or by sestrin binding promise effective regulation of Keap1 levels and stabilization of Nrf2 with consequent transcription of its anti-oxidant program as needed (Fig. 4).

MiT/TFE transcription factors require ATG5/ATG9 for regulation of lysosomal/autophagy protein expression in mitophagy

Already from Fig. 1, one can see that the lysosome plays a central role in the regulation of autophagy and metabolism (*24,26*). The lysosome is integrally involved in the initiation of autophagy and, of course, lysosomal degradative capacity must also be regulated according to demand. The transcription factors mainly delegated for this purpose are in the MiT/TFE family, basic helix-loop-helix leucine zipper transcription

factors binding to the E-box core sequence (CANNTG) (*91*). This family includes the frequently studied transcription factor EB (TFEB). Members of this family associate with the Rag GTPase proteins and are phosphorylated by active mTORC1 on the lysosomal outer surface, being subsequently sequestered in the cytosol bound to the chaperone 14-3-3. In response to nutrient starvation; however, inactivation of mTORC1 is rapidly followed by TFEB dephosphorylation, its translocation into the nucleus and transactivation of numerous genes for autophagy and for lysosomal biogenesis (Fig. 5). These include lysosomal acid lipase, the proton pump v-ATPase, p62 and ATG9 (*26,92*).

Just how closely TFEB and other MiT/TFE transcription factors are integrated with the process of autophagy was recently documented (*8*). These investigators demonstrated that for mitophagy, a special form of autophagy modified for the degradation of dysfunctional mitochondria, TFEB translocation into the nucleus required the functions of both ATG5 and ATG9 (Fig. 5). Mitophagy aims to achieve lysosomal degradation of dysfunctional mitochondria, i.e. comparatively large organelles demanding significant upregulation of autophagic and lysosomal capacity. It is a form of selective autophagy and an essential mitochondrial quality control function (*93*). Following loss of the mitochondrial membrane potential or accumulation of misfolded proteins in a mitochondrion, PTEN-induced kinase 1 (PINK1) accumulates on the outer mitochondrial membrane where it recruits from the cytosol the E3 ubiquitin ligase, parkin, concomitantly activating/phosphorylating this latent enzyme to ubiquitinate proteins on the outer mitochondrial membrane, thereby marking the damaged organelle for binding by autophagy receptor/adaptors (Fig. 5). Parkin activation takes place as a two-step event; PINK1 phosphorylates parkin on ser65 in its ubiquitin-like domain, and in parallel, also phosphorylates ubiquitin (*94,95*). Though

autophagy receptors/adaptors such as p62 and optineurin are known to bind the ubiquitin chains on damaged mitochondria, recent results have established that the NDP52 and optineurin, not p62, are the primary receptors/adaptors for PINK1- and parkin-mediated mitophagy, providing the bridge to lipidated LC3 (*96*). Furthermore, these receptors are responsible for the recruitment of ULK1/2, DFCP1 and WIPII (not shown in Fig. 5) to early phagophores forming adjacent to the mitochondria (*96*).

That TFEB activation, translocation and functional transactivation of needed target genes should require parkin, ATG5 and ATG9 illustrates the interdependence between early stages of phagophore construction (*97-99*) and the projected transcriptional needs of an ambitious degradative project like mitophagy (*8*). Noteworthy is the fact that such interdependence is not seen when TFEB activation occurs after nutrient starvation, a stimulus requiring much less autophagic/lysosomal biogenesis. In this case, TFEB activation, nuclear translocation and transcriptional activity are ATG5-, ATG9- and parkin-independent (*8*).

Autophagy and the NFκB/IKK activation are interdependent

NFκB can transactivate many autophagy-relevant genes, including *Beclin 1*. Significantly, NFκB plays a central role in transactivating genes involved in inflammatory responses and the general tendency of NFκB-regulated transcription is pro-autophagic. Several years ago, it became clear that that signaling pathways leading to NFkB activation also overlap with autophagy (*100-102*). The IκB kinase (IKK) brings about the activation of NFκB by committing IκB to proteasomal degradation. For IKK activation, TGF-β activated kinase 1 (TAK1) and its binding proteins, TAB2 and TAB3 are required. However, since TAB2 and TAB3 exist as complexes with the

autophagy protein Beclin 1, TAK1 and Beclin 1 are competitors. Beclin 1 has many other interactions, all of which limit its availability, but upon its release from these complexes, it can initiate autophagy and concomitantly, with the availability of TAB2 and TAB3, the IKK-dependent activation of NFκB can begin (Fig. 6).

