THÈSE

PRÉSENTÉE POUR L'OBTENTION DU GRADE DE:

DOCTEUR DE L'UNIVERSITÉ LILLE 1 SCIENCES ET TECHNOLOGIES

ECOLE DOCTORALE BIOLOGIE SANTÉ LILLE NORD DE FRANCE

DISCIPLINE: ASPECTS MOLÉCULAIRES ET CELLULAIRES DE LA BIOLOGIE

Calcium, Calcium-permeable channels and autophagy modulators in control of autophagy and cancer

Thèse dirigée par le Professeur Natalia Prevarskaya et co-dirigée par le Dr. Christian Slomianny présentée et soutenue publiquement par

Maya YASSINE

3 Décembre 2013

Membres du jury:

Dr. Dominique Legrand, Université Lille 1	Président
Pr. Geert Bultynck, University of Leuven (Belgium)	Rapporteur
Pr. Fabrice Matifat, Université d'Amiens	Rapporteur
Pr. Mergny Djavaheri, Université Bordeaux Ségalen	Examinateur
Dr. Christian SLOMIANNY, Université Lille 1	Examinateur
Pr. Natalia PREVARSKAYA, Université Lille 1	Examinateur

Thèse réalisée au sein du Laboratoire de Physiologie Cellulaire INSERM U1003, Université Lille 1, Villeneuve d'Ascq, France

Abstract

Autophagy is a tightly regulated cellular pathway the main purpose of which is lysosomal degradation and subsequent recycling of cytoplasmic material to maintain normal cellular homeostasis. Defects in autophagy are linked to a variety of pathological states, including cancer. Cancer is the disease associated with abnormal tissue growth following an alteration in such fundamental cellular processes as apoptosis, proliferation, differentiation, migration and autophagy.

Calcium is a ubiquitous secondary messenger which regulates plethora of physiological and pathological processes such as aging, neurodegeneration and cancer. The role of calcium and calcium-permeable channels in cancer is well-established, whereas the information about molecular nature of channels regulating autophagy and the mechanisms of this regulation is still limited. The role of autophagy in cancer is complex, as it can promote both tumor prevention and survival/treatment resistance. Elevated autophagy is often detected in cancer cells in response to radiation and chemotherapy. Furthermore, autophagy seems to contribute to the therapeutic resistance of some cancers. It's now clear that modulation of autophagy has a great potential in cancer diagnosis and treatment.

Our findings identified intracellular calcium as an important regulator of autophagy. We propose a possible link between calcium, calcium permeable ion channels, autopohagy and cancer progression. Further, our results revealed a new autophagy modulator ML-9 as an attractive tool for targeting autophagy in cancer therapy.

Résumé

L'autophagie est une voie cellulaire strictement régulée dont le but principal est la dégradation lysosomale et le recyclage ultérieur du matériel cytoplasmique afin de maintenir l'homéostasie cellulaire normale. Des défauts dans l'autophagie sont liés à une variété d'états pathologiques, dont le cancer. Le cancer est une maladie associée aux modifications des processus cellulaires fondamentaux tels que l'apoptose, la prolifération, la différenciation, la migration et l'autophagie.

Le calcium est un second messager ubiquitaire qui régule une série de processus physiologiques et pathologiques tels que le vieillissement, la neurodégénérescence et le cancer. Si le rôle du calcium et des canaux calciques dans le cancer est bien établi, l'information sur la nature moléculaire des canaux régulant l'autophagie ainsi que les mécanismes de cette régulation reste encore limitée. Le rôle de l'autophagie dans le cancer est complexe. En effet, elle peut favoriser à la fois la prévention tumorale, le résistance aux traitements et la survie des patients. Autophagie est souvent détectée dans les cellules cancéreuses en réponse aux expositions aux rayons et la chimiothérapie. De plus, l'autophagie semble contribuer à la résistance thérapeutique de certains cancers. Il est maintenant bien établi que la modulation de l'autophagie peut potentiellement contribuer à la mise en œuvre des méthodes de traitement du cancer.

Dans cette étude, nos travaux ont permis d'identifier le calcium intracellulaire, comme un régulateur important de l'autophagie. Ainsi, nous proposons un lien possible entre le calcium, les canaux calciques, l'autophagie et la progression du cancer. De plus, nous avons mis en évidence un nouveau modulateur de l'autophagie, le ML-9. Cet outil pourrait potentiellement cibler l'autophagie et être utilisé dans le traitement des cancers.

To my mother's soul

family

&

husband

Madame and Sir Jury members,

I am very thankful and grateful to all of you for accepting to be part of my jury. I thank you for the time you spent in reading and editing my thesis. I also thank you for your criticism because without it I cannot improve and be motivated to give the best I can. I would also like to thank you for the research work you have done to enrich our knowledge in science, each in her/his field of specialty. Finally, I hope I will be up to your expectations.

Dr. Dominique Legrand, it is a great honor for me to have you as a president of my jury. I am sincerely thankful to you.

Pr. Geert Bultynck, thank you for the time you have dedicated to me in your first visit to our laboratory and thank you again and again for the time and effort you will offer as a rapporteur of my thesis. I appreciate it very much.

Pr. Fabrice Matifat, I would like to thank you for being my rapporteur and for the time you will dedicate to me for improving my manuscript. Your comments are of great value to me.

Pr. Mergny Djavaheri, I would like to thank you for accepting to be part of my jury. I am honored to have you as an examinateur.

Dr. Christian Slomianny, I want to thank you for your patience and help all through my Ph-D study. Thank you for being there for me as an instructor and a mentor.

Pr. Natalia Prevarskaya, thank you is not enough to describe my gratitude and appreciation for your guidance and surveillance of my work and also for life making decisions.

I cannot finish without saying thank you, for ERASMUS MUNDUS and Hariri foundation for giving me the chance to do my Ph-D studies in France and for ***every member*** of our team in the Laboratory Physiologie Cellulaire for adding more to my scientific knowledge and mastering of techniques. Finally, I would like to express my biggest thanks to ***Artem*** and ***Katya***, I would not have done it without you!

Outline

Glossary

2-APB	2-aminodiethyldiphenyl borate
3-MA	3-methyl adenine
AC	adenylyl cyclase
ADPR	adenosine diphosphoribose
AMPK	adenosine monophosphate activated protein kinase
AR	androgen receptor
ATCC	american type culture collection
ATG	autophagy related protein
Atg	autophagy related gene
ATP	adenosine 5'-triphosphate
Ca2+	calcium
[Ca2+]i	intracellular calcium
cd2+	cadmium
CAD	CRAC activation domain
CAI	Carboxyamidotriazole
cAMP	3'5' cyclic adenosine monophosphate
CaN	calcineurin
CREB	cAMP response element binding protein
CICR	calcium induced calcium release
CMA	chaperone mediated autophagy

CQ	chloroquine
DAG	diacylglycerol
DAPK	dependent death associated protein kinase
DMEM	dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DSCR1	Down's syndrome critical region 1
DYRK1A	dual-specificity tyrosine-phosphorylated and regulated kinase 1A
EDTA	éthylène diamine tétraacétique
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
GFP	green fluorescent protein
HEK-293	human embryonic kidney 293 cells
ICRAC	calcium release activated calcium current
IGF	Insulin like growth factor
IP3	inositol triphosphate
IP3R	inositol triphosphate receptor
LC3	light chain 3
Li 3+	Lithium
LNCaP	Lymph node carcinoma of the prostate
LRRK2	leucine-rich repeat kinase-2
MAPK	mitogen activated protein kinase
MCU	mitochondrial calcium uniporter

MCUR1	mitochondrial calcium uniporter regulator 1
MLCK	myosin light chain kinase
MPT	mitochondrial permeability transition
MPTP	Mitochondrial Permeability Transition Pore
mtor	mammalian target of rapamycin
MTS	3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium salt
MW	molecular weight
NAADP	nicotinic acid adenine dinucleotide phosphate
NE	neuroendocrine
NF-AT	nuclear factor of activated T cell
Orai1	calcium release activated calcium channel protein1
PBS	phosphate buffered saline
PC3	prostate adenocarcinoma
PE	phosphatidylethanolamine
PI3K	phosphoinositide 3 kinase
PIP3	phosphatidylinositol (3,4,5)
PLC	phospholipase C
PM	plasma membrane
PMCA	plasma membrane calcium ATPases
prApe1	precursor form of aminopeptidase I
PSA	prostate specific antigen

PTEN	phosphatase and TENsin
PTP	permeability transition pore
Rb	retinoblastoma
Rheb	Ras homology enriched in brain
ROCs	receptor operated channels
RT-PCR	reverse transcription polymerase chain reaction
RyR	ryanodine receptor
SAM	sterile α-motif
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	sarcoendoplasmic reticular calcium ATPases
siRNA	small interfering Ribonucleic acid
SOAR	STIM-Orai activating region
SOC	Store operated calcium
SOCE	store operated calcium entry
STIM1	Stromal interaction molecule 1
TG	thapsigargin
TPCs	two pore channels
TRP	transient receptor potential
TRPA	transient receptor potential ankyrin-like
TRPC	transient receptor potential canonical
TRPML	transient receptor potential mucolipin
TRPM	transient receptor potential melastatin

TRPP	transient receptor potential polycystin
TRPV	transient receptor potential valinoid
tor	target of rapamycin
Tsc	tuberous sclerosis complex
VEGF	vascular endothelial growth factor
VGCCs	voltage-gated calcium channels
VOCs	voltage operated channels
XeB	xestospongin

Introduction

I- Cancer

A- Hallmarks of Cancer

Cancer affects the young and old, the rich and poor, men, women and children without discrimination and represents a burden on patients, families and societies. Cancer is considered to be a chronic disease formed due to dynamic changes in the genome, where the oncogenes have dominant gain of function as compared to tumor suppressor genes with recessive loss of function. It is a succession of genetic alterations causing the change of normal cells to malignant cancers. Even though, the human body has different mechanisms for maintaining the genome and DNA sequence information in order, like the presence of checkpoints that act at critical times, cancer can still develop due to malfunction of components of this genome. Thus, causing genome instability and generation of mutant cells (Kreuzaler et al., 2012).

Cancer is one of the leading causes of death in the world, particularly in developing countries. In 2008, cancer accounted for 7.6 million deaths which is equivalent to 13% of deaths in the world. Moreover, the deaths from cancer are projected to continue to rise to over 13.1 million in 2030 worldwide (http://www.who.int/cancer/en/index.html). Lung cancer causes the most cancer deaths each year. It is followed in death's frequency by prostate cancer, with approximately one out of six men are being diagnosed with prostate cancer worldwide. The research done in this thesis was conducted mainly on prostate cancer cells, so there will be a focus on cancer in general and prostate cancer in specific. Prostate cancer can develop fast due to metastasis leading to eventual death of about 250,000 men yearly. In 1990, the prostate specific antigen was discovered which lead to the diagnosis of this cancer in its early stages paving the way for the ability to stop cancer in its early form, significantly increasing chances of arresting disease in its benign stage (Lilja et al., 2012). Since that time, intensive research was done to treat cancer in its all stages. However, further investigations must be done to reach a proper understanding of changes that lead to the progression from a pre-cancerous lesion to a malignant prostate tumor. These changes could be a result of various cellular interactions.

There are more than 100 types of cancer, each has specific genotype. However, most of these genotypes can be classified in terms of six alterations in physiology that characterize

malignant growth (Hanahan and Weinberg, 2011). The six essential alterations and hallmarks of cancer are as the following and as shown in (**Figure 1).**

- Sustained proliferation: Cancer cells secrete mitogens as well intrinsic growth factors represented by neuroendocrine (NE) differentiated cells. NE differentiation occurs in carcinomas such as colorectal, lung, breast and prostate. The ability of tumor cells to produce their own growth signals through the activation of Ras oncogene makes them independent of obtaining growth factors from other cells within the tissues or induces neighboring cells to secrete growth signals so as these tumor cells can proliferate. Normal cells cannot proliferate without such stimulatory signals (Hanahan and Weinberg, 2000). However, cancer cells can also activate signaling pathways operating downstream of growth receptors (Hanahan and Weinberg, 2011). Cancer cell genomes show somatic mutations in certain human tumors causing activation of signaling pathways usually stimulated by activated growth factor receptors (Davies and Samuels, 2010). Cancer tumors could also show catalytic mutations in phosphoinositide 3-kinase (PI3K) pathway, causing hyperactivity in PI3K signaling including Akt that is its key transducer (Jiang and Liu, 2009).

It is also worth noting that defects in negative feedback mechanisms that are usually responsible in attenuating proilerative signaling enhances proliferation in tumors (Wertz and Dixit, 2010). An example to this would be RAS oncoprotein. Ras is oncogenic not due its hyperactivation but due to mutations in its genes compromising Ras GTPase activity that usually operates as a negative feedback mechanism. Another mechanism involves PTEN phosphatase which usually degrades PI3K product phosphatidylinositol (3,4,5)triphosphate (PIP3) and thus counteracts PI3K (Jiang and Liu, 2009). During tumorigenesis, loss of function mutations in PTEN amplifies PI3K signaling and thus sustain proliferation. Phosphate and TENsin (PTEN) expression is usually lost by promoter methylation in human tumors (Yuan and Catley, 2008).

Another example is mammalian target of rapamycin (mtor) kinase, the cell growth and metabolism regulator. Activation of mtor by negative feedback loop inhibits PI3K signaling (Sudarsanam and Johnson, 2010). However, when mtor is inhibited in cancer cells, the activity of PI3K is increased due to the loss of negative feedback, thus achieving sustained proliferation in those cells.

Finally, in some cases the ever increasing expression of some oncoproteins like RAS does not result in increased cancer proliferation but rather non proliferative but viable state known as senescence (Collado and Serrano, 2010).

- Evading gorwth suppressors: Tissue homeostasis depends on the action of multiple mitogens and extracellular growth inhibitory signals that limit proliferation by inducing proliferation arrest, apoptosis, differentiation or senescence. Cancer cells are able to escape such anti proliferative signals through the disruption of retinoblastoma (Rb) and P53 protein pathways. The loss of Rb suppressor and liberation of transcription genes that usually control the progression from G1 to S phase allows cell proliferation regardless of antigrowth signals that demands cells to be quiescent. Therefore, when Rb, the gatekeeper of cell cycle progression, is not present proliferation is sustained (Hanahan and Weinberg, 2000). Inhibiting proliferation depends on other factors as well. P53 senses abnormalities , stress and if the genomic damage is enormous P53 prevents cell cycle progression until the damage is removed or apoptosis takes place (Burkhar and Sage, 2008). Moreover, cancer cells overexpress c-myc oncoprotein which allows the impairement of differentiation and promote growth.

Finally, it has been shown that cell to cell contact formed by dense populations of cells suppress cell proliferation. However, this cell to cell contact inhibition is abolished in various types of cancer cells where the mechanisms of contact inhibition are being investigated (Hanahan and Weinberg, 2011).

- Resisting cell death: The ability of tumor cells to expand in number depends not only on the proliferation rate of the cells but also on the rate of cell death by the programmed cell death, apoptosis. Cancer cells are able to resist apoptosis through production of survival factors and through mutation in the tumor suppressor p53 protein. The inactivation of p53 protein is found in more than 50 % of human cancers and results in defects in the apoptotic machinery (Hanahan and Weinberg, 2000). In fact, apotosis is attenuated in tumors that become malignant and resistant to therapies (Adams and Cory, 2007). Tumors can escape apoptosis by increasing the expression of antiapoptotic regulators like Bcl-2 and/ or down regulating the proapoptotic factors like Bax, Bim and Puma. Recent research has shown interconnections between the pathways governing apoptosis, autophagy and celluar

homeostatsis. For example, they share PI3K, AKT and mtor pathway. It could be that nutrient deprivation, radiotherapy and other stresses can induce elevated levels of autophagy that could be the reason for cancer cells survival due to the cytoprotective role of autophagy (Hanahan and Weinberg, 2011).

Another form of cell death that can be programmed is necrosis. Necrotic cell death, unlike apoptosis and autophagy, releases inflammatory signals into the surrounding tissues. Evidences show that immune inflammatory cells can be tumor promoting allowing cancer cell proliferation, angiogenesis and invasiveness. Thus, these tumor promoting inflammatory cells can be beneficial for the survival of cancer cells (White et al., 2010; Levine and Kroemer, 2008).

- Enabling replicative immortality: Normal cells possess an intrinsic program that limits their proliferative growth to 60, 70 divisions. The prevention of chromosomal telomere shortening during the replication of cancer cells due to the enhanced activity of telomerase makes tumor cells immortal and that is to multiply without limit (Hanahan and Weinberg, 2000). Cells are considered immortal when they emerge from a population in crisis/dying which have escaped senescence and thus exhibited an unlimited replicative potential (Hanahan and Weinberg, 2011).

- Inducing angiogenesis: The presence of oxygen and nutrients and evacuating carbondioxide and metabolic wastes are crucial events for keeping cell integrity. Thus, for tumors to progress they must stimulate the growth of new blood vessels from the endothelium of the existing vasculature which is known as angiogenesis. During tumor development, tumors activate the angiogenic switch from vascular quiescence by playing around with the balance between angiogenesis inducers and inhibitors especially with increasing the production of the vascular endothelial growth factor (VEGF) (Hanahan and Weinberg, 2000). Other proangiogenic signals, such as fibroblast growth factor family help sustain tumor angiogenesis (Baeriswyl and Christofori, 2009). Recently, it has been shown that cells originating from the bone marrow have a role in pathological angiogenesis associated with tumor growth, such cells include macrophages and neutrophils (Qian and Pollard, 2010).

- Activating invasion and metastasis: Tumor cells can penetrate blood or lymphatic vessels, circulate through the intravascular system and then proliferate at another site; the process

known as metastasis. Metastasis is the cause of 90 % of human cancer deaths. The most important alteration observed in cancer metastasis concerning the cell-environment interaction is the inactivation of E-cadherin, a cell to cell interaction molecule found on epithelial cells. Most of the epithelial cancers have inactive E-cadherin or beta catenin genes, transcriptional repression or proteolysis of cadherin extracellular domain (Hanahan and Weinberg, 2000). Another factor that affects the invasive and metastatic property of cancer is protease genes that are up regulated and protease inhibitor genes that are down regulated. It is thought that successful invasion and metastasis occurrence requires the involvement of all of the above mentioned hallmarks of cancer (Hanahan and Weinberg, 2000). Novawadays, the process of invasion and metastasis is known as an invasion-metastasis cascade beginning with a local invasion, intravasation of cancer cells into nearby blood and lymphatic vessels , transit of cancer cells through the lymphatic systems, followed by extravasation and that is by the escape of cancer cells into the parenchyma of distant tissues, the formation of small nodules of cancer cells known as micrometastasis and followed by the last step known as colonization through the growth of microscopic lesions into macroscopic tumors (Talmadge and Fidler, 2010).

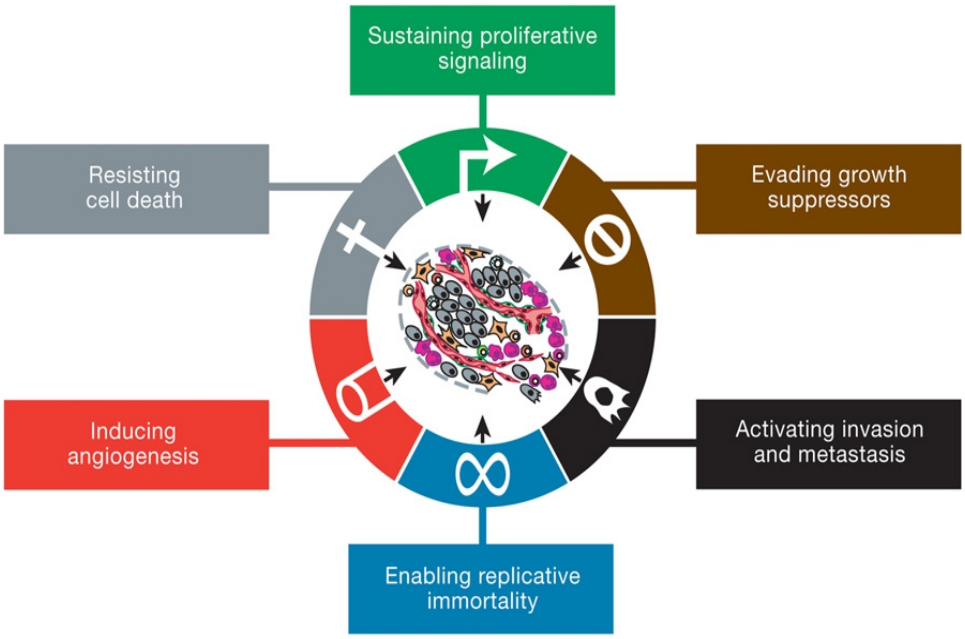
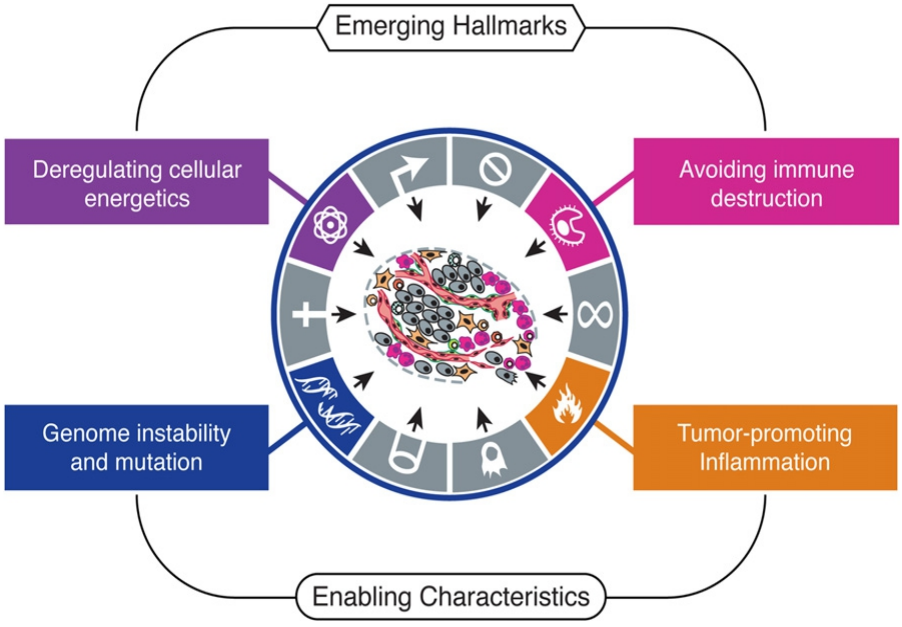


Figure 1: The hallmarks of cancer (Hanahan and Weinberg, 2011).

