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Comparison of the induction and regulation of autophagy in proliferating and senescent cancer cells

Srovnání indukce a regulace autofagocytózy v proliferujících a senescentních nádorových buňkách

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 5.8.2014

Podpis

Na tomto místě bych chtěl poděkovat Dr. Ladislavu Anděrovi za umožnění vypracování a vedení této práce. Dík patří také ostatním členům laboratoře Buněčné signalizace a apoptózy Ústavu molekulární genetiky Akademie věd, zejména pak Janu Švadlenkovi a Zuzaně Naháckéí kteří mě naučili většinu metodik a nesčetnými konzultacemi dále rozvíjeli mé vědecké myšlení. V neposlední řadě děkuji své rodině a přítelkyni za velkou podporu během celého studia.

Content

Abstract

Autophagy, senescence and apoptosis are tightly linked processes which together determine the fate of cells in response to various stresses. There is ample evidence supporting the notion that senescent cells are highly dependent on autophagy and this process is here much more intensive than in nonsenescent cells. Autophagy may to some extent compensate increased energetic and metabolic demands of senescent cells and also helps with removal of toxic products such as oxidized proteins, protein aggregates and damaged organelles resulting from an overloaded metabolism of some senescent cells. In addition, some studies reported the need of autophagy for the adoption of senescent phenotype. However, there are also studies with seemingly contradictory results claiming that increased autophagy prevents or delays cellular senescence. Relationship of autophagy to apoptosis is similarly ambivalent. Whereas intact autophagy is necessary for the cell, while slightly increased autophagy still has a rather positive impact, excessive autophagy may lead to degradation of critical components necessary for cell function and survival and can trigger one of the modes of programmed cell death. In the first part of this work, we focused on the analysis of autophagic response in senescent and proliferating pancreatic cancer cells PANC-1. We observed up to 5-fold lower level of autophagic response, represented here as the number of autophagosomes per cell, in senescent cells. In the second part PANC-1 cells along with the cells of mammary carcinoma T47D were treated with three different inducers of autophagy, EBSS, rapamycin and metformin. We then tested whether the treated cells modulate their sensitivity to selected apoptogens TRAIL, FasL, or MitoVes. Results showed that EBSS-starved PANC-1 cells became significantly more sensitive to these apoptogens than the control cells. Subsequent analysis of surface death receptors revealed increased expression of the TRAIL receptor DR5, likely contributing to their increased sensitivity to TRAIL. The same effect, however, was not completely confirmed in the second cell line, T47D, where the increase in percentage of dead cells was observed only in the induction of cell death by MitoVes, while in the case of Fas ligand, EBSS together with other inducers of autophagy acted rather cytoprotectively.

Abstrakt

Autofagie, senescence a apoptóza jsou velmi těsně propojené děje, které společně určují osud buňky v reakci na rozličné stresy. Existuje množství důkazů podporujících tezi, že senescentní buňky jsou na autofagii vysoce závislé a tento proces v nich probíhá intenzivněji, než v nesenescentních buňkách. Autofagie může do určité míry kompenzovat zvýšené energetické a metabolické nároky senescentních buněk a též pomáhá odstraňovat toxické produkty jako jsou oxidované proteiny, proteinové agregáty a poškozené organely, vznikající jako důsledek přetíženého metabolismu některých senescentních buněk. Navíc některé studie prokázaly nezbytnost autofagie i pro samotné navození senescentního fenotypu. Stejně tak však existují i studie se zdánlivě protichůdnými výsledky, které tvrdí, že zvýšená autofagie naopak brání navození buněčné senescence. Vztah autofagie k apoptóze je podobně dvojaký. Neporušená schopnost autofagie je pro buňku nezbytná, přičemž mírně zvýšená autofagie má na buňku stále spíše pozitivní dopady. V případě velmi silně zvýšené až excesivní autofagie však může dojít ke kritické degradaci komponent důležitých pro další fungování buňky a to může vést až ke spuštění některého z programů buněčné smrti. V první části této práce jsme se zaměřili na porovnání autofagické odpovědi mezi proliferujícími a senescentními buňkami rakoviny slinivky PANC-1. V senescentních buňkách jsme pozorovali až pětinásobně nižší úroveň autofagické odpovědi, vyjádřenou zde počtem autofagosomů na jednu buňku. V druhé části této práce jsme u buněk PANC-1 společně s buňkami prsního karcinomu T47D, iniciovali autofagickou odpověď pomocí tří rozdílných induktorů autofagie, EBSS, rapamycinu a metforminu. Poté jsme testovali, zda takto ošetřené buňky zvýší, či sníží svou citlivost k některému z apoptogenů TRAIL, FasL, či MitoVes. Výsledky ukázaly, že PANC-1 buňky, které byly předinkubovány s EBSS médiem umíraly výrazně více než kontrolní buňky. Následná analýza povrchových receptorů smrti ukázala možný vliv zvýšené exprese receptoru DR5 na zvýšené umírání buněk, zejména v případě TRAILu. Stejný efekt se však nepodařilo zcela potvrdit u druhé buněčné linie, T47D, kde jsme zvýšený počet mrtvých buněk pozorovali pouze při indukci buněčné smrti pomocí MitoVesu, přičemž v případě Fas ligandu EBSS společně s dalšími induktory autofagie působil spíše cytoprotektivně.

List of abbreviations

4E-BP1 - Eukaryotic translation initiation factor 4E-binding protein 1

Alfy - Autophagy-linked FYVE protein

AMBRA1 - Activating molecule in Beclin1-regulated autophagy

AMPK - 5' adenosine monophosphate-activated protein kinase

ASPP2 - N-terminal apoptosis-stimulating of p53 protein 2

ATG - Autophagy related

ATG3 - Autophagy-related protein 3

ATG4A - Autophagy-related protein 4A

ATG4B - Autophagy-related protein 4B

ATG4C - Autophagy-related protein 4C

ATG4D - Autophagy-related protein 4D

ATG5 - Autophagy-related protein 5

ATG7 - Autophagy-related protein 7

ATG8L - Autophagy-related 8-like protein

ATG9 - Autophagy-related protein 9

ATG9A - Autophagy-related protein 9A

ATG9B - Autophagy-related protein 9B

ATG10 - Autophagy-related protein 10

ATG12 - Autophagy-related protein 12

ATG13 - Autophagy-related protein 13

ATG14L/Barkor - Autophagy-related protein 14-like protein /Beclin 1-associated autophagy-related key regulator

ATG16L - Autophagy related 16-like protein

ATG16L1 - Autophagy related 16-1-like protein

ATG16L2 - Autophagy related 16-1-like protein

ATM - Ataxia telangiectasia mutated

Bad - Bcl-2-associated death promoter

Bax - Bcl-2-associated X protein

BAG - Bcl-2-associated athanogene

BAG1 - Bcl-2-associated athanogene 1

BAG3 - Bcl-2-associated athanogene 3

Bcl-2 - B-cell lymphoma 2

Beclin1/BECN1 - Coiled-coil myosin-like BCL2-interacting protein

BH3 - Bcl-2 homology domain 3

Bif-1/SH3GLB1 - Bax-interacting factor 1/SH3 domain-containing GRB2-like protein B1

Bim - Bcl-2-like protein 11

Bnip3 - BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

Bnip3L - BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like

c-FLIP - Cellular FLIP

C12orf44/ATG101 - Chromosome 12 Open Reading Frame 44/Autophagy-Related Protein 101

CCCP - Carbonyl cyanide m-chlorophenylhydrazone

CMA - Chaperone-mediated autophagy

Cpt - Camptothecin

DcR1 - Decoy receptor 1

DcR2 - Decoy receptor 2

DFCP - Double FYVE domain-containing protein

DR4 - Death receptor 4

DR5 - Death receptor 5

DRAM - Damage-regulated autophagy modulator

FADD - FAS-associated death domain protein

FasL - Fas ligand

FBD - FIP200 binding domain

FoxO3 - Forkhead box protein O3

FoxO3A - Forkhead box protein O3

GABARAP - Gamma-aminobutyric acid receptor-associated protein

GAP - GTPase-activating protein

GATE16 - Golgi-associated ATPase enhancer of 16 kDa

GLB1 - Galactosidase beta 1

HDM2 - Human double minute 2 protein

hsc70 - Heat shock-cognate protein of 70KDa

IAP - Inhibitor of apoptosis

iDISC - Intracellular death-inducing signaling complex

IGF-BP3 - Insulin-like growth factor-binding protein 3

IGFBP7 - Insulin-like growth factor-binding protein 37

IL6 - Interleukin 6

IL8 - Interleukin 8

LAMP1 - Lysosome-associated membrane protein type 1

LAMP2A - Lysosome-associated membrane protein type 2A

LC3 - Light chain 3 (the whole name of the protein is microtubule-associated protein light chain 3)

LC3A - Light chain 3A protein

LC3B - Light chain 3B protein

LC3-I - Light chain 3-I

LC3-II - Light chain3-II

LIR - LC3-interacting region

LKB1 - Liver kinase B1

Mcl-1 - Induced myeloid leukemia cell differentiation protein

MitoVES - Mitochondrially targeted vitamin E succinate

MMP3 - Matrix metalloproteinase-3

mTOR - Mammalian target of rapamycin / mechanistic target of rapamycin

mTORC1 - Mammalian target of rapamycin complex1

mTORC2 - Mammalian target of rapamycin complex 2

MAPK - Mitogen-activated protein kinase

N-BAR - N-Bin-amphiphysin-rvs

NBR1 - Neighbor of BRCA1 gene 1

OIS - Oncogene-induced senescence

p150 - Eukaryotic initiation factor 4F subunit p150

p27 - Cyclin-dependent kinase inhibitor p27

p300 - Histone acetyltransferase p300

p38IP - p38-interacting protein

p53 - Tumor suppressor p53

p62/SQSTM1 - Phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa /Sequestosome-1

PAS - preautophagosomal structure

PB1 - Phox and Bem1p

PDK4 - Pyruvate dehydrogenase kinase isoenzyme 4

PI3P - Phosphatidylinositol 3-phosphate

PIK3R4 - Phosphoinositide 3-kinase regulatory subunit 4

PKA - Protein kinase A

PML - Promyelocytic leukemia

PtdIns3K - Phosphatidylinositol 3-kinase

PTEN - Phosphatase and tensin homolog

Puma - p53 upregulated modulator of apoptosis

Rab7 - Ras-related protein Rab-7

Rab9 - Ras-related protein Rab-7

Rag - Ras-related GTP-binding protein

RagA - Ras-related GTP-binding protein A

RagB - Ras-related GTP-binding protein B

RagC - Ras-related GTP-binding protein C

RagB - Ras-related GTP-binding protein D

Raptor - Regulatory-associated protein of mTOR

RAS - Renin-angiotensin system

Rb - Retinoblastoma protein

RB1CC1/FIP200 - RB1-Inducible Coiled-Coil 1 /FAK family kinase-interacting protein of 200 kDa

Rheb - Ras homolog enriched in brain

ROS - Reactive oxygen species

Rubicon - Run domain Beclin-1 interacting and cysteine-rich containing protein

S6K1 - Ribosomal protein S6 kinase beta-1

SASP - Senescence-associated secretory phenotype

SH3 - Src homology 3

SNARE - Soluble NSF attachment protein receptor

STX17 - Syntaxin 17

TAK1 - Transforming growth factor-beta–activated kinase 1

TASCC - TOR-autophagy spatial coupling compartment

TFEB - Transcription factor EB

TGN46 - Trans-Golgi network integral membrane protein 2

TIS - Therapy induced senescence

TP53INP2 - Tumor protein p53-inducible nuclear protein 2

TFEB - Transcription factor EB

TRAIL - TNF-related apoptosis-inducing ligand

TSC1 - Tuberous sclerosis 1 (hamartin)

TSC2 - Tuberous sclerosis 2 (tuberin)

UBA - Ubiquitin-associated domain

Ubl - Ubiquitin-like

ULK - Unc-51-like kinase

ULK1 - Unc-51-like kinase 1

ULK2 - Unc-51-like kinase 2

ULK3 - Unc-51-like kinase 3

UVRAG - UV radiation resistance-associated gene protein

VMP1 - Vacuole membrane protein 1

VPS34/PIK3C3 - Vacuolar protein sorting-associated protein 45 / Phosphatidylinositol 3-kinase catalytic subunit type 3

WIPI1 - WD repeat domain phosphoinositide-interacting protein 1

WIPI2 - WD repeat domain phosphoinositide-interacting protein 2

ZFYVE1/DFCP1 - Zinc finger FYVE domain-containing protein 1 / Double FYVE-containing protein 1

Introduction

Macroautophagy, or just autophagy, is an essential catabolic process by which cells using double-membrane vesicles called autophagosomes sequester portions of cytoplasm or organelles and recycle them in lysosomes. Mostly, it has a major beneficial and cytoprotective effect, as it relieves cells from various adverse conditions such as nutrient and energetic deprivation, hypoxia and cumulation of damaged proteins or organelles. Excessive autophagy may however significantly contribute to cell death, if not directly trigger it.

Diverse stresses, whose most common feature is at the final stage DNA damage may induce a state of very stable cell cycle arrest called senescence. This state is often characterized by morphological reconstitution, changes in expression and secretion of specific set of molecules which is called senescence-associated secretory phenotype (SASP). Senescence is an example of antagonistic pleiotropy, as it functions as anti-tumor barrier in early age, but as the number of senescent cells accumulate in organism with age, the detrimental effects, such as high expression of proinflammatory cytokines, begins to outweigh their initial payoff. Specific removal of senescent cells from organisms is now the subject of great efforts. Several studies suggested that senescent cells are highly dependent on autophagy, because of their increased energetic and nutrient needs. In the first part of this work we addressed this question in greater detail on a cell line derived from pancreatic cancer, by measuring kinetics of autophagy response in proliferating and senescent cancer cells.

In the second part of the work we examined the effect of diversely increased autophagy on sensitivity of two different cancer cell lines to various apoptotic stimuli.

1 Literature review

1.1 Autophagy

Autophagy is a highly conserved catabolic process in which proteins, portions of cytosol and even the whole organelles are, using various mechanisms, delivered into lysosomes for breakdown and recycling of the resulting macromolecules. It relieves cells from various stresses like nutrient deprivation, oxidative stress, cumulation of misfolded or aggregated proteins, damaged organelles, hypoxia and last but not least eliminates intracellular pathogens, thereby helping to maintain cellular homeostasis. The term autophagy was firstly introduced by Christian de Duve during his research of mitochondria degradation within lysosomes in rat liver more than fifty years ago. A special subset of genes necessary for the process called Autophagy-specific genes (ATGs) was discovered in 90s in yeast and since then the amount of knowledge about the topic rapidly expanded. There are three types of autophagy: Chaperone-mediated autophagy (CMA), microautophagy and most importantly predominantly used macroautophagy.

1.1.1 Chaperone-mediated autophagy

The main feature of CMA is that proteins degraded this way are individually selected. The heat shock-cognate protein of 70KDa (hsc70) recognizes a specific amino acid motif (KFERQ) in their amino acid sequence and targets them to surface of lysosome, where it interacts with the cytosolic tail of the single-span membrane protein LAMP2A. Cargo-bound Hsc70-LAMP2 complex then associates with other proteins in a multiprotein complex, which is required for translocation into lysosome. The substrate can bind to the receptor while still in a folded state, but in order to cross the lysosomal membrane, the substrate needs to undergo unfolding, which is a process mediated probably by hsc70 and some other cochaperones. CMA is also induced by starvation, but the temporal kinetics is different compared to macroautophagy (Cuervo and Wong 2014).

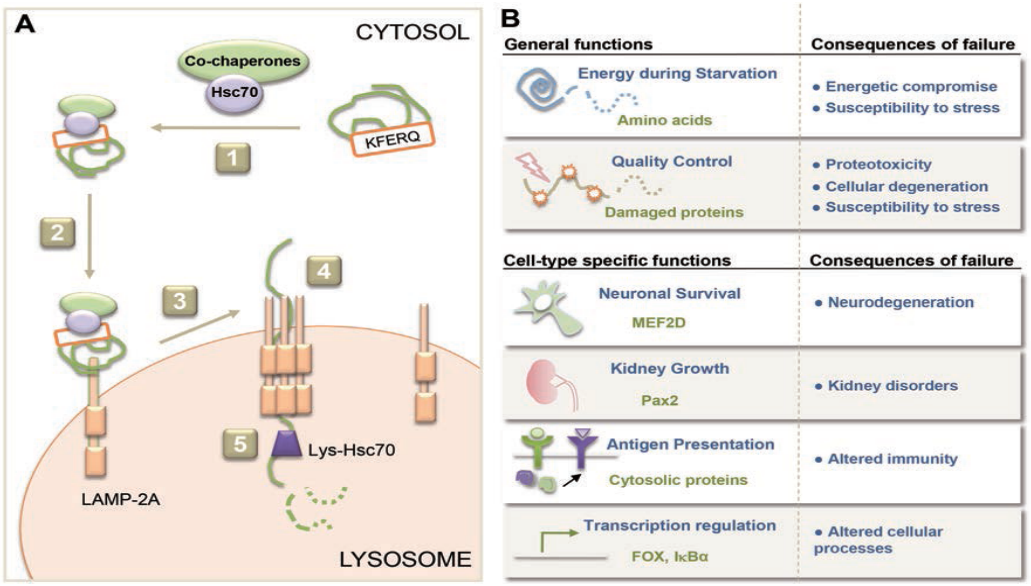


Figure 1. Steps and physiological functions of CMA. **(A)** Proteins degraded by CMA are identified in the cytosol by a chaperone complex that, upon binding to the targeting motif in the substrate protein (1), brings it to the surface of lysosomes (2).Binding of the substrate to the cytosolic tail of the receptor protein LAMP-2A promotes LAMP-2A multimerization to form a translocation complex (3). Upon unfolding, the substrate proteins cross the lysosomal membrane (4) assisted by a luminal chaperone and reach the lysosomal matrix where they undergo complete degradation (5). **(B)** General and cell-type specific functions of CMA and consequences of CMA failure in different organs and systems (Cuervo and Wong 2014).