Surprisingly, not only does autophagy require the release of Beclin 1 together with IKK activation, but also reciprocally, autophagy must be functional in order for NFκB activation to occur (*103*). Starvation or drugs like the mTORC1 inhibitor rapamycin or the p53 inhibitor pifithrin-α which stimulate autophagy also strongly stimulate activation of IKK, followed by the translocation of NFκB/p65Rel into the nucleus and its activation. In wild-type MEF, the activation of the canonical NFκB pathway must be accompanied by initiation of functional autophagy (*103*); thus, in *Atg5*- or *Atg7*-deficient MEF, the NFκB pathway remains largely inactive (Fig. 6). Here again clearly, there is interdependence for function between autophagy and a transcription factor important for stress response and especially inflammatory response.

Concluding Remarks

Several lines of evidence indicate that autophagy exists as a pathway closely integrated with the stress response network, because, while autophagy is a major functional target of stress responses, it is also influences the transcription factor activity regulating these responses. We can recognize various ways in which individual ATG proteins can modulate and amplify the activities across the entire stress network.

The “connectedness” of ATG proteins in the network is important for understanding the complexity of the choices available to the cell following induction of

autophagy. Beclin 1 plays essential roles in many membrane trafficking pathways (*17,18*), competes with NFκB activation (*102,103*) and serves as a balance point between apoptosis and autophagy. Similarly, ATG5 can be degraded by calpain to produce a pro-apoptotic fragment (*104,105*), but also, importantly, ATG5 upregulation can act to induce p53 expression and activation (*68,69*), setting off a stress response together with the initiation of autophagy. ATG7, besides functioning as an E1-ligase in the conjugation reactions producing ATG12-ATG5 and LC3-PE, serves as an essential accessory protein required for p53-dependent transactivation of $p21^{CDKN1A}$ and growth arrest in DDR (*35*), but also subtly represses p53-dependent *Puma*, *Noxa* and *Bax* transactivation (*35*) and interacts with acetylated $FOXO_1$ to induce autophagy (*78,79*).

In a clinical context, unfortunately, all these examples of integration between autophagy and stress responses underline how difficult it will be to intervene selectively with drugs against the helpful survival facility which autophagy provides to established tumor cells (*106,107*). While the stress response and autophagy are evolutionary programs which help to raise the bar against early oncogenesis, in practice they also assure some degree of chemotherapy resistance in established tumors. To date, no protocol has been devised which could eliminate autophagy without incapacitating stress responses and increasing the long-term oncogenic risks. It is therefore the hope that further investigation in this area will help to achieve this goal in the near future.

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Figure Legends

Fig. 1. Pathways regulating stress response and ultimately mTORC1. Red arrows delineate the pathways which are activated after DNA damage or oxidative stress, leading finally to the initiation of autophagy. At the site of DNA double strand breaks, monomer ATM phosphorylates and activates Chk2, which then phosphorylates p53, leading to its tetramerization and activation. The transactivation of *p21*^{CDKN1A} establishes cell cycle arrest, while other p53 transactivation targets, pro-autophagic proteins like sestrins and DRAM, metabolic effectors such as TIGAR, but also proteins like Puma, Noxa and Bax which potentially induce apoptosis, can all be contribute. P53 plays a complex role, because after DNA damage, activated p53 is also translocated to the cytoplasm where it instigates via LKB1 the phosphorylation/activation of AMPK.

Cells regulate mTORC1 with multiple sensor pathways. Under all stress conditions, AMPK plays a central role because it determines the phosphorylation/activation of TSC1/2 (P), regulating Rheb GTPase and thus its conversion to the inactive, GDP-bound form whiuch causes mTORC1 inactivation and displacement from its lysosomal tethers. This relieves the inhibitory block on autophagy (X). AMPK is able to detect energy charge (see green inhibitory arrow on AMPK leading from glucose and O_2). Growth factor (GF) stimulation is essential for maintaining TSC1/2 in an inactive state, antagonizing the role of AMPK [see GF, growth factor Receptor (R), PI3K and AKT with green inhibitory arrows in the lower left quadrant]. Furthermore, a sufficiency of essential amino acids is required for mTORC1 tethering to the Rag GTPases; as with Rheb inactivation, a lack of amino acids causes mTORC1 displacement and inactivation. Thus, mTORC1 is displacement by any of these sensor pathways causes a fundamental “switch” which triggers the spontaneous initiation of autophagy, but also is felt overall in the cell as shift from anabolic to catabolic metabolism.