B- Enabling charactersistics and emerging hallmarks

All of the above mentioned hallmarks of cancer are aquired functional capabilities that allow the cancer cells to grow, survive, proliferate and spread. However, their acquisition is made possible by two enabling characteristics **(Figure 2)**. The first is genome instability and mutation. The development of genomic instability in cancer cells generates rare mutations including rearrangement of chromosomes all of which leads to the formation of hallmark capabilities as stated above. The second one is tumor promoting inflammation where the inflammatory state of premalignant and malignant lesions that are formed by cells of the immune system could promote tumor progression by various means (Hanahan and Weinberg, 2011).

Moreover, it was proposed that other hallmarks could be added to the list since may facilitate the development and progression of many human cancers and thus would be considered as emerging hallmarks of cancer as also shown in Figure 2. Two of which were thought to be compelling (Negrini et al., 2010). The first involves reprogramming of cellular energy metabolism in order to support continuous cell growth and proliferation. The second involves active evasion by cancer cells from attack and elimination by immune cells (Hanahan and Weinberg, 2011).



<u>Figure 2:</u> Emerging hallmarks and enabling characteristics (Hanahan and Weinberg, 2011).

C- The tumor microenvironment

It is not enough to study tumors by cancer cell characteristics of individual cell types within it but also by studying the whole tumor microenvironment that cancer cells construct during tumorigenesis. The tumor is not just a group of homogeneous cancer cells as shown **Figure 3** but rather includes a subclass of neoplastic cells within tumors known as cancer stem cells (Cho and Clarke, 2008). For example, in carcinomas the neoplastic epithelial cells constitute the parenchyma that is different from the mesenchymal cells that forms the tumor associated stroma. The multiple stromal cell types create a succession of tumor microenvironments that change as tumors invade normal tissues. Therefore, the stromal cell types and the extracellular matrix change during primary, invasive and metastatic growth (Singh et al., 2010).

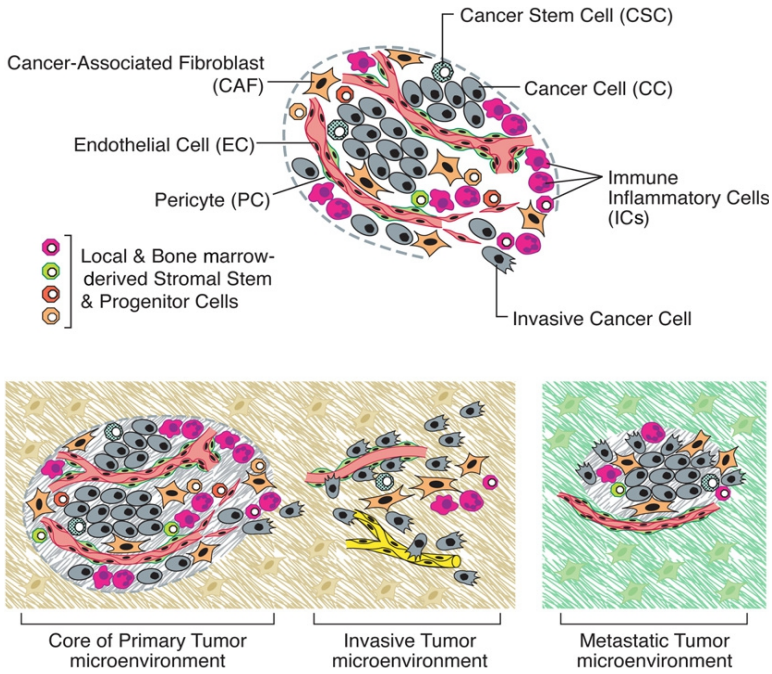


Figure 3: The cells of the tumor microenvironment (Hanahan and Weinberg, 2011).

II- Calcium in physiology and pathology

A- Calcium Signaling in cell survival and cell death

Each cell type has a special signaling pathway for delivering calcium signals with the spatial and temporal properties corresponding to its specific function and physiology. Signaling pathways have one thing in common and that is the ability to generate calcium transients (Berridge and Roderick et al., 2003). A transient is a small increase in free intracellular calcium. it is organized into regular oscillations. An increase in intracellular calcium can be due to calcium entry or release from internal stores. Inositol triphosphate (IP3) has roles in integrating the process of calcium entry and release. It can act locally at membrane to promote calcium entry through a conformational coupling mechanism and that is through stimulating inositol triphosphate receptors (IP3Rs) that are coupled to entry channels. IP3 can also indirectly activate calcium entry by stimulating uncoupled IP3Rs in junctional zones to switch on store operated calcium (SOC) pathway to ensure that internal stores remain filled. However, the calcium that enters is taken mostly by endoplasmic reticulum (ER) increasing calcium in lumen. When there is overload in internal stores, calcium is released through increasing the sensitivity of uncoupled IP3R to participate in regenerative release of calcium. Whatever is the entry mechanism, the important issue is that entry brings calcium into the cell before a spike during an oscillation. Therefore, calcium response starts with IP3 induced calcium entry and when there is luminal overload there will be regenerative calcium release or a spike (Berridge, 2004). Moreover, calcium entry can be activated by other ways as well.

Calcium signaling plays a significant role in cell survival and cell death. The tight interplay between ER and mitochondria is a key determinant of cell function and survival through the control of intracellular calcium signaling. The physical platform for the association between the ER and mitochondria is a domain of the ER called the mitochondrial associated membrane (Higo et al., 2010). It is crucial for highly efficient transmission of calcium from the ER to mitochondria, thus controlling fundamental processes involved in energy production and also determining cell fate by triggering or preventing apoptosis.

Normal ER to mitochondria calcium signaling should remain within a relatively small physiological range (Higo et al., 2010). Overload of calcium uptake by the mitochondria

stimulates the calcium sensitive matrix dehydrogenases and thus mitochondrial adenosine 5' trisphosphate (ATP) production. Mitochondrial calcium overload was for a long time recognized as a determinant in apoptotic and necrotic cell death and was related to the role of the mitochondrial permeation transition pore (PTP). Persistent PTP opening is followed by mitochondrial depolarization and calcium release, cessation of oxidative phosphorylation, matrix swelling with inner membrane remodeling, and eventually outer membrane rupture with release of cytochrome c and other apoptotic proteins (Bernardi et al., 2007). On the other hand, sufficient calcium supply by constitutive activity of the IP3R is needed to provide calcium to the mitochondria for efficient oxygen consumption and ATP production (Cardenas et al., 2010 and Higo et al., 2010). In the absence of this calcium transfer, cells are prone to activation of autophagy to sustain their survival (Cardenas et al., 2010).

Physiological calcium influx is essential for cell survival by virtue of all the cellular responses mediated by calcium from gene regulation to neurotransmission. Calcium influx is required to maintain all forms of calcium signals from puffs to oscillations to calcium waves (Lee et al., 2010). Yet, excessive calcium influx is highly toxic leading to cell death. This has been widely documented for cell death programs in neurons (Berliocchi et al., 2005 and Berna-Erro et al., 2009) and in blood cells (Kummerow et al., 2009). Calcium toxicity and cell death due to calcium influx activated by excessive receptor stimulation and by other cell stressors lead to several pathologies (Petersen et al., 2009).

B- Role of calcium in physiology

Calcium signals could be of two functions due to spatial localization of calcium: elementary activating physiological cellular processes in the channels like membrane excitability, mitochondrial metabolism, vesicle secretion, smooth muscle relaxation, mitosis or it could be deeper through channels producing an intracellular calcium wave that cause a global action on the cell like fertilization, smooth, skeletal and cardiac muscle contractions, liver metabolism, gene transcription and cell proliferation also it produces intercellular global calcium wave that spreads from one cell to the other enhancing their communication (Berridge and Lipp et al., 1998).

According to the physiological function of the cell, different frequency modulation of calcium can be used. For example, in order for the muscle of the heart to contract, cells should increase the frequency of calcium sparks and activate gene transcription. Cells have also developed special ways to sense the frequency and duration of calcium oscillations or signals through the action of calcium dependent transcription factor cAMP response element binding protein (CREB) and enzymes like Calmodulin-dependent protein kinase II, the down stream effector of calcium sensor protein Calmodulin. Cells are also able to sense the changes in amplitude of calcium signaling. For example, calcium oscillator can be driven for 10-20 hours during the physiological process of fertilization due to the activation of phospholipase C that produces IP3. Later in fertilization, calcium signals are important for the differentiation of specific cell types and in development of heart, for example, they help in the production of the heart's septum and valves through calcium sensitive protein phosphatase calcineurin (CaN) activating the transcription of nuclear factor of activated T cell (NF-AT) (Clapham 2007).

Calcium signaling plays also an important role in the development of nervous system. In case of neurons, when a signal is received calcium enters the spines from external sources through receptor operated channels (ROCs) or voltage operated channels (VOCs) and from internal sources like channels activated by IP3 to amplify the signal. Moreover, in case of electrical signals, calcium entering through VOCs causes the release of neurotransmitters and these signals are usually fast. However, localized calcium release from ER has a role in neurons excitability (Berridge and Lipp et al., 1998). In addition, in case of learning and memory, calcium activates 3',5'-cyclic adensosine monophosphate (cAMP) and mitogen activated protein kinase (MAPK) and PI3K signaling pathways to control synaptic strength. Calcium signaling is also involved in the proliferation of immune cells in response to foreign antigens producing IP3 causing the release of calcium from internal stores that are refilled by SOC in order to maintain a prolonged increase of calcium. This increase also causes the activation of NF-AT (Berridge 2012).

The stability of calcium signaling depends on the transcription of genes responsible for regulating the calcium signaling pathway. For example, NF-AT activates the transcription of genes like endothelin, the transient receptor potential (TRP) family member canonical TRP

channel 3 (TRPC3) and TRPC6, IP3R, NF-AT2, Down's syndrome critical region 1 (DSCR1), dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) and the large conductance K+ channels. When there is increase in intracellular calcium, CREB activates the expression of the anti-apoptotic factor Bcl-2 which can inhibit calcium release by the IP3Rs and activated CaN dephosphorylates NF-AT **(Figure 4)** (Berridge 2012).

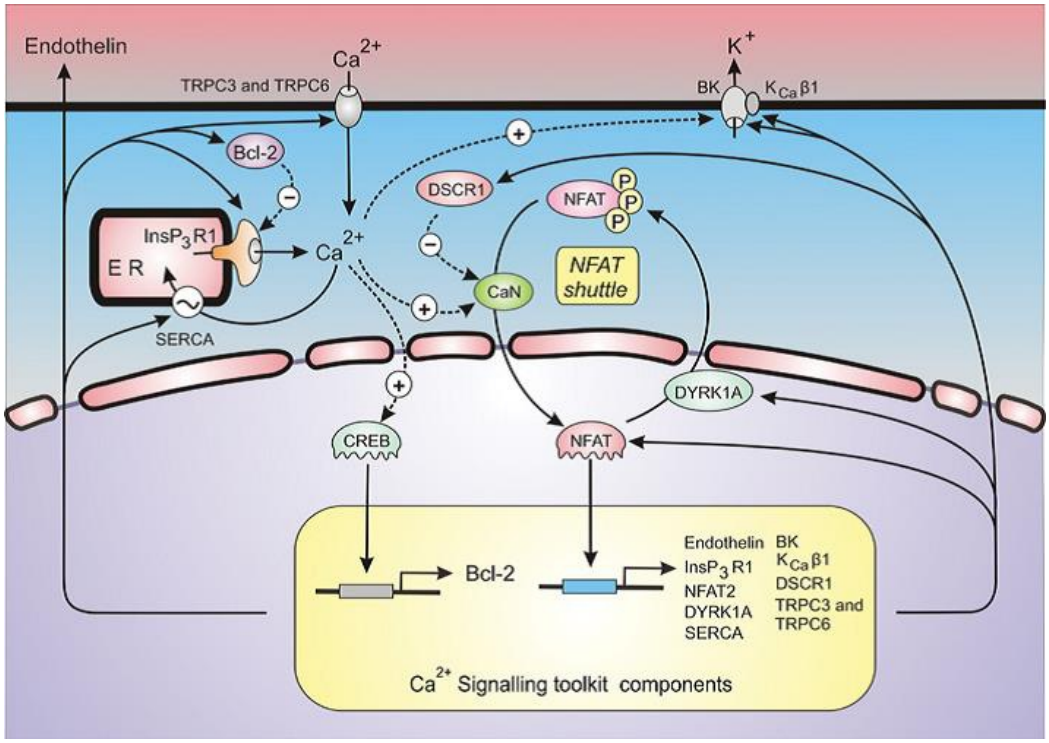


Figure 4: Calcium dependent transcription factors and calcium signaling components (Berridge 2012).

C- Role of calcium in pathology

The role of calcium in many cell signaling pathways lead to calcium homeostasis characterized by a steady state of calcium influx, efflux and storage. In fact, each cell whether normal or pathological is characterized by a special calcium signaling mechanism depending on its duration, frequency, amplitude and cellular localization. Calcium signaling through IP3 is very important in the control mechanisms in most cell types. In non-excitable cells, it is involved in cell activation. In excitable cells, the fast-acting voltage-sensitive calcium entry ways provide calcium signal for cell activation, whereas the IP3 calcium pathway modulates

the intensity of calcium signaling. Many of the serious diseases in humans seem to result from defects in calcium signaling especially defects in the function of IP3 calcium signaling pathway in controlling the activity of excitable cells such as cardiac cells in heart diseases and neurons in neurological diseases (Berridge, 2012) and cancer. Moreover, deregulated cytosolic calcium concentration obtained due to abnormalities or mutations in calcium channels, calcium transporters, calcium pumps and calcium binding proteins can induce multiple pathologies.

In case of heart diseases, there is an increase in cardiac hormones like endothelin and adrenergic agents that leads to the elevated production of IP3 causing an increase in the force of heart contractions and fast heart beats due to the increase in calcium release. Also, cardiac hypertrophy and congestive heart failure are induced by hormones that operate through the IP3 calcium pathway where IP3 triggers elevation in nuclear calcium signaling (Berridge, 2006). Remodeling of calcium signaling and defects in IP3 calcium pathway can lead to severe neural diseases like Alzheimer's disease, bipolar disorders and schizophrenia. For example, in Alzheimer's disease, it is thought that abnormal amyloid metabolism remodels calcium signaling homoeostasis which causes the progressive decline in memory and the increase in neuronal cell apoptosis. Elevation of calcium in the resting levels due to the increased activity of IP3 receptors is detected in this disease (Berridge, 2012). However, irregular calcium entry through SOC leads to autoimmune diseases (Izquierdo et al., 2013) and kidney diseases (Pochynyuk et al., 2013). Further, decreased calcium in lumen of ER may lead to diseases that have gained less attention so far but yet are serious as the above mentioned and may induce apoptosis and cell death (Mekahli et al., 2011).

Moreover, deregulation in calcium homeostasis leads serious diseases like cancer. Cancer is caused by defects in the mechanisms underlying cell proliferation, apoptosis and migration. Calcium is central to the proper functioning of all of these mechanisms since disruption in calcium homeostasis and altered expression and or function of calcium channels lead to promotion and progression of cancer cells. It is unquestionable now that calcium channels and transporters play a role in all stages of cancer disease from initiation to metastasis since is being involved in nearly all of the hallmarks of cancer (Pederson et al., 2013).

Calcium signaling of long durations is important for growth signals in many cells like in case of fertilization. Defects in calcium signaling can cause problems in cell growth leading to cancer. It is known that in cancer cells calcium requirement is reduced for cell growth (Berridge 1993). The role of calcium is well established in many cell signaling pathways involved in carcinogenesis like cell prferation, differentiation and apoptosis (Prevarskaya et al., 2011). The involvement of calcium permeable channels in regulation of cellular proliferation and apoptosis has been established since 1980. The transformation of a normal cell into a cancer cell is affected by defects in calcium pumps, sodium/calcium exchangers and calcium channels, which lead to abnormal cell growth and abnormal death mechanisms (Prevarskaya et al., 2007). Calcium release from internal stores has a role in impairing apoptosis in prostate cancer (Shapovalov et al., 2013).

III- Calcium regulatory systems

A- Calcium pumps and exchangers

ATPase pumps actively moves calcium into the ER through sarcoendoplasmic reticular calcium ATPases (SERCA) pumps or moves it out of the cell through plasma membrane calcium ATPases (PMCA) pumps. These pumps have low capacities for calcium but high affinities which enables them to keep the low resting levels of calcium at about 100nM (Strehler and Treiman, 2004).

The sodium/calcium exchangers and the sodium/calcium potassium exchangers exchange one calcium ion for three sodium ions or cotransport one potassium ion with one calcium ion in exchange for four sodium ions. The sodium/calcium exchangers have low affinities for calcium but high capacities and this enables them to function at the beginning of the recovery process in order to quickly remove calcium. Therefore, the PMCAs are effective at maintaining low internal calcium over long durations, whereas sodium/calcium exchangers and sodium/calcium potassium exchangers can make the rapid adjustments needed during generation of action potentials (Hilgemann et al., 2006).

B- Channels

The sources of calcium at the cellular level could be internal from calcium stores or channels on different organelles or could be from external sources through channels on plasma membrane. Calcium signals that originate from channels in the plasma membrane or internal stores constitute the major blocks in calcium signaling.

1. Calcium channels of intracellular membranes

The two major classes of intracellular calcium channels involved in regulation of cell fate and release of calcium from internal stores are IP3Rs and Ryanodine receptor (RyR) families. These channels are activated by calcium, enabling these release channels to excite each other to create intracellular calcium waves during the globalization of calcium signals.

a- IP3R

IP3R is a membrane glycoprotein complex which represents a dominant second messenger leading to the release of calcium from intracellular stores. IP3Rs constitute the network of ER or sarcoplasmic reticulum the major calcium stores, where they control cellular processes like excitation-contraction coupling, apoptosis and autophagy (Shapovalov et al., 2013). IP3Rs increase intracellular calcium from 100nM to about 1µM. They are activated upon binding to IP3 produced by G protein coupled receptors and protein tyrosine kinase linked receptors that are coupled to different phospholipase C isoforms which hyrolyzes PIP2 into IP3 and diacylglycerol (DAG). IP3 can bind to the ER membrane calcium permeable IP3Rs and cause them to open realeasing the stored calcium into the cytosol (Clapham., 2007). Three homologues genes: ITPR1, ITPR2 and ITPR3 encode for the three IP3R types. The three types are IP3R1, IP3R2 and IP3R3. They have multiple splice variants in mammalian cells including humans. The isoforms have distinct and overlapping patterns of expression with most cells expressing more than one isoform. The IP3Rs are ubiquitously expressed (Foskett et al., 2007). The diversity of IP3R expression is impressive, but the functional implications of this diversity, both at the single channel as well as cellular levels, are still only poorly explored. This diversity suggests that cells require distinct IP3Rs to regulate specific functions. Cerebellar Purkinje neurons express the type 1 isoform predominately,

whereas insulin secreting βcells express primarily the type 3 channel (Taylor et al., 1999), and cardiac myocytes express predominately the type 2 isoform (Perez et al., 1997).

IP3R channels depend on various ligands such as IP3, ATP, and cytosolic calcium allowling the investigation of spatiotemporal phenomena such as the spatial transmission of calcium signals within and between cells by calcium waves (Siekmann et al., 2012).

b- RyR

RyR channels are found in skeletal and smooth muscle cells as well as neurons. It is the major cellular mediator of calcium induced calcium release (CICR) in mammals. It is activated by nucleotide messenger, cyclic ADP-ribose that target RYRs on ER to ensure intracellular calcium release and can also be gated via protein-protein interaction with plasma membrane (PM) voltage gated calcium channels, a mechanism characteristics of skeletal muscles (Lee et al., 2011). There is 3 isoforms: RyR1 found in skeletal muscles, RyR2 found in smooth muscles of heart and RyR3 found in brain. Like the IP3R, RyRs are massive tetrameric channels permeant to calcium. The primary natural agonist of RyR is calcium like most calcium permeant channels where higher cytoplasmic calcium near the mouth of the channel inhibits gating and prevents calcium overload. Eighty percent of the RyR's mass is cytoplasmic, where it interacts with several binding proteins. RyRs may interact with members of the TRPC family (Darszon et al., 2011). In physiological conditions; IP3 or RyR receptors stimulate the activaton of store operated channels (Capoid et al., 2011).

c- Other channels of intracellular membrane

There is a novel class of endolysosomally localized nicotinic acid adenine dinucleotide phosphate (NAADP) sensitive two pore channels (TPCs). It is a small family of two members that forms cation selective ion channels. They are found within the endolysosomal system as calcium channels that release calcium in response to NAADP (Berridge 2012). Calcium released locally from the endolysosomes can be further coupled to the realease from the ER via CICR, thereby inducing global calcium signals. Notably, in lymphokine activated killer cells, NAADP was shown to increase lysosome related intracellular calcium signaling, as well as migration suggesting a role of NAADP in calcium dependent migratory behaviours (Rah et al., 2010).