1.1.2 Microautophagy

Mechanistically, microautophagy stands between two other types. It uses invagination of lysosomal membrane to directly engulf cytoplasmic cargo into lysosome and sequester it . It can be selective or nonselective and it also responds to similar spectrum of autophagy inductors as the other two types. Moreover, microautophagy coordinates its actions with macroautophagy and CMA and complements them (Li et al., 2012).

1.1.3 Macroautophagy

Macroutophagy is by far the most-studied and also a dominant type of autophagy. The data presented in this Thesis are exclusively related to this type of autophagy. In literature, the term "Macroautophagy" is freely interchangeable with "Autophagy" as well as it is further in this work.

The specific trait of this major type of autophagy is the formation of double membrane vesicles called autophagosomes in the cytoplasm and their subsequent fusion

with lysosomes. Very briefly (more detail description follows below), after autophagy-inducing stimulus, there is a strong accumulation of autophagy-associated protein at specific sites in the cytoplasm. An isolation membrane, also called the phagophore, which is an initial stage of autophagosome is being formed in the initial stages of this process. The phagophore expands and engulf portion of cytoplasm (which may also contain organelles). Finally, as already matured double membrane particle called autophagosome, it fuses with lysosome, where its cargo is hydrolyzed along with the inner membrane of autophagosome and produced amino acids, lipids and nucleotides are released into cytoplasm for their reuse. The whole process is relatively rapid. It is estimated that one cycle takes 5-10 minutes from the initiation of phagophore formation to the lysosomal degradation of autophagosome (Mizushima et al., 2001 ; Fujita et al., 2008). The details of this process with its main participants and regulation pathways are discussed in greater detail further in the text.

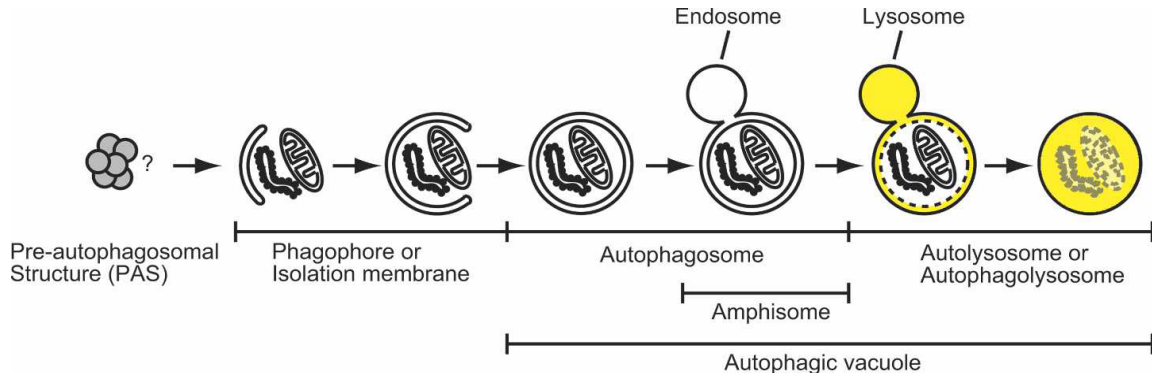


Figure 2. The process of macroautophagy in mammalian cells. A portion of cytoplasm, including organelles, is enclosed by a phagophore or isolation membrane to form an autophagosome. The outer membrane of the autophagosome subsequently fuses with the endosome and then the lysosome, and the internal material is degraded. In yeast, autophagosomes are generated from the PAS, which has not yet been identified in mammalian cells. The nomenclature for various autophagic structures is indicated (Mizushima 2007).

1.2 Origin of autophagosomes

Apparently, there are multiple autophagy initiation sites in mammalian cells. Moreover, it seems that there are also multiple sources from which autophagosomal membrane can be derived. Among possible donors we can find the outer mitochondrial membrane (Hailey et al., 2010), cytoplasmic membrane (Ravikumar et al., 2010) and most often also endoplasmic reticulum membrane (Axe et al., 2008 ; Ylä-Anttila et al., 2009 ; Hayashi-Nishino et al., 2009). There is evidence, that an PI3P-binding protein DFCP (double FYVE domain-containing protein), which is also an early marker of autophagy localizes (unlike others PI3P-binding proteins, which targets to lysosome) to ER and Golgi membranes in an amino acid starvation-dependent manner and colocalizes here with other essential autophagy proteins (Axe et al., 2008). Furthermore, LC3-positive isolation membranes emerged from the DFPC-positive puncta. These were named omegasomes due to their similarity with the letter omega. The process of emerging and connections of this structures with ER membranes were confirmed by 3D tomography (Ylä-Anttila et al., 2009 ; Hayashi-Nishino et al., 2009). Consistently with this, was shown, that proteins of the autophagy core machinery also localize to the ER. (Fan et al., 2011 ; Koyama-Honda et al., 2013).

1.3 The core complexes in autophagy

This term refers to a group of proteins which are almost indispensable for proper autophagosome formation and the progress of autophagy at all. We can divide them into 4 subgroups: proteins of the ULK complex, proteins of the Phosphatidylinositol 3-kinase (PtdIns3K) complex, the transmembrane proteins and the ubiquitin-like conjugation systems. They are in time-dependent hierarchical manner recruited to the isolation membrane.

1.3.1 Ulk complex

Probably the most upstream system of autophagy core machinery is the complex assembled around ULK kinase (Itakura and Mizushima, 2010), which consists of four main proteins :ULK1/2, ATG13, RB1CC1/FIP200 (Ganley et al., 2009 ; Hara et al., 2008)

and C12orf44/ATG101 (Mercer et al., 2009 ; Hosokawa et al., 2009).

ULK1 and ULK2 are protein kinases, which share significant homology. Although they both can bind the same binding partners, it seems that ULK1 is the crucial one for the induction of autophagy, while ULK2 may represent some redundancy in autophagy system (Chan et al., 2007). Another homolog, ULK3 was shown to play significant role in autophagy in some specific cases and will be discussed further in the text.

Atg13 binds both ULK1 and ULK2 and mediates the interaction of the ULK proteins with FIP200. The binding of Atg13 stabilizes and activates ULK and facilitates the phosphorylation of FIP200 by ULK. (Jung et al., 2009)

Both FIP200 and ATG13 are critical for correct localization of ULK1 to the pre-autophagosome and stability of ULK1 protein. They also individually enhance ULK1 kinase activity, but both are required for maximal stimulation. (Ganley et al., 2009)

Atg101 interacts with ULK1 most likely through direct interaction with Atg13. It stabilizes the expression of Atg13, probably by protecting Atg13 from proteasomal degradation. It is also important for keeping the basal phosphorylation of Atg13 and ULK1 (Hosokawa et al., 2009; Mercer et al., 2009).

Whereas formation of the ULK1-Atg13-FIP200 complex and its interaction with Atg101 is nutrient status- independent, (Hosokawa et al., 2009 ; Hosokawa et al., 2009) the association of probably the most important autophagy regulating kinase mTORC1, with this complex strongly depends on it. Under nutrient rich conditions mTORC1 is incorporated into the ULK1-Atg13-FIP200 complex through interaction between its Raptor subunit and ULK1 and phosphorylates ULK1 and ATG13. Their mTORC1-mediated phosphorylation blocks further steps in autophagy and thus has major inhibitory impact on initiation of autophagic process. Atg13 is further phosphorylated by ULK1. Upon starvation, mTORC1 dissociates from the complex and Ulk1 is dephosphorylated, which leads to ULK1 activation and phosphorylation of downstream substrates (Hosokawa et al., 2009 ; Ganley et al., 2009).

Ulk1 can also be directly activated by another, for autophagy almost equally important kinase, AMPK which phosphorylates its Ser 317 and Ser 777. Under nutrient sufficiency, high mTOR activity prevents Ulk1 activation by phosphorylating Ulk1 Ser 757 and disrupting the interaction between Ulk1 and AMPK, whereas during starvation this does not occur (Kim et al., 2011).

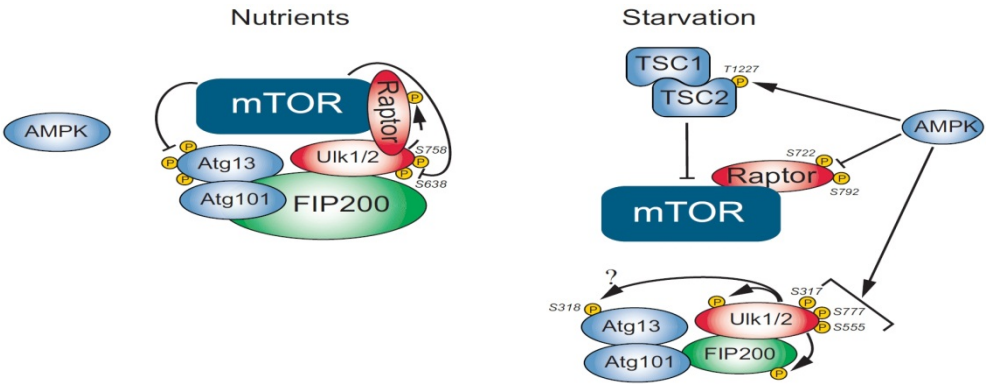


Figure 3. mTORC1 downstream signaling and autophagy regulation. Ulk1 and Ulk2 form a stable complex with Atg13, FIP200 and Atg101. Under nutrient-rich conditions mTORC1 phosphorylates Ulk1/2 and Atg13, thereby inhibiting the Ulk1/2 kinase activity and complex stability. In response to starvation, the mTORC1-dependent phosphorylation sites in Ulk1/2 are rapidly dephosphorylated, and Ulk1/2 autophosphorylates and phosphorylates Atg13 and FIP200 resulting in translocation of the entire complex to the pre-autophagosomal membrane and induction of autophagy. Alternatively, Ulk1/2 is phosphorylated by AMPK and thereby activated. In addition, AMPK indirectly leads to the induction of autophagy by inhibiting mTORC1 through phosphorylation of Raptor or TSC2 (Tchevkina and Komelkov 2012).

However, ULK1 can impose a negative feedback effect on both kinases. In the case of mTOR, ULK1 phosphorylates its Raptor subunit and thereby inhibits its activity in a manner different from its standard mode of activation/inhibition through TSC2. (Jung et al., 2011) This could probably serve to intensify the autophagic process. On the other hand, Ulk1/2 can phosphorylate all three subunits of AMPK and thereby negatively regulate its activity, which may lead to autophagy attenuation (Löffler et al., 2011).

Although the eminent importance of ULK1 complex in autophagy induction is well known, the exact effect on downstream proteins is being uncovered in the last few years. Among its most important targets are proteins from PtdIns3K complexes.

1.3.2 Phosphatidylinositol 3-kinases complexes

For the progression of autophagy and recruitment of other PI3P-binding proteins, e.g. WIPI1 and WIPI2 (Proikas-Cezanne et al., 2004 ; Polson et al., 2010) or ZFYVE1/DFCP1 (Axe et al., 2008) to the sites of autophagosome formation is necessary PI3 kinases-mediated generation of phosphatidylinositol 3-phosphate.

Mammals have two main classes of phosphatidylinositol 3-kinases: class I PtdIns3K and class III PtdIns3K. Complexes containing class III PtdIns3K are essential for

autophagosome formation. They are at least three and consist of the conserved core composed of class III PtdIns3K VPS34/PIK3C3, regulatory Ser/Thr kinase p150/PIK3R4 , regulatory protein Beclin1/BECN1 and complex-specific proteins ATG14L/Barkor or UVRAG, which along with other proteins mediate many vital interactions leading to autophagy promotion (Itakura et al., 2008 ; Liang et al., 2006 ; Matsunaga et al., 2009 ; Zhong et al., 2009).

In the centre of complex regulatory network stands Beclin1, interacting with PI3K complexes either directly or through some mediator proteins. In addition, Beclin-1 can also be inhibited by the interaction with antiapoptotic protein Bcl-2 (Pattingre et al., 2005). Interactions with other proteins from the Bcl-2 family, which are important not only for autophagy induction itself, but particularly for the autophagy-apoptosis interplay will be discussed in other part.

Beclin 1-VPS34 complex is tethered to the cytoskeleton through interaction between the Beclin 1-interacting protein AMBRA1 and dynein light chains 1/2. When autophagy is induced, ULK1 phosphorylates AMBRA1, releasing the complex from dynein. Its subsequent relocalization to the endoplasmic reticulum enables autophagosome nucleation. Therefore, AMBRA1 constitutes a direct regulatory link between ULK1 and Beclin 1-VPS34 (Di Bartolomeo et al., 2010).

Following autophagy induction for expample by amino-acid starvation or mTOR inhibition, the activated ULK1 phosphorylates Beclin-1 on Ser 14, thereby enhancing the activity of the ATG14L-containing VPS34 complexes. Phosphorylation of Beclin-1 Ser 14 by ULK is required for full induction of autophagy in mammalian cells (Russel et al., 2013).

Two other proteins from the complex with mostly regulatory function are ATG14L and UVRAG. They bind to the Vps34 complex through Beclin1 in a mutually exclusive manner (Matsunaga et al., 2009 ; Itakura et al., 2008).

ATG14L localizes to the isolation membrane and ER in nutrient rich conditions. During starvation it colocalizes with ATG16L and LC3 positive structures independently of the interaction with Vps34 complex. (Itakura et al., 2008 ; Matsunaga et al., 2009). It possibly directs class III PtdIns3K complex to the phagophore to initiate the production of PI3P and subsequent recruitment of other autophagy proteins. Moreover Atg14L determines differential regulation (either inhibition or activation) of different Vps34 complexes in response to glucose starvation.

As for the UVRAG, very important seems to be its interaction of with Bif-

1/SH3GLB1. In response to nutrient deprivation Bif-1 colocalizes with Atg5, LC3 and ATG9. It contains amino-terminal N-BAR domain and a carboxy-terminal SH3 domain and shows membrane binding and bending activites. Furthermore, Bif-1-positive, crescent-shaped small vesicles expand by recruiting and fusing with Atg9-positive small membranes to complete autophagosome formation (Takahashi et al., 2007 ; Takahashi et al., 2009), which is likely one of the mechanisms, how autophagosomal membrane curvature is formed.

Another autophagy-promoting activity of UVRAG is its interaction with C Vps complex, a key component of the endosomal fusion machinery. The UVRAG-class-C-Vps complexes accelerate endosome-lysosome fusion, resulting in rapid degradation of endocytic cargo. This interaction stimulates Rab7 GTPase activity and autophagosome fusion with late endosomes/lysosomes, thereby enhancing delivery and degradation of autophagic cargo. Remarkably, autophagosome/endosome maturation mediated by the UVRAG-class-C-Vps complex is genetically separable from UVRAG-Beclin1-mediated autophagosome formation. These facts indicate that UVRAG functions as a multivalent trafficking effector that regulates not only two important steps of autophagy - autophagosome formation and maturation - but also endosomal fusion, which concomitantly enhances transport of autophagic and endocytic cargo to the degradative compartments (Liang et al., 2008).

In contrast, UVRAG participates also in complex with Rubicon protein which localizes to endosome/lysosome, reduces Vps34 activity and downregulates autophagy (Matsunaga et al., 2009 ; Zhong et al., 2009).

The aforementioned processes (the autophagy-promoting ones) lead to establishment of a platform for further expansion of growing phagophore. The next step is elongation of the isolation membrane, which requires delivery of substantial amount of membrane material. In this process participate autophagy-specific transmembrane proteins ATG9 and VMP1.

1.3.3 ATG9

ATG9 is a transmembrane protein occuring in mammalian cells in two forms: ATG9A and ATG9B. Both are functional in autophagosome formation, but differs by its cell type-specific expression. Whereas ATG9A is expressed ubiquitously, ATG9B is highly expressed especially in placenta and pituitary glands (Yamada et al., 2005). ATG9 is

thought to participate in membrane delivery from donor sources to growing autophagosomes. Under nutrient-rich conditions it localizes to trans-Golgi network and late endosomes and, where it colocalizes with TGN46, the cation-independent mannose-6-phosphate receptor, Rab7 and Rab9. Amino acid starvation or rapamycin treatment causes a redistribution of ATG9 from the trans-Golgi network to peripheral, endosomal membranes, which are positive for the autophagosomal marker GFP-LC3. This redistribution requires ULK1 and PI3K activity and is probably essential for autophagosome formation and delivery of a membrane to growing phagophore (Young et al., 2006).

Another possible mode of autophagy regulation could act through ATG9. MAPK p38alpha can inhibit ATG9-binding partner p38IP and so block autophagy (Webber and Tooze 2010).

1.3.4 VMP1

VMP1 is another transmembrane protein essential for autophagosome formation. Its overexpression is sufficient to trigger autophagy, whereas its downregulation inhibits formation of autophagosome. During starvation or after rapamycin treatment VMP1 colocalizes with LC3, and interacts with Beclin 1 (Ropolo et al., 2007). It is thought that it may play a part in recruiting another ATG proteins (LC3, Beclin1) to growing autophagosome, in part also via scaffold protein TP53INP2 (Nowak et al., 2009).

1.3.5 Ubiquitin-like conjugation systems

Maturation of autophagosome requires two ubiquitin-like systems. The name was derived from a strong resemblance of some of participating proteins to ubiquitin and from a similarity of reactions to ubiquitination. There are two systems: ATG12-ATG5 and LC3.