ROS accumulation resulting from metabolic imbalance or mitochondrial damage allows oxidized (ox) ATM dimers to phosphorylate and activate AMPK directly with consequent TSC1/2 activation, and Rheb and mTORC1 inactivation. Interestingly, after glucose starvation, AMPK can also send a stress signal upstream, phosphorylating and activating p53 (see yellow arrow), setting off a stress response, and potentially inducing mitochondrial (“Mito”) damage and apoptosis (see yellow dotted arrow).

Under stress-free conditions, cytoplasmic p53 (cyto-p53; upper right quadrant) regulates cell metabolism by modulating the pentose phosphate pathway (PPP; green inhibitory arrow) and by inhibiting autophagy “tonically” by complexing with FIP200 (green inhibitory arrow).

Fig. 2. ATG5 and ATG7 impact on p53 pathways in stress response. ATG7 is an essential accessory protein for the activation of p53-dependent transactivation of *p21*^{CDKN1A} leading to cell cycle arrest (see light blue arrows). ATG7-p53 complexes are to be found in both cytoplasm and nucleus of normal cells. Ectopic ATG7 expression induces autophagy.

Ectopic ATG5 expression induces autophagy, too, but it also stimulates p53 upregulation, activation and transactivation of *p21*^{CDKN1A}. The question mark (?) points to a speculative stimulatory loop which may be responsible for such p53 upregulaion/activation after ectopic ATG5 expression. This proposal is based on the positive feed-forward loop between AMPK, which acts to phosphorylate p53. This is followed by p53-dependent transactivation of sestrin and a resulting potentiation of AMPK activation (see red arrows). Furthermore, ATG5 is found in significant amounts in the nucleus where, if overexpressed, it binds to survivin/BIRC5. There, ATG5, by competing with Aurora B, interferes with the function of the chromosome passenger complex at the centromere, inducing mitotic catastrophe.