Also several subtypes of mammalian TRP channel are located within the membranes of intracellular organelles and mediate calcium release in mammalian cells like transient receptor potential cation channel mucolipin subfamily member 2 (TRPML), transient receptor potential melastatin (TRPM) and transient receptor potential valinoid (TRPV) as shown (**Figure 5).**

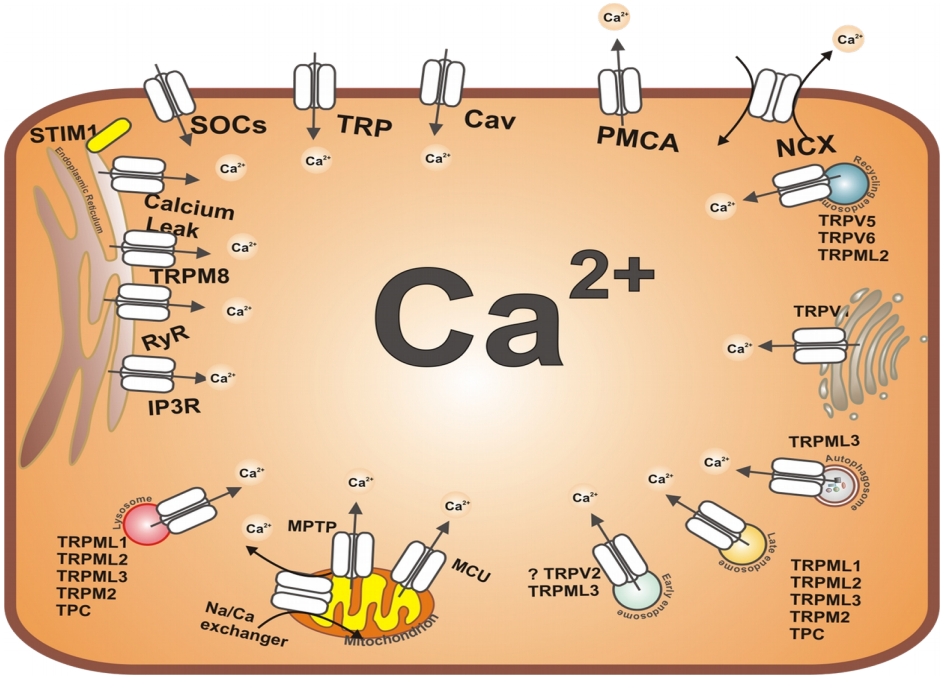


Figure 5: Cytosolic calcium regulation (Yassine Maya)

2. Plasma membrane calcium permeable channels

a- SOC channels

Refilling of intracellular stores, typically the ER, occurs via store operated calcium entry (SOCE). The decrease in ER calcium activates SOC channels in the plasma membrane, leading to influx of extracellular calcium (Muller et al., 2014).

SOC channels are located in the plasma membrane of non-excitable cells. Normally, when ER calcium content decreases below threshold it triggers stromal interaction molecule 1 (STIM1) to relocate to PM and to oligomerize to interact with Orai1 (calcium release activated calcium channel protein 1) protein which is the first step in SOCE activation. The first calcium current regulated by store depletion was named the calcium release activated

calcium current (ICRAC). TRP channels were the first channels thought to produce ICRAC current through SOC activity (Darszon et al., 2011). Many scientific studies have shown that SOC activity is triggered by blocking SERCA pump that causes the gradual store depletion of calcium. SOCE is involved in muscle contraction, exocytosis and sperm chemotaxis. The physiological and pathological importance of SOCE has been implicated in many diseases, especially in immune disorders and cancer since it has a role in apoptosis, proliferatiion, invasion, migration and metastasis regulation (Chen et al., 2013).

a.1 STIM1 protein

Stromal interacting molecule 1 (STIM1) is a transmembrane protein integral to the ER membrane that forms the ER calcium sensing component (Muller et al., 2014). However, at least two families of proteins mediate the associated entry of calcium across the PM and they are the TRP channels and Orai proteins (Liou et al., 2005). Upon activation of the IP3R, the calcium concentration in the ER decreases, that is sensed by STIM1 through its EF hand domain. STIM1 activates Orai1 in the PM via protein-protein interaction. STIM molecules, STIM1 and STIM2, are the single transmembrane proteins that are mainly localized in the ER membrane. The important functional domains of STIM1 include a canonical EF hand calcium-binding domain and a sterile α-motif (SAM) protein interaction domain in the luminal N-terminal end, and a STIM-Orai activating region (SOAR), which is similar to the CRAC activation domain (CAD), in the cytoplasmic C-terminal end (Chen et al., 2010). The EF hand domain enables STIM1 to sense small decreases in ER luminal calcium concentration, whereas the SAM domain mediates the STIM1 oligomerization. When ER calcium is depleted, STIM1 proteins oligomerize into multiple punctae and redistribute to the PM, known as the ER-PM junctions as shown in **(Figure 6)**. Following puncta formation, STIM1 directly interacts with SOCE channels in the plasma membrane, either TRP channels or pores formed by Orai proteins (Huang et al., 2011). Triggered by STIM1 these channels open, allowing influx of Ca21 and subsequent refilling of stores through uptake of calcium ions into the ER by SERCA pumps. Although STIM2 molecule is similar in the overall structure and properties to STIM1, such as in ER localization, luminal calcium binding and redistribution to puncta at ER-plasma membrane junctions upon calcium store depletion, its role in SOCE activation is still not clear (Soboloff et al., 2012).

a.2 Orai1 protein

Mammals possess three types of Orai proteins (Orai1, 2 and 3) of which Orai1 is the most characterized. Although all three Orai proteins form functional SOC channels when co-expressed with STIM1, they differ in tissue distribution and in the selectivity and calcium conductivity. Orai1 protein, a four transmembrane domain calcium channel in the PM, translocates to the STIM1-containing ER-plasma membrane junctions following store depletion and opens to mediate calcium entry as shown in **(Figure 6)**. The opening of the Orai1 calcium channel is mediated by the direct physical interaction between the cytoplasmic C-terminal domain of Orai1 and the cytoplasmic C-terminal SOAR/CAD domain of STIM1.

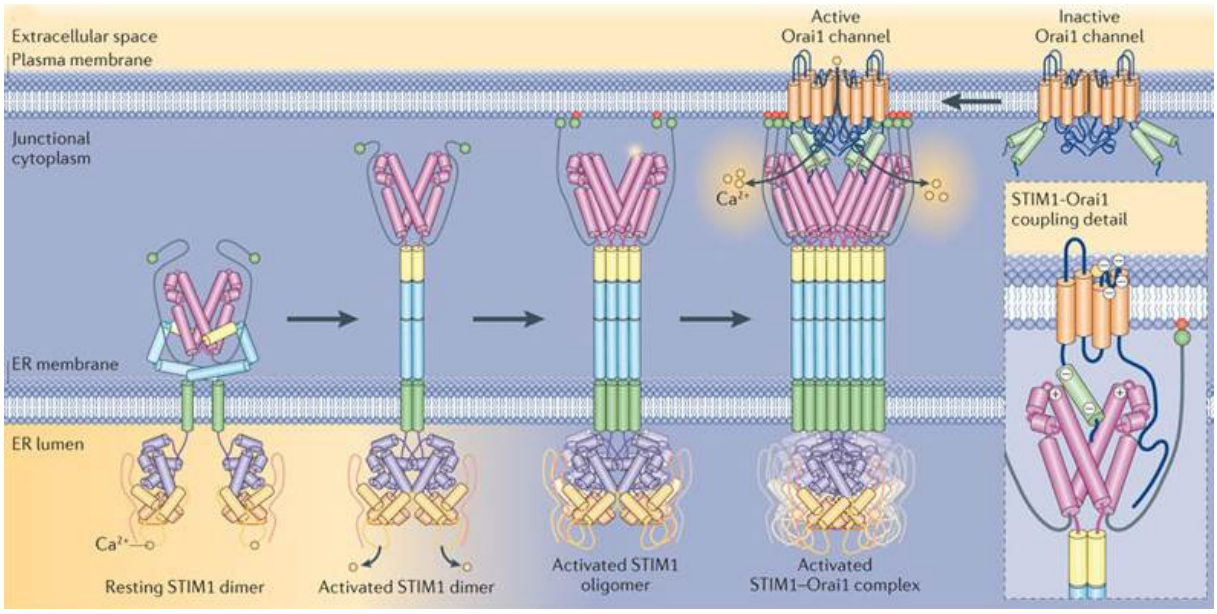


Figure 6: STIM1 dependent SOCE activation (Soboloff et al., 2012).

b- Voltage gated calcium channels

Voltage gated calcium channels (VGCCs) are commonly associated with excitable cells and physiological processes such as neurotransmitter release, excitation-contraction coupling or hormone secretion and are activated by depolarizing membrane potentials. They are classified as slow, intermediate and high voltage activated channels. For example, L-type calcium channel (Long-Lasting) responsible for long lasting action potential in cardiac cells and dendrites and P-type calcium channel (Purkinje) found in Purkinje neurons, Q-type

calcium channel found in cerebellar cells and N-type calcium channel (Neural/Non-L) found in brain and peripheral nervous system are high voltage activated channels. R-type calcium channel (Residual) found in cerebellar cells and some neurons are intermediate voltage activated calcium channels and T-type calcium channel (Transient) found in neurons, cells that have pacemaker activity and bone cells are low voltage activated channels (Murata et al., 2005).

c- Ligand gated calcium channels

They are transmembrane ion channels that open to allow ions like calcium to cross the membrane as activated by ligands such neurotransmitters. These proteins are typically composed of at least two different domains: a transmembrane domain which includes the ion pore, and an extracellular domain which includes the ligand binding site. They are classified into 3 subfamilies Cys-loop receptors, Ionotropic glutamate receptors and ATP-gated channels. They are involved in fast chemical synaptic transmission in the nervous system but they can also be found extrasynaptically as well as outside the nervous system (Murata et al., 2005). The cys-loop receptors are named after a characteristic loop formed by a disulfide bond between two cysteine residues in the N terminal extracellular domain. They are subdivided with respect to the type of ion that they conduct anionic or cationic and further into families defined by the endogenous ligand. The ionotropic glutamate receptors, for example NMDA, bind the neurotransmitter glutamate. They form tetramers with each subunit consisting of an extracellular amino terminal domain that involves tetramer assembly, an extracellular ligand binding domain that binds glutamate, and a transmembrane domain that forms the ion channel. ATP gated channels open in response to binding ATP. They form trimers with two transmembrane helices per subunit and both the C and N termini are on the intracellular side. P2X purinergic ionotropic receptor families have several members that open in response to the binding of extracellular ATP. Ligand gated calcium channels are involved in several cellular processes like cardiac contraction, chronic pain and apoptosis (Connolly et al., 2004).

d- TRP channels

I will be talking in more detail about these channels because of our laboratory interest in the effect of some members of TRP channels (and SOC channels) in prostate cancer. TRP channels are mammalian homologues of the Drosophila TRP channel gene encoding a family of around twenty eight ion channel proteins that are further sub grouped according to homology and function: TRPC (canonical), TRPV (valinoid), TRPM (melastatin), TRPML (mucolipin), TRPA (ankyrin-like) and TRPP (polycystin). These channels are structurally related to a superfamily of voltage-gated channel proteins. The channel subunits have six transmembrane segments that form tetramers of non-selective cationic channels, which allow for the influx of calcium ions into the cell, and therefore they are involved in several cellular regulatory mechanisms. The S6 segment or domain is considered to be the most conserved and the most important for gating of these channels **(Figure 7).** Activity of most of the TRP channels is modulated by voltage or ligands. TRP channels can be activated by chemicals, mechanical and physical stimulus as well as changes in surrounding environment. The activity of TRP channels can cause cells depolarization from their resting potentials and increase their intracellular calcium (Clapham et al., 2001).

Although most members of TRP channels are cationic non selective, they have been considered to be the first candidates for store SOC channels activity. It could be hypothetically because of their direct gating by IP3R, formation of a second messenger that diffuses from ER to the channels and the exocytotic fusion of vesicles containing SOC channels. In short, TRP channels exhibit various sensory properties but regulating calcium homeostasis in the cell is being the most significant deciding the fate of the cell simply by regulating the balance between cellular proliferation and apoptosis. Increased expression of TRP channels in PM often promotes calcium dependent proliferation leading to inhibition of apoptosis in affected cells and tumorigenesis (Roderick et al., 2008). To date, TRP activity linked to cancer progression included only alterations in expression levels of TRP channels rather than mutations (Shapovalov et al., 2013). Therefore, deregulation of calcium permeable channels is linked to a number of diseases such as cancer making them a therapeutic target.

d.1 TRPV

The TRPV family has five members grouped into three subfamilies: TRPV1 and TRPV2 are the vanilloid receptors. TRPV4 is the osm-9-like and TRPV5 and TRPV6 are the calcium selective channels.

TRPV1 was isolated by cloning from a rat dorsal-root-ganglion library using the hot pepper compound capsaicin and is sensitive to heat. It is found in brain, spinal cord and sensory neurons. TRPV2 is, like TRPV1, more permeable to calcium ions than to sodium ions. It is present in neurons, spinal cord, spleen and lung. It is sensitive to heat and growth factors. TRPV4 is present in liver, kidney, heart and nervous system and is activated by reducing the extracellular osmolarity leading to cell swelling. TRPV5 is present in the intestine, kidney and placenta. It is highly calcium selective and its co-localization with 1,25 dihyroxyvitamin D3 suggests a role in regulation of low intracellular calcium. TRPV6 is present in intestine, placenta, kidney, prostate and salivary gland. It has physical properties like ICRAC and is highly calcium selective. It is activated by low intracellular calcium and inactivated by higher. It is suggested that more than 50 % of TRPV6 current is activated by IP3 and thapsigargin mediated store depletion in the presence of low intracellular calcium (Yue et al., 2001). However, concerning its gating whole cell patch clumping experiments were not able to show a corresponding whole cell current related to TRPV6 under normal conditions. Therefore, experiments were to be done via fura-2 measurements.

d.2 TRPC

The TRPC family is divided into four subfamilies according to homology and function: TRPC1, TRPC4, 5, TRPC3, 6, 7 and TRPC2. TRPC1 is supposed to be the first member of the TRP family to be an ion channel. It is a non-selective cationic channel. The second TRPC family TRPC4, 5 form homomeric cation channels. TRPC4 is present in the brain, testis, placenta, adrenal gland and endothelial cells where TRPC5 is found only in brain. They are proposed to be regulated by receptor-operated stimulus or store depletion but it is still unclear (Clapham et al., 2001). The third TRPC family, consisting of TRPC3, 6 ,7 are cationic non-selective channels present mainly in brain and muscles. TRPC3 is proposed to be activated by IP3R suggesting a possible link between SOCs and TRPs (Berridge 2004) and the membrane-bound DAG whereas TRPC6,7 are activated by DAG.

d.3 TRPM

The TRPM family has eight members divided into four groups according to sequence homology. The first subgroup includes the founding member TRPM1 (melastatin) and TRPM3. The second subgroup includes TRPM7 and TRPM6. The third subgroup has TRPM2 and TRPM8. The fourth subgroup has TRPM5 and TRPM4. This family of channels is non-selective.

TRPM7 is the first member to be identified as an ion channel in this family. It is present in the kidney, heart, liver, spleen, lung and brain. Its activity depends on the kinase activity of its own carboxy terminal kinase. TRPM2 is present in fetal and adult brain. TRPM 8 was first cloned as a gene specific to prostate whose expression is increased in prostate cancer in the presence of high levels of androgen. It is sensitive to cold and methanol and has an established role as a chemical sensor in the peripheral nervous system. Moreover, an interesting property of TRPM8 is that it can exist in multiple splice variant. The full length form is normally found on the PM while the shorter isoforms are localized to the ER being as a calcium release channel (Bidaux et al., 2007). The mechanisms which determine the localization of TRPM8 and its physiological role is not fully clarified. Moreover, TRPM2 on plasma membrane is responsible for calcium influx. Cyclic ADP ribose dependent calcium influx via TRPM2 causes insulin secretion (Lee et al., 2011).

d.4 TRPML

TRPML comprises a group of three evolutionarily related proteins that belongs to the large family of transient receptor potential ion channels. The three members of the TRPML ("ML" for mucolipin) sub-family are not extremely well characterized. TRPML1 is known to be localized in late endosomes. This subunit also contains a lipase domain between its S1 and S2 segments. While the function of this domain is unknown it has been proposed that it is involved in channel regulation. Physiological studies have described TRPML1 channels as proton leak channels in lysosomes responsible for preventing these organelles from becoming too acidic. TRPML2 and TRPML3 are more poorly characterized than TRPML1 (Nilius et al., 2007).

d.5 TRPA

The only member of the TRPA sub-family, TRPA1, contains 14 N-terminal ankyrin repeats and is believed to function as a mechanical stress sensor. It is expressed in the dorsal root ganglion, trigeminal ganglion, and hair cells. It is temperature sensitive with some reports claiming that it is activated by noxiously cold stimuli. TRPA1 is known to be activated by compounds capable of forming covalent chemical bonds with the protein's cystein like isothiocyanates, which are the pungent chemicals found in food substances (Nilius et al., 2007).

d.6 TRPP

TRPP is a family of transient receptor potential ion channels which when mutated can cause polycystic kidney disease. All of the members of this group contain a coiled coil region in their C-terminus involved in the interaction with the polycystin-1 group. TRPP1 and TRPP3 form constitutively active cation-selective ion channels that are permeable to calcium. TRPP2 has also been implicated in sour taste perception (Nilius et al., 2007).

e- Arachidonate regulated calcium entry channels

They are activated in response to receptor mediated derivation of arachidonic acid and thus can be classified as second messenger operated channels. They cause oscillatory calcium signals in various cells. The activation of these channels requires STIM1 that is always present on the PM in addition to Orai1 and Orai3 which constitute the pore of the channel (Prevarskaya et al., 2011).

IV- Calcium channels and cancer

If to consider invasion and metastasis as the major hallmark of cancer, it is worth noting that calcium and the calcium permeable channels are crucial regulators of cell ability to migrate (Prevarskaya et al., 2011). They also play important roles in the other hallmarks of cancer.

Differentiation to occur requires calcium signaling. Lymph node carcinoma of the prostate (LNCaP) cells require T type voltage dependent calcium channels for their differentiation. The TRP family member TRPM8 promotes the secretion of mitogens in several cancers like breast, colon and skin cancers. Due to excessive mitogen secretion, cancer cells become sensitive to them and thus develop an uncontrolled proliferation because of altered expression or function of receptors and ion channels that receive these signals. Moreover, evading growth suppressors by malignant cells results from their altered calcium homeostasis including expression of calcium permeable ion channels (Prevarskaya et al., 2007) **(Figure 7)**. Also, to evade apoptosis, cancer cells must reduce or stop calcium influx either by down regulating the expression of calcium permeable channels and or the signaling pathways that lead to their activation. For example, hormone-refractory apoptosis-resistant phenotypes of prostate cancer cells are characterized by reduced levels of SOCE that prevents calcium overload in response to pro-apoptotic stimuli (Prevarskaya et al., 2010). In addition, ionotropic purinergic P2X7 receptors belonging to the ligand gated calcium permeable channels play an important role in evading apoptosis of some cancer cells. In cervical cancer cells, decreased P2X7 expression together with the dominant-negative action of a short P2X7 splice variant results in the down regulation of the functional P2X7 receptor, thereby preventing calcium influx needed for apoptosis induction. Calcium influx through voltage gated channels or TRP members promote the activation of telomerase making tumor cells immortal. Calcium permeable channels and calcium signaling in general are important for the process of angiogenesis (Prevarskaya et al., 2007). Usually, the morphological and adherence changes observed during cell migration are accompanied by changes in intracellular calcium concentration. Also, the calcium permeable TRP channels like TRPC, TRPV1 are involved in increased motility and invasion in fibrosarcoma and hepatoblastoma cells respectively and TRPV2 is involved in increased migration and invasion in prostate cancer cells. SOCE constituents Orai1 and STIM1 are involved in increased migration and higher rate of focal adhesion turn over in breast cancer cells. Further, IP3R and RyRs responsible for calcium release both showed increase in migration in several types of cancer cells (Prevarskaya et al., 2011)**.**

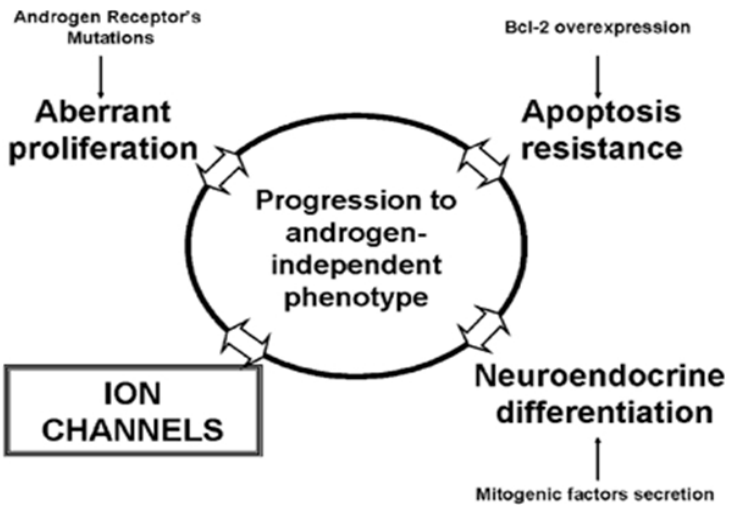


Figure 7: Schematic representation of the malignant transformation of prostatic cells resulting from enhanced proliferation, NE differentiation, and apoptosis resistance (Prevarskaya et al., 2007).