1.3.5.1 ATG12-ATG5 system

Whole functional complex at this stage of autophagy is formed by a dimer of two covalently bound proteins ATG12-ATG5, which are noncovalently associated with

ATG16L1. First, ATG12 forms an intermediate with Cys 572 of ATG7 , which is an essential E1-like enzyme for the ATG12-ATG5 conjugation system. (Tanida et al., 2001) It is followed by E2-like conjugation of ATG12 with ATG10 (Mizushima et al., 2002) and subsequently, covalent conjugation via an isopeptide bond between the C-terminal glycine of ATG12 and lysine-130 of ATG5. (Mizushima et al., 1998) The ATG12-ATG5 conjugate can then interact with WD-repeat protein ATG16L1 (Mizushima et al., 2003) and dimerize (Fujita et al., 2009). ATG16L is expressed in two isoforms in mammalian cells, out of which the second, ATG16L2, despite it´s capability to form the same complex as ATG16L1, is not recruited to phagophores and therefore does not possess the ability to mediate canonical autophagy (Ishibashi et al., 2011).

In conjunction with ATG12-ATG5, ATG16L associates with the autophagic isolation membrane for the duration of autophagosome formation and it seems, that is also responsible for targeting of the complex. (Mizushima et al., 2003)

Very important could be recently discovered interaction of ATG16L1 with ULK1 complex, more specifically interaction of FIP200-binding domain (FBD) of ATG16L1 with ULK1 complex subunit FIP200. ATG16L1 with deleted FBD is defective in mediating amino acid starvation-induced autophagy, which requires ULK1 complex, but it still retains its function in supporting glucose deprivation-induced autophagy, which is ULK1 complex-independent process. (Gammoh et al., 2013 ; Nishimura et al., 2013).

Additional modes of regulation enter the autophagic regulatory network on this level. One of them is a specific interaction of ATG16L with small Golgi-resident GTPase Rab33A/B in a GTP-dependent manner (Itoh et al., 2008). The second one is possible autophagy-inhibiting acetylation of ATG5, ATG7, ATG12 and LC3 by acetyltransferase p300 (Lee et al., 2009).

Membrane localization of ATG12-ATG5-ATG16L complex also determines the localization and lipidation of LC3, the central protein of the second ubiquitin-like system (Fujita et al., 2008).

1.3.5.2 ATG8 system

Mammals have at least 4 homologs of ATG8: LC3, GABARAP, GATE16 and ATG8L. The best characterized is LC3 family. It can be further divided into 3 subfamilies: LC3A, LC3B

and LC3C, where LC3B has a primary role in autophagy and the name is freely interchangeable with LC3 if not specified otherwise.

Another important participants in this Ubl-system are ATG4, ATG3 and ATG7. ATG4 has in mammals 4 isoforms: ATG4A, ATG4B,ATG4C and ATG4D, where he most important seems to be ATG4B.

LC3 can be found in three forms: newly synthesized precursor LC3, LC3-I, which is cytosolic and LC3-II, which is membrane bound. LC3-II is present both inside and outside of autophagosomes and is currently the most widely used marker of autophagosome. Newly synthesized LC3 is subjected to posttranslational removal of the C-terminal 22 amino acids by ATG4B which creates LC3-I with an exposed C-terminal glycine (Kabeya et al., 2000 ; Kirisako et al., 2000 ; Kabeya et al., 2004). It is followed by the conjugation of the glycine to cystein of ATG7, which acts here as an E1-like enzyme. LC3-I activated this way is then transferred to an E2-like enzyme ATG3, which mediates its final conjugation to phosphatidylethanolamine. (Ichimura et al., 2000 ; Tanida et al., 2001 ; Tanida et al., 2002). This way, a fraction of LC3-I is turned into LC3-II (also known as LC3-PE). The amount of LC3-II can be in some cases correlated with the extent of autophagosome formation, but is strongly tissue and cell-dependent (Kabeya et al., 2000 ; Mizushima et al., 2004). LC3, GABARAP, GATE16 and ATG8L sre subjected to similar modifications, and are thought to act in later stages of autophagosome formation (Tanida et al., 2001 ; Tanida et al., 2002 ;Tanida et al., 2006).

As mentioned above, there is a strong dependence of LC3 localization and processing on ATG5-ATG12-ATG16L complex, which may serve as an E3 like enzyme in LC3 system. Interestingly, ATG5-ATG12 and ATG16L-dependent lipidation of LC3 in cells, where these three components were overexpressed and targeted to membranes was phosphatidylinositol 3-phosphate- and starvation signal-independent, whereas usually this lipidation depends on PI3K activity. The interaction of ATG12 with ATG3 further supports the central role of the first ubiquitin-like conjugation system in LC3 processing. (Fujita et al., 2008)

Interestingly, LC3 can be regulated by direct phosphorylation by PKA in response to various stimuli. This phosphorylation has an inhibitory character, as it reduces LC3 recruitment to autophagosomes (Cherra et al., 2010).

1.3.6 Proteins involved in cargo selection

Macroautophagy is often defined as a bulk-degradation process, where a portion of cytoplasm is enveloped in double membrane vesicle and the content is after fusion with lysosome degraded. It could be therefore tempting to imagine selection of the "bulk" as a nonselective, random process. However there are specific markers on the cargo, which are recognized by receptor-adapter proteins of autophagic machinery.

Probably the best explored one is multifunctional scaffold protein p62/SQSTM1. It contains at least three domains which determine its function in autophagy. Ubiquitin-binding domain (UBA) binds ubiquitin-tagged proteins or protein aggregates, LC3-interacting region (LIR) interacts with proteins from LC3 and GABARAP family and is therefore responsible for recruiting p62 into autophagosomes, and PB1 domain which gives p62 the ability to form polymers and so increase the number of ubiquitinated proteins delivered into autophagosomes on one molecule of LC3 (Bjørkøy et al., 2005 ; Pankiv et al., 2007 ; Shvets et al., 2008 ; Ichimura et al., 2008).

Nbr-1 and Alfy are two other adaptors involved in clearance of ubiquitinated protein aggregates. NBR1 contains an UBA and two LIR domains. It can act both independently or in concert with p62.

Alfy,a phosphatidylinositol 3-phosphate-binding protein, is recruited to intracellular inclusions and scaffolds a complex between p62-positive proteins and the autophagic effectors ATG5, ATG12, ATG16L, and LC3. Both p62 and ALFY localize to nuclear PML bodies (Kirkin et al., 2009 ; Filimonenko et al., 2010 ; Clausen et al., 2010). All three proteins are involved in binding, and clearance of ubiquitinated proteins and their aggregates with probably often overlapping functions. Besides p62 was also shown to participate in autophagy of organelles, namely peroxisomes (Kim et al., 2008) and mitochondria (Geisler et al., 2010).

1.3.7 Fusion with lysosome

After autophagosome enwrapped the cargo, the last step is to fuse with lysosome. Involvement of SNARE proteins in this process was long presumed, but the final evidence pointing to an autophagy specific SNARE protein was still missing. Itakura et al. identified syntaxin 17 (Stx17), autophagosomal SNARE required for fusion with lysosome. StX17 localizes to the outer membrane of completed autophagosomes but not to an unclosed

intermediate, which prevents fusion of isolation membrane with lysosome (Itakura et al., 2012).

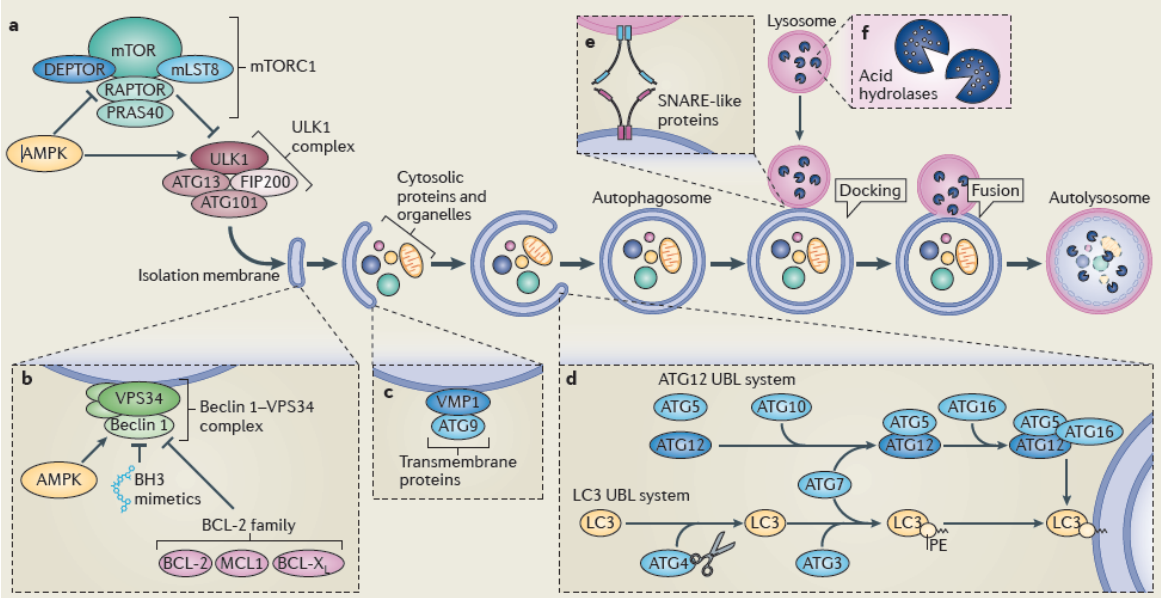


Figure 4. Basic molecular machinery of autophagy (Mariño et al., 2014).

1.4 Main signalling pathways in autophagy

There are two major pathways that respond to nutrient status of the cell. These are mTOR pathway, responsible mostly for sensing amino acids availability and AMPK pathway, sensing especially glucose level. As briefly outlined above, there can be several crosstalks between these two pathways. In addition, p53 can interfere either positively or negatively with these pathways and thus coordinate autophagic response during various cellular stresses with other essential processes.

1.4.1 mTOR

mTOR is is a serine/threonin kinase involved in processes like cell growth, cell survival, transcription, translation. In the context of autophagy and this work, the most important is its central tole in sensing nutrient status of the cell, especially availability of amino acids. Activated mTOR strongly suppresses autophagy in nutrient rich conditions. The abbreviation refers to "mammalian target of rapamycin" or "mechanistic target of

rapamycin", as it was derived from the interaction of mTOR with macrolid antibiotics rapamycin, which impose strong inhibitory effect on mTOR (Heitman et al., 1991). There are two complexes, called mTORC1 and mTORC2. They differ in the subunit composition and therefore also in exact function. For autophagy mTORC1 is the one with more profound impact.

Although there are more possible modes of mTOR activation, involving PKA and MAPK pathways , in the context of autophagy one of them is principal - the regulation through Rag GTPases, Rheb and TSC1/TSC2. There are four mammalian Rag proteins: RagA, RagB, RagC, RagD. They form dimers and interact with Raptor subunit of mTORC1 mediating its relocalization to lysosomal surface in the presence of amino acids. The lysosomal targeting and recruitment is mediated by the complex of endosomal/lysosomal adapters collectively referred to as the Ragulator complex, which also serves as a guanine nucleotide exchange factor for Rag proteins (Kim et al., 2008 ; Sancak et al., 2008 ; Sancak et al., 2010). Positioned near lysosome, mTOR can be activated by another GTPase, Rheb, which is here also located. This interaction is crucial for mTOR activation (Garami et al., 2003 ; Inoki et al., 2003). For activating mTOR, Rheb itself must be relieved from inhibitory effect of its upstream regulator TSC1/TSC2 complex, which serves as GTPase-activating protein for Rheb. This could be achieved for example by Akt-dependent phosphorylation of TSC2, which disrupts its interaction with TSC1 and inactivates it (Tee et al., 2003 ; Inoki et al., 2002 ; Gao et al., 2002).

On the other hand, when mTOR is inhibited due to lack of amino acids, autophagy is induced, which results in autophagosome-lysosome fusion, sequestration of engulfed proteins and release of recycled amino acids. Prolonged starvation can lead to increase of amino acids to the level, which is sufficient to reactivate mTOR. Increased mTOR activity attenuates autophagy and generates proto-lysosomal tubules and vesicles that extrude from autolysosomes and ultimately mature into functional lysosomes, thereby restoring the full complement of lysosomes in the cell (Yu et al., 2010).

Besides the already discussed regulation through ULK-complex, mTORC1 is capable to modulate autophagy in other ways. One of them is inactivation of the ATG14-containing Vps34 kinase complexes by phosphorylation of ATG14, which results into inhibition of autophagy (Yuan et al., 2013).

Under nutrient rich condition mTORC1 colocalizes on the lysosomal membrane with TFEB, a transcription factor that drives expression of autophagy and lysosomal genes and phoshorylates it thus promoting its association with members of the YWHA (14-3-3)

family. TFEB is hereby being retained in cytosol. Starvation or pharmacological mTORC1 inhibition causes dissociation of the TFEB/YWHA complex and rapid transport of TFEB to the nucleus where it increases transcription of multiple genes implicated in autophagy and lysosomal function (Settembre et al., 2012 ; Martina et al., 2012).

1.4.2 AMPK

AMPK is a protein kinase composed of three subunits, which has a central role in sensing energy level of a cell represented by AMP to ATP ratio.

Three upstream kinases may phosphorylate and activate AMPK: TAK1, LKB1 and Ca2+/calmodulin-dependent protein kinase (Momcilovic et al., 2006 ; Hong et al. 2003 ; Woods et al., 2003). In autophagy it is connected mainly with glucose withdrawal and it can promote the process through various mechanisms.

AMPK´s autophagy-promoting activities are frequently connected with the inhibition of mTOR pathway. This can be achieved in one way through phosphorylation of TSC2, which enhances its Rheb-inhibiting activity, thus preventing mTOR activation. Furthermore, TSC2 and its phosphorylation by AMPK protects cells from energy deprivation-induced apoptosis (Inoki et al., 2003).

AMPK can also directly phosphorylate the mTOR binding partner Raptor on two well-conserved serine residues, and this phosphorylation induces 14-3-3 binding to Raptor. The phosphorylation of Raptor by AMPK is required for the inhibition of mTORC1 and cell-cycle arrest induced by energy stress (Gwinn et al., 2008).

Another major mode of action is through ULK complex. In addition to aforementioned activating AMPK- mediated phosphorylations of ser 317 and ser 777 (Kim et al., 2011), several other relevant phosphorylation sites have been identified. Among them ser 555, which is necessary for binding of 14-3-3 protein (Markus et al., 2011). Presence of these protein in AMPK-ULK1-mTORC1 complex coincides with raptor Ser 792 phosphorylation and mTOR inactivation (Lee et al 2010).

Recent study revealed an intriguing mechanism of direct phosphorylation of Vps34. During starvation AMPK stimulate function of autophagy specific (ATG14- or UVRAG-containing Vps34 complexes). Concomitantly, AMPK inhibit function of non-autophagic Vps34 complexes (Kim et al., 2013).

Furthermore, AMPK phosphorylates and stabilizes p27, which also lead to

autophagy induction (Liang et al., 2007).

1.4.3 p53

p53 as a master houskeeper plays a major role in signalling networks of senescence, apoptosis and autophagy and thus it is considered as a central "hub" where many crosstalks take place.

p53 can impose either activation or inhibitory effect on autophagy induction in starved cells. This discrimination relies largely on current subcellular localization of p53. Cytoplasmic localization is associated with inhibitory effect on autophagy, which is caused by direct binding of p53 to FIP200 subunit of ULK complex (Morselli et al., 2011). Inducers of autophagy like starvation, rapamycin treatment and ER-stress caused proteasome-mediated degradation of p53 in HDM2-dependent manner. Inhibition of cytoplasmic p53 degradation prevented the activation of autophagy in several cell lines. Nuclear localization on the other hand not only fails to inhibit autophagy, but is rather associated with autophagy activation through initiation of transcription of target genes (Tasdemir et al., 2008 ; Morselli et al., 2008).

Among these belong beta1 and beta2 subunits of the AMPK, TSC2 , PTEN and IGF-BP3. Each of them negatively regulates mTOR pathway after stress. Expression of these genes is however highly cell type and tissue-specific, with with the TSC2 and PTEN proteins being coordinately regulated in those tissues that use insulin-dependent energy metabolism e. g. skeletal muscle, heart, white fat, liver, and kidney (Feng et al., 2007).

Genotoxic stress-induced p53-dependent upregulation has been observed also for the AMPK activators sestrins 1 and 2, products of two p53 target genes. Sestrin1 and Sestrin2, activate AMPK and target it to phosphorylate TSC2 and stimulate its GAP activity, thereby inhibiting mTOR. Correspondingly, Sestrin2-deficient mice fail to inhibit mTOR signalling upon genotoxic challenge. (Budanov and Karin, 2008)

During glucose deprivation, AMPK can phosphorylate p53 on serine 15 and thus trigger p21/Waf1 expression and G1/S checkpoint. This cell-cycle arrest occurs despite continued amino acid availability and active mTOR, but is completely reversible upon prompt restoration of glucose level in case that the duration of the deprivation period does not exceed some threshold value. Persistent activation of AMPK actually leads to accelerated p53-dependent senescence (Jones et al., 2005).

Many other more or less direct or indirect p53 autophagy-influencing interactions were indicated, which is not surprising considering the known complexity of p53 interactome. One of these partners is damage-regulated autophagy modulator (DRAM).

It is a p53 target gene encoding lysosomal protein, through which is p53 able to induce autophagy. DRAM also plays a role in p53-mediated apoptosis. (Crighton et al., 2006).

p53 also activates gene expression of proapoptotic proteins of the Bcl-2 family, such as Puma, Bad and Bax, which were also shown to induce autophagy.

1.5 Senescence

Cellular senescence is a state of permanent cell cycle arrest associated with extensive cellular remodelling, changes in gene expression and an altered secretory pathway. It is a cellular response to many stressors including gama- or UV-irradiation, oxidative stress, activated oncogenes, and chemotherapy, which in most cases cause DNA damage. Cell in response triggers many effectors like DNA damage-response, senescence-associated secretory phenotype chromatin remodelling and possibly autophagy. There are two signalling pathways which are considered to be master regulators of senescence: p53-p21 and p16/Rb (Pawlikowski et al., 2013).