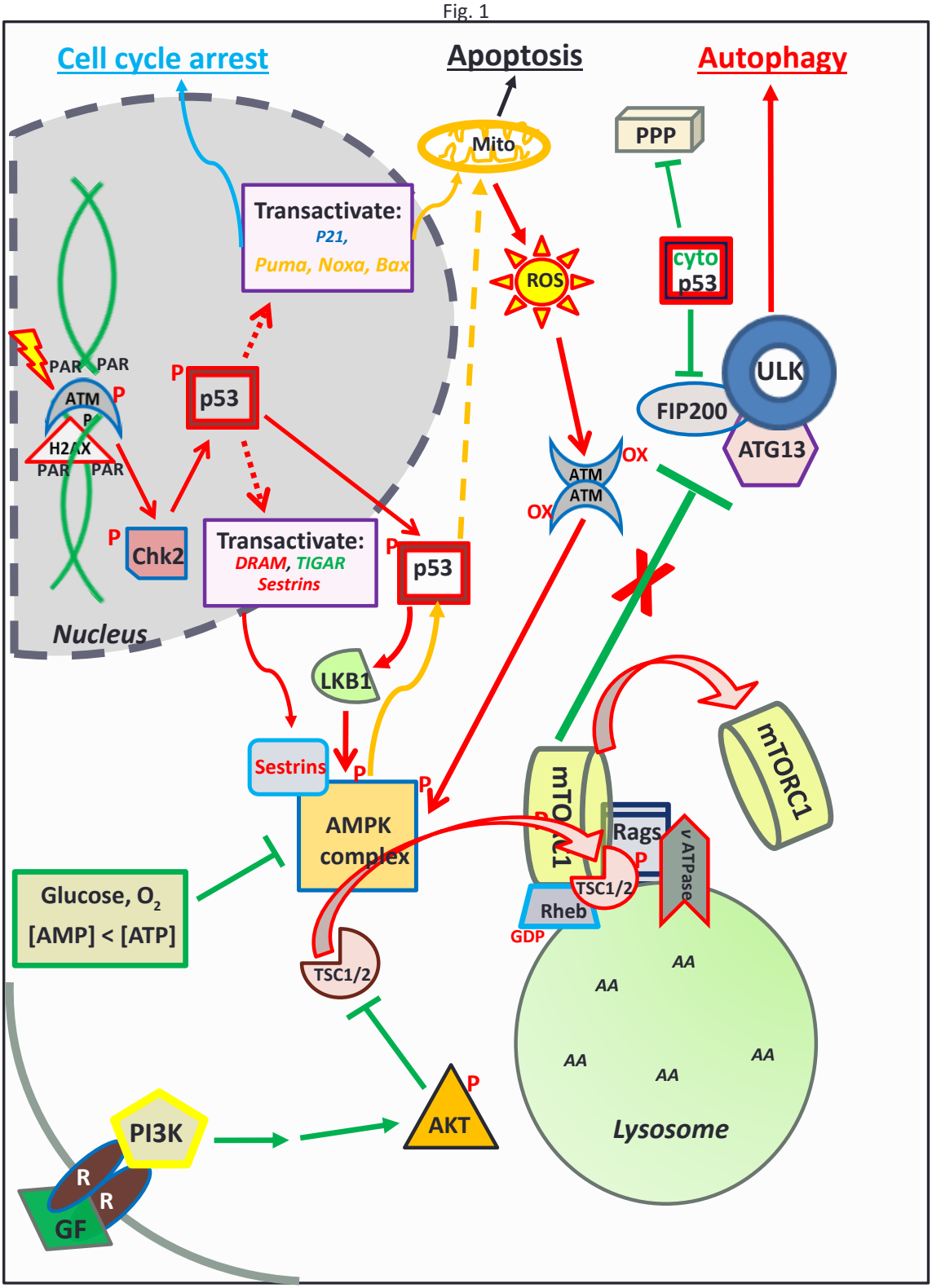
Fig. 3. A feedback regulation by ATG7 on FOXO$_1$. FOXO3 transcription factor activity antagonizes FOXO1 expression in the nucleus; thus negatively regulating autophagy. Following an inactivation of the Sirt2 deacetylase caused by oxidative stress, acetylated cytoplasmic FOXO1 accumulates in the cytoplasm forming a complex with ATG7 which is retained in the cytoplasm. Autophagy is induced by the acetylated FOXO1/ATG7 complex. This mechanism for autophagy induction is believed to suppress tumor xenograft growth in nude mice in an autophagy-dependent manner.

Fig. 4. By regulating Keap1 degradation, p62-dependent selective autophagy creates feedback loops for Nrf2 amplification in stress response pathways. Because *SQSTM1*, the gene for p62, is transactivated by Nrf2, a feed forward loop is established: when ROS levels rise; Nrf2 plays an important role in regulating genes needed for anti-oxidant (Anti-ox) proteins. Keap1 as an ubiquitin ligase which regulates levels of the Nrf2 transcription factor, determining its proteasomal degradation. However, since the autophagic cargo receptor, p62, can complex with Keap1, this complex, together with aggregates (Ag) of other ubiquitinated proteins, are sequestered at the developing phagophore (Ph) and degraded. Interestingly, the affinity of p62 for Keap1 is increased following its activation by sestrin binding (not shown) or following phosphorylation (P) by mTORC1 and other unknown kinases, thus regulating the rate of Keap1 degradation in autophagy.

Fig. 5. MiT/TFE transcription factors require parkin, ATG5 and ATG9 for regulation of autophagic/lysosomal gene expression in mitophagy. Mitophagy represents a massive, selective autophagic degradation requiring increased resources in both autophagic and lysosomal proteins. Their expression depends on the MiT/TFE transcription factors (here exemplified by TFEB). These transcription factors are retained in the cytoplasm with the chaperone 14-3-3 after their phosphorylation by active mTORC1. Following mitochondrial dysfunction with accompanying ROS production, the PINK1 kinase recruits the E3 ubiquitin ligase, parkin, to the outer mitochondrial membrane where it ubiquinates numerous mitochondrial surface proteins. These then can serve as targets for autophagy receptor/adaptors, bridging to the LC3 on the phagophore (Ph). TFEB activation and translocation into the nucleus take place in a parkin, ATG5 and ATG9-dependent manner which allows successful transactivation of numerous lysosomal and autophagy genes. The role of ATG5 and ATG9 in contributing to the formation of the early phagophore is the probable mechanism for their role in TFEB translocation though some evidence suggests that both ATGs also enter into the nucleus.[8] Glucose deprivation also stimulates TFEB activation and translocation, but is ATG5 and ATG9 independent.