A- TRP channels involved in prostate cancer

TRP channels activity is affected by cell's integrity which is modulated during carcinogenesis. This could be the reason for the role played by some of the TRP channels in prostate cancer progression and could characterize the cancer cell phenotype of the tumor. **(Figure 8)**.

1) TRPM

TRPM8 is the specific TRP channel with the most studies done and found in literature concerning prostate cancer**.** The initial identification of the channel happened in prostate since it was highly expressed. It was found in epithelial cells, apical epithelial cells and smooth muscles of human prostate. Further studies, have shown the importance of this channel in the progression of prostate cancer, where it was shown that the expression level of this channel was increased in cancerous cells (Bidaux et al., 2005) and when it is

overexpressed on both ER and PM it increases apoptosis and inhibits the migration of the prostate adenocarcinoma3 (PC3)TRPM8 cells. It inhibits migration in PC3 cells upon activation by icilin or the prostate specific antigen (PSA) (Gkika et al., 2010). Other study shows that TRPM8 inhibits migration in PC3 cells through inactivation of focal adhesion kinase (Yang et al., 2003). TRPM8 expression level is affected by prostate cancer progression since its expression is regulated by androgen receptors in prostate. Its expression level is decreased in androgen insensitive cells. It has been proposed that according to the balance between the expression levels of the isoforms of this channel on PM or the ER, TRPM8 may shift the calcium homeostasis in the cells toward proliferation or apoptosis of prostate tissue, so it is all about the balance in gene expression. The activation of the shorter isoform of TRPM8 located on ER by cold or menthol in LNCaP cells resulted in ER store depletion followed by SOCE. It is believed that increased activity of the shorter isoforms increases proliferation and shift cell cycle to malignant transformation (Prevarskaya et al., 2007). Finally, TRPM8 role in cell migration inhibition makes it a “Good” channel in prostate cancer fighting with more to be uncovered about the role that is played by TRPM8 shorter isoform.

There is increasing evidence that TRPM2 is involved in prostate cancer where its expression is elevated. Selective knock down of TRPM2 inhibits the growth of prostate cancer cells but not normal ones. Further, it was shown that this channel is relocated from the PM to the nucleus in PC3 and DU-145 prostate cell lines inhibiting nuclear ribosylation (Zeng et al., 2010).

Further, TRPM1, TRPM4 and TRPM5 channels regulate cell survival and proliferation of cancerous cells other than prostate. Various expression levels were shown for TRPM4 channel in androgen independent prostate. TRPM7 channel is up-regulated in breast cancer increasing the proliferation rate of cells (Shapovalov et al., 2011).

2) TRPC

TRPC1 channel is involved in prostate cancer but its expression is decreased in the androgen independent phase of progression. It plays a proproliferative role in normal cells and a pro-apoptotic role in transformed cells. It is also possible that the colocalization of TRPC1 with different receptors could lead to a difference in function of TRPC1 at different stages of prostate cancer progression (Thebault et al., 2006).

The other TRPC subfamily member that is involved in prostate cancer is TRPC3. It is reported that after prolonged store depletion (24-48 hrs) in LNCaP prostate cancer cells, TRPC3 channel expression level is increased. Further, suppression of TRPC3 affects the antiapoptotic signaling in DU-145 prostate cancer cells. TRPC4 is thought to cause ATP induced growth arrest in prostate cancer cells and is thought to be possibly involved in SOCE in addition to, its pro-apoptotic action. Further, it was shown that the activation of calcium/calmodulin/CaN dependent transcription factor NF-AT cell pathway leads to proliferation in the primary human prostate cancer epithelial cells by calcium entry through TRPC6 (Thebault et al.,2006).

3) TRPV

The most member of TRPV subfamily to be studied and focused on its role in prostate cancer is TRPV6. It is expressed in epithelial cells of several organs and is involved in trancellular calcium movement. The activity of this channel depends on vitamin D, progesterone, estrogen, tamoxifen all of which modulating proliferation and survival of cancer cells (Bolanz et al., 2008). TRPV6 is proposed to be a prognostic marker of prostate cancer progression. Its expression is increased in LNCaP and PC3 cells as the cancer is moving toward its late stages regardless of the presence of androgen, more or less, depending on the metastasis of the cancer. However, some studies showed that TRPV6 channel is not expressed in androgen independent prostate cancer cell lines like PC3 and DU-145, thus leaving androgen dependence of TRPV6 an open question. TRPV6 has a pro-proliferative and anti-apoptotic effects in cancer tissues, mainly this being related to supplemental calcium entry necessary for influencing cell cycle regulators (Lehen'kyi et al., 2012). Lehen'ky showed that TRPV6 is directly involved in the control of proliferation as its silencing slowed down the proliferation rate and decreased the accumulation of LNCaP cells in the S phase of cell cycle. Last but not least, TRPV6 ion channel has been thought of as a possible participant in SOCE and was proposed to be one of the primary pathways responsible for the calcium uptake in prostate cancer. In short, TRPV6 could be considered a "Bad" guy in prostate cancer, whose inhibition could be good for controlling the progression of prostate cancer (Gkika et al., 2010).

TRPV1 is thought to be an important player in controlling prostate cancer progression. It is expressed in LNCaP and PC3 cells and in human prostate tissue. Its expression is increased with the progression of the cancer in order to suppress the tumor by inducing apoptosis through causing an intracellular overload of calcium (Lehen'kyi et al., 2007).

TRPV2 is elevated in androgen independent PC3 and DU-145 cells in metastatic stages facilitating cell migration and invasion. Silencing of this channel drastically reduces the migration of prostate cancer cells, whereas its overexpression increases their migration. The mechanism of action of TRPV2 in invasion would be through maintaining an elevated level of cytosolic calcium in cancer cells derived from castration resistant tumors due to its constitutive channel activity (Monet et al., 2010). Further, activation of TRPV2 by lysophospholipids stimulates calcium influx and increases the migration potential of prostate cancer cells (Hao et al., 2007).

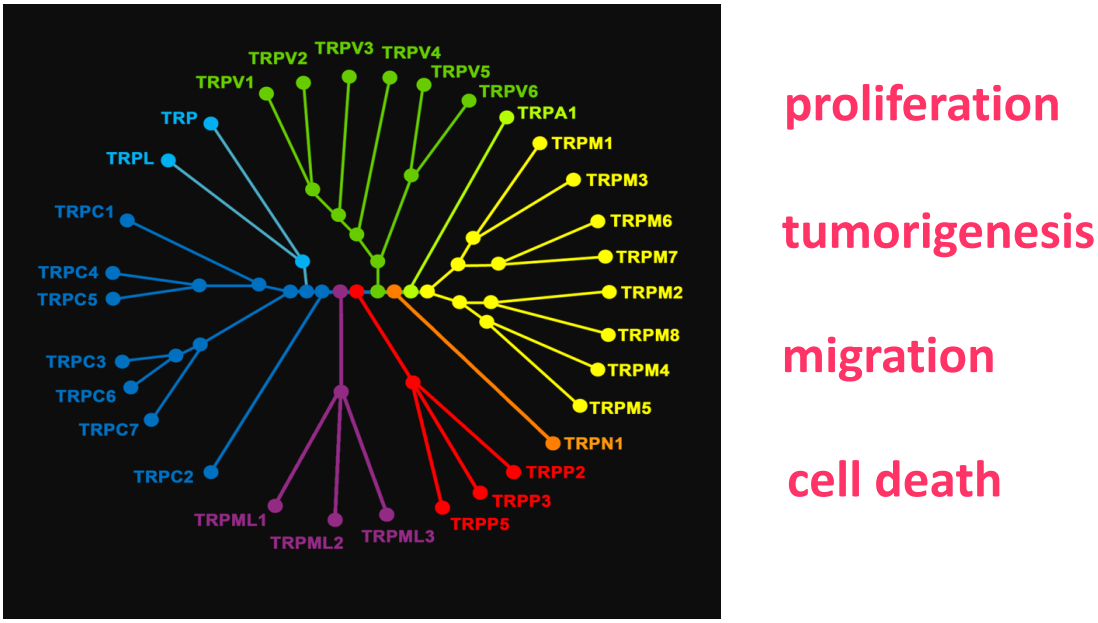


Figure 8: TRP channels affects cancer progression (Yassine Maya)

B- SOC channels and prostate cancer

Mostly, when apoptosis is triggered by excessive calcium, SOCE is thought to be involved since a prolonged calcium signaling is needed. However, it is not always the case that the over activity of SOCE is the only reason for triggering apoptosis because apoptosis itself can be triggered through different pathways. Cancerous cells often have reduction in

SOCE entry to prevent calcium overload and therefore inhibit apoptosis. In prostate, Orai1 acts as a source of calcium influx triggering apoptosis. Studies have shown that the down regulation of Orai1 and thus SOCE prevents the cells from undergoing cell death in prostate cancer cells (Huang et al., 2011). Moreover, the coupling of STIM1 and Orai1 is important for the pro-apoptotic effects of SOCE.

In general, in all cancer cells calcium homeostasis is remodeled during tumor progression since calcium influx is increased through STIM1 and Orai1 upregulation. STIM1 and Orai1 through interactions between actomyosin and focal adhesion turnover cause cell migration. STIM1 also influences cancer cell proliferation through cell cycle regulators p21 and cdc25C. Additionally, STIM1 mediated SOCE plays an important role in tumor angiogenesis by production of VEGF from cancer cells, or cell cycle progression of vascular endothelial cells (Abdullaev et al., 2008) as shown in **(Figure 9)**.

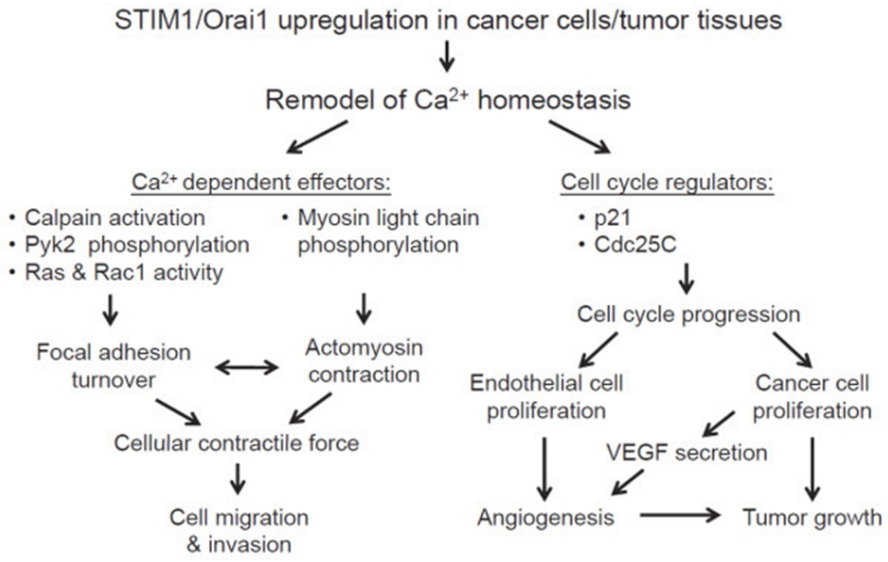


Figure 9: STIM1/Orai1-mediated calcium signaling in tumor biology (Chen et al., 2013).

C- IP3R and RyRs and prostate cancer

In prostate cancer, there exist two modes of calcium entry associated with calcium release from ER through SOC and IP3R (Figure 10). Special treatment of LNCaP with cadmium ion showed increased proliferation due to increased levels of intracellular IP3 and cytosolic

calcium. RyR channels also affect calcium homeostasis in prostate cancer cells. Their stimulation by RyR specific agonist promoted calcium release from Ryanodine sensitive stores and stimulated apoptosis of prostate cancer cells (Charbel C et al., 2013).

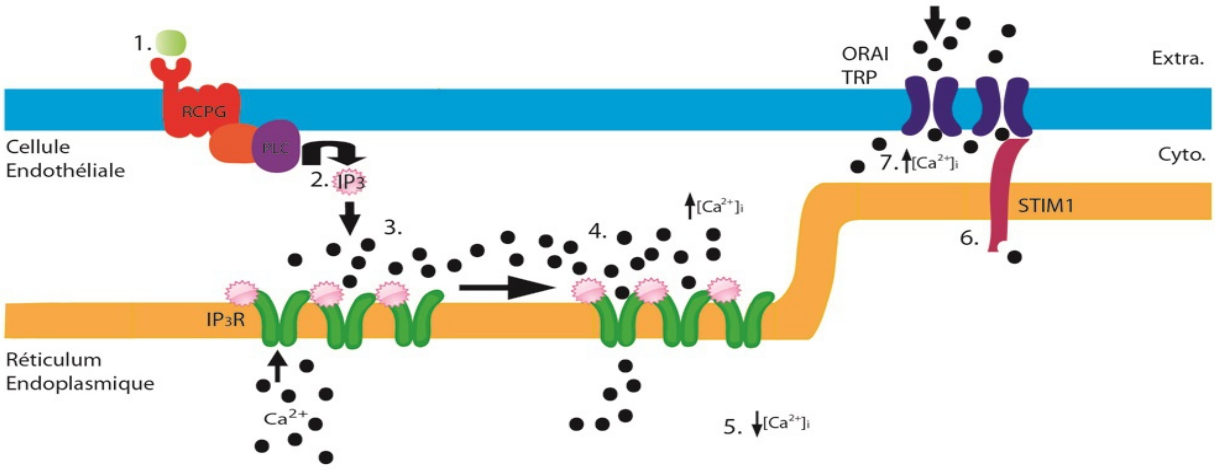


Figure 10: Major calcium entry pathways through SOC and IP3R (Charbel C et al., 2013)

D- VGCCs and prostate cancer

These channels that are associated with excitable cells also have a role in regulating cell proliferation and cell death in prostate cancer. A fraction of epithelial rat prostate cells that undergo apoptosis in absence of androgen was found to be sensitive to VGCC blockers. Many trials to find VGCC current in cancerous cells was not succeeding until a low voltage activated calcium current, related to T-type calcium channel was detected in undifferentiated LNCaP cells. This channel was shown to be overexpressed during NE differentiation (Gackière et al., 2013). Moreover, in prostate cancer PC3 cells, the peptide ghrelin induced overexpression of T-type VGCCs and triggered apoptosis. In a recent study, it was shown that T-type channels in coordination with potassium channels are involved in proliferation of prostate cancer cells . The T type channel could be involved in the stimulation of mitogenic factor secretion and could therefore be a target for therapeutic strategies (Mariot et al., 2002).

V- Calcium channels and drug therapy

Deregulation of above mentioned calcium permeable channels is linked to cancer and thus makes them an interesting therapeutic target.

To start with calcium channels and treatment for heart arrhythmias, the IP3 induced cardiac arrhythmias can be prevented by adding the channel inhibitor 2-aminodiethyldiphenyl borate (2-APB), which is known to act by blocking calcium release from the IP3R. The fact that 2-APB can completely restore the normal calcium transients suggests that the IP3R may be a very significant target for the development of drugs to prevent the cardiac arrhythmia that are one of the main causes of sudden heart death (Berridge 2012). Moreover, Calcium channel blockers are being tested in clinical trials for their effect in delaying the onset and progression of Alzheimer disease. Memantine, a current treatment used in this disease, exerts some of its beneficial effects by blocking calcium entry into neurons (Goodison et al., 2012).

Calcium channels are attractive tools for drug therapy. TRP channels can be good candidates since they have extracellular domains that can bind ligands or antibodies and they affect important steps in prostate cancer progression such as proliferation, apoptosis and migration. Indeed drugs can affect ion channel function through several different mechanisms ranging from the classic pore block, over allosteric regulation due to specific drug binding to the target protein, to the allosteric regulation that arises from more nonspecific drug induced changes in lipid packing adjacent to the protein and in bilayer material properties (Prevarskaya et al., 2010). This area of research is developing as the potential for the pharmacological modulation of channels is one of the key advantages over other targets, which may be restricted to siRNA or gene therapy approaches. For instance, the newly identified carboxamide WS-12 is the most potent TRPM8 agonist and could constitute a good candidate for drug development. In addition, the development of humanized inhibitory antibodies to extracellular domains of TRPV6 and TRPV2 channels may provide the desired specificity for a successful use in vivo. Finally, in vivo and on human trials will have to define the real potentials and limitations of TRP channels as a drug targets and until then more research should be done (Hippert et al., 2006).

Since most channels are not cancer specific and are found in different tissues, they should be targeted selectively because the pharmacological impairment of channel function could be toxic to normal cells. A possible solution might be the coupling of a drug to a targeting moiety to produce a drug derivative that can only be activated inside tumors. For instance, the so called “smart bomb” for prostate cancer that combines the SERCA inhibitor thapsigargin (TG), which induces apoptosis through the activation of ER stress and calcium entry pathways, with a targeting peptide representing the substrate for prostate cancer specific serine protease. Such an inactive prodrug has been shown to demonstrate preferential toxicity only to PSA producing prostate cancer cells (Prevarskaya et al., 2010).

VI- Autophagy, a mechanism depending on calcium and playing a role in cancer

A- History and definition

The word “autophagy” was invented by Christian de Duve, the discoverer of lysosomes, when he observed autophagosomes and realized that cells could degrade their own components. He was the first to prove the involvement of lysosomes in the autophagy machinery. The word “autophagy” was invented on the occasion of the Ciba Foundation Symposium on Lysosomes, which took place in London on February 12–14, 1963. When Christian de Duve introduced the term autophagy, he did not imagine that later, it would become a major topic in biology and that a new journal would be entitled *Autophagy* (Martens et al., 2013).

Autophagy is a Greek word that means “self-eating”. It is a catabolic process, in eukaryotic cells, that delivers cytoplasmic material to the lysosomes for degradation and reuse by the cells. When cells are starving they can digest protein aggregates, long lived proteins and organelles to release amino acids to maintain cellular homeostasis. At the beginning, scientists were able to study autophagy by examining autophagosomes and their fusion with lysosomes using electron microscopes. However, the auotophagy field has developed and new methods for detecting autophagy were used starting from the 1990’s (Klionsky et al., 2007).

B- Types of autophagy

There is three types of autophagy:

- Macroautophagy and is usually referred to as autophagy.
- Chaperone mediated autophagy (CMA) involves the direct translocation of cytosolic proteins across the lysosomal membrane which requires protein unfolding by chaperone proteins.
- Microautophagy involves inward invagination of lysosomal membrane which delivers a small portion of cytoplasm into the lysosomal lumen.

C- Autophagy machinery

Autophagy is conserved from yeast to mammals and is marked by the formation of autophagosomes. When autophagy is initiated, a small vesicular sac called the isolation membrane or phagophore expands and encloses a portion of cytoplasm, which results in the formation of a double membraned structure, the autophagosome. Then, the outer membrane of the autophagosome fuses with lysosome to form an autolysosome, leading to the degradation of the enclosed materials together with the inner autophagosomal membrane and like this the cell would be consuming part of itself. The endosome, which is the result of endocytosis, can also fuse with the autophagosome to form an amphisome before fusion with the lysosome and in this case the cell would be consuming materials from outside of the cell. Amino acids and other small molecules that are generated by autophagic degradation are delivered back to the cytosol for recycling or energy production (Klionsky, 2007).

The autophagy related gene (Atg) ULK/Atg1 kinase complex, the autophagy specific PI3K complex, Beclin1/Vps34 complex and their related proteins are important for the nucleation step, whereas the Atg12- and microtubule associated protein light chain 3 (LC3) conjugation systems are important for the elongation step **(Figure 11)**. However, there is a still unanswered question related to the origin of the autophagosome membrane. Some studies have indicated that the origin of the membrane is the ER, mitochondria, golgi, plasma membrane and endosomes. A recent study, suggests a role for the ER (Axe et al., 2008).

Another study shows that the outer membrane of the mitochondria with some parts from the ER are the major source for the autophagososmal membrane (Castro Obregon, 2010).

Other proteins required for autophagosome-lysosomal fusion, lysosomal acidification, and lysosomal digestion are involved in autophagy. To date, only LC3, a mammalian homolog of yeast Atg8, is known to exist on autophagosomes, and therefore, this protein is considered a marker for autophagosomes. The identification of the autophagic machinery has greatly facilitated the detection of autophagy through LC3 based biochemical and microscopic techniques, as well as the ability to experimentally manipulate the autophagy pathway through knockout or knockdown of autophagy genes. The autophagy pathway can also be manipulated with agents that regulate autophagosome formation or their degradation (Axe et al., 2008).