1.6 Autophagy and senescence

Autophagy and senescence were shown to be highly interconnected processes, acting together or in relation to each other in response to various cellular stresses.
First solid data connecting autophagy and senescence were published about 10 years ago showing that in senescent fibroblasts there is an increase in size of autophagic vacuoles and a clustering of otherwise scattered dots of lysosome-linked enzyme beta-galactosidase at the level of autophagic vacuoles (Gerland et al., 2003).

1.6.1 Autophagy as switch between senescence and apoptosis

Experiment on human umbilical vein endothelial cells (HUVECs) suggested possible

involvement of autophagy in deciding whether cells induce apoptosis or enter senescence. In this case pharmacological suppression of autophagy prevented development of premature senescence but did lead to the enhanced rate of apoptosis in HUVECs exposed to extracellular stress, such as advanced glycation end products. Pharmacological induction of autophagy resulted in reciprocal changes (Patschan et al., 2008).

Very similar results shown setup with DNA damage-induced senecence and Oncogene-induced senescence. Also in this cases, senescence was prevented and apoptosis was elevated by inhibiting autophagy (Singh et al., 2012 ; Guo et al., 2012).

1.6.2 Autophagy as a mediator of senescence

Role of autophagy as senescence-promoting process was further supported in the pivotal work by Young and colleagues . They presented strong evidence, that oncogene-induced senescence and with slight differences also DNA damage-induced senescence are accompanied by elevated autophagic activity. After induction of senescence by overexpressed Ras there is approximately 2 days lasting "mitotic phase" (not present in DNA damage-induced senescence) accompanied by rapid cellular division. Around day 5-6 cells reach "senescence phase". The gap between is called "transition phase", during which operate effectors of senescence and cells undergo vast changes in morphology, gene expression, secretory profile and other important characteristics. In addition to transcription upregulation, an efficient mechanism for protein turnover is needed. Autophagy, measured as LC3-II level, reached its peak during this transition phase along with significant increase in the degradation of long-lived proteins and overall rapid protein turnover (Young et al., 2009).

1.6.3 mTORC1 and mTORC2 activities reflect initiation of autophagy in senescing cells

In agreement with it, mTORC1 activity increased in response to the mitotic signal and subsequently decreased during the transition phase. Furthermore mTORC2, acting probably at least in part through the ATG protein transcription-activating mTORC2-AKT-FoxO3a pathway paralleled with its activity the kinetics of mTORC1 (Young et al., 2009).

1.6.4 FOXO3 and unique expression profile of senescent cells

Deeper investigation of transcriptional profile of genes involved in autophagy and senescence shown, that a subset of ATG genes and other autophagy regulators, including direct targets of FoxO3a like LC3B, Bnip3 and Bnip3L, were initially diminished during the mitotic phase, where FoxO3a was highly phosphorylated. As the level of phosphorylation of FoxO3a resulting in its activation, the suppression of these genes was removed and hence they were up-regulated during the transition to the senescence phase.

The ATG gene expression profile of the senescent cells was unique, since amino acid starvation, which mimics a physiological trigger of autophagy, did not induce a similar expression pattern of ATG genes compared to nonsenescent (Young et al., 2009).

Many lysosomal genes showed a similar expression pattern to the up-regulated ATG genes as well (Young et al., 2009). One such gene was GLB1, whose product is believed to be responsible for senescence-associated beta-galactosidase (SA-beta-gal) activity (Lee et al. 2006). It is possible, that SA-beta-gal activity, and perhaps GLB1 up-regulation might ,at least in part, also reflect the activity of autophagy.

Involvement of FoxO3A was further supported by revealing its mode of action through transcriptional upregulation of the expression of PDK4, which resulted in blocking ATP generation, increase of AMP: ATP ratio followed by AMPK activation and mTOR inhibition. It is worth noting that the increase of AMPK activity is not a consequence of the upregulation of LKB1 expression as FoxO3A activation did not change LKB1 level (Guo et al., 2012).

1.6.5 SASP expression kinetics and autophagy

Senescence-associated secreted factors, such as IL6, IL8, IGFBP7, and MMP3, also showed similar expression kinetics, while other senescence-associated genes showed a rather steady increase over the time course. Strikingly, impairment of autophagy delayed IL6 and IL8 production. This effect was not observed in cells with knockdown of Rb, suggesting that the inhibition of IL6 and IL8 production is not a consequence of delayed senescence. The requirement of autophagy for the efficient production of these factors indicates that autophagy can modulate the functional activity of senescent cells and

adoption of the senescent cell- specific SASP (Young et al., 2009).

1.6.6 Role of ULK3 in senescence

Very interesting results related to senescence point to the involvement of ULK kinases in this process. Whereas in most cases ULK1 appears to be the main ATG1 homolog in mammalian autophagy, with occasional and partly redundant function, the data obtained from senescent cells point to ULK3 as to the more important in autophagy-senescence context. Stable overexpression of ULK3 was sufficient to induce autophagy as well as premature senescence, further supporting a causative role for active autophagy in senescence (Young et al., 2009)

1.6.7 Autophagy and senescence bypass

The importance of autophagy for senescence was further emphasized by the fact that defects in autophagy may lead to a substantial increase in bypass of senescence, even to a higher levels than those seen in cells with depleted Rb protein. On the other hand, when the senescent state is established, inhibition or dysfunction of autophagy is unable to reverse it. These results, obtained in vitro on human diploid fibroblasts BJ and IMR90, were further supported by data obtained from in vivo chemically- induced murine papillomas (Young et al., 2009).

Experiments with chemotherapy-induced senescence on breast tumor cells MCF-7 and colon carcinoma cells HCT-116 again shown a close association between autophagy and senescence and their collateral regulation via common signalling pathways. The cell lines were treated with clinically relevant concentrations of camptothecin and doxorubicin, which resulted in the induction of autophagy and senescence. After supression of ROS generation and modulation of ATM, p53 and p21 activity, both autophagy and senescence were suppressed. Consistently with previous data, inhibition of autophagy alone by pharmacological and genetic approaches could not entirely abrogate the senescence response, which was only reduced and/or delayed (Goehe et al., 2012).

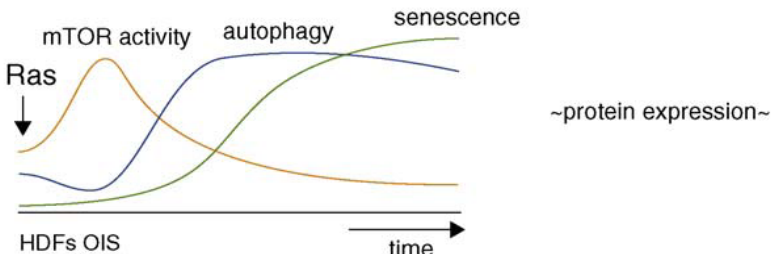


Figure 5. Time kinetics of mTOR activity, autophagy response and senescence onset after constitutive Ras activation (Young and Narita 2010).

Autophagy as a possible mediator of cellular-senescence process was further confirmed on other cell lines using different inductors of senescence. These include biliary epithelial cells (Sasaki et al., 2010), cancer-associated fibroblasts, breast cancer cells (Capparelli et al., 2012) and others. Although autophagy is considered to have antitumorigenic effect in healthy cells, whereas in transformed cells it can promote tumor growth, then mediating senescence is likely another possible mode of antitumorigenic effect, even in already transformed cells (Yang et al., 2013 ; Liu et al., 2014). However, there are results showing a completely opposite role for autophagy in senescence.

1.6.8 Autophagy as protection against senescence

Kang et al. demonstrated that autophagy impairment (by depletion of ATG7 and ATG12, or Lamp2) induces premature senescence in primary human diploid fibroblasts (AIPS and RS strains). In addition, several important components that have been implicated in the mTOR pathway (S6K1, p-S6, and 4E-BP1) or autophagy pathway (Beclin-1, ATG7, p62 monomer, and ATG12-ATG5 conjugates) were downregulated in senescent cells. Furthermore, they shown that p53 inhibition or ROS scavenging can delay premature senescence, induced by autophagy impairment and can also reverse the changes seen in the mTOR and autophagy pathways. Possible explanation may be that autophagy impairment can induce senescence in human primary fibroblasts through activation of the p53 tumor suppressor pathway due to increased ROS generation, perhaps from an accumulation of dysfunctional mitochondria, which are usually eliminated by mitophagy (Kang et al., 2011).

Very recent study conducted again on HUVECs but with quite different conclusions concerning autophagy-senescence relation , shown that exposure of cells to high concentrations of glucose, causing activation of renin-angiotensin system (RAS) and mitochondrial damage lead to apoptosis, senescence and autophagy. Interestingly, by impairing autophagy, senescence and apoptosis were accelerated. Same results were obtained using high glucose-mimicking agent angiotensin II or proton gradient uncoupler CCCP (Chen et al., 2014).

Even in OIS, autophagy was shown to have dual role. In mouse embryonic fibroblasts it determines the cellular response to oncogenic RAS in a way, where elevated autophagy leads to a bypass of RAS-induced senescence and a reduction in, or a lack of, autophagy enhances it. Important role was suggested for N-terminal Apoptosis-stimulating of p53 protein 2 (ASPP2), which mediates RAS-induced senescence and inhibits autophagy by competing with ATG16 to bind ATG5-ATG12 and thus preventing ATG16-ATG5-ATG12 complex formation (Wang et al., 2012).

Consistently with this opinion, inhibition of mTORC1 (but not mTORC2) was shown to delay the onset of senescence in replicatively senescent fibroblasts and disrupt Ras-induced senescence. Furthermore, some aspects of senescence (e.g. drop of p21, p53 levels, partial suppression of cytokine secretion) were even partly reversed (Kolesnichenko et al., 2012).

It is obvious that the autophagy-senescence relationship is far from being clear. The definition of exact circumstances and factors causing autophagy to act in one or the other way is highly desirable. One possible interesting link could be the one with SASP.

1.6.9 Autophagy and SASP

Production of SASP is highly demanding in terms of nutrients and energy. It was thought, that autophagy may also help to cope with such increased demands. Dörr et al. addressed this question on a clinically interesting model of chemotherapy- induced (cyclophosphamide and doxorubicin) cellular senescence (TIS) on mouse lymphoma cells in vivo. After treatment and the onset of senescence, the SASP producing cells, exhibited increased glucose uptake and much higher ATP production. It is linked at least in part with massive proteotoxic, UPR and ER stress caused most probably by the production of large

amounts of secretory molecules. Unlike senescent models that lacks a strong SASP response, those with SASP are much more sensitive to blocking glucose utilization or autophagy, which led to their selective elimination through caspase-12 and caspase-3 mediated endoplasmic reticulum related apoptosis (Dörr et al., 2013).

SASP producing senescent cells however not only need catabolic pathways to recycle nutrients or get rid of damaging protein aggregates or organelles, but also properly activated translation. These are processes, that often go against each other, at least on the level of regulation. It would be beneficial and for senescent cells maybe almost necessary to have both these processes activated extensively at the same time. A distinct cellular compartment located at the trans-Golgi called the TOR-autophagy spatial coupling compartment (TASCC) may serve to this purpose. During Ras-induced senescence of primary fibroblasts IMR90 there accumulates mTOR and (auto)lysosomes. mTOR recruitment to the TASCC is amino acid- and Rag GTPase-dependent and disruption of mTOR localization to the TASCC suppressed interleukin-6/8 synthesis, which could be considered as markers of SASP. Autolysosome-derived amino acids enhance mTOR recruitment to the TASCC. The TASCC formation was observed in mouse papillomas, during macrophage differentiation and in glomerular podocytes. The cells all displayed increased protein secretion,. (Narita et al., 2011).

Direct link of mTOR with regulation of SASP was quite recently demonstrated on its involvement in sensitization of cells to IL-6 by positively regulating soluble interleukin 6 receptor expression and switching IL-6 non-responder cells into IL-6 responder cells (Garbers et al., 2013).

1.6.10 Autophagy is preferred catabolic pathway in senescence

There are two main protein-recycling pathways: proteasomal and autophagic. BAG1 and BAG3, the Hsc/Hsp70 co-chaperones and modulators of protein quality control, regulate this pathways and are reciprocally regulated during cellular aging (replicative senescence). BAG1 is essential for effective proteasome degradation, while BAG3 for autophagic. A switch from BAG1 to BAG3 determines that replicatively senescent cells use more intensively the macroautophagic system for turnover of polyubiquitinated proteins. This increased macroautophagic flux is regulated by BAG3 in concert with the ubiquitin-binding protein p62/SQSTM1. This finding further supports the view of

autophagy as an essential process for senescent cells (Gamerdinger et al., 2009).

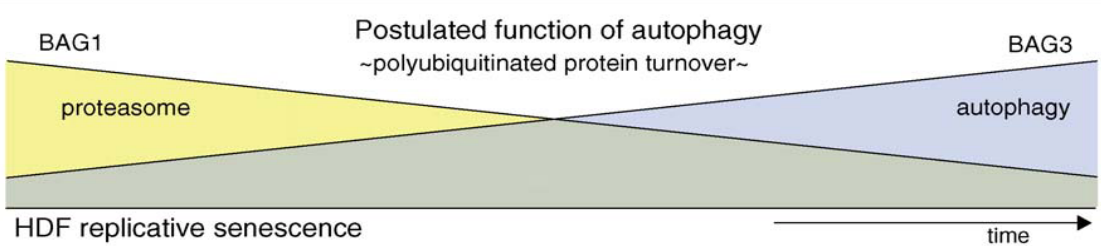


Figure 6. Shift from proteasomal to autophagic degradation pathway during replicative senecence (Young and Narita 2010).

1.7 Autophagy as a mode of cell death

Autophagy, in contrast to its extensive cytoprotective effect, was according to some experimental results showing dying cells with massive autophagic vacuolization, considered as a possible mode of cell death, also called "autophagic cell death" or "cell death type II" (Shen and Codogno., 2011). However this opinion is now being revised, because unlike other types of cell-death, there are almost no clear data marking autophagy as one of the modes of cell death. Rather than considering of autophagy as a separate type of cell death, it was proposed to be a process that may in some cases contribute to cell death by another means (apoptosis, necrosis, necroptosis), which is much better supported by current evidence (Shen and Codogno., 2011 ; Shen et al., 2012).

1.8 Autophagy and apoptosis

Both autophagy and apotosis are responses to some cellular stresses. Whereas autophagy is considered mainly as a prosurvival mechanism, apoptosis on the other hand is by definition a process leading to cell death. So far, many crosstalks were defined suggesting multivalent nature of this relation. First, autophagy can act as a predecessor of apoptosis. Here it represents probably the futile attempt of the cell to save itself in reaction to the stress, which ultimately proves fatal. Second, autophagy may inhibit apoptosis, representing the case, where damage was not critical and cytoprotective effect of autophagy was sufficient

to avert cell death. Third is an inverse relationship, where apoptotic response is so pronounced, that it interferes with autophagic response. And the last one is autophagy as a promoter of apoptosis and this is precisely, what is at the center of our interest.

1.8.1 Autophagy as an apoptotic promoter

Several possible mechanisms of how can autophagy contribute to cell death have been identified so far. One of them is modified version of the concept " eating itself to death", here presented with accent on degradation of specific cellular components followed by apoptotic death. The second one is direct involvement of ATG proteins in proapoptotic cascade and the last one is possible function of some autophagic structures as a platform for recruitment of apoptotic effectors.

1.8.1.1 Phagophore as a platform for caspase-8 activation

The information we have about the exact mechanisms by which autophagy can promote apoptosis are still very fragmentary. One of these possible connections could be autophagy-dependent activation of caspase-8 and initiation of the apoptotic cascade. In cells treated with SKI-I, a pan-sphingosine kinase inhibitor, and bortezomib, a proteasome inhibitor, autophagy is induced concomitantly with caspase-8 activation. Caspase-8 forms a complex with Atg5 and colocalizes with LC3 and p62. Moreover, FADD, an adaptor protein for caspase-8 activation, associates with Atg5 on Atg16L- and LC3-positive autophagosomal membranes and loss of FADD suppresses cell death. Inhibition of autophagosome formation by depletion of Atg5 or Atg3 results in a marked suppression of caspase-8 activation and apoptosis. Although caspase-8 self-association depends on p62/SQSTM1, its self-processing requires the autophagosomal membrane. These results could indicate that under certain conditions, the autophagosomal membrane may serve as a platform for an intracellular death-inducing signaling complex (iDISC) that recruits self-associated caspase-8 to initiate the caspase-8/-3 cascade (Young et al., 2012). What is the exact nature of these conditions needs to be revealed, as it is not a standard mode of caspase-8 activation. Other data in contrast suggest, that it is inhibition of autophagy, what promotes caspase-8 activation (Amir et al., 2013).

1.8.1.2 Apoptosis promotion through autophagy-mediated sequestration of vital cellular components

It was shown, that excessive autophagy could contribute to cell death by sequestering vital components to the point where it is no longer bearable. This was once considered to be possible mechanism of autophagic cell death, because of the experiment, where cells with inhibited caspases showed increased ROS level, membrane oxidation and loss of plasma membrane integrity. Inhibition of autophagy blocked ROS accumulation cell death. The cause of abnormal ROS accumulation was the selective degradation of the major enzymatic ROS scavenger, catalase (Yu et al., 2006). However the final execution of cellular rupture was probably mediated by the means of necrosis.

A similar mechanism could contribute also to apoptosis through degradation of IAPs, which was proven in a specific case of oogenesis in Drosophila melanogaster (Nezis et al., 2010).