Fig. 6. Functions required for NFκB transcription factor activation overlap with the induction of autophagy, because, for NFκB activation, a functional autophagic pathway is required. NFκB is normally retained the cytoplasm, bound to its inhibitor, IκB. NFκB is activated by the IKK complex which displaces the IκB for proteasomal degradation. For IKK function, however, the TGF-β activating kinase 1 (TAK1) is required, together with its co-factors TAB2 and TAB3. Since these two co-factors exist as complexes with Beclin 1, which itself is in an equilibrium with Bcl-2 as well, NFκB activation can only occur in parallel with autophagy as a consequence of shifts in the equilibria of TAB2 and TAB3 with Beclin 1, making them available for IKK activation, but at the same time releasing free Beclin 1 to initiate autophagy.

Fig. 1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Nucleus
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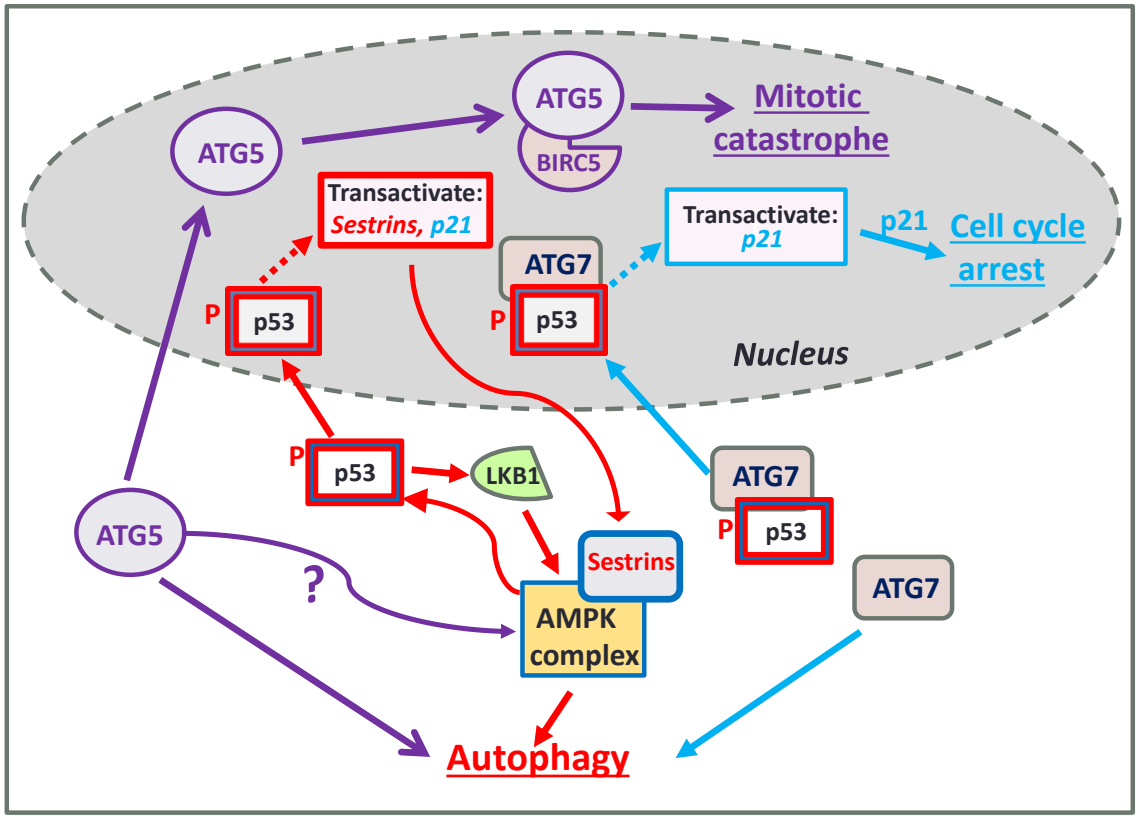
Fig. 2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Cell cycle arrest
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P
p53
Nucleus
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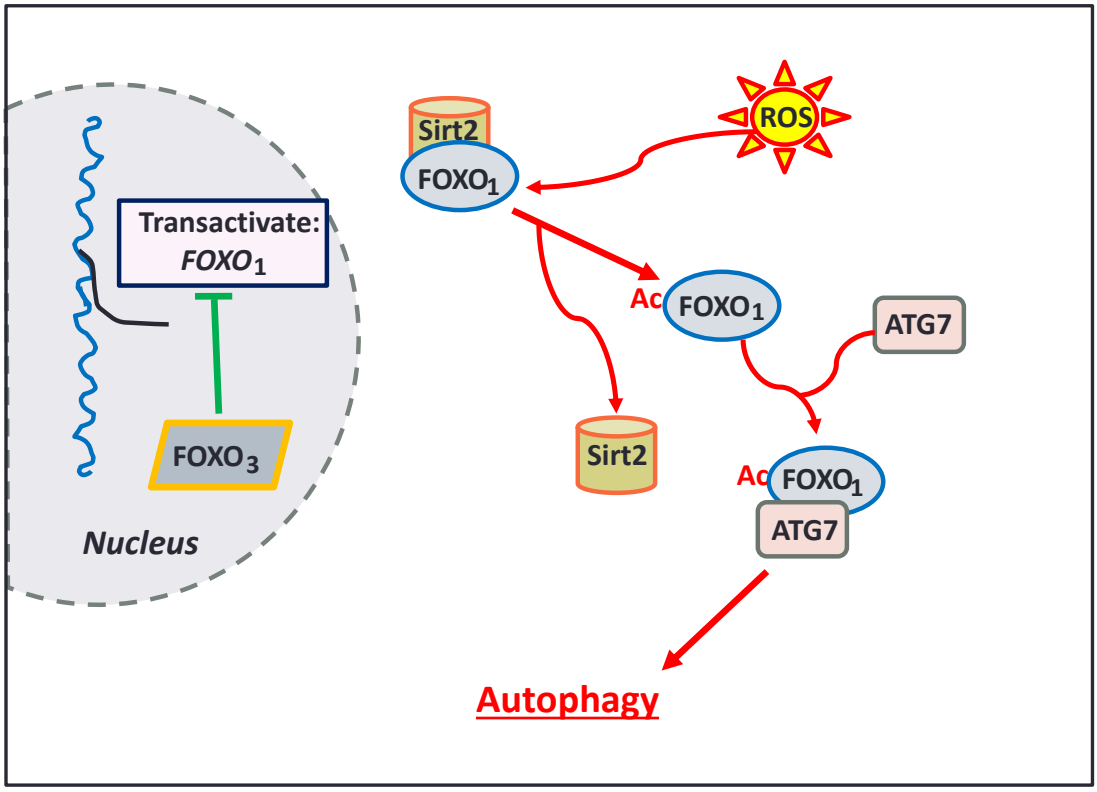
ATG5
?
Sestrins
AMPK complex
ATG7
Autophagy