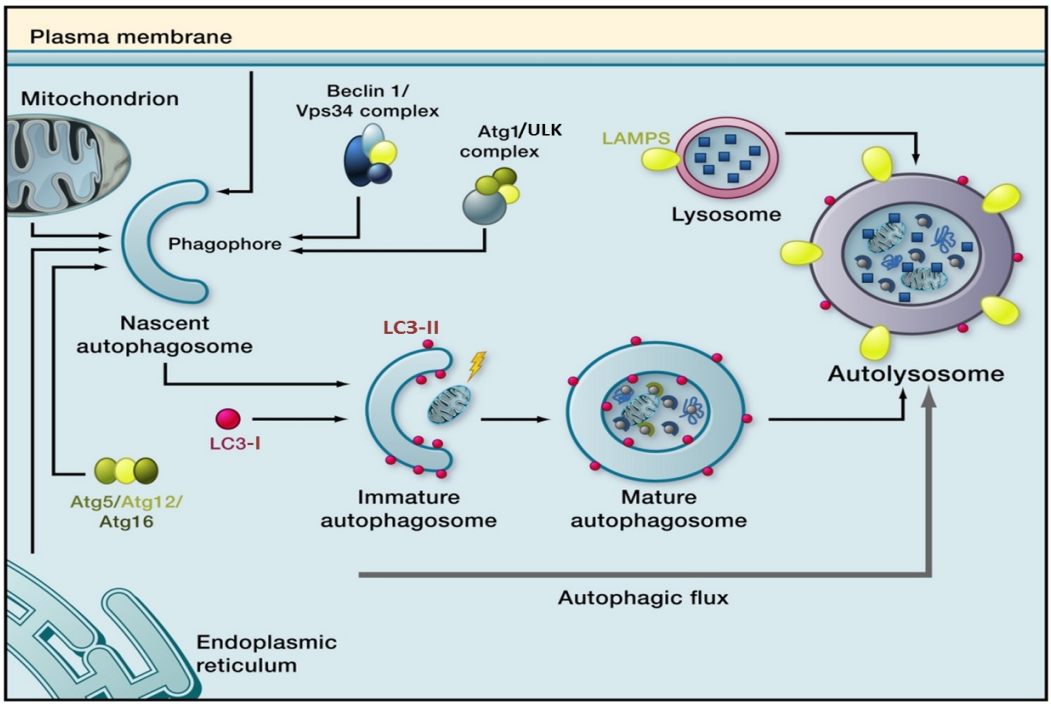


Figure 11: The autophagic machinery (Rabinowitz and White, 2010).

1. Autophagy inhibitors

Several pharmacological and genetic approaches inhibit autophagy. As autophagosome formation requires class III PI3K activity, one of the most commonly used pharmacological agents to inhibit autophagy in vitro involves the use of PI3K inhibitors such

as wortmannin, LY294002, or 3-methyladeninie (3-MA) shown **(Figure 12).** However, it should be noted that all of these reagents can inhibit both class I PI3K activity which inhibits autophagy as well as class III PI3K activity which is required for autophagy (Ravikumar et al., 2004), and some of the PI3K inhibitors, such as wortmannin, also inhibit mtor, an autophagy inhibitory molecule by targeting its ATP binding site. Moreover, PI3K both class I and class III regulate diverse cell signaling and membrane trafficking processes, these PI3K inhibitors are not autophagy specific. Another concern is that 3-MA, which is used at very high concentrations to inhibit autophagy (usually 10 mM), can target other kinases and affect other cellular processes. Indeed, 3-MA can suppress proteolysis even in Atg5-deficient cells, suggesting that its effects on protein degradation extend beyond its role in autophagy inhibition (Mizushima et al., 2008). Although PI3K inhibitors block the formation of autophagosomes, the other major pharmacological inhibitors in experimental use block the later stages of autophagy. More specific inhibition of the autophagy pathway can be achieved by knockout or knockdown of different autophagy genes. To date, autophagy deficiency or reduction has been confirmed in cells lacking Atg3, Atg5 and Beclin 1 (Sou et al., 2008).

2. Autophagy activators

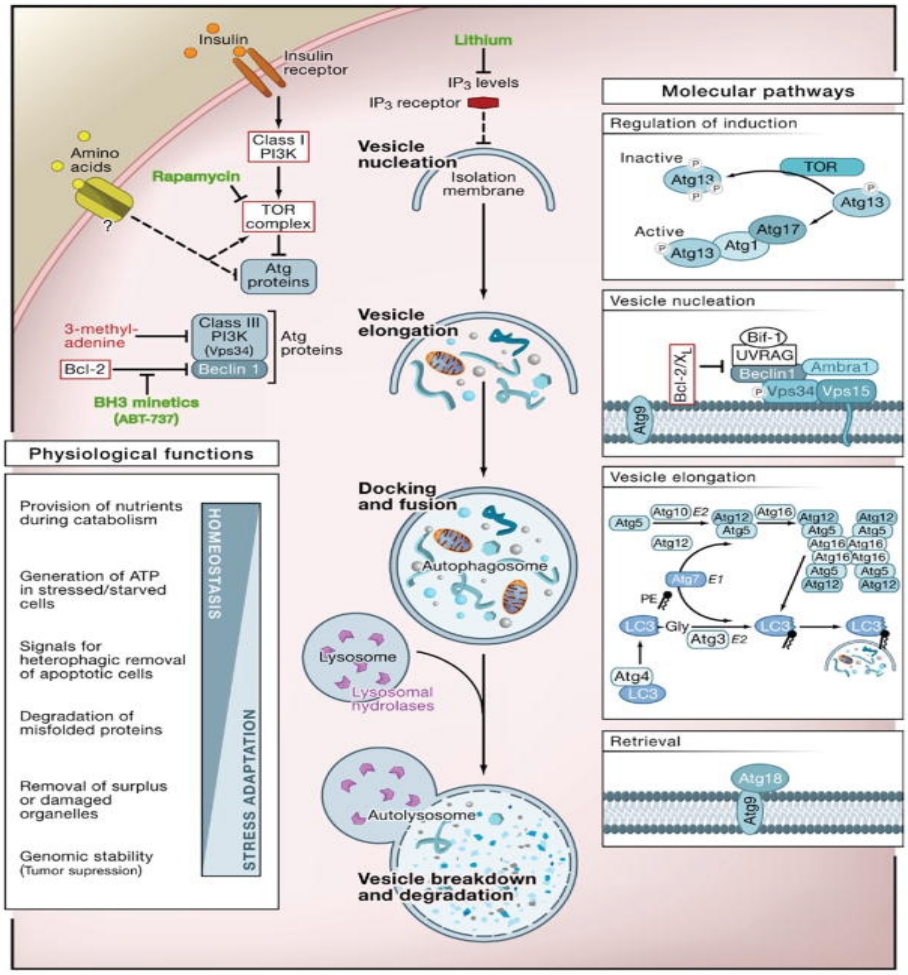
There is growing interest in activators of autophagy, not only for research purposes and also for therapeutic purposes. Similar to the case with autophagy inhibitors, there are several different methods to activate autophagy, but they lack complete specificity for the autophagy pathway.

a- mtor dependent pathway

Autophagy usually takes place in the cell in normal basal conditions to ensure cell integrity. Eventhough, the best known physiological inducer of autophagy is starvation, which induces such effects both in vitro and in vivo. In most cell lines, autophagy induction can be observed within 1 hr of amino acid withdrawal; a notable exception is that certain tumor cell lines may be resistant to starvation induced autophagy. Another way to activate autophagy is through the modulation of nutrient sensing signaling pathways. The best target is mtor, which is a potent suppressor of autophagy. Rapamycin, an inhibitor of mtor, activates autophagy both in vitro and in vivo (Ravikumar et al., 2004) **(Figure 12).** Mammalian Atg 13 ,

ULK1 and ULK2 have recently been identified as direct targets of mtor. Atg13 binds ULK1/ULK2 so they would interact with FIP200. Under nutrient rich conditions, mtor is part of this complex. Inhibition of mtor, leads to its dissociation from the complex and results in partial dephosphorylation of Atg 13 and ULK1/2 which leads to the activation of ULK1/2 and thus phosphorylation of FIP200 and thus induction of autophagy (Ravikumar et al., 2009).

Insulin like growth factor signaling activates mtor via PI3K and Akt. Activation of adenosine monophosphate activated protein kinase (AMPK) inhibits mtor activity by tuberous sclerosis complex (Tsc)1, Tsc2 and Ras homology enriched in brain (Rheb) (Hall et al., 2008). p53, a commonly mutated gene in human cancers, can positively and negatively regulate autophagy via the mtor pathway. Activated P53 can stimulate autophagy by activating AMPK or by upregulating PTEN and Tsc1. Genetic or chemical inhibition of p53 can also activates autophagy (Levine and Abrams, 2008).



<u>Figure12:</u> Autophagic regulatory pathways that have been targeted pharmacologically for experimental or clinical purposes. Inhibitors and activators of autophagy are shown in red and green, respectively (Kroemer et al., 2008).

b- mtor independent pathway

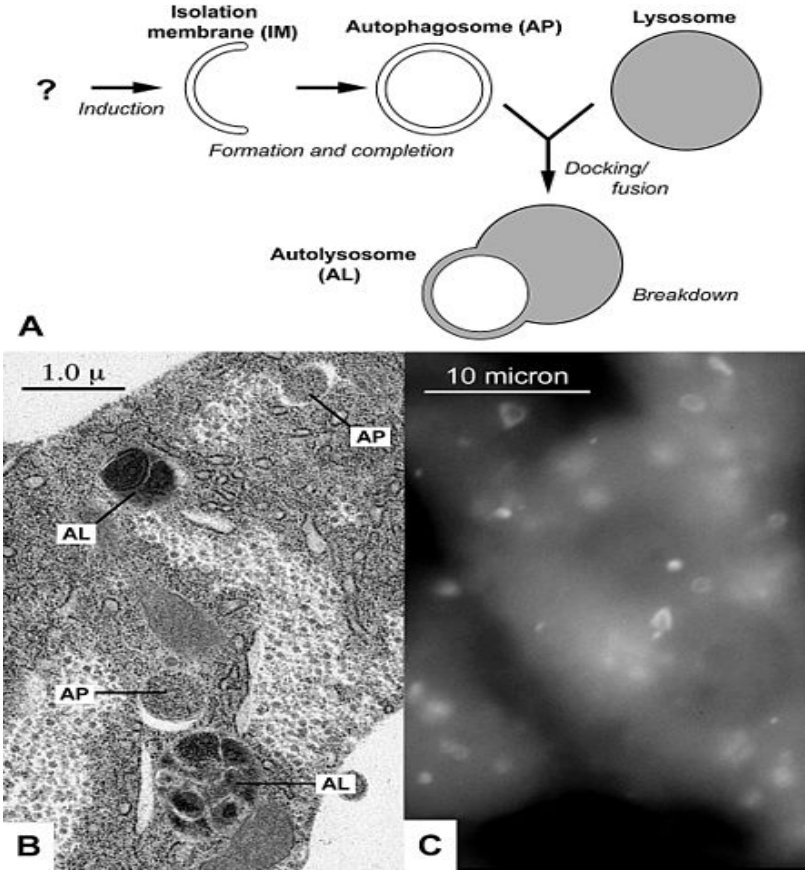
Several mtor independent autophagy activators have also been reported like Lithium **(Figure 12)**, a drug used to treat neurodegenerative diseases induces autophagy through inhibition of inositol monophosphatase which is mtor independent (Sarkar et al., 2005).

D- Methods for autophagy detection

The following methods detect different stages of the autophagy pathway like autophagosome, autolysosome, autophagic degradation products. One should use more than one method concurrently in order to determine whether an increase in any of the autophagic markers in the pathway represents a true increase in autophagic degradation or just a block in the completion of the autophagic pathway.

1. Electron microscopy

Mammalian autophagy was discovered in 1950's by electron microscopists during their work on lysosomes. An autophagosome is defined as a double membraned structure containing undigested cytoplasmic contents, which has not fused with a lysosome as shown in **(Figure 13)**. Although this may be the case in "bulk autophagy," there is increasing evidence for organelle-specific autophagy, including pexophagy, mitophagy, ribophagy, and reticulophagy (Van der Vaart et al., 2008). In contrast to an autophagosome containing cellular cargo which is usually easy to identify, the distinction of autolysosomes from other cellular membranous compartments is often more difficult. The autolysosome is an organelle generated by the fusion of an autophagosome and a lysosome, an endosome can also be involved, which has a single limiting membrane and contains cytoplasmic materials at various stages of degradation. At early stages, the inside materials can be recognized as having originated from cytoplasm. However, if degradation proceeds too far, it is not easy to determine whether the inside materials are of intracellular origin (Mizushima et al., 2010).



<u>Figure 13:</u> Autophagososmes and autolysosomes under the electron microscope (Kroemer et al., 2008)

2. Fluorescence microscopy

The mammalian protein of autophagy LC3 is considered a widely used marker for autophagososmes. Among the three LC3 isoforms (A,B and C), LC3B is the most widely used (Klionsky et al., 2012) . Soon after synthesis, formed LC3 is processed at its C terminus by Atg4 and becomes LC3-I, which has a glycine residue at the C-terminal end. LC3-I is after being conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE) by a ubiquitination-like enzymatic reaction. In contrast to the cytoplasmic localization of LC3-I, LC3-II is found in both the outer and inner membranes of the autophagosome. After fusion with the lysosome, LC3 on the outer membrane is cleaved off by Atg4 and LC3 on the inner membrane is degraded by lysosomal enzymes, resulting in very low LC3 content in the autolysosome. Thus, endogenous LC3 or green fluorescent protein (GFP)-LC3 is visualized by fluorescence microscopy either as a diffuse cytoplasmic pool or as punctate structures that represent autophagosomes. Although the number of punctate LC3 or GFP-LC3 structures per

cell is usually an accurate measure of autophagosome number, this assay has some difficulties. The number of punctate structures can be counted visually or automatically using computerized software image analysis programs (Mizushima et al., 2010) **(Figure 14A-B).**

Another assay that helps in autophagy detection requires the use of fluorescence microscopy and that is measuring florescence in acidic compartments **(Figure 14C)** like delivery of mRFP-GFP-LC3 to the lysososomes.

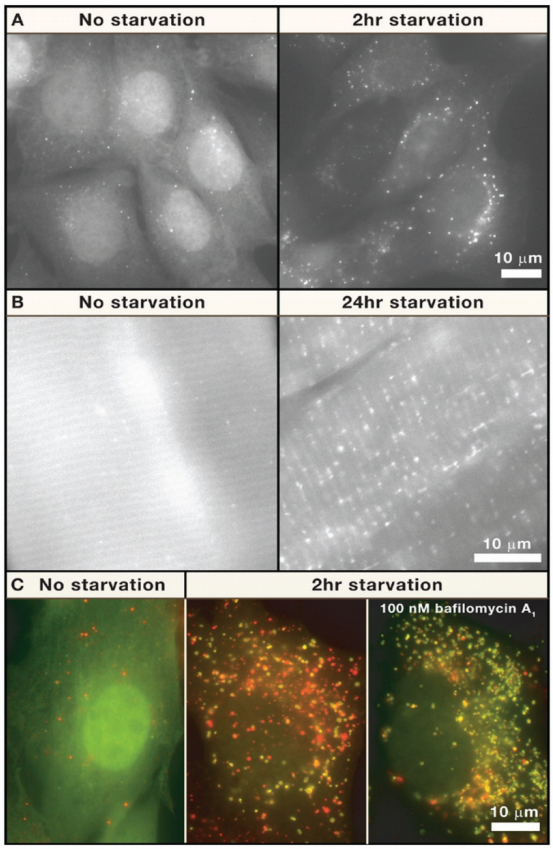


Figure 14: GFP-LC3 and mRFP-GFP-LC3 Puncta Formation Assays. (A) NIH 3T3 cells in culture stably expressing GFP-LC3 with (right) or without (left) 2 hr of starvation (depletion of both amino acids and serum). There is not only an increase in GFPLC3 puncta number, but also a decrease in total GFP-LC3 fluorescent signals during the 2 hr incubation period. (B) Example of an analysis of GFP-LC3 transgenic mice. Skeletal muscle and heart muscle samples were prepared from GFP-LC3 transgenic mice before or after 24 hr of starvation. (C) Mouse embryonic fibroblasts expressing mRFP-GFP-LC3 (left) were subjected to starvation treatment (2 hr) with (right) or without (middle) 100 nM bafilomycin A1 to inhibit autophagosome/lysosome fusion. Note that both yellow (autophagosome) and red (autolysosome) puncta increase in the middle panel, whereas most puncta in the right panel (Mizushima et al., 2010).

3. Biochemistry Assays

LC3 can be also detected in biochemical assays to assess autophagosome numbers. The conversion from endogenous LC3-I to LC3-II and from GFP-LC3-I to GFP-LC3-II can be detected by immunoblotting with antibodies against LC3 and GFP, respectively. Although the actual molecular weight (MW) of LC3-II is larger than that of LC3-I, LC3-II (apparent MW is 14 kD) migrates faster than LC3-I (apparent MW is 16 kD) in SDS-PAGE because of extreme hydrophobicity of LC3-II. The amount of LC3-II usually correlates well with the number of autophagosomes. However, when components of the autophagic machinery are inactivated due to genetic or pharmacological reasons, it is still possible that autophagy is suppressed even if LC3-II is detected. In such cases, other approaches including GFP-LC3 labeling methods and autophagic flux assays are required to study autophagic activity.

4. Detecting autophagic flux

a- LC3 turnover assays

One of the principal methods to measure autophagic flux is the monitoring of LC3 turnover, which is based on the observation that LC3-II is degraded in autolysosomes. If cells are treated with lysosomotropic reagents such as ammonium chloride, chloroquine (CQ), or bafilomycin A1, which inhibit acidification inside the lysosome or inhibit autophagosome-lysosome fusion, or with inhibitors of lysosomal proteases such as E64d and pepstatin A, the degradation of LC3-II is blocked, resulting in the accumulation of LC3-II (Tanida et al., 2005). Accordingly, the differences in the amount of LC3-II between samples in the presence and absence of lysosomal inhibitors represent the amount of LC3 that is delivered to lysosomes for degradation and that is what is called autophagic flux (Klionsky et al., 2012).

For example, levels of LC3-II are increased by treatment with CQ even under nonstarvation conditions **(compare lanes 1 and 3**, **Figure 15A**). However, the difference in LC3-II levels in the presence and absence of CQ is larger under starvation conditions **(compare lanes 3 and 4 Figure 15B),** indicating that autophagic flux is increased during starvation.

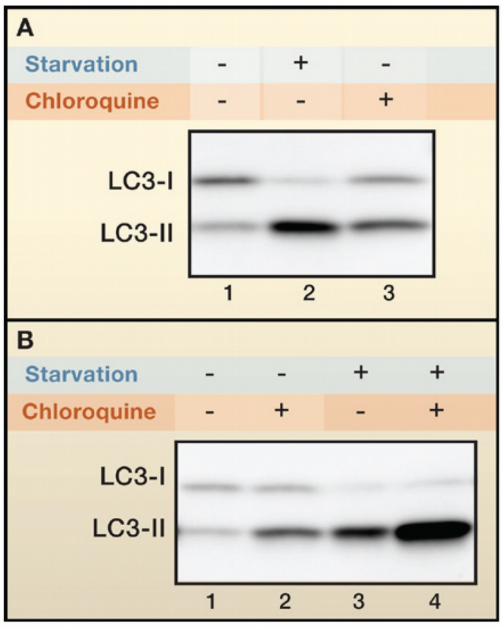


Figure 15: LC3 turn over assays. (A) Steady-state levels of LC3 expression. Mouse embryonic fibroblasts were cultured in regular Dulbecco's modified Eagle's medium (DMEM) culture medium (lane 1), DMEM without amino acids and serum (lane 2), and regular DMEM containing 20 μM CQ (lane 3) for 1 hr. Cell lysates were subjected to immunoblot analysis with an anti-LC3 antibody. The positions of LC3-I and LC3-II are indicated. (B) LC3 turnover assay for measuring autophagic flux. Cells were cultured as in (A), and the difference in LC3-II levels between samples with and without CQ was compared under nonstarvation and starvation conditions. (Mizushima et al., 2010).

b- Degradation of selective substrates

Besides LC3, levels of other autophagy substrates can be used to study autophagic flux. Recent studies have revealed that several specific substrates are preferentially degraded by autophagy, of which the best studied example is p62 (also known as SQSTM1/sequestome 1). p62 is selectively incorporated into autophagosomes through direct binding to LC3 and is degraded by autophagy; thus, the total cellular expression levels of p62 inversely correlate with autophagic activity. For example, a starvation-induced reduction in p62 levels is not observed in autophagy-deficient cells but instead p62 accumulates (Yoshimori et al, 2007). Overall, this assay seems quite promising.

c- Delivery of mRFP-GFP-LC3 to the Lysosome

Another useful method to measure autophagic flux is based on the concept of lysosomal quenching of GFP in GFP labeled autophagic substrates such as LC3. GFP is a stably folded protein and relatively resistant to lysosomal proteases. However, the low PH inside the lysosome quenches the fluorescent signal of GFP, which makes it difficult to trace the delivery of GFP-LC3 to lysosomes. Most GFP-LC3 punctate signals do not colocalize with lysosomes (Kabeya et al., 2000). In contrast, RFP (and other red fluorescent proteins, such as mCherry) exhibits more stable fluorescence in acidic compartments and mRFP-LC3 can readily be detected in autolysosomes. By exploiting the difference in the nature of these two fluorescent proteins (like lysosomal quenching of GFP fluorescence versus lysosomal stability of RFP fluorescence), autophagic flux can be morphologically traced with an mRFP-GFP-LC3 tandem construct **(Figure 14C).** With this novel construct, autophagosomes and autolysosomes are labeled with yellow (mRFP and GFP) and red (mRFP only) signals. If autophagic flux is increased, both yellow and red punctae are increased. However, if autophagosome maturation into autolysosomes is blocked, only yellow punctae are increased without a concomitant increase in red punctae (Mizushima et al., 2010).