1.8.1.3 Participation of ATG proteins in apoptosis induction

As was said before, some autophagy-related proteins can directly participate in the complex signalling network of programmed cell deaths. ATG12 was suggested to be a positive mediator of mitochondrial apoptosis directly regulating the apoptotic pathway by binding and inactivating prosurvival Bcl-2 family members, including Bcl-2 and Mcl-1. The binding occurs independently of Atg5 or Atg3 and requires a unique BH3-like motif in Atg12. In apoptotic cells, knockdown of Atg12 inhibited Bax activation and cytochrome c release, while ectopic expression of Atg12 antagonized the antiapoptotic activity of Mcl-1 (Rubinstein et al., 2011).

In another experiment on murine hepatoma cells, examining effects of photosensitizing agents that damage lysosomes, was shown, that ATG7-deficiency not only led to presumed suppression of autophagy, but also suppressed the apoptotic response. An ATG7-deficiency did not prevent immediate lysosomal photodamage, however, unlike wild-type and ATG5-deficient cells, the lysosomes of ATG7-deficient were able to recover lysosomal gradient within and therefore never underwent permeabilization (Kessel et al., 2012).Yet, it is known that lysosome permeabilization may lead to apoptosis (Boya et al., 2003).

2 Aims of the thesis

The first aim of this thesis was to examine and compare the extent and kinetics of autophagic response in proliferating and senescent PANC-1 cells.

The second aim was to analyze potential changes in sensitivity of PANC-1 and T47D cells with enhanced autophagy to various apoptogenes.

The specific aims are:

1. Preparation of PANC-1 cells stably expressing recombinant EGFP-LC3 and LAMP1-RFP.

2. Preparation of stable senescent culture of PANC-1 cells.

3. Quantification of the number of autophagosomes in proliferating and senescent PANC-1 cells.

4. Analysis of apoptotic response triggered by three different apoptogenes TRAIL, FasL and MitoVes after induction of autophagy (pretreatment with either EBSS medium or rapamycin or metformin).

3 Materials and methods

3.1 Reagents and drugs

3.1.1 Autophagy inducers

EBSS (Sigma-Aldrich) - Earle's Balanced Salt Solution, amino acid-free medium, strong inducer of autophagy

Rapamycin (Sigma-Aldrich) - potent inhibitor of mTOR, final concentration in medium used for induction: 10nM

Metformin (Sigma-Aldrich) - activator of AMPK, final concentration in medium used for induction: 100μM

3.1.2 Autophagy inhibitors

Chloroquine (Sigma Aldrich) - a lysosomotropic agent that prevents endosomal acidification, raises the lysosomal pH, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation. It is used for assessing autophagic flux. Working concentration 70μM..

3.1.3 Apoptosis inducers

TRAIL (Apronex) - TNF-related apoptosis-inducing ligand, binds receptors DR4, DR5, DcR1 and DcR2 and induces apoptosis in an caspase-8-dependent manner, final concentration in medium used for induction: 100ng/ml

FasL (ENZOLS) - Fas ligand, induces trimerization of Fas receptor, which triggers apoptosis, final concentration in medium used for induction: 100ng/ml

MitoVES (provided by Jiri Neuzil, Institute of Biotechnology ASCR) - Mitochondrially targeted vitamin E succinate, inhibits succinate dehydrogenase activity of mitochondrial complex II and causes apoptosis through generation of ROS, final concentration in medium used for induction: 10μM

3.2 Cell cultures and cultivation media

PANC-1 (Human pancreatic carcinoma, epithelial-like cell line) were obtained from American Type Culture Collection (ATCC) and cultivated in DMEM with 10% FBS. The cells overexpress heregulin/human epidermal growth-factor receptor 2 (HER2/neu) oncogene (which is present in 60-70% of human pancreatic carcinomas), express mutant p53 and are estrogen receptor (ER) negative. Cells are also negative for MUC4 (Sialomucin complex SMC, an intramembrane ligand for ErbB2) and positive for Smad4, a TGF beta signalling cascade protein inactivated in human gastrointestinal cancers (Abcam).

T47D (Human ductal breast epithelial tumor cell line) were obtained from American Type Culture Collection (ATCC) and cultivated in DMEM with 10% FBS. T47D cells carry receptors for a variety of steroids and calcitonin. They express mutant tumor suppressor protein p53 protein. Under normal culturing conditions, they express progesterone receptor constitutively and are responsive to estrogen (Abcam).

HEK-293 (Human Embryonic Kidney 293 cells) were obtained from ATCC and cultivated in DMEM with 10% FBS. They express SV40 large T antigens and were used for the production of recombinant lentiviruses.

3.3 Preparation of senescent cancer cells culture

As an inducer of senescence was used DNA topoisomerase I inhibitor camptothecin, which induces DNA damage through DNA breaks introduced by the nicking activity of DNA topoisomerase I, while the rejoining activity is blocked. Cells were planted in DMEM supplemented with 10% FBS and antibiotics the day before treatment, so they reached approximately 60% confluency at the moment of treatment. Camptothecin was added to the final concentration in the cultivation medium 25nM. Fully senescent cell culture was established approximately on the day 7 of the treatment.

3.4 Preparation of lentiviral expression vectors

Used chemicals and enzymes

EcoRI (Thermo scientific), BamHI (Thermo scientific), T4 DNA Ligase (Thermo scientific)

FastAP thermosensitive alkaline phosphatase (Thermo scientific), Yellow Tango buffer (Thermo scientific), T4 Ligase buffer (Thermo scientific)

Commercial kits and bacteria

Zymoclean gel DNA recovery kit (Zymo research), Zyppy plasmid miniprep kit (Zymo research), JET Star (Genomed), TOP10 competent Escherichia coli (Invitrogen)

Plasmids

pEGFP-LC3 (Addgene), pCDHNeo (Addgene)

Using restriction endonucleases EcoRI and BamHI, an EGFP-LC3-II fragment was excised from pEGFP-LC3 plasmid according to the enzyme manufacturer's protocol (Thermo Scientific). The size of the excised fragment was checked on DNA gel electrophoresis and after isolation using Zymoclean gel DNA recovery kit was DNA fragment ligated into lentiviral plasmid pCDH-Neo using T4 DNA ligase. Competent E.coli were transformed with the plasmid and plasmid DNA from selected colonies was isolated using Zyppy plasmid miniprep kit. The recombinant pCDH-Neo-EGFP-LC3 was then amplified in 100 ml E.coli culture and purified using JET Star midiprep kit.

3.5 Production of recombinant lentiviruses

Cells, vectors and chemicals

HEK 293 TN cultivated in DMEM + ATB + 10%FBS

pCDH-Neo-EGFP-LC3, pLJM1- LAMP1-mRFP-FLAG (Addgene), lentiviral packaging vectors: pMD2G , psPAX (both Addgene), PEG/it Virus Precipitation Solution (SBI, LV810A-1)

Transfection reagents

2 M calcium phosphate , *2xBBS solution (pH 6,95)*

HEK293 TN were seeded into 10cm tissue dishes and cultivated to 50-60% confluency. Just before transfection, the cultivation medium was exchanged.

For one transfection we prepared two sterile Eppendorf tubes: the first with 500 µl of 2x BBS and the second with 500 µl of 0,25M $CaCl_2$. Calculated amount of DNA (expression and packaging plasmids) was added into the eppendorf with $CaCl_2$ and mixed by vortex. $CaCl_2$ containing DNA packaging and lentivirus plasmid was dropwise mixed with the 2xBBS in the second eppendorf and incubated at the room temperature for 15 min. Then the solution was spread all over the dish with HEK293T cells. Cells were then cultivated in incubator with 3% CO_2 atmosphere for approximately 6 hours, washed with warm PBS, and the fresh medium (DMEM+ATB+FBS) was added. Cells were cultivated for additional 36hrs and after that was the medium harvested and centrifugated (3000rpm/15 minutes) to remove floating cells and debris. Supernatants were transferred into new tubes, amount of PEG/it Virus Precipitation Solution equivalent to 1/5 of the volume was added and tubes were stored in 4°C for approximately 16 hours. The supernatants were then centrifuged at 1500 × g for 30 minutes at 4°C. After the centrifugation, all traces of fluid were removed by aspiration and each pellet containing recombinant lentiviruses was resuspended in 500µl of PBS and in aliquots stored at -80°C.

3.6 Transduction of target cells

PANC-1 and T47D were split the day before transduction on a 24-well plate in order to get 60% confluency at the time of lentivirus addition. Various titer of recombinant lentiviruses were added to each well in order to get different intensities of transduction. Cells were then incubated for 24h and after that washed two times with PBS. Finally, fresh medium with either puromycin (selection of pLJM1- LAMP1-mRFP-FLAG containing cells) or neomycin (selection of pCDH-Neo-EGFP-LC3 containing cells) was added. Cells that survived selection were analyzed by fluorescence microscopy and for further experiments were selected those cultures with mid-high homogenous expression of recombinant fluorescent proteins. For the preparation of double transductants EGFP-LC3-expressing cells were transduced with LAMP1-mRFP-expressing lentiviruses and selected in the

medium containing both neomycin and puromycin.

3.7 Imunocytochemistry and confocal microscopy

Chemicals

1% paraformaldehyde in PBS

Moviol/DAPI

Immersion oil for microscopy

Instruments

Leica SP5

Cells were planted into 24 well plates with a coverslip at the bottom of each well in order to reach 50-70% confluency at the time of fixation. Because of visualising fluorescence of the native EGFP/RFP we used very mild modification of standard fixation protocol. After reaching the appropriate confluency, the medium was aspirated and cells were washed 2 times with PBS. A solution of 1% high grade methanol free paraformaldehyde cooled to 4°C was added to the wells and the whole plate let to stay for 8-10 minutes in a fridge. Immediately after that cells were washed 2 times with PBS. Coverslips were immediately mounted with a drop of mounting medium containing DAPI (Moviol/DAPI), sealed with nail polish to prevent drying and movement and visualised on confocal microscope Leica SP5.

3.8 Western Blotting

Antibodies

LC3B (Cell signalling), Bim (Cell Signaling), Mcl-1 (BD Biosciences), Actin (Santa Cruz), Anti-Flag (Sigma-Aldrich)

Others

BCA Protein Assay Kit (Pierce Biotechnology), 40% acrylamide, SDS PAGE gel and running buffers, SDS PAGE lysis buffer

Miniprotean II electrophoresis unit, Hoefer SemiPhor semi-dry transfer unit

Cells were grown on 6-well plates. After reaching the appropriate confluency, they were treated with different agents and left in 5% CO2 incubator. After the given period, wells were washed twice with cooled PBS and lysed with 1x nonreducing lysis buffer. Lysates were transferred into 200µl pipette tips with filter intersecting a cover of 1,5ml eppendorf tubes and centrifuged (13200rpm, 2 minutes). The filtered lysates were heated for 2 minutes at 95°C. Concentration of proteins was measured by BCA assay using BCA Protein Assay Kit and samples were diluted to the same concentration. Subsequently, dithiothreitol and bromphenol blue were added to the final concentrations 0,5% and 0,05% respectively and samples were denatured at 95°C for 10 minutes.

Equal amounts of proteins (around 40µg) were then resolved by SDS-PAGE on 12% or 15% acrylamide gels. Proteins were then transferred from the gel to nitrocellulose membrane using Hoefer SemiPhor semi-dry transfer unit (20 V, 90 min).

The membrane was blocked in 5% milk for western blotting diluted in PBS/Tween for 30 minutes. After that, the membrane was incubated with given primary antibody according to the manufacturer´s recommendations. Subsequently, the membrane was washed five times with PBS/Tween and incubated with HRP-conjugated antibody diluted 1:5000 in PBS/Tween with 1% milk for westen blotting for 1 hour. Proteins were finally detected using SuperSignal West Femto Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions.

3.9 Detection of apoptotic cells by flow cytometry

Chemicals

Annexin V binding buffer (Apronex)

Annexin V-FITC (Apronex) - a phosphatidylserine-binding protein with conjugated fluorophor used for staining apoptotic cells.

Hoechst 33258 - a fluorescent dye, which enters dead or almost dead cells and binds to their DNA. It is used for staining late apoptotic/necrotic cells.

Instruments

LSR-II (BD Biosciences)

FlowJo software (Tree Star)

On day one, cells (PANC-1, T47D) were seeded into 24-well plates in order to get 50-70% confluency at the time of autophagy-inducing treatment. On day two, the cells were first treated with autophagy-inducing agents (replacement of DMEM with EBSS, or with DMEM with Rapamycin, or DMEM with Metformin). After five hours, apoptotic inducers were added and plates were kept in 5% CO_2 incubator for 15 hours. On day three, medium with floating cells was transferred into round-bottomed 96-well plate. Floating cells were centrifuged (900rpm, 4°C, 5 min) and medium removed. Adherent cells in 24-well plates were washed with PBS, trypsinized, added to the appropriate wells in the 96-well plate and centrifuged. Pellets were resuspended in 200µl Annexin V binding buffer, centrifuged (1400rpm, 4°C, 5 min) and again resuspended in 100µl Annexin V binding buffer containing 0,5µl Annexin V-FITC. Cells were incubated for 20 minutes in dark on ice. Just before measurement, 10µl of Hoechst 33258 was added to the final concentration 4 µg/ml. The samples were measured on a flow cytometer LSR-II and evaluated using FlowJo software.

3.10 Staining and measurement of death receptors

Chemicals
PBS-GA (PBS + 0,2% gelatine + 0,1% NaN_3)
Hoechst 33258

Antibodies
DR4 (Exbio)
DR5 (Exbio)
Fas (Sigma-Aldrich)

Instruments
LSR-II (BD Biosciences)
FlowJo software (Tree Star)

Cells were grown in 6-well plates. After reaching 70% confluency, 18 hours before harvesting, part of them was treated with autophagy-inducing agents (EBSS, Rapamycin, Metformin). Another part was treated 5 hours before harvesting in the same way. After 5 hours, cells were trypsinized and each 6-well was divided into four 96-wells in round-bottomed 96-well plate. Cells were centrifuged (900rpm, 4°C, 5 min), washed twice with 200µl of cooled PBS, resuspended in 100µl of cooled PBS-GA, again centrifuged and resuspended in 20µl of cooled PBS-GA with respective primary antibodies (concentration 2µg/µl). Cells were incubated 30 minutes on ice and after that washed twice with 200µl of cooled PBS-GA and pelleted by centrifugation. Pellets were resuspended in 20µl of cooled PBS-GA with secondary antibody (4µl/2000µl PBS-GA). After a 20-minute incubation, cells were washed twice with 200µl cooled PBS-GA and finally resuspended in 90µl of PBS-GA. Just before the measurement, 10µl of Hoechst 33258 was added to each well to the final concentration 4µg/ml. Samples were measured on a flow cytometer LSR-II and evaluated using FlowJo software.

3.11 High-throughput analysis of autophagy kinetics

Materials

black 96-well plates with clear bottom (Greiner Bio-One)

Instruments

Operetta (Perkin-Elmer)

Harmony software (Perkin-Elmer)

PANC-1 cells were seeded in black clear-bottom 96-well plates. Part of them was treated with camptothecin 7 days prior to the experiment itself in order to get fully established senescent phenotype on the day of measurement. Approximately one hour before measurement, autophagy was induced using EBSS, Rapamycin (final concentration 10nM) and Metformin (final concentration 100µM). Cells in 12 fields per well were analyzed using a Operetta high-content imaging system. The number of autophagosomes and lysosomal clumps was determined using Harmony software.

4 Results

4.1 Effects of Camptothecin on PANC1 cell line

For the induction of cancer cells senescence, camptothecin, a potent inducer of DNA damage, was added to cells at various confluency in several concentrations ranging from 10nM to 100nM. Whereas the lowest concentrations very often failed to induce senescence, especially when added to more confluent cells, concentrations higher than approximately 50nM (depending on confluency) resulted in almost complete apoptosis. The optimal concentration for the induction of senescence was in most cases 25nM. Cells were incubated with camptothecin for 7 days and continuously assessed using light microscope. After 7 days, the whole culture was considered fully senescent, showing typically flattened morphology (Figure 7).

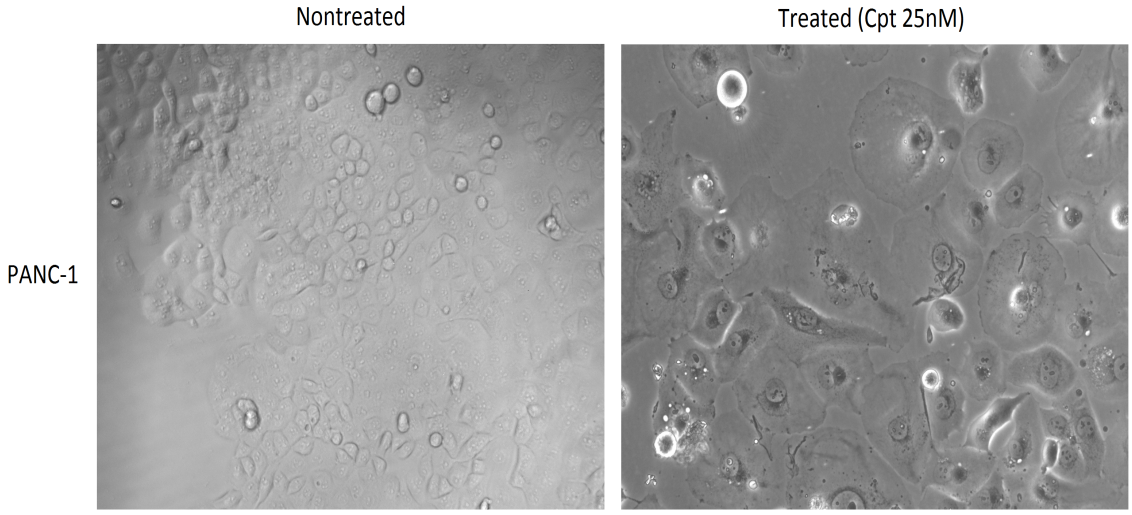


Figure 7. Changes in cellular morphology between senescent and nonsenescent cells. PANC-1 cells were treated with 25nM camptothecin for 7 days. During that time they adopted clearly recognizable senescent phenotype.