Fig. 3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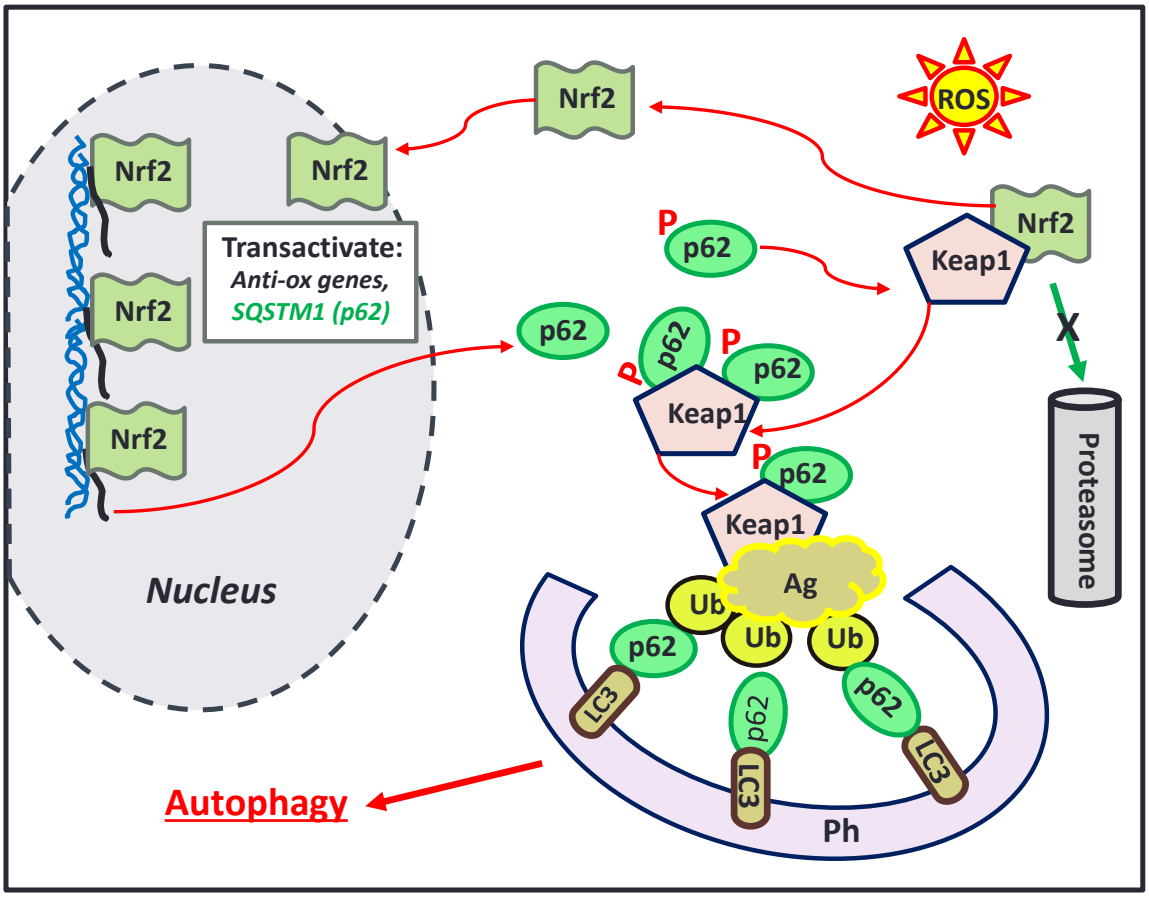
Autophagy

Fig. 4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P
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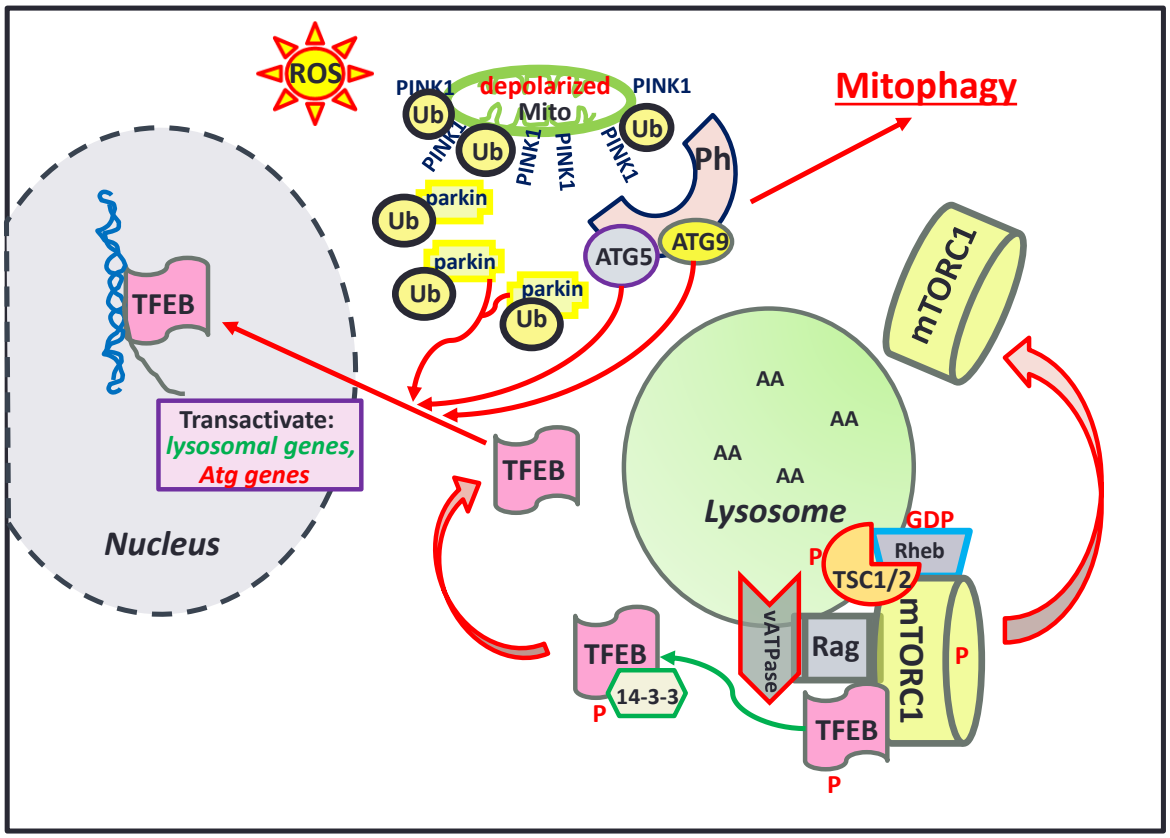
Nucleus
Autophagy
Ph