VII- The role of autophagy in physiology and pathology

The process of autophagy takes place in the cells at all times but at basal conditions. Normally autophagy removes damaged proteins and organelles to sustain homeostasis and as to say to keep quality control of the cell. However, induced autophagy like in case of starvation or stress, removes bulk intracellular components to be degraded and recycled providing building blocks for metabolic pathways and for synthesis of required stress response proteins needed to keep cell's integrity. Failure in autophagy leads to accumulation of reactive oxygen species, metabolic deficiency and toxicity all of which can make the cell susceptible to various diseases and the initiation of tumor development (Ravikumar et al., 2009).

A- Autophagy in physiology

Genetic targeting of key autophagy regulators in the mouse germ line has further highlighted the importance of autophagy for normal mammalian development. For example, Atg5 deficient mice were born but died within 1 day of birth due to a dependence on autophagy of the heart and diaphragm for nutrients and energy during the immediate postnatal period (Barth et al., 2010). Similar phenotypes were observed for mice lacking Atg7 or Atg3. Further, tissue-specific knockout of Atg5 or Atg7 in brain led to the neurodegeneration and difficulties in motor function, while loss of Beclin 1 (Atg6) resulted in early embryonic lethality characterized by reduced embryo size. In addition, fertilization induces autophagy. Autophagy defective oocytes derived from oocyte specific Atg 5 knockout mice failed to develop beyond the four and eight cell stages if they were fertilized by Atg5-null sperm (Tsukamoto et al., 2008). Autophagy also appears to regulate the clearance of apoptotic corpses during development (Qu et al., 2007). Further, autophagy is considered to be a good way for clearing the body from intracellular bacteria, viruses and protozoans but may promote pathogen replication at some conditions. It participates in antigen formation as part of the adaptive immune response (Ravikumar et al., 2009). In the heart, it has a house keeping role under normal conditions and it is enhanced during cardiac aging and acute ischemia. It prevents accumulation of toxic proteins that may result in physiological dysfunction. It prevents liver dysfunction by removal of nonfunctional ER that results from accumulation of aggregate prone proteins. Moreover, autophagy removes associated protein aggregates such as mutant huntingtin which causes Huntington's disease, and mutant forms of α-synuclein which causes forms of familial Parkinson's disease. The clearance of such proteins is impaired when autophagy is compromised. This function of autophagy might also be relevant physiologically, because protein aggregates form in the brain and some other tissues from endogenous 'normal' proteins in Atg5 or Atg7 knockout mice, which have compromised autophagy. Such a mechanism might also contribute to other neurodegenerative diseases. For instance, recent studies suggest that autophagosome-lysosome fusion is impaired in a form of fronto-temporal dementia that is caused by mutations in a gene in the ESCRT complex, and by mutations in the dynein-complex component dynactin that cause a form of motor neuron disease (Munch et al., 2004). This

will impair the clearance of autophagic substrates and predispose cells to aggregate formation **(Figure 16)**.

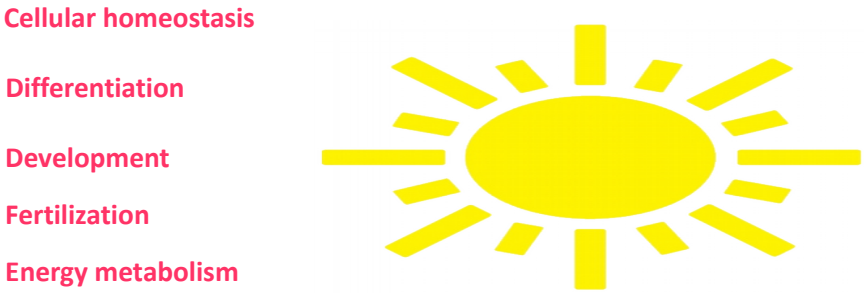


Figure 16: The role of autophagy in health (Yassine Maya)

B- Autophagy in cancer

Research has become more interested in knowing the relationship between autophagy and cancer especially its role in regulation of cancer development and progression. In addition to the role that autophagy plays in defining the destiny of cells upon introduction to anticancer therapy.

The regulation of autophagy overlaps signaling pathways that regulate tumorigenesis. Several tumor suppressor genes involved in the upstream inhibition of tor signaling, including PTEN, Tsc1, Tsc2, dependent death associated protein kinase (DAPK) and LKB1 stimulate autophagy and, conversely, tor activating oncogene products such as bcl-2 class I PI3K and Akt inhibit autophagy (Kroemer and Levine 2008). Indeed, a number of autophagy related proteins (ATG) including beclin1, ATG5, ATG4and ATG7 have been shown to be tumor suppressors (Kondratskyi and Yassine et al., 2013). One of the possible mechanisms for the anti-tumorigenic functions of autophagy is based on its cytoprotective role.

However, evidence for the importance of autophagy in protecting against nutritional stress comes from studies where tumor cells are deprived of survival factors, lead to an increase in autophagy that prevented the cells from dying. Moreover, when autophagy is prevented under these conditions, the cells undergo apoptosis (Boya et al., 2005). Thus, when tumor cells are starved, autophagy stops them from dying by inhibiting apoptosis. Autophagy keeps tumor cells alive when limited angiogenesis leads to nutrient deprivation

and hypoxia; therefore, we would expect that increased autophagy would promote the growth of solid tumors and Ras driven cancers, whereas reduced autophagy might provide a useful way to limit established tumor growth**.** Suppression of essential autophagy proteins was shown to inhibit cell growth indicating that autophagy maintains tumor cell survival and suggesting that blocking autophagy in estabished tumors and tumors that are addicted to autophagy, such as Ras-driven cancers, may be an effective treatment **(Figure 17)**. Thus, Inhibition of autophagy in already established tumor cells has been shown to enhance the efficacy of anticancer drugs, supporting its role in cytoprotection (Mathew and White., 2011).

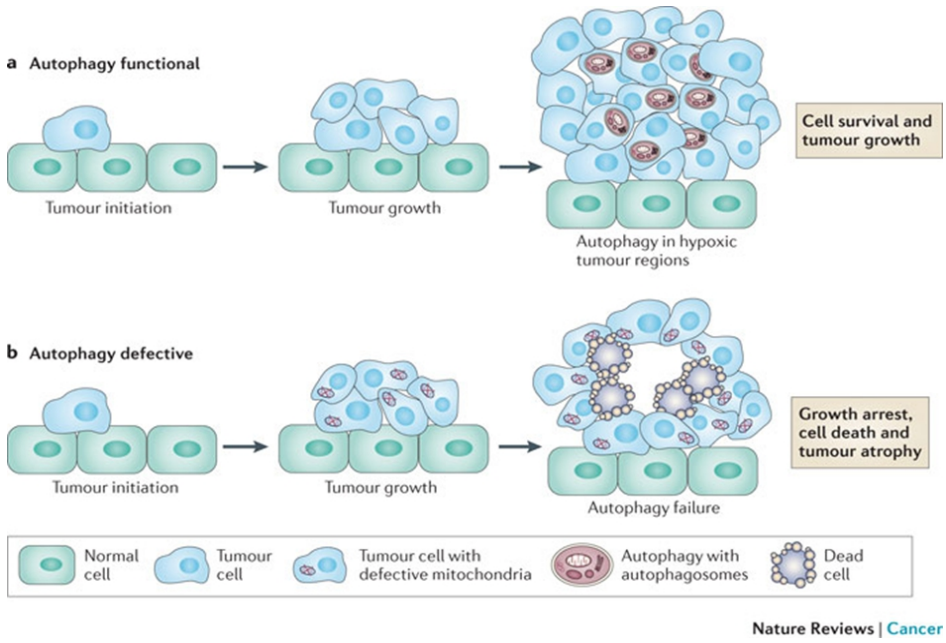


Figure 17: The role of autophagy in supporting the growth of aggressive cancers (White et al., 2012).

Knowing that autophagy helps tumors in advanced stages to grow and survive, it can also have a preventive anticancer role and that is by inhibiting early tumor fromation **(Figure 18 a**). It is a matter of balance between oncogenesis and tumor suppression in early and established tumors. Studies indicate an anticancer role for autophagy. The autophagy gene Beclin 1 (which is part of a type III PI3K complex) required for autophagic vesicle formation, is a tumor suppressor in mice and is lost in human breast, ovarian, and other tumors (Kondo et al., 2005). 75% of ovarian cancers, 50% of breast cancers and 40% of prostate cancers are haploinsufficient for beclin 1 (White et al., 2012). Also, another autophagy gene ATG5 acts as

a tumor suppressor in leukemia and is required for maintaining cell survival and proliferation (Kreuzaler et al., 2012). The molecular link between defective autophagy and tumorigenesis is the accumulation of the autophagy substrate p62 that leads to production of reactive oxygen species and DNA damage causing DNA instability (Zhineng et al., 2011) inducing cellular stress and increasing the potential of tumor development. Moreover, p53 and PTEN, two of the most commonly mutated tumor suppressor genes, both induce autophagy. Conversely, the oncogenic protein Bcl-2 directly interacts with Beclin 1 to inhibit autophagy (Pattingre et al., 2005). Because oncogenes can inhibit autophagy and tumor suppressors induce autophagy this suggests that autophagy serves an anticancer role. The mechanism through which autophagy inhibits tumor development is unclear. Possibilities include limiting tumor cell growth or reducing mutagenesis or other damage caused by reactive oxygen species by removal of damaged mitochondria and other organelles. Alternatively, autophagy may kill developing tumor cells. In support of this idea, a cell death pathway that involves both autophagy and apoptosis is suggesting that autophagy prevents early tumor development. Therefore, increasing autophagy to prevent tumor formation in persons with risk for cancer and reducing it if a tumor is already established would be a good idea **(Figure 18)**.

Many anticancer agents have been reported to induce autophagy, leading to the suggestion that autophagic cell death may be an important mechanism of tumor cell killing by these agents. However, autophagic cell death remains a debatable issue since autophagy occurs in tumor cells before their death and it more known as a protective mechanism by cells and not killing. It could only mean that the chemotherapeutic drugs induced autophagy and then the cells died. In short, “It has become clear with increased research that autophagy constitutes a futile attempt of dying cells to adapt to lethal stress rather than a mechanism to execute a cell death program”, as said by Kroemer (Kroemer et al., 2011).

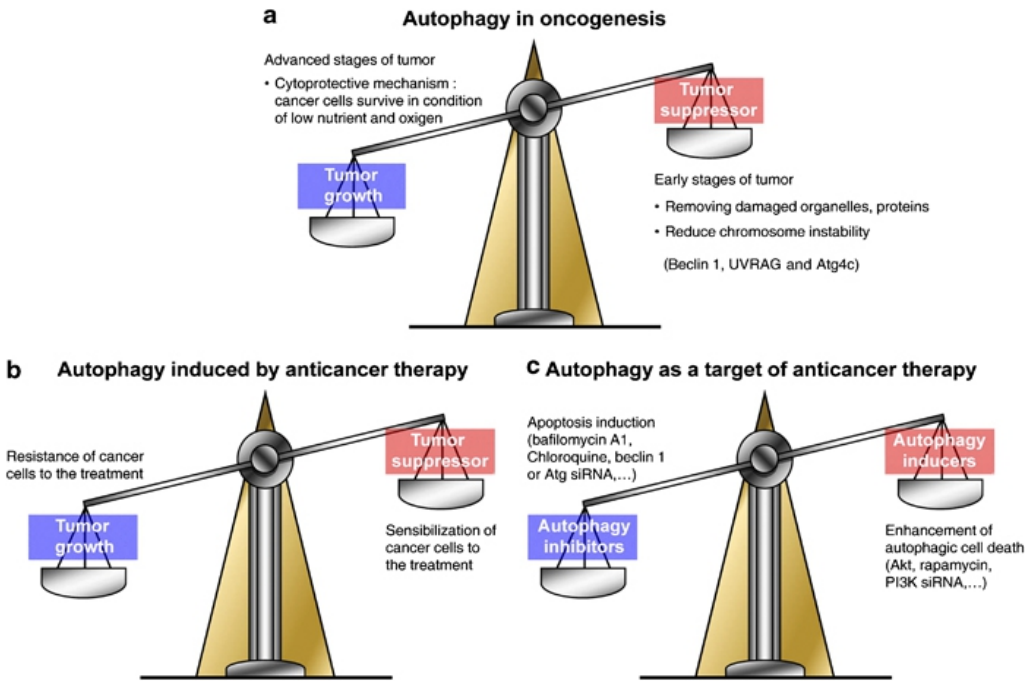


Figure 18: **Autophagy and cancer progression and cancer treatment (Kroemer et al., 2011)**

VIII- The dual role of calcium in autophagy

It is worth emphasizing that cancer and autophagy are considered to be both calcium dependent mechanisms, the idea behind the work done in this thesis. Recent findings identified intracellular calcium as a key regulator of both basal (Cardenas et al., 2010) and induced (Hoyer-Hansen et al., 2007) autophagy. The complex role for calcium in autophagy regulation has become obvious since 1993, when the first report linking autophagy and intracellularly sequestered calcium was published (Gordon et al., 1993). Indeed, Gordon et al. demonstrated that decrease as well as increase in cytosolic calcium levels inhibited autophagy in rat hepatocytes (Gordon et al., 1993). And till now, the data on the mechanisms by which calcium controls autophagy remain rather controversial. Several groups reported inhibitory actions of calcium on autophagy, while another proposed mechanisms for calcium to activate autophagy (Decuypere et al., 2011a; Cardenas and Foskett, 2012; Parys et al., 2012). Indeed, Hoyer-Hansen and colleagues provided evidence that a rise in the free cytosolic calcium is a potent inducer of macroautophagy (Hoyer-Hansen et al., 2007). They demonstrated that calcium mobilizing agents, namely vitamin D3, thapsigargin, ATP and

ionomycin, stimulate autophagy via a signaling pathway involving calcium activated kinase CAMKK-beta, which directly activates AMPK to inhibit mTOR (Hoyer-Hansen et al., 2007). Recently, this pathway was shown to be required for amyloid-beta peptide induced autophagosome formation (Son et al., 2012). Calcium/CAMKK-beta/AMPK pathway, although mTOR-independent, has been found to be involved in the leucine-rich repeat kinase-2 (LRRK2) induced autophagy (Gomez-Suaga et al., 2012). Authors proposed the mechanism in which LRRK2 activates NAADP receptors, in particular TPC2, leading to calcium mobilization from acidic stores that in turn stimulates calcium induced calcium release from ER and subsequent CAMKK-beta/AMPK pathway activation (Gomez-Suaga et al., 2012). In all the cases discussed above, buffering of cytosolic calcium with BAPTA-AM effectively inhibited autophagosomes accumulation, confirming the role of calcium. Another evidence supporting stimulatory effect of calcium on autophagy is that exogenously introduced calcium in the form of calcium phosphate precipitates induces macroautophagy, which is Beclin1, Atg5 and PI3K class III dependent (Gao et al., 2008). This effect could be antagonized by extra- or intra-cellular calcium chelation.

In line with activatory role of calcium in autophagy, Sakaki et al. showed that (Ca2+) calcium dependent activation of protein kinase Ctheta is required for ER-stress induced autophagy but not for starvation induced autophagy (Sakaki et al., 2008).

In another studies Ca2+/calmodulin DAPK was shown to positively regulate autophagy in a Beclin1 dependent manner. Thus, DAPK phosphorylates Beclin1, thereby promoting its dissociation from Bcl-XL and Bcl-2 inhibitory proteins (Zalckvar et al., 2009 a,b).

On the contrary, Khan et al suggested that basal autophagic flux may be negatively regulated by IP3R-dependent Ca2+ release from the ER (Khan and Joseph, 2010). The authors proposed a mechanism in which cytosolic Ca2+ elevation acts to maintain an elevated mtor complex 1 activity through AMPK independent pathway (Khan and Joseph, 2010).

Studies have shown that mtor independent pathway that is marked by reduced levels of IP3, is regulated by intracellular Ca2+ levels cAMP, and have revealed additional drugs that might induce autophagy, such as verapamil, an L-type Ca2+-channel antagonist, and clonidine, an imidazoline receptor agonist that reduces cAMP levels (Williams et al., 2008). In this pathway, autophagy is inhibited when intracellular cAMP levels are increased by adenylyl

cyclase (AC), which influences autophagy by activating the guanine nucleotide exchange factor Epac. Epac then activates Rap2B, which inhibits autophagy by activating phospholipase C (PLCε), resulting in the production of IP3, which mediates the release of Ca2+ from ER stores. Increased intracytosolic Ca2+ blocks autophagy by activating calpains that are a family of Ca2+-dependent cysteine proteases which mediate their effects on autophagy through Gsα, which is activated after calpain cleavage. This, in turn, increases AC activity to increase cAMP levels, thus forming a loop.

Furthermore, amino acids were shown to induce an increase in [Ca2+]i, supposedly through the influx of extracellular Ca2+, which acts to enhance the binding of Ca2+/CaM to hVps34, resulting in mtor activation (Gulati et al., 2008). This pathway could also lead to autophagy inhibition.

Thus, calcium is likely to have different regulatory effects on autophagy **(Figure 19)**. It depends on spatial and temporal parameters of calcium signaling, nutrient and growth factor availability, as well as pathology (cancer, neurodegenerative disorders, inflammation etc.) (Decuypere et al., 2011).

IX- The role of known calcium permeable channels in autophagy regulation.

A- IP3R, autophagy and mitochonrial bioenergetics

Modulation of calcium permeable channels expression/function affects intracellular Ca2+ concentrations and, consequently calcium dependent processes, such as proliferation, apoptosis and autophagy. The role of calcium-permeable channels for proliferation and apoptosis is largely recognized (Monteith et al., 2007; Flourakis and Prevarskaya, 2009; Prevarskaya et al., 2010; Dubois et al., 2013), whereas the information about molecular nature of channels regulating autophagy and the mechanisms of this regulation is still limited.

Most reports considering calcium permeable channels as autophagy regulators focused on IP3R, the main intracellular Ca2+ release channel (Parys et al., 2012). Together these reports suggested a complex role for IP3R, since both stimulatory as well as inhibitory functions for IP3R toward autophagy have been described. Thus, in one paper it was suggested that cadmium (Cd2+) induces autophagy through elevation of cytosolic calcium via

IP3R and subsequent extracellular signal-regulated kinase (ERK) activation (Wang et al., 2008). As a proof for this, the authors showed that 2-APB, a blocker of IP3R, suppressed, while knockdown of CaN, a putative IP3R inhibitor, increased Cd2+ induced autophagy (Wang et al., 2008). However, 2-APB was shown to modulate a number of TRP channels, SERCA pump and SOCs (Peppiatt et al., 2003; Clapham, 2007) and as to CaN, its role in the IP3R regulation is debated at present (Bultynck et al., 2003).

In another study, IP3R was shown to be required for differentiation factor DIF-induced autophagic cell death in Dictyostelium discoideum (Lam et al., 2008). Through random insertional mutagenesis, the authors showed that inactivation of the iplA gene, the only gene encoding an IP3R in this organism, prevented autophagic cell death (Lam et al., 2008).

The stimulatory role of IP3R on starvation induced autophagy has been recently shown (Decuypere et al., 2011b). The authors showed that the Ca2+ chelator BAPTA-AM as well as the IP3R inhibitor xestospongin B abolished starvation induced increase in LC3 lipidation and GFP-LC3-puncta formation. Moreover, starvation lead to IP3R sensitization through increased Beclin1 binding to the IP3R (Decuypere et al., 2011b).

To date, most reports on IP3R-dependent regulation of autophagy suggest inhibitory role for IP3R toward autophagy (Parys et al., 2012). Thus, lithium (Li+) was found to induce mtor independent autophagy through inhibition of inositol monophosphatase and further decrease in IP3 levels (Sarkar et al., 2005). Consistently, another study demonstrated that IP3R inhibitor xestospongin (XeB) or IP3R knockdown induced autophagy in HeLa cells (Criollo et al., 2007). One of the mechanisms, by which XeB and starvation induce autophagy was proposed by Vicencio et al. (2009). The authors suggested that XeB and nutrient starvation disrupt a molecular complex formed by the IP3R, Beclin 1 and Bcl-2, and presented evidence that the IP3R represses autophagy through Bcl-2-mediated binding of Beclin 1, thus suggesting Ca2+-independent mechanism (Vicencio et al., 2009).