4.2 Temporal kinetics of autophagy response in PANC-1 cells

For the detection of autophagy and analysis of the kinetics of autophagic response we transduced PANC-1 cells with fusion proteins EGFP-LC3 and LAMP1-RFP.

LC3 is associated with late autophagosomes and remains bound to them till its lysosomal degradation in autophagolysosomes. It is the most widely used intrinsic autophagy-monitoring protein (Klionsky et al., 2012). EGFP-LC3 therefore allows us to detect and to some extent track single autophagosomes using advanced methods of fluorescence microscopy.

LAMP1 is a glycoprotein associated with lysosomal membrane, therefore we used its RFP-fused form to detect lysosomes. Moreover, because lysosomes form clearly distinguishable clusters (approximately one per cell) located near the nucleus, we used number of these lysosomal clusters to determine the number of cells. These transductions allowed us to conduct live-cell imaging experiments without fixation or antibody staining.

In cells cultured in DMEM + 10% FBS was autophagy induced by several approaches, most notably by their starvation in serum-less EBSS medium. The culture medium was removed and replaced by nutrient-poor medium EBSS approximately 30 minutes before measurement. We chose EBSS as an autophagy inducer, because it closely represents the natural state of nutrient (specifically aminoacid) shortage. In our experimental setup it also showed the best autophagy-inducing capability compared to other used autophagy inducers, namely rapamycin and metformin, in given concetrations as documented by more efficient processing of EGFP-LC3 in PANC-1 and also T47D cell (Figure 8) Weaker detection of EGFP-LC3B-I does not mean lower expression of LC3B but represents more rapid lipidation of nonlipidated form producing more EGFP-LC3B-II, which is also rapidly degraded after the fusion of autophagosome with lysosome. Inhibition of the fusion step with chloroquine resulted in the accumulation of autophagosomes and thus EGFP-LC3B-II form in all cells. Typical example of EGFP-LC3 foci (autophagosomes) and LAMP1-RFP foci (lysosomal clusters) is shown in Figure 9.

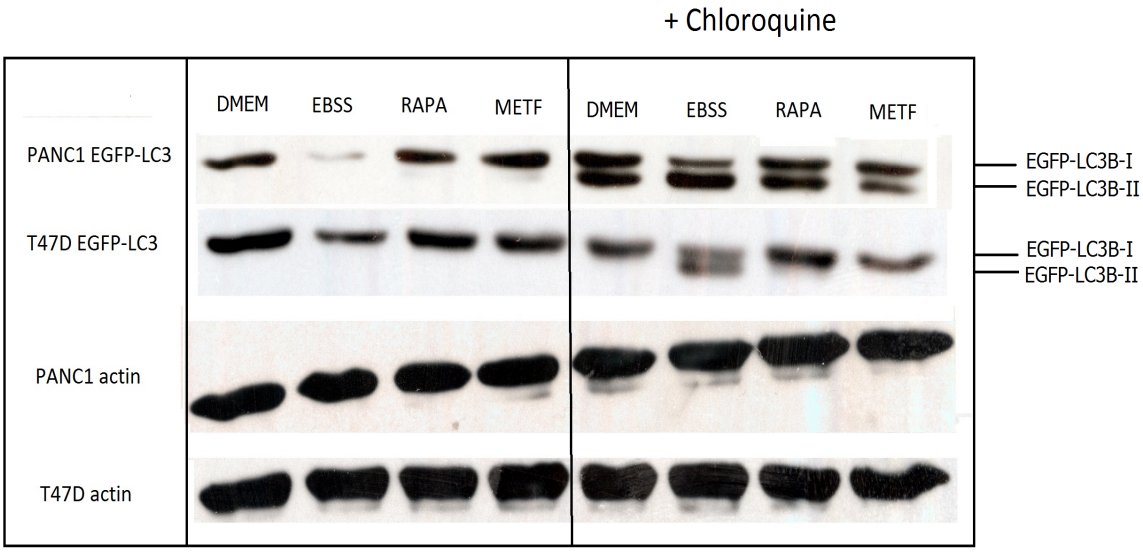


Figure 8. Various capabilities of autophagy inducers to induce autophagy in PANC-1 and T47D cell lines. Cells were treated with either EBSS for 5 hours, or 10nm rapamycin for 5 hours or 100μM metformin for 8 hours to induce autophagy, or left in DMEM for control. Chloroquine, an inhibitor of autophagosome-lysosome fusion was added to half the samples in the final concentration 70μM.. EGFP-LC3B-I bands represent a nonlipidated cytosolic form of processed LC3B, while EGFP-LC3B-II bands represent its lipidated, membrane bound form.

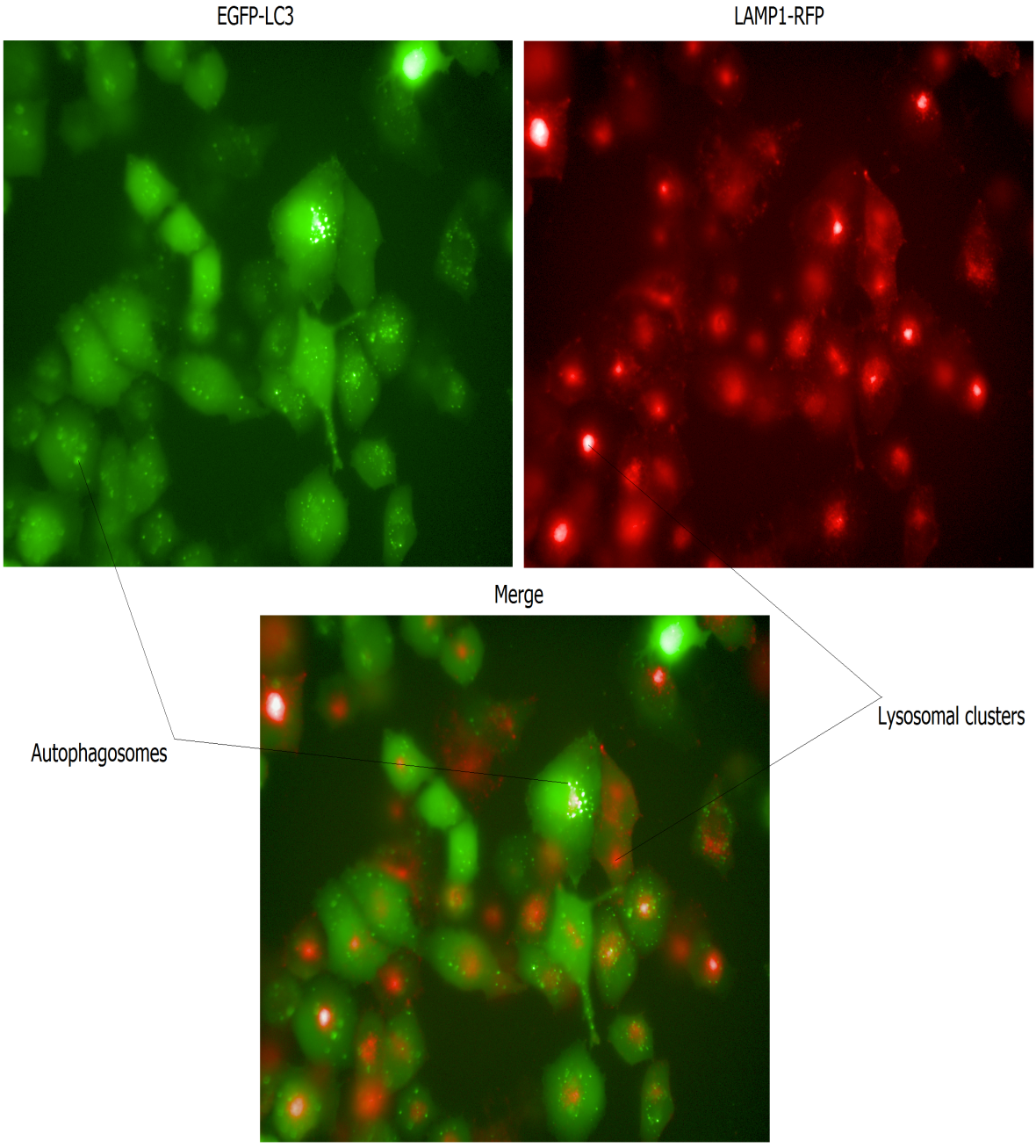


Figure 9. Autophagosomes and lysosomal clusters. PANC-1 cells were starved in EBSS for 1 hour. EGFP-LC3 and LAMP1-RFP foci were detected using Operetta high-throughput screening system.

To quantify the accumulation of autophagosomes we used the high throughput screening system Operetta in combination with the integrated image analysis software Harmony, both from Perkin–Elmer. To allow appropriate automated image analysis we developed within the Harmony software an algorithm for recognizing single autophagosomes and aforementioned lysosomal clusters.

The number of autophagosomes per cell was calculated as the number of all detected autophagosomes divided by the number of all detected lysosomal clusters in the particular image. The final result is the average from three independent experiments.

Our results document, that the autophagic response is triggered swiftly after EBSS-induced starvation. After one hour, approximately 20 autophagosomes per cell were observable. This number raised to almost 50 autophagosomes per cell after 24 hours. Interestingly, after five hours of EBSS treatment, there was relatively small, but highly reproducible drop in the number of autophagosomes interrupting otherwise rising trend of autophagosome count during prolonged starvation.

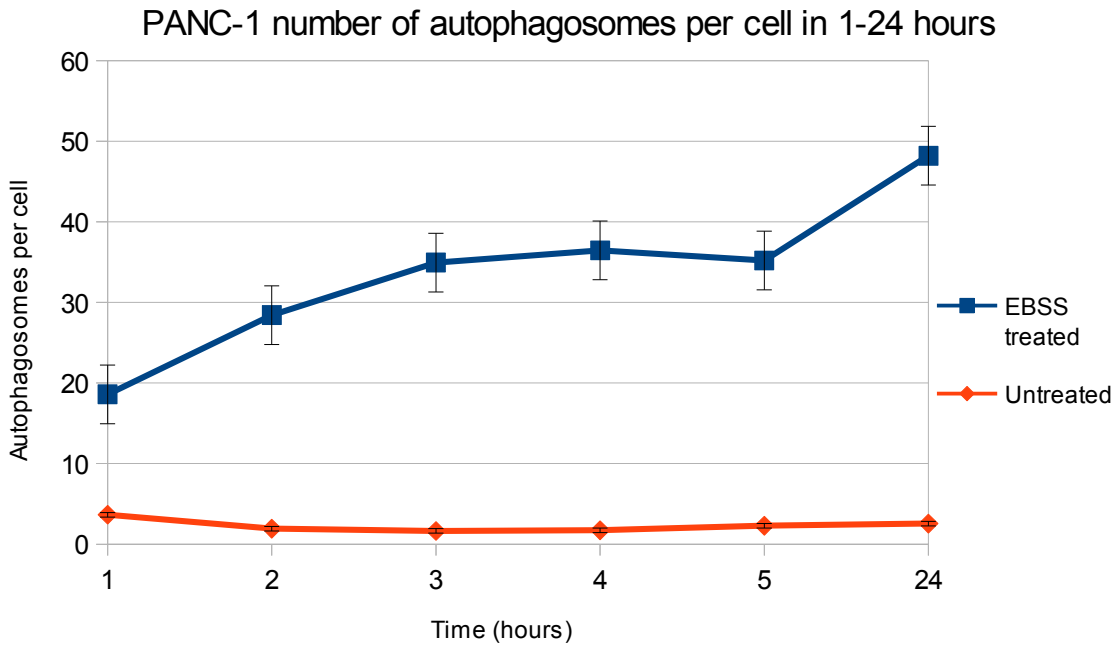


Figure 10. Increase in the number of autophagosomes in time in PANC-1 cell line. Cells were cultured in DMEM which was 1 hour before measurement exchanged for EBSS to induce autophagy. Number of autophagosomes was evaluated using Operetta high throughput screening system and Harmony software from Perkin-Elmer. The results are the average of three independent experiments. The error bars indicate average ± SD.

4.3 Temporal kinetics of autophagy response in PANC-1 senescent cells

To determine whether there is a significant difference in the kinetics of autophagosomes formation between senescent and nonsenescent PANC-1 cells, EGFP-LC3 and LAMP1-RFP transduced senescent PANC-1 cells were prepared using 25nM camptothecin for 7 days and left for one day in DMEM to recover. Approximately 1 hour before measurement, DMEM was exchanged for EBSS and cells were examined in a similar way as nonsenescent cells using Operetta high throughput screening system and the same algorithm for automated image analysis in Harmony software.

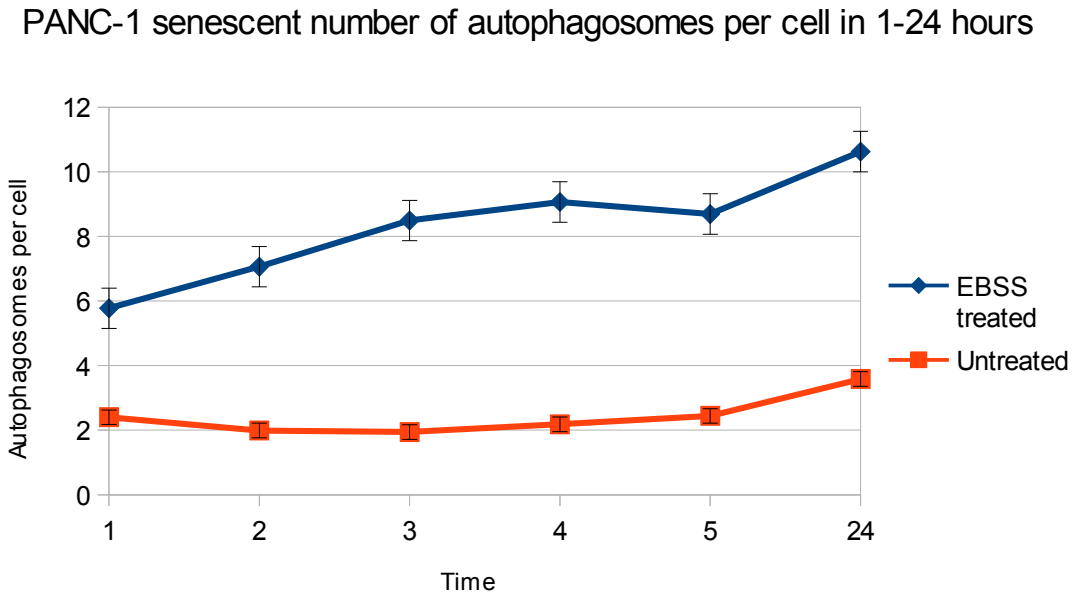


Figure 11. Increase in the number of autophagosomes in time in PANC-1 senescent cells. Senescence was induced by 25nM camptothecin. Approximately 1 hour before measurement standard DMEM medium was exchanged for EBSS to induce autophagy. Number of autophagosomes was evaluated using Operetta high throughput screening system and Harmony software from Perkin-Elmer. The results are the average of three independent experiments. The error bars indicate average ± SD.

4.4 EBSS induces stronger autophagic response in nonsenescent cells

Senescent PANC-1 cells showed in contrast to their proliferating counterparts substantially decreased number of autophagosomes. Only about 6 autophagosomes per cell were observable after one hour of incubation with EBSS and approximately 11 autophagosomes after 24 hours of incubation, which is more than 3 times or more than 4 times lower number compared to the same time points in nonsenescent cells. Interestingly, we also observed similar decrease in number of autophagosomes after five hours of EBSS treatment.

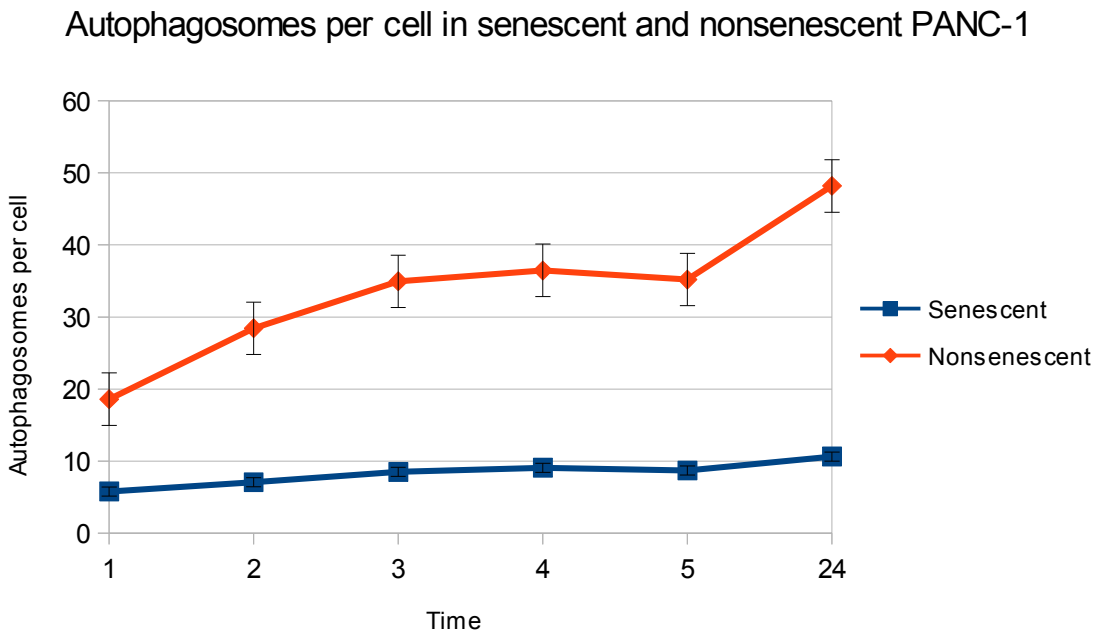


Figure 12. Comparison of number of autophagosomes in PANC-1 senescent and nonsenescent cells. Nonsenescent cells exhibit substantially increased number of autophagosomes in time. The error bars indicate average ± SD.