Fig. 5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Lysosome
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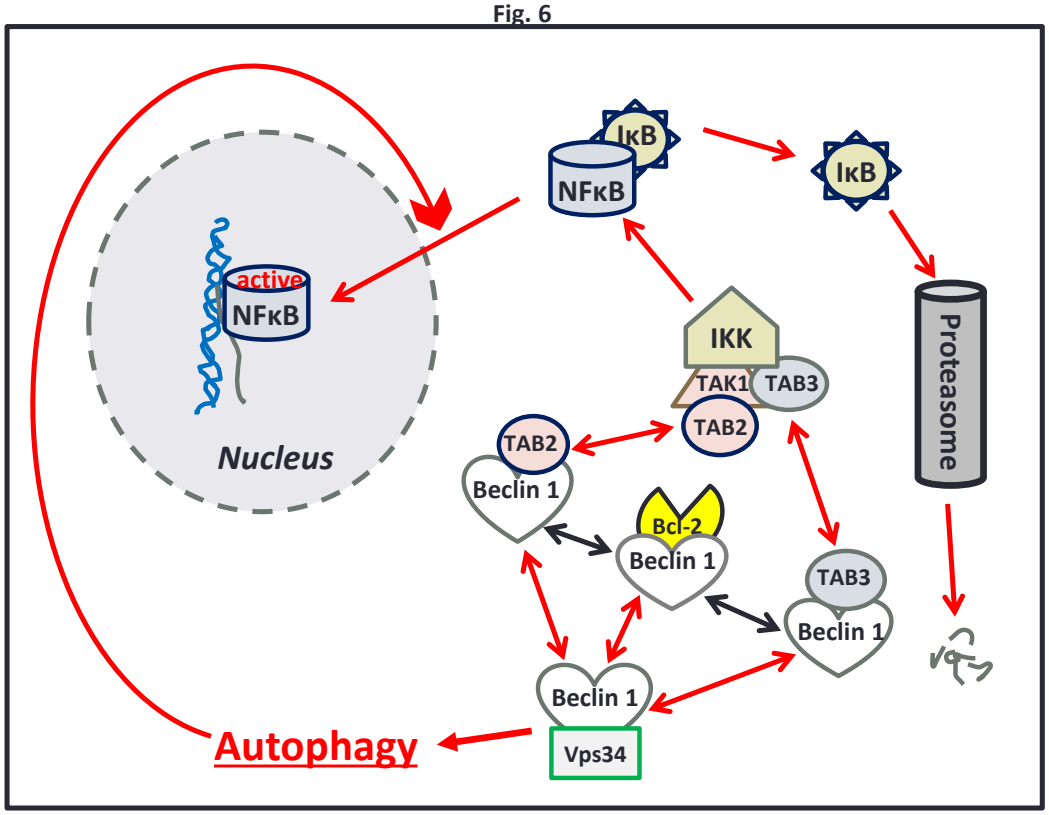
Fig. 6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