As three IP3R isoforms exist, some groups studied the impact of IP3R on autophagy in the triple IP3R-deficient DT40 cells (Cardenas et al., 2010; Khan and Joseph, 2010). These cells demonstrate higher basal autophagy levels, compared to wild-type. Interestingly, expression of IP3R3, but not of ryanodine receptor type 2, rescued elevated autophagy in these cells (Cardenas et al., 2010). In contrast, expression of Ca2+ impermeable mutant

D2550A-IP3R3 failed to suppress constitutive autophagy, suggesting a necessity of the Ca2+-release activity for IP3R. The authors proposed the mechanism in which constitutive IP3R mediated Ca2+ release and uptake of this Ca2+ by mitochondria is fundamentally required to maintain mitochondrial bioenergetics and ATP production in resting cells thereby suppressing autophagy. Absence of this Ca2+ transfer results in inhibition of pyruvate dehydrogenase and activation of AMPK, which activates prosurvival macroautophagy in mTOR independent manner (Cardenas et al., 2010).

B- TRPML1

Along with IP3R some other calcium permeable channels were shown to be involved in autophagy regulation. Among them, TRPML1, also known as mucolipin-1, ubiquitously expressed TRP channel primarily localized to the late endosomal and lysosomal compartments (Zeevi et al., 2009; Cheng et al., 2010). Direct patch-clamp of enlarged lysosomes revealed that TRPML1 is a Ca2+ permeable channel (Dong et al., 2008). The main physiological function of TRPML1 channel is considered to serve as a late endosomal/lysosomal Ca2+ release channel. Loss of function mutations in the human TRPML1 gene result in mucolipidosis type IV, a neurodegenerative lysosomal storage disorder characterized by mental retardation and retinal degeneration (Bach, 2001; Altarescu et al., 2002). Fibroblasts from mucolipidosis type IV patients exhibit enlarged vacuoles with accumulated lipids and acid mucopolysacharides, suggesting the role for TRPML1 in trafficking of proteins and lipids (Riedel et al., 1985; Goldin et al., 1999; Slaugenhaupt et al., 1999). Loss of TRPML1 has been shown to be accompanied by impairment in the lysosomal pH, accumulation of autophagosomes and abnormal mitochondria, accumulation and aggregation of p62 and ubiquitin proteins, all of which suggested a defective autophagy (Jennings et al., 2006; Soyombo et al., 2006; Vergarajauregui et al., 2008; Curcio-Morelli et al., 2010). Indeed, several studies have proposed TRPML1 as autophagy regulator (Vergarajauregui et al., 2008; Venugopal et al., 2009; Curcio-Morelli et al., 2010; Wong et al., 2012a; Venkatachalam et al., 2013). Vergarajauregui et al. showed that accumulation of autophagosomes in TRPML1-deficient fibroblasts obtained from mucolipidosis type IV patients was due to increased Beclin 1 dependent autophagosome formation and delayed fusion of autophagosomes with late endosomes/lysosomes. The authors claimed that

TRPML1 is necessary for efficient fusion of both autophagosomes and late endosomes with lysosomes although it is not clear if the Ca2+ channel function of the TRPML1 is essential here (Vergarajauregui et al., 2008). In another study, group of S. Slaugenhaupt showed that CMA is impaired in mucolipidosis type IV fibroblasts (Venugopal et al., 2009). The authors showed that TRPML1 directly interacts with Hsc70 and Hsp40, members of molecular chaperone complex required for CMA, and hypothesized that this interaction may be required for intralysosomal Hsc70 to facilitate the translocation of CMA substrate proteins across the lysosomal membrane. The authors alo speculated that TRPML1 channel activity is required for CMA (Venugopal et al., 2009).

In 2010, same group investigated macroautophagy in neurons isolated from cerebellum of TRPML1−/− mouse embryos (Curcio-Morelli et al., 2010). These cells displayed higher levels of basal autophagy markers compared to wild-type ones. In addition, LC3-II clearance was affected in these cells, suggesting impairment of lysosomal function. However, the link between observed defects in autophagy and functionality of TRPML1 as a Ca2+ permeable channel is missing. Recently, Wong et al showed that Drosophila TRPML is required for tor complex 1 activation (Wong et al., 2012a). Authors demonstrated defects in amphisomes/lysosomes fusion and elevated late endosomal/lysosomal Ca2+ levels in flies lacking TRPML1. Authors also showed decreased tor complex 1 activity and increased induction of autophagy in TRPML1− mutants. Moreover, authors suggested that tor complex 1 regulates the subcellular localization of TRPML1. Thus, this study points out to TRPML1 as a Ca2+ channel present in amphisomes which releases luminal Ca2+ to facilitate Ca2+ dependent fusion of amphisomes with lysosomes (Wong et al., 2012a).

C- TRPML3

In addition to TRPML1, another member of mucolipin family, TRPML3 has been shown to be involved in autophagy regulation. In contrast to TRPML1, TRPML3 exhibits more restrictive tissue distribution, and is primarily localized to early as well as late endosomes/lysosomes and less to the PM (Zeevi et al., 2009; Cheng et al., 2010).

It has been shown that overexpression of TRPML3 leads to increased autophagy in HeLa cells (Kim et al., 2009). Moreover, TRPML3 is recruited to autophagosomes upon induction of autophagy. Additionally, expression of dominant negative mutant TRPML3 or

knockdown of endogenous TRPML3 by siRNA reduces autophagy. Thus, it has been proposed that TRPML3 provides Ca2+ that is required for fusion and fission events in autophagy (Kim et al., 2009). Further, heteromultimerization of TRPML channels was shown to affect autophagy (Zeevi et al., 2010).

D- TRPV1

Also, TRPV1 was proposed to regulate autophagy in thymocytes (Farfariello et al., 2012). The authors showed that capsaicin, an activator of TRPV1, induces Beclin 1 dependent accumulation of LC3-II protein. This effect can be antagonized by capsazepine, a blocker of TRPV1 and compound C, an AMPK inhibitor, suggesting AMPK involvement. The authors proposed that capsaicin induced autophagy is calcium dependent, as cotreatment with EDTA markedly reduced LC3-II accumulation. Moreover, it was shown that capsaicin induces accumulation of ATG4C and triggers its oxidation in a ROS dependent manner, thus regulating LC3 lipidation levels (Farfariello et al., 2012). However, capsaicin was shown to have TRPV1-independent effects, such as inhibition of VGCCs (Hagenacker et al., 2005), cancer cell growth inhibition and apoptosis induction (Mori et al., 2006; Chow et al., 2007). Additionally, upon prolonged exposure to capsaicin, TRPV1 desensitization occurs and its activity decreases (Caterina et al., 1997). Thus, additional experiments using more specific agonists and antagonists as well as siRNA knockdown are needed to confirm the role of TRPV1 in autophagy regulation. It would be interesting as well to compare the effect of capsaicin on autophagy in TRPV1-expressing and TRPV1-null cells.

E- L type calcium channels and autophagy

Some ion channels, which do not belong to the family of TRP channels, were also proposed to regulate autophagy. Williams et al. found that L-type calcium channels antagonists, namely verapamil, loperamide, nimodipine, nitrendipine and amiodarone induce mtor independent autophagy (Williams et al., 2008). Conversely, the L-type Ca2+ channel agonist (±)-BAY K 8644 that increases cytosolic Ca2+ levels, inhibits autophagy. Authors demonstrated that elevated cytosolic Ca2+, presumably due to activity of L-type calcium channels on the plasma membrane, can activate calpains, a family of Ca2+-dependent cysteine proteases, which cleave and activate the α-subunit of heterotrimeric G proteins Gsα. Gsα activation, in turn, increases adenylyl cyclase activity leading to increase in

cAMP levels. Next, elevated intracellular cAMP levels negatively regulate autophagy by promoting IP3 production via cAMP-Epac-Rap2B-PLC-ε pathway. Finally, IP3, via IP3R influence cytosolic Ca2+ levels, which can again activate calpains, thus creating a potential positive feedback loop for autophagy inhibition (Williams et al., 2008).

Again, it is important to mention that although several different L-type calcium channel inhibitors as well as agonist were used in the study, the data showing the effect of siRNA mediated knockdown of L-type calcium channels and/or channel-dead mutants on autophagy are missing. It would be interesting as well to check the effect of these inhibitors on "negative control" cells lacking L-type calcium channels. In addition, verapamil is known to passively diffuse into the lysosome, where it becomes protonated and could cause an increase in lysosomal pH (Lemieux et al., 2004). This could lead to the inhibition of lysosome function and thus block fusion with the autophagosome.

F- Two pore channels and autophagy

TPCs have been also proposed to regulate autophagy (Pereira et al., 2011; Gomez-Suaga et al., 2012). Mammalian TPC family comprise two members TPC1 and TPC2, widely expressed in humans and localized intracellularly on endolysosomes, with TPC2 being specifically targeted to lysosomes. Several groups proposed TPC as a mediator of endolysosomal calcium release in response to the elevation of the second messenger, NAADP (Calcraft et al., 2009; Galione et al., 2009). Recently Pereira et al. demonstrated that NAADP stimulates autophagy via TPCs in rat astrocytes (Pereira et al., 2011). The authors showed that NAADP mediated increase in the number of LC3-GFP puncta was reduced in cells, transfected with dominant negative TPC2 L265P construct, suggesting the importance of TPC2 for autophagy (Pereira et al., 2011). TPC2 channel has been also proposed to be involved in LRRK2 induced autophagy (Gomez-Suaga et al., 2012).

G- Mitochondrial calcium uptake channel and autophagy

The mitochondrial calcium uptake (MCU) that was recently identified as a channel responsible for mitochondrial Ca2+ uptake (Baughman et al., 2011; De Stefani et al., 2011) has been demonstrated to have the role in autophagy regulation. Indeed, Cardenas et al. showed that the uniporter inhibitor Ru360 inhibited cell O2 consumption rate, activated

AMPK, and induced autophagy (Cardenas et al., 2010). In line with these data, MCUR1 (mitochondrial calcium uniporter regulator 1) was shown to regulate autophagy (Mallilankaraman et al., 2012). MCUR1 represents an integral membrane protein that is required for MCU-dependent mitochondrial Ca2+ uptake. Knockdown of MCUR1 in HeLa and HEK293T cells reduced cell O2 consumption rate, activated AMPK, and induced macroautophagy (Mallilankaraman et al., 2012). Importantly, stable knockdown of MCU in HeLa cells elicited essentially the same effects, confirming the regulatory role for MCU toward autophagy (Mallilankaraman et al., 2012).

MPTP has been also suggested to be implicated in autophagy regulation. Elmore et al. proposed that mitochondrial permeability transition (MPT) initiates autophagy in rat hepatocytes. Although the mechanism by which the MPT signals autophagic sequestration was not investigated in this work, the authors hypothesized that factors released from the mitochondrial intermembrane space as a consequence of MPT could stimulate autophagy (Elmore et al., 2001). It is not clear if the Ca2+ release channel function of the (mitochondrial permeability transition pore). MPTP is essential here as well. A functional MPTP was also shown to be required for starvation-induced mitochondrial autophagy (Carreira et al., 2010). The authors demonstrated that starvation induced mitochondrial depolarization in cardiac cells. This depolarization was prevented by cyclosporin A (MPT inhibitor). Further, the authors showed that cyclophilin D a component of the MPTP, is required for mitochondrial removal by starvation-induced autophagy. Interestingly, cardiomyocytes from cyclophilin D deficient mice failed to upregulate autophagy in response to nutrient deprivation, suggesting that MPTP is essential here (Carreira et al., 2010). Again the role of calcium and the importance of calcium permeability for MPTP in the regulation of autophagy were not assessed in this study.

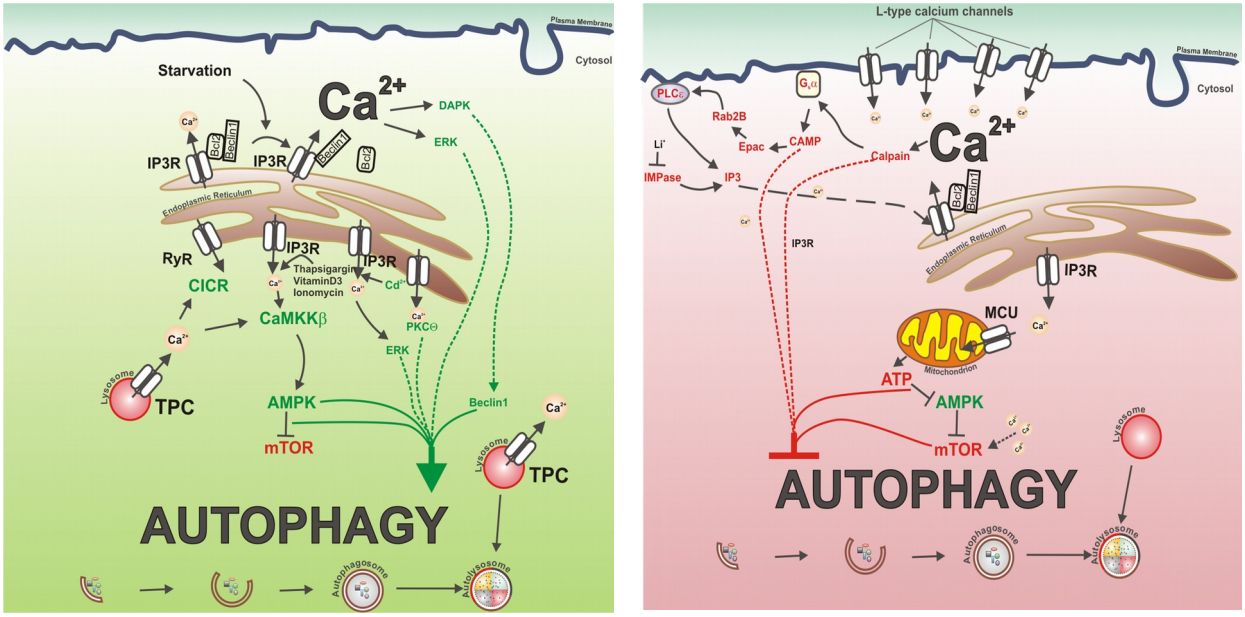


Figure 19: Stimulatory and inhibitory functions for calcium towards autophagy (Yassine Maya).

X- The implication of autophagic and calcium modulators in cancer treatment

A- Autophagy modulators

For a long time, it was suggested that autophagy acts as a tumor suppressor in several cancers. However, it is not the case in established tumors where it acts as a mechanism to support the survival of established cancer cells.

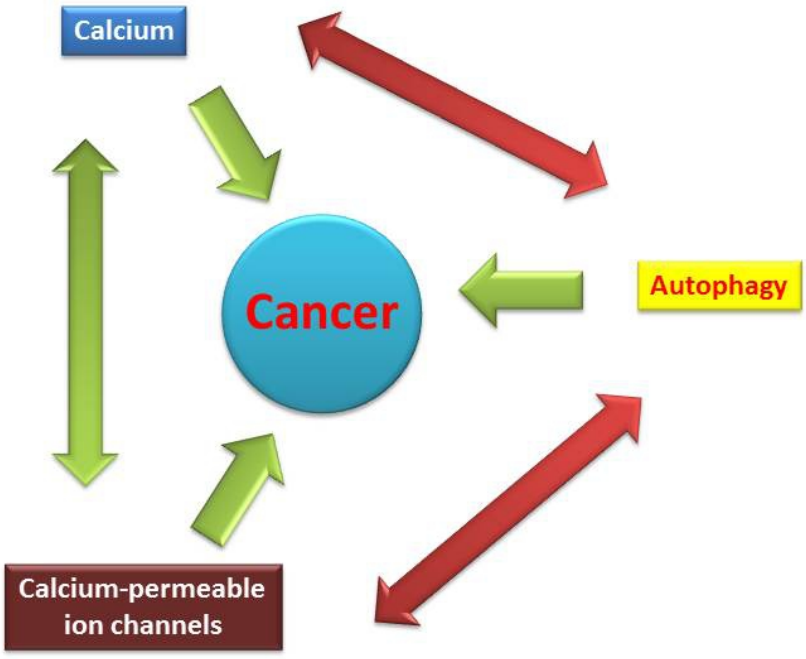
Thus, it is not surprising that inhibition of autophagy sensitize tumor cells to chemotherapy treatments (Guo et al., 2012; Selvakumaran et al., 2013). Examples include camptothecin in breast cancer and cisplatin in oesophageal cancer cell line and proteasome inhibitors in prostate cancer cells and monoclonal antibodies in breast cancer cells (Kreuzaler et al., 2012). Therefore, autophagy inhibition as an adjuvant to chemotherapy represents a promising strategy in the treatment of some cancers (Amaravadi et al., 2011; White, 2012). Indeed, more than 20 clinical trials are currently evaluating the efficacy of CQ and hydroxychloroquine, autophagy inhibitors, in treatment of different cancers either in monotherapy or in combination with other anticancer agents. The preliminary results of many of these trials show apparent anti-tumor activity (Yang et al., 2011b; Kimura et al.,

2013). In addition, CQ and hydroxychloroquine another autophagy inhibitors, including 3-MA, bafilomycin A1 and pepstatin A have been shown to enhance the anti-tumor efficacy of chemotherapeutic drugs both *in vitro* and *in vivo* (Hsu et al., 2009; Li et al., 2010;Cheongetal.,2012;Lamoureux and Zoubeidi,2013). However, it should be noted that all these autophagy inhibitors are not specific and can modulate other cellular processes, such as endocytosis, lysosomal function etc. Hence, unexpected side effects could occur when treating patients with these drugs. Therefore, more specific and potent autophagy inhibitors are clearly needed. Thus, figuring out whether to stimulate or inhibit autophagy in each particular case will provide a powerful approach to treat cancer.

B- Calcium channel modulators

Calcium channels are considered to be important players in cancer regulation. There are signs of promising developments of calcium channel modulators in clinical applications concerning cancer treatment. The importance of calcium modulators is highlighted till nowadays by the use of immunosuppressant drugs such as cyclosporine that works by inhibiting calcium dependent activation of NFAT factors. Also, SOC channels in PM might be the target of calcium influx inhibitor that has been in clinical trials to slow down the growth of malignant cancers (Berridge 2012). Carboxyamidotriazole (CAI) is a cytostatic inhibitor of non-voltage-operated calcium channels and calcium channel-mediated signaling pathways that suppress angiogenesis, tumor growth, invasion and metastasis, and is already in clinical trials as an orally active antineoplastic agent (http://clinicaltrials.gov/). Moreover, the T type calcium channel inhibitor mibefradil reduces cell viability and clonogenic potential as well as induces apoptosis in glioblastoma mutliforme brain tumors that are usually resistant to radio and chemotherapy. As a result of using such inhibitor, reduced phosphorylation of AKt is observed suggesting inhibition of mtor complex 2/Akt pathway and thus making T type calcium channels a new molecular target for tumor therapy (Valerie et al., 2013). In addition, the results of experiments done by Zhang et al using LNCaP cells indicate that TRPM8 is required for cell survival. These results indicate that TRPM8 is an important determinator of calcium homeostasis in prostate epithelial cells and may be a potential target for the action of drugs in the management of prostate cancer using TRPM8's antagonist capsazepine (Zhang et al 2012). Therefore, more channel blockers and modulators should be investigated.

Objectives



Cancer has been identified as a chronic disease. In an attempt to battle this disease the world health organization have developed many programs to improve early screening and find therapies to improve survival and quality of life for the patients in late stages of cancer. For example, prostate cancer moves from androgen dependent to androgen independent stage and metastasize fast which makes it harder for treatment.

The role of calcium in controlling mechanisms that underlie cancer is well established. Defects in the cellular pathway autophagy lead to several diseases including cancer. Regardless of the complex role that autophagy plays in cancer; it was shown that modulation of autophagy has a great impact on cancer prognosis and treatment. This makes autophagy an interesting target for cancer therapy. In addition, recent findings identified the importance of intracellular calcium in autophagy regulation.

Due to the fact that the role of calcium and calcium permeable channels are well studied in cancer but still limited in the field of autophagy. In addition, both calcium and autophagy are implicated in cancer progression. We were interested in this thesis to study calcium dependent mechanisms of autophagy regulation and its importance in treating cancer. The three factors we studied were:

- The possibility of involvement of calcium permeable channels in autophagy regulation in prostate cancer.
- The link between calcium, calcium permeable channels, autophagy and cancer progression and therapy of prostate cancer.
- The use of autophagy modulators to enhance the efficacy of prostate cancer treatment.

Materials and Methods

I. Cellular culture

A- Cell Lines

1) Cancerous cell Lines

In this study, different prostatic cell lines were used:

- The androgen dependent **LNCaP** cells were purchased from the American Type Culture Collection (ATCC). LNCaP are a cell line of human cells used in the cancer field. LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells derived from the left supraclavicular lymph node metastasis from a 50 year old caucasian male in 1977. The LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma. The LNCaP cells grow readily in vitro in a medium rich with androgens and their doubling time is 60 hr. They have specific androgen and estrogen receptors. LNCaP cells also express PSA.

- The androgen independent **PC3** cells were purchased from ATCC. The PC3 cell lines were established in 1979 from bone metastasis of grade IV of prostate cancer in a 62-year-old Caucasian male. These cells do not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factors. They can grow and proliferate in the absence of androgens since they do not have a functional androgen receptor. They do not express PSA. PC3 cells have high metastatic potential compared to DU-145 cells and to LNCaP cells which have low metastatic potential.

- The androgen independent **DU-145** cells were purchased from ATCC. The DU-145 cell line was derived from brain metastasis. DU-145 are not hormone sensitive and do not express PSA. DU-145 cells have moderate metastatic potential.