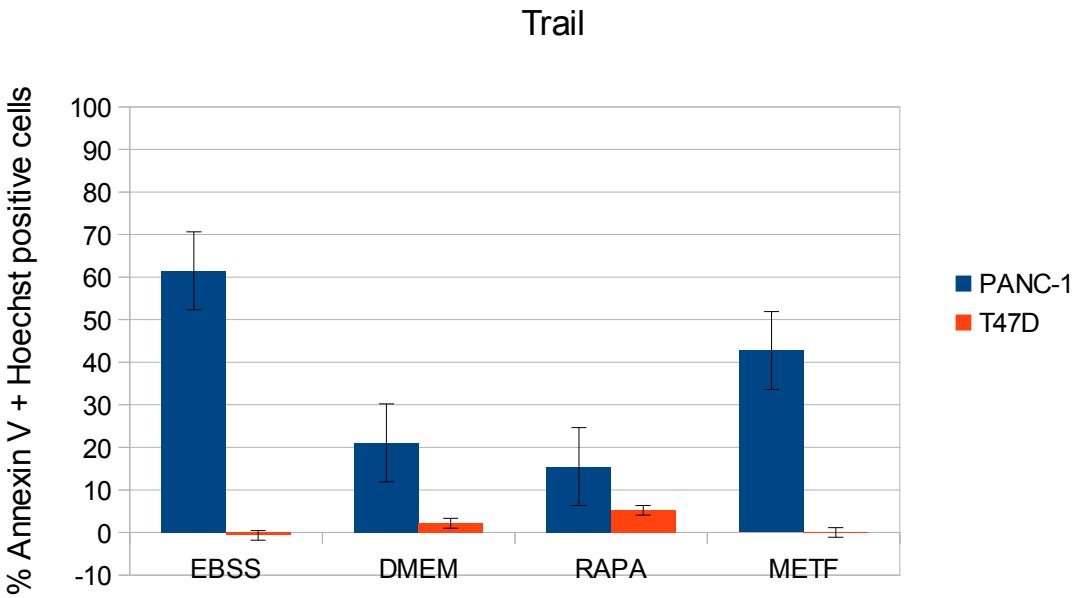
4.5 Different sensitivity of proliferating and autophagic PANC-1 and T47D cells to inducers of apoptosis

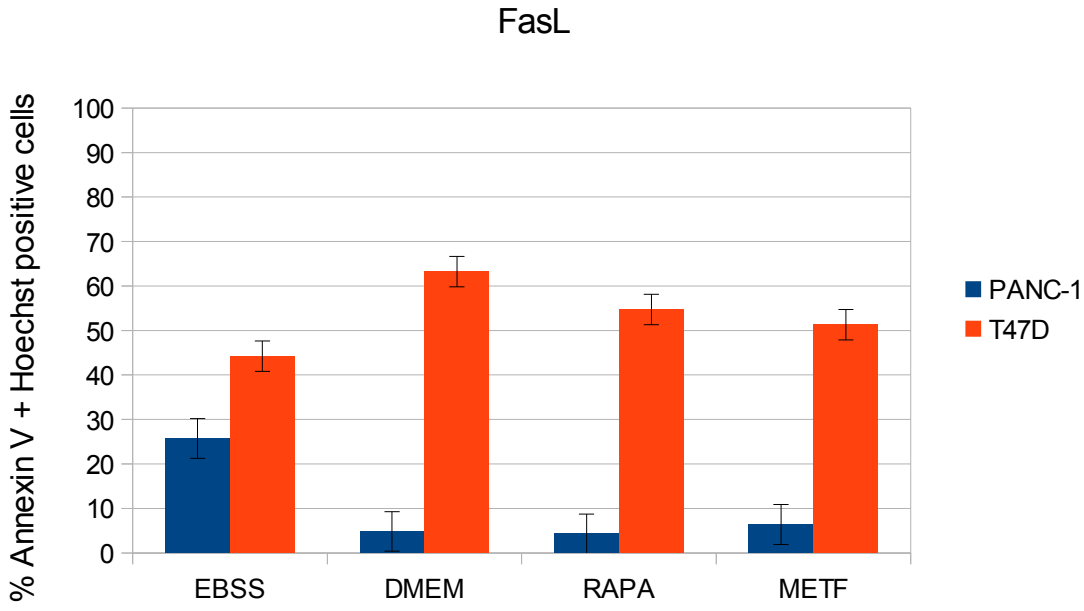
We were also interested if there are differences in sensitivity of PANC-1 and T47D cells with induced autophagy to certain apoptotic triggers. To determine an effect of various inducers of autophagy on cellular sensitivity to specific apoptogens, PANC-1 and T47D cells were first pretreated with autophagy-inducing agents EBSS, Rapamycin and Metformin. Rapamycin is a potent inhibitor of mTOR and widely used inducer of autophagy mimicking, at least partially, lack of amino acids. Metformin inhibits AMPK which could be considered to mimic glucose shortage. Concentrations and time of pretreatment before adding apoptogens were set as following: for rapamycin 10nM and 5 hours, for EBSS 5 hours and for metformin 100μM and 8 hours. After that, cells were treated with different apoptogens for 18 hours. Concentrations were 100ng/ml for Trail, 100ng/ml for Fas ligand and 10μM for MitoVes. We used Annexin V-FITC assay with Hoechst 33258 to stain apoptotic and late apoptotic/necrotic cells and quantified them by flow cytometry.

TRAIL induced apoptosis of more than 60% EBSS-starved, autophagic PANC-1 cells and more than 40% apoptosis in the case of metformin pretreatment, which is 3 times and 2 times more than in control represented here by the cells incubated in DMEM. Rapamycin on the other hand shown mild cytoprotective effect. The situation in T47D cells was completely different, as we observed almost no apoptotic cells in all cases where the cells were treated with TRAIL. Treatment of PANC-1 cells with FasL resulted to a 25% of dead cells incubated in EBSS, while only a few percent of cells died in proliferating cells or cells treated with rapamycin or metformin. In T47D cells, highest apoptotic response was observed in control (DMEM only) samples, where all three autophagy inducers shown mild cytoprotective effect. MitoVes caused a massive 70% apoptosis in both EBSS-starved cell lines. Interestingly, whereas in PANC-1 cells pretreated with rapamycin, metformin or DMEM-only, the quantity of dead cells was very low - similarly around 10%, in T47D cells this number was much higher ranging from approximately 43% in the case of metformin to around 56% in the cells treated with rapamycin.

To summarize this, autophagy induced by EBSS strongly sensitizes PANC-1 cells to apoptosis triggered by all three apoptosis inducers we used. With the exception of metformin treatment followed by apoptosis induction by Trail, no other autophagy induction imposed significant effect in terms of sensitization to apoptosis. In T47D is the

situation completely different. Not only they seem to be completely resistant to Trail-induced apoptosis whether autophagy is induced or not, but also treating these cells with any of our autophagy inductors makes them slightly more resistant to FasL induced apoptosis. Moreover, the highest degree of such protection is imposed by EBSS pretreatment, which by contrast slightly sensitizes the same cell line to MitoVes induced apoptosis.





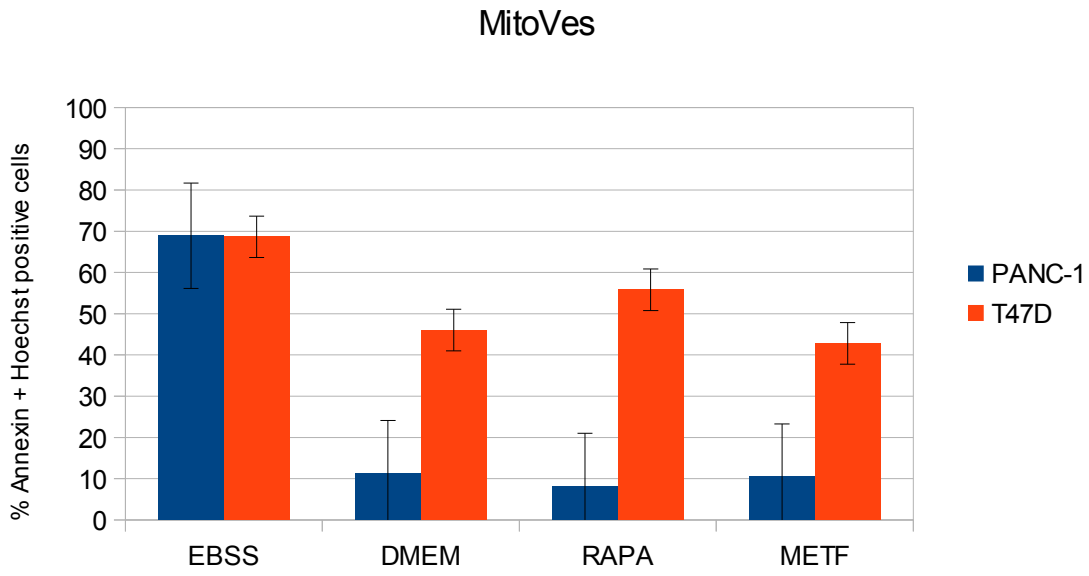


Figure 13. Comparison of sensitivity of PANC-1 and T47D cells to various apoptotic stimuli after different autophagy-inducing pretreatments. Cells were incubated with either EBSS for 5 hours, or 10nm rapamycin for 5 hours or 100 μM metformin for 8 hours to induce autophagy, or left in DMEM for control. After that, apoptotic inducers Trail (100ng/ml), FasL (100ng/ml) and MitoVes (10μM) were added and let incubate for 18 hours. Apoptosis was determined by total percentage of Annexin V and Hoechst 33258 positive cells by fluorescence-activated cell sorting (FACS). The results are the average of three independent experiments. The error bars indicate average ± SD.

4.6 Different expression of surface death receptors in PANC-1 and T47D cells with induced autophagy

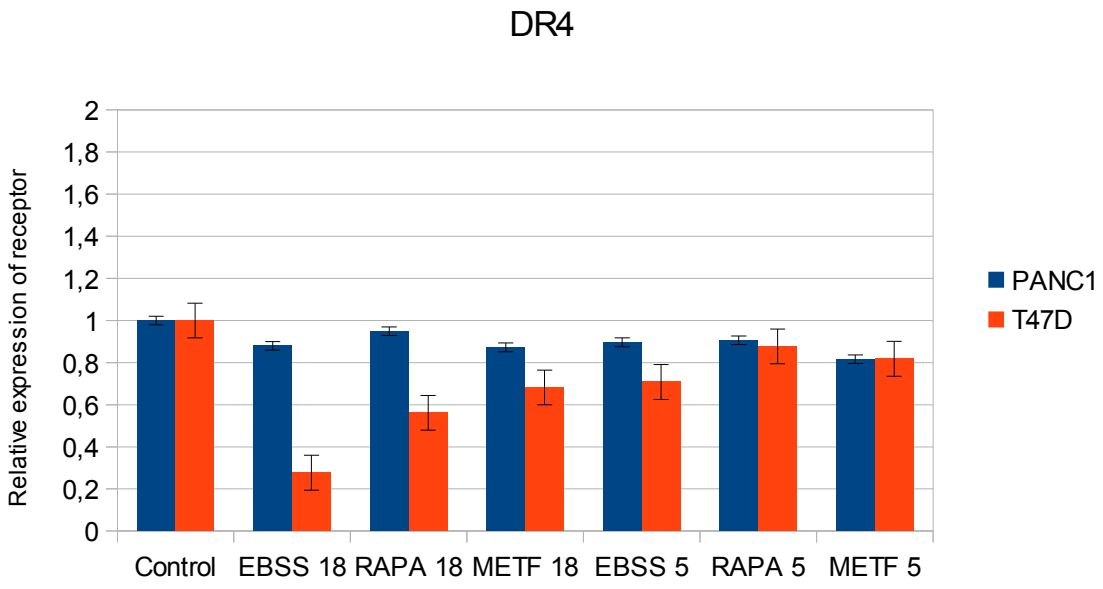
We further asked, whether increased and prolonged autophagy could contribute to sensitizing or desensitizing PANC-1 and T47D cell lines to apoptosis through changes in DR4, DR5 and Fas receptors levels. To answer this question, we treated cells with autophagy inducers EBSS, rapamycin and metformin for 5 hours or 18 hours. After that we stained cell surface death receptors with appropriate antibodies and analyzed them by flow cytometry.

DR4 receptor level was decreased in both cell lines in all pro-autophagic treatments notably more in T47D cells, especially in longer lasting treatments. The most striking was in this respect treatment with EBSS for 18 hours. It caused DR4 to drop to only 28% compared to control.

Expression of DR5 was, in contrast to DR4, markedly increased (1.8 fold) in PANC-1 cells treated with EBSS for 18 hours, but significantly decreased in T47D cells, likely reflecting cell-specific conditions. Rapamycin and metformin did not have such strong effect on TRAIL receptors expression as EBSS did (Fig. XX).

Fas receptor expression showed relatively clear patterns in both cell lines. In PANC-1, in all cases there was a slight increase not exceeding 10% while in T47D there was mild decrease in all cases, reaching minimum of approximately 80% again in the case of EBSS 18 setup.

In general,T47D cells exhibit overall decrease of all examined receptors in almost all used autophagy-inducing treatments with the exception of DR5 after metformin treatment. In PANC-1, the situation is more complicated with overall decrease of expression DR4, overall increase of FasR expression and combination of both increase and decrease of expression of DR5.



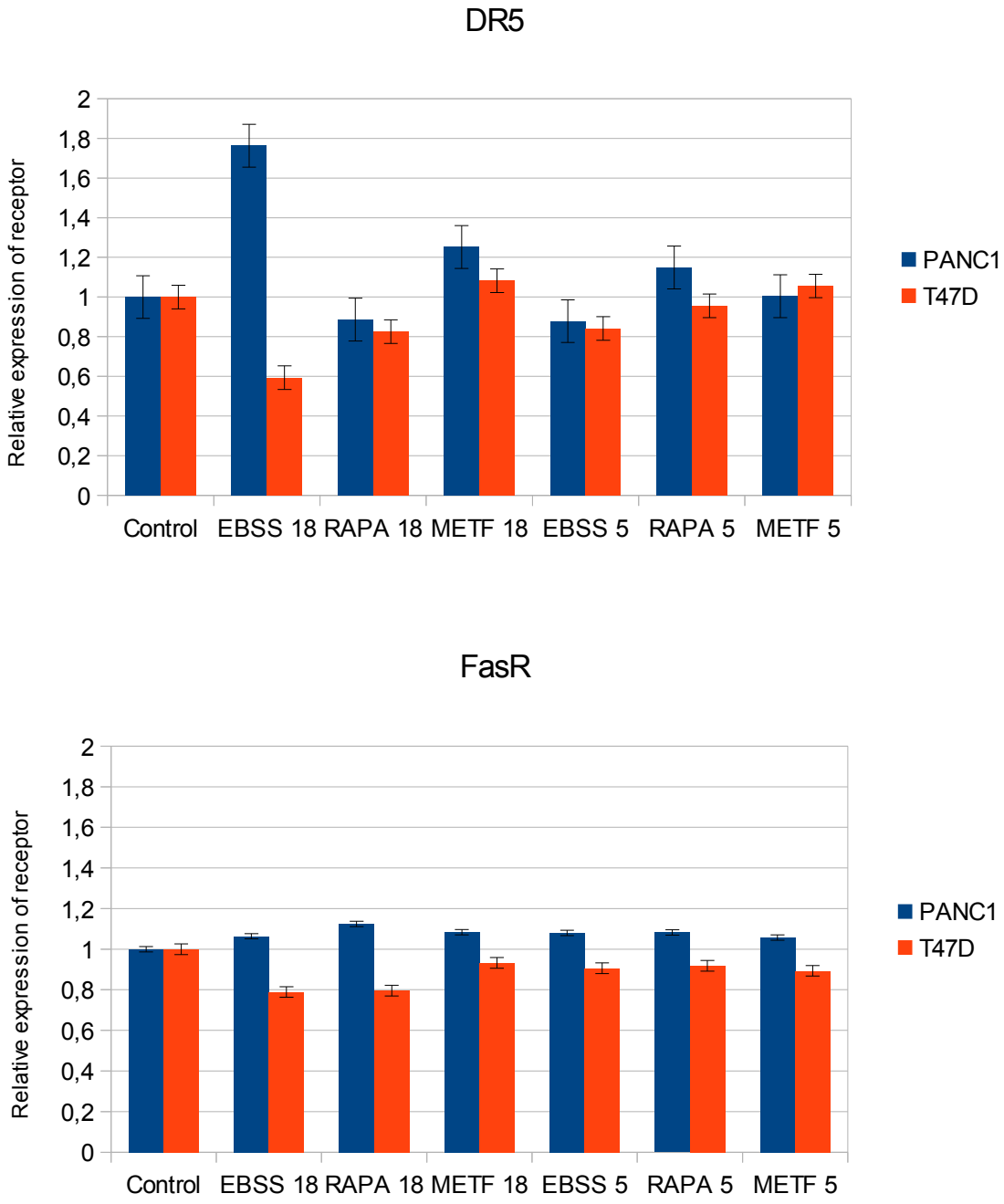


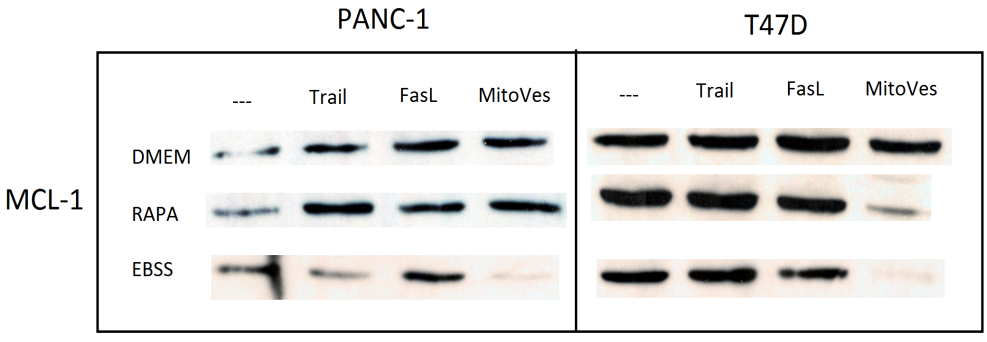
Figure 14. Relative expression of surface death receptors DR4, DR5 and FasR in PANC-1 and T47D cell lines. Cells were treated with particular autophagy inducers for 5hours or 18 hours and analyzed by flow cytometry. Control with assigned value of 1 (= 100%) represents the level of the appropriate receptor in nontreated (DMEM only) cells. The results are the average of three independent experiments. The error bars indicate average ± SD.

4.7 Different expression of Mcl-1 and Bim proteins in PANC-1 and T47D cells with induced autophagy

We further examined the expression of two proteins involved in apoptotic signalling, the antiapoptotic Mcl-1 and proapoptotic Bim, under conditions of induced autophagy. Cells were first pretreated with EBSS or 10nM rapamycin for 5 hours and then apoptosis inducers were added to the following final concentrations: 100ng/ml for Trail, 100ng/ml for Fas ligand and 10μM for MitoVes. After 18 hours cells were harvested and protein expression analyzed by western blot according to the protocol described in chapter 3.8.

Expression of antiapoptotic Mcl-1 was significantly increased in cells treated with each of apoptosis inducers when preincubated with rapamycin or DMEM (representing low or no induction of autophagy). Interestingly, when preincubated with strongly pro-autophagic EBSS, Mcl-1 expression decreased in Trail or MitoVES treated cells, while it stayed unchanged in FasL-treated PANC-1 cells. In T47D cells, the most significant changes in expression were caused by MitoVes on cells preincubated with rapamycin and even more with EBSS.

Bim expression was markedly decreased by exposure to FasL and MitoVes in rapamycin treated PANC-1 cells and even more by MitoVes in EBSS treated PANC-1. T47D cells shown slightly different pattern, but the lowest levels of expression were again achieved in combinations of EBSS + MitoVes and rapamycin + MitoVes.



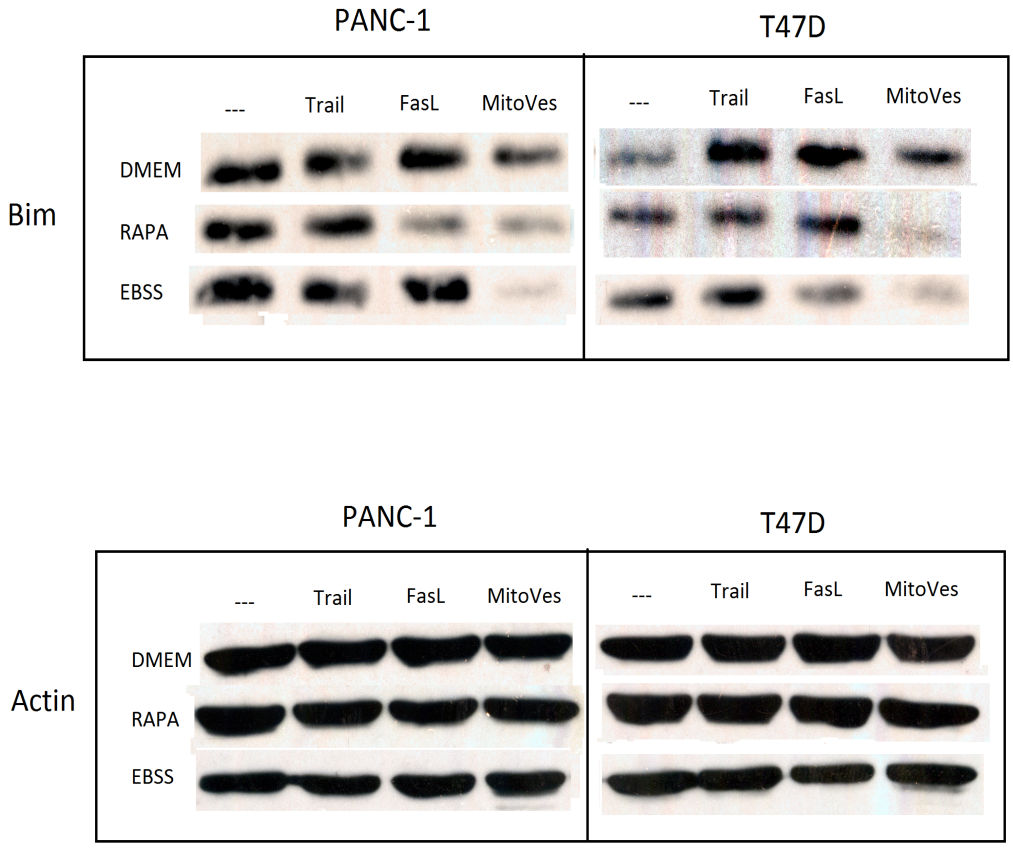


Figure 15. Mcl-1 and Bim expression in PANC-1 and T47D with various autophagic state. Autophagy was induced by EBSS or 10nM rapamycin. Cells were then treated with three different apoptosis inducers: Trail (100ng/ml), Fas ligand (100ng/ml) and MitoVes (10μM) and protein expression was analyzed by western blotting.

5 Discussion

Increased numbers of senescent cells in various tissues and organs may pose a threat to their proper function and contribute to accelerated aging. Specific removing of senescent cells is therefore currently a burgeoning area of research. It is a matter of wide discussion whether autophagy promotes or hinders adoption of senescent phenotype and whether senescent cells rely heavily on autophagy to satisfy their increased energetic and metabolic needs or conversely increased autophagy could be somehow detrimental for them (see chapter 1.6).

In the first part of this work we addressed some of these aspects by exact quantification of the number of autophagosomes per cell and its progression in time after autophagy induction in proliferating and senescent PANC-1 cells. For this purpose we transduced pancreatic cancer cell line PANC-1 with two fused proteins EGFP-LC3 and LAMP1-RFP. The former one allowed us to detect single autophagosomes, whereas the latter one allowed us to determine the number of cells and thus calculate the number of autophagosomes per cell. Measurement and analysis itself were performed using advanced high throughput screening system Operetta with Harmony software.

First, we needed to choose the most suitable autophagy inducer, so we tested three commonly used: EBSS, Rapamycin and Metformin for their capability to trigger autophagic response. EBSS proved the best autophagy-inducing ability as demonstrated by western blotting of processed LC3, confocal microscopy and Operetta screening (Data not shown). Moreover we believe, that the treatment with EBSS well simulates amino acid depletion and thus conditions in which autophagy is triggered in vivo. Chemical inducers of autophagy rapamycin and metformin performed less efficiently and a question is whether used concentrations of rapamycin and metformin were sufficient to trigger relevant autophagic response. Rapamycin was used at various concentrations in a number of studies (Li et al., 2013 ; Moad et al., 2013 ; Dutta et al., 2013). In respect to some clinical studies reporting rapamycin toxicity (Kaplan et al., 1999 ; Barlow et al., 2012 ; Marti et al., 2005), especially in higher doses, we decided to use as low concentration as possible, while still capable of inducing autophagy. A problem may be that different cell types may not adequately respond to such low concentrations. Taking all this into account, we decided to use 10nM rapamycin in all our experiments. As for metformin, which has very good clinical references regarding possible side effects, used concentration was

100μM, however it showed only weak autophagy-inducing ability.

For the above reasons we used solely EBSS to induce autophagy in the first part of our work. Our results document, that in PANC-1 cells number of autophagosomes increases rapidly after treatment. One hour after addition of EBSS more than 18 autophagosomes per cell were observable, compared to 3.5 in nontreated cells. This number raised to 28 in treated compared to only 2 autophagosomes in nontreated, representing a 14-fold increase after only 2 hours of autophagy induction. We believe, that the slight drop from 3.5 autophagosomes per cell in time point 1h to an average of 2 autophagosomes per cell in the rest of time points is some kind of artefact caused by residual autophagic response in timepoint 1h. Autophagy could have been partially triggered during switching cultivation medium and brief washing of the cells with PBS before adding fresh DMEM and gradually faded during 1-2 hours. In our setup, where the experiment was terminated after 24 hours, cells contained the highest number of autophagosomes - 48 - just in time point 24h. It represented a 19-fold increase. Currently unanswered question is whether this represents a state at least close to maximum capacity of a given cell type, or whether another significant increase is possible. We also observed mild, but highly reproducible decrease in the number of autophagosomes in time point 5h, which we are currently unable to convincingly explain. It could be a consequence of transient deficit of necessary components involved in autophagy machinery.

The earliest time from the beginning of the treatment from which we have data was 30 minutes (Data not shown). Autophagy was already substantially increased by that time. We speculate, that to reach such level even less time than 30 minutes is sufficient. Perhaps the most determining is the time needed for generation of single autophagosome, which is something between 5-10 minutes (Mizushima et al., 2001 ; Fujita et al., 2008).

The main goal in this part of the work was however to determine the differences in autophagic response measured by the number of formed autophagosomes between proliferating and senescent PANC-1 cells. To accomplish this, we exposed PANC-1 cells to 25nM camptothecin. This DNA damaging quinoline alkaloid was reported to induce senescence via inhibition of DNA topoisomerase I, specifically the rejoining step of the breakage-reunion reaction thus creating double-strand breaks. (Hsiang et al., 1985 ; Liu et al., 2000) After 7 days of camptothecin treatment, senescent phenotype was considered to be fully established, with the cells exhibiting typically senescent morphology. Subsequently they were subjected to the same treatment and analysis as nonsenescent cells. Results shown similar rising trend with the same kind of mild drop in time point 5h, but the

total number of autophagosomes per cell in senescent cells was dramatically decreased in cells with induced autophagy. Nonsenescent cells exhibited almost three times more autophagosomes after one hour of treatment. This ratio raised to almost 5:1 in a favor of nonsenescent cells after 24 hours of EBSS treatment. On the other hand, control senescent cells shown virtually the same values as nonsenescent control cells, implicating that the main difference does not refer to the increased basal level of autophagy but rather capability of the cells to respond to autophagic stimuli or perhaps their maximum capacity of autophagic response.

These findings are in contrary to data suggesting higher dependence of senescent cells on autophagy (see sections 1.6.9 and 1.6.10), but it is good to keep in mind, that most of the results of this type can vary dramatically depending on the cell type and due to pleiotropic effects of most autophagy inducers. Further experiments on different cell lines and deeper investigation is needed before some general conclusions can be made.

The exact impact of autophagy on cell death is still unclear with publications reporting its strong cytoprotective effect but also its negative role in cell survival. In the second part of the work, we addressed a question whether increased autophagy could sensitize PANC-1 and T47D cancer cells to apoptosis triggered by various apoptotic inducers and whether there is some difference in such sensitization when different autophagic inducers are used for pretreatment. For this purpose, cells were exposed to either EBSS, or 10nM rapamycin or 100μM metformin for given time and then treated with TRAIL or FasL or MitoVes. We clearly observed that the selected cell lines respond quite differently to apoptotic treatments independently of autophagy induction. PANC-1 showed strong resistance to all three apoptogens. This resistance is well documented in literature, in many cases along with responsible mechanisms (Khanbolooki et al., 2006 ; Elnemr et al., 2001 ; Shabaik et al., 2013). Interestingly, EBSS treatment largely enhanced PANC-1 sensitivity to selected apoptogens, most significantly to MitoVES – more than 6-fold. This cell death-enhancing effect of EBSS was observed also when apoptosis was induced by Fas ligand and TRAIL, where it caused 5-fold respectively 3-fold increase. Such enhancement was not observed in other autophagy-inducing treatments, only in TRAIL-induced apoptosis metformin performed as a weak sensitizer. It is in accordance with data recently obtained by Zhang et al. on human bladder cancer cells, where metformin enhanced TRAIL-induced apoptosis through mTOR/S6K1-mediated downregulation of c-FLIP (Zhang et al., 2014). These data along with study conducted by Zhao et al. (Zhao et al., 2013) suggest, that this kind of

enhancement may not be a consequence of autophagic process per se, but rather transcriptional activities of mTOR. In combination with two other apoptosis inducers, however, metformin did not exhibit any significant effect in PANC-1 cells. Rapamycin even showed a very small, almost negligible antiapoptotic effect, which definitely is not surprise due to a plethora of reports pointing to its cytoprotective functions. Death receptors-induced apoptosis is initiated at their receptors and thus we analyzed expression of surface death receptors DR4, DR5 and Fas receptor. Expression of DR4 was overally slightly reduced and expression of Fas receptor overally slightly increased in all cases, probably not much reflecting change in cell death. DR5 on the other hand with 1.8-fold increase in expression after 18 hours of EBSS treatment could have significantly contributed to EBSS-mediated sensitization of PANC-1 cells to TRAIL. Similar, but much lower increase was detected also when cells were treated with metformin for 18 hours, further supporting role of DR5 receptor in sensitizing of PANC-1 cells.

As mentioned above, situation in T47D cells was completely different. This cell line showed almost complete resistance to TRAIL induced apoptosis which is in agreement with several papers reporting strong TRAIL resistance in breast cancer cells including T47D (Zhang and Zhang 2008 ; Guseva et al., 2008). Situation in FasL treatment was quite opposite, as the mortality of the cells reached over 60% in control DMEM-only cells with all used autophagy inducers lowering this value and therefore imposing antiapoptotic effect. According to various studies (Guseva et al., 2008 ; Keane et al., 1996) , such strong impact of FasL is not surprising. Also our results shown high expression of Fas receptor in T47D cells (Data not shown). Cytoprotective effects of autophagy on breast cancer cell lines have been documented in few studies (Ravikumar et al., 2006 ; Thomas et al., 2011) and our results support it well in the case of Fas ligand-induced apoptosis, but not in the case of MitoVes (ROS) -induced apoptosis, where rapamycin and especially EBSS pretreatment significantly enhanced MitoVES-induced apoptosis. Analysis of the expression of surface receptors shed some light into this. Except subtle increase of DR5 after metformin treatment, expression of all three receptors was downregulated in all cases. This downregulation was especially pronounced when cells were treated with EBSS (probably just simple consequence of stronger autophagic response). These results corelate with cell death rates in FasL treatment but not in MitoVes-treated cells arguing that apoptosis in the case of MitoVes is relatively independent on surface receptors expression. That is in an agreement with the mechanism of MitoVes action, which triggers apoptosis through generation of ROS and intrinsic apoptotic pathway.

We also examined expression of two proteins involved in autophagy and apoptosis, the antiapoptotic Mcl-1 (Germain et al., 2011) and the proapoptotic Bim (Gillings et al., 2009). However the expression of proteins did not show any apparent correlation with previously observed results in neither of our cell lines and deeper analysis is therefore required to provide appropriate explanation.

Taken all the observations together, it seems that concomitant exposure of PANC-1 cells and to a lesser extent also T47D cells to particular apoptogene and strong autophagic response caused by amino acid shortage leads to substantial increase in cellular mortality. We hypothesize, that lack of available nutrients (amino acids) leads to reduced ability of the cell to cope with apoptogenic stress, notably when it is caused by apoptogenes that ultimately lead to cell damage, as it is in the case of MitoVes. Cancer cells with increased metabolic demands could be especially prone to this kind of combined approach and if these results are confirmed in vivo it could possibly constitute new possibilities for treatment of certain cancers. One of the most-prospective advantages of this kind of (chemo)therapy enhancement is that the "enhancing agent" is not an agent at all, but it could be mimicked by some kind of diet restrictions. However it is good to keep in mind, that such simple extrapolation of results from cell culture to complex organism is always problematic and a lot of further research needs to be done.

6 Conclusions

1) Using lentiviral vectors we prepared EGFP-LC3 and LAMP1-RFP double-transduced pancreatic cancer PANC-1 cells and used them for the analysis of single autophagosomes and autophagy kinetics inside living cells during 24 hours period.

2) We analyzed the cellular autophagic response to three inducers of autophagy: EBSS, rapamycin and metformin and found out that EBSS is the strongest inducer of autophagy in our system.

3) We set up a protocol for induction of senescence in PANC-1 cancer cells using their 7-day treatment with 25nM camptothecin.

4) We determined and compared autophagic response represented here as a number of detected autophagosomes per cell, in proliferating and senescent PANC-1 cells. We observed a dramatic decrease of number of autophagosomes in senescent PANC-1 cells in all time points compared to proliferating PANC-1 cells. The disparity reached its maximal value at the last measured time point (24 hours), senescent cells showed here only 1/5 of the number of autophagosomes detected in proliferating PANC1 cells.

5) We analyzed changes in sensitivity of pancreatic cancer PANC-1 cells and breast carcinoma T47D cells to three diferent apoptogens TRAIL, FasL and MitoVes after pretreatment with autophagic inducers EBSS, rapamycin and metformin. We observed significant increase in the number of dead cells in response to all apoptogens in PANC-1 cells pretreated with EBSS. Likely the amino acid deprivation may pose additional and under certain circumstances unbearable burden for already stressed cancer cells. However results obtained from T47D cells not always complied with PANC-1 cells reflecting cell type–specific response. EBSS enhanced apoptosis/necrosis only in case of MitoVes, while in case of FasL acted as well as other autophagy inducers cytoprotectively.

6) We quantified the cell surface expression of death receptors DR4, DR5 and FasR and observed significant 1.8-fold increase in expression of DR5 in PANC-1 cells , which probably contributed to the increase in cell death after TRAIL treatment. T47D showed

overally decreased levels of receptors, where the lowest value was when the cells were treated with strongest autophagy inducer EBSS, probably reflecting magnitude of degradation caused by autophagy and affecting most of the proteins in cell.

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