- Several pancreatic cancer cell lines were also used that were purchased from ATCC.

2) Transformed cell lines

Human Embryonic Kidney 293 (HEK-293) cells were generated in the early 70s by transformation of cultures of normal human embryonic kidney cells with sheared

adenovirus. HEK-293 cells are very easy to grow and transfect very readily and are of interest to us because they have low expression levels of endogenous ion channels on plasma membranes so they can serve as a standard cell model.

B- Culture conditions

1) Media used

Prostate cancer cell lines LNCaP, PC3 and DU-145 were cultured in RPMI 1640 medium (31870, Gibco-Life Technologies) supplemented with 5 mM L-glutamine (25030, Gibco) and 10% fetal bovine serum (F7524, Sigma). In case of starvation, no fetal bovine serum was added to the medium. HEK-293 cells were cultured in Dulbecco's minimal essential medium DMEM+GlutaMAX (31966, Invitrogen, Life Technologies Inc.) supplemented with 10% fetal bovine serum (F7524, Sigma). Pancreatic cancer cell lines ASPC1 and BxPC3 were cultured in RPMI 1640 medium (31870, Gibco-Life Technologies) supplemented with 5 mM L-glutamine (25030, Gibco) and 10% FCS (PAA Gold).

The cells were placed in incubator of 37°C and 5% CO2. When adherent cells reach around 80 % confluency, they are to be detached from incubator flasks using Trypsin-EDTA, 0.25%, ICN, USA. The effect of Trypsin is inhibited by the addition of complete culture media to the cells to be placed in new T75 flasks (50,000 cells/ml) after splitting is done using Trypsin. Culture media is to be changed every two days.

2) Cell conservation (Freezing down and thawing)

After trypsinization, 1 ml of culture freezing media (Recovery Cell Culture Freezing Medium, Gibco) is to be added on around one million of cells needed to be conserved and frozen down in special freezing tubes. Then, the tubes are placed in a special freezing box filled with isopropanol and kept in -80°C for overnight. The isolation of the tubes in the isopropanol box allows the cells in frozen media to loose around 1° per minute and therefore, allowing the gradual freezing down of cells. If frozen cells are to be kept conserved for long periods of time, cells in their freezing media should be put in cryotubes and conserved in liquid nitrogen tanks.

It is preferred to place the frozen tube of cells in a water bath of about 37°C in case of thawing, for several minutes. The cells are then transferred to T25 or T75 flasks of complete

medium so as to grow. Media is adviced to be changed the second day.

II- Measuring cell viability

A- Cell proliferation Assay

Cells were seeded at 10000 cells/well on 96-well plates in normal medium. The cells were treated either with DMSO or the indicated concentrations of treatments for up to 72 h in full or serum-starved media. Cell viability was monitored using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega), on the basis of the cellular conversion of the colorimetric reagent MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] into soluble formazan by dehydrogenase enzymes found only in metabolically active cells. Following treatment the cells were incubated with reagent solution and absorbance was recorded at 490 nm wavelength using an ELISA plate reader (Molecular Devices).

B- Trypan blue

The cell viability was also assessed by trypan blue exclusion assay. After treatment with either DMSO or the required treatment, the cells were collected by trypsin-EDTA, incubated with trypan blue and the viable cells as well as dead (blue) cells were counted using hemcytometer.

III- Transient and stable transfection

LNCaP cells stably expressing eGFP-LC3 (LNCaP-eGFP-LC3) were generated by stable transfection with eGFP-LC3 plasmid (kind gift from Prof. Geert Bultynck KU Leuven, Belgium) using Xtremegene HP transfection reagent (Qiagen) and selection with 500 µg/mL G418 (Sigma-Aldrich).

LNCaP cells were transiently transfected with mCherry-eGFP-LC3B construct (kindly provided by Prof. Terje Johansen Institute of Medical Biology, University of Tromso, Norway) using Nucleofector technology (Lonza) as described by the manufacturer. Briefly, 1 million of cells were transfected with 2 µg of mCherry-eGFP-LC3B plasmid and seeded on tissue culture dishes with cover glass bottom (FluoroDish, FD35, World Presicion Instruments, Inc.). Two

days after plating cells were used for treatments and subsequent confocal imaging.

LNCaP cells were transfected with 40 nM of siRNA against STIM1 (Eurogentec) adjust the total volume to 100µL using medium without fetal bovine serum and mix with 6 µl Hyperfect transfection reagent using the lipofection technique (Qiagen Inc.) usually 5 minutes is the incubation time at room temperature so the complex of transfection is formed, following the manufacturer's instructions.

IV- Immunodetection

A- Extraction of proteins

Cells were washed with cold PBS and lysed in ice-cold buffer containing:
1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1% Sodium deoxycholate, 10 mM PO4Na2/K buffer, a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail PhosSTOP (Roche). The lysates were centrifuged at 15,000×g at 4°C for 15 minutes to remove cell debris and supernatant protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology).

B- Westernblot

30 µg of total protein were subjected to SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) for an around 2 h migration of 50 mA intensity fixed per one gel followed by transfer to PVDF membranes using the Trans-Blot® SD semi-dry transfer cell (Bio-Rad) at 25 volts and 50 mA per gel for a minimum of 1h30. The membranes were blocked in a 5% fat-free milk containing TNT buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were next incubated overnight at 4°C with primary antibodies, and then for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase. After washing, the membranes were processed for chemiluminescence detection using Luminata Western HRP substrate (Milipore). Image J software was employed for quantitative analysis.

Primary antibody	Company	Dilution	Secondary antibody	Molecular weight (kDa)
Beta actine	Sigma	1/2000	Mouse	42
Akt 193H12	Cell signaling	1/1000	Rabbit	60
pAkt(Ser 473)	Cell signaling	1/1000	Rabbit	60
mtor	Cell signaling	1/1000	Rabbit	289
pmTOR(Ser2448)	Cell Signaling	1/1000	Rabbit	289
LC3 B (L7543)	Sigma	1/1667	Rabbit	16 and 18
P62 (GP62-C)	PROGEN	1/1000	Pig	62
Beclin1	Sigma	1/1000	Rabbit	60
pPERK(Thr 981)	Santa Cruz	1/200	Rabbit	125
Orai1	ProScience	1/500	Mouse	55
STIM1	BD transduction	1/250	Mouse	84
TRPV6	Santa Cruz	1/200	Rabbit	85 and 100
TRPC1	Novus	1/250	Rabbit	80
Lamp2 sc-18822	Santa Cruz	1/250	Mouse	120

Table 1: List of primary antibodies used to study protein expression

C- Flurorescence microscopy

LNCaP-GFP-LC3 cells were grown on glass coverslips. Following treatments cells were rinsed with PBS, fixed with 4% paraformaldehyde-1X PBS for 15 min, washed with PBS and incubated with DAPI for 10 min. After three washes with PBS the slides were mounted with Mowiol® on glass slides and subjected to subsequent fluorescence analysis using Zeiss Axiovert microscope.

V- Electron microscopy

Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for at least 30 minutes at 4°C. After fixation, the specimens were thoroughly washed in 0.1 M cacodylate buffer and then postfixed with 1% osmium tetroxide in the same buffer for 1 hour

at room temperature, stained with 2% uranyl acetate in distilled water for 15 minutes, dehydrated in graded acetonitrile, and embedded in Epon. Ultrathin sections (80 to 90 nm thick) were cut on a Leica UC7, transferred on 150-mesh grids and contrasted with 2% uranyl acetate solution and Reynolds lead citrate solution. The electron micrographs were taken with a Hitachi H600 transmission electron microscope at 75 kV accelerating voltage. For immuno-electron microscopy, the cells were fixed overnight at 4°C in 8% paraformaldehyde and 0.05 % glutaraldehyde in PBS buffer, thoroughly washed in the same buffer and infused in sucrose 2.3 M containing 20% polyvinyl pyrrolidone 10000 in phosphate buffer 0.1 M. The pellets were mounted on microtome supports and rapidly frozen in melting nitrogen. Ultrathin sections of about 90–100 nm were obtained using a Leica UC7 cryoultramicrotome equipped with a FC7 device. Sections were first incubated in blocking medium (0.05 M glycine, 5% fish gelatine in 0.1 M PBS buffer) for 30 min. The grids were incubated with the antibody anti-LAMP2 for 1 hour at 37°C or overnight at 4°C. After washing, sections were incubated at room temperature for 30 min in the secondary gold conjugates (18 nm, Jackson Immuno Research) diluted in the same buffer. Following a final thorough wash in PBS alone, the grids were fixed in 2% glutaraldehyde for 10 min at room temperature and washed in water. After staining with 0.5% uranyl acetate in 1.5% methyl cellulose, sections were observed on a Hitachi H600 transmission electron microscope at 75 kV accelerating voltage.

VI- Confocal microscopy and acridine orange staining

Live cell images were obtained using confocal laser scanning microscope (LSM 700, Carl Zeiss MicroImaging GmbH) with a Plan Apochromat 40×/1.3 numerical aperture oil immersion objective, 340 nm and equipped with a CO2 and thermocontrolled chamber. The images were analyzed in Zeiss LSM Image Browser software and prepared for publication in Adobe Photoshop.

LNCaP cells were seeded on tissue culture dishes with cover glass bottom (FluoroDish, FD35, World Presicion Instruments, Inc.). Two days after plating cells were treated with normal, serum-starved or certain treatment containing medium for 12 h. At the end of treatments acridine orange was added to the cells (1 μg/ml final concentration) for 15 min in 37°C. Then the cells were washed 2 times with appropriate medium and subjected to confocal imaging.

VII- Calcium Imaging

LNCaP cells were grown on glass coverslips to carry out calcium imaging experiments. Changes in [Ca2+]i in LNCaP cells were imaged using the high-affinity fluorescent Ca2+ indicator fluo-4, which was loaded by 1-hour incubation of the cells with 5 µM fluo-4 acetoxymethyl ester followed by 1-hour wash to allow time for de-esterification. Confocal [Ca2+]I imaging was performed as described previously.55 Briefly, experimental chambers with the cells were placed on the stage of Axiovert 200M inverted microscope attached to a LSM 510 META laser-scanning unit (Zeiss, Oberkochen, Germany). The x-y confocal images of the fluo-4 fluorescence were acquired at 0.15 Hz using a Zeiss plan-Apochromat 40× 1.3 N.A. objective. Fluo-4 fluorescence was excited by the 488 nm line of a 500 mW argon ion laser (Laser-Fertigung, Hamburg, Germany) and was captured at wavelengths above 505 nm. The illumination intensity was attenuated to 0.6% with an acousto-optical tunable filter (Zeiss, Oberkochen). In all the experiments the photomultiplier gain was set at 688. To optimise signal quality the pinhole was set to provide a confocal optical section <4 µm. The composition of the Ca2+/Mg2+-free external solution used during experiments was (in mM): 140 NaCl, 5KCl, 5 Glucose, 10 Hepes (pH 7.4). Alternatively, ratiometric dye Fura-2/AM was used as a Ca2+ indicator. LNCaP cells were loaded with 2 µM Fura-2/AM for 45 min at 37oC and 5%CO2 in RPMI medium and subsequently washed three times with external solution containing (in mM): 140 NaCl, 5KCl, 1 MgCl2, 2 CaCl2, 5 Glucose, 10 Hepes (pH 7.4). The coverslip was then transferred in a perfusion chamber on the stage of Nikon Eclipse Ti microscope. Fluorescence was alternatively excited at 340 and 380 nm with a monochromator (Polychrome IV, TILL Photonics Gmbh) and captured at 510 nm by a QImaging CCD camera (QImaging). Acquisition and analysis was performed with the MetaFluor 7.7.5.0 software (Molecular Devices Corp.).

VIII- RT-PCR

RNA was isolated using the guanidium thiocyanate–phenol–chloroform extraction procedure, reverse transcribed using moloney murine leukemia (MuLV) reverse transcriptase (Perkin Elmer, Courtaboeuf, France). DNA amplification conditions included the initial denaturation step of 7 min at 950C, and 36 cycles of 30 s at 950C, 30 s at 600C, and 30 s at 720C, and finally 7 min at 720C.

IX- Statistical analysis

Data were analyzed using Origin 7.0 (Microcal Software). Results are expressed as mean±SEM. Statistical analysis was performed using Student's t-test, and $p<0.05$ was considered as significant. Asterisks denote: * - $p<0.05$, and ** - $p<0.01$.

Results

Article 1:

Identification of ML-9 as a lysosomotropic agent targeting autophagy and cell death (Accepted in *Cell Death and Disease* journal, 2014).

The experimental contribution to the article:

Cell culture and transfection of cells

Cell viability (MTS assay and trpan blue counting)

Westernblotting

Immunocytochemistry and fluorescence microscopy

Confocal microscopy

Contributed to the manuscript of the article in the introduction, results and discussion sections.

Identification of ML-9 as a lysosomotropic agent targeting autophagy and cell death

Running title: **ML-9 targets autophagy and cell death**

Artem Kondratskyi[1§], Maya Yassine[1§], Christian Slomianny[1], Kateryna Kondratska[1], Dmitri Gordienko[1,2], Etienne Dewailly[1], V'yacheslav Lehen'kyi[1], Roman Skryma[1], Natalia Prevarskaya[1*]

[1] Inserm, U-1003, Equipe labellisée par la Ligue Nationale contre le cancer. Laboratory of Excellence, Ion Channels Science and Therapeutics, Université Lille 1, Villeneuve d'Ascq, France.

[2] Present address: Laboratory of Molecular Pharmacology and Biophysics of Cell Signalling, Bogomoletz Institute of Physiology; State Key Laboratory of Molecular and Cell Biology, Kiev, Ukraine

§These authors contributed equally to this work

*Correspondence to: Professor Natalia Prevarskaya; Laboratory of Cell Physiology INSERM U 1003; Bat. SN 3, UFR de Biologie Université Lille 1, 59655 Villeneuve d'Asq cedex, France; Tel: +(33) 3 20 33 60 18; Fax: +(33) 3 20 43 40 66; E-mail: Natacha.Prevarskaya@univ-lille1.fr

Abstract

The growing number of studies suggested that inhibition of autophagy enhances the efficacy of Akt kinase inhibitors in cancer therapy. Here we provide evidence that ML-9, a widely used inhibitor of Akt kinase, MLCK and stromal interaction molecule 1 (STIM1) represents the "two-in-one" compound which stimulates autophagosome formation (by downregulating Akt/mTOR pathway) and inhibits their degradation (by acting like a lysosomotropic agent and increasing lysosomal pH). We show that ML-9 as a monotherapy effectively induces prostate cancer cell death associated with the accumulation of autophagic vacuoles. Further, ML-9 enhances the anticancer activity of docetaxel, suggesting its potential application as an adjuvant to existing anticancer chemotherapy. Altogether our results revealed the complex effect of ML-9 on autophagy and indentified ML-9 as an attractive tool for targeting autophagy in cancer therapy through dual inhibition of both the Akt pathway and autophagy.

Key words: ML-9, autophagy, cell death, lysosomotropic agents, calcium

Abbreviations: STIM1, stromal interaction molecule 1; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; MLCK, myosin light-chain kinase; GFP, green fluorescent protein; LNCaP, lymph node carcinoma of the prostate; ATG, autophagy-related gene; TEM, transmission electron microscopy; Lamp2, lysosomal-associated membrane protein 2; HEK-293, human embryonic kidney 293; CQ, chloroquine; 3-MA, 3-Methyladenine; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; TG, thapsigargin; SOCE, store operated calcium entry; PERK, protein kinase RNA-like endoplasmic reticulum kinase; BAPTA/AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis/acetoxymethyl ester; AR, androgen receptor; siRNA, small interfering RNA; PARP, poly (ADP-ribose) polymerase.

Introduction

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway is a vital pathway that is implicated in a variety of cellular processes, deregulation of which could contribute to the malignant transformation. Accordingly, this pathway has been linked to tumorigenesis and resistance to anticancer therapy in different cancers.[1-2] Considering this, a number of novel inhibitors targeting PI3K, Akt or mTOR are currently being investigated in clinical trials for cancer therapy. However, it was reported that inhibition of the different components of the PI3K/Akt/mTOR pathway often stimulates prosurvival processes, such as autophagy, limiting the anticancer efficacy of the aforementioned inhibitors.[3-4]

Autophagy, a cellular process comprising degradation and recycling of protein aggregates, long-lived proteins and damaged organelles, is essential to maintain cellular homeostasis.[5-6] At basal and stress conditions autophagy assures cytoprotective response to support cell survival. In contrast, a growing number of studies suggests that autophagy may contribute to cell death.[7] Defects in autophagy are linked to a number of pathological states including cancer.[8] Indeed, accumulated evidence suggests that autophagy inhibition facilitates apoptotic cell death, inhibits tumor cells growth and sensitize tumor cells to chemotherapy treatments.[9-13]

Recently, it was proposed that inhibition of autophagy represents a prospective strategy to increase the therapeutic efficacy of Akt inhibitors.[14-16]

A naphthalene sulphonamide derivative, 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9) is a membrane permeable organic substance (Supplementary Figure S1) which is widely used as an inhibitor of Akt kinase,[17-18] myosin light-chain kinase (MLCK) [19] and stromal interaction molecule 1 (STIM1).[20] ML-9 has been previously shown to promote apoptotic cell death and inhibit invasion and adhesion in certain cancer cell lines. [21-23] Recently, ML-9 was identified as a compound that increases the number of GFP-LC3 vesicles in human glioblastoma H4 cells.[24]

Given the increasing interest in development of novel autophagy modulators as well as inhibitors of the Akt/mTOR pathway for cancer treatment, we investigated the mechanisms of autophagy regulation by ML-9 and assessed its potential application as an anticancer drug.

Results

ML-9 stimulates autophagy by downregulating Akt/mTOR pathway in prostate cancer cells. Akt phosphorylation is widely established to be one of the key-stone events involved in the regulation of prostate cancer cell survival.[25-28] Since ML-9 has been suggested to be the potent inhibitor of Akt phosphorylation in other cell models, we firstly checked for this function of ML-9 in prostate cancer cells. In LNCaP cells ML-9 (30 μM) reduced the phosphorylation of Akt kinase suggesting its inhibition (Figure 1a). Since mTOR kinase acts downstream of Akt kinase, we further examined the effect of ML-9 on mTOR activity. We found that phosphorylation of mTOR was significantly reduced by ML-9 in a dose-dependent manner which suggests the inhibition of mTOR kinase activity (Figure 1b). As mTOR kinase represents a central player in autophagy regulation we next tested the effect of ML-9 on autophagy by analyzing the levels of endogenous LC3-II protein, the most widely used marker for autophagosomes.[29] ML-9 significantly increased LC3-II levels in LNCaP cells in a dose- and time-dependent manner (Figures 1c and d). In addition, in LNCaP cells stably expressing eGFP-LC3 (LNCaP-eGFP-LC3), treatment with ML-9 resulted in accumulation of eGFP-LC3 puncta (Figure 1e).

Further, transmission electron microscopy (TEM) analysis revealed the increased number of large autophagosome-like and autolysosome-like vacuoles in ML-9-treated cells compared to the control ones (Figure 1f). Moreover, the accumulation of multi-lamellar membrane structures, small vesicles, cytoplasmic material and organelles at different stages of degradation has been detected within the lumen of the numerous expanded vacuoles after ML-9 treatment (Figure1f). Importantly, we have noted that

apparent autolysosomes were significantly greater in number than autophagosomes (Figure 1f and Supplementary Figure S2). In addition, immuno-TEM analysis showed the presence of immunogold-labeled lysosomal protein Lamp2 in these vacuoles (Supplementary Figure S3). These findings suggest autophagic/lysosomal origin of these vacuoles.

ML-9 also caused an increase in LC3-II levels in prostate cancer PC-3 cells, human embryonic kidney HEK-293 cells, pancreatic cancer ASPC1 and BxPC3 cells, but not in DU-145 cells lacking full-length ATG5 [30] (Supplementary Figure S4a and S4b), suggesting the necessity of the functional autophagic pathway.

Overall, these data suggest that ML-9 stimulates autophagy and induces accumulation of autophagic vacuoles and functional autophagic machinery is required for this.

ML-9 blocks autophagic flux. An accumulation of autophagic vacuoles can be the consequence of two different processes: increased autophagosome formation and/or block in autophagosome maturation/degradation. Therefore, we analyzed autophagic flux in LNCaP cells using the lysosome inhibitor chloroquine (CQ). We showed that at concentrations up to 20 μM ML-9 induced LC3-II accumulation without inhibiting autophagic flux, as clear increase in LC3-II levels was detected upon addition of CQ (50 μM for 1h). In contrast, CQ failed to increase LC3-II levels induced by higher concentrated ML-9 (30 μM -50 μM) (Figure 2a). In addition, combined treatment of LNCaP cells with ML-9 (30 μM) and bafilomycin A1 (another inhibitor of lysosomal acidification) did not elevate LC3-II levels compared to the treatment with ML-9 alone (Supplementary Figure S5). These results suggest that at concentrations >30 μM ML-9 effectively inhibits autophagic flux.

Consistent with immunoblotting experiments CQ failed to enhance ML-9-induced eGFP-LC3 puncta accumulation in LNCaP-eGFP-LC3 cells, while a clear increase in eGFP-LC3 puncta has been observed in basal and serum-starved conditions (Figure 2b).

We next confirmed these results by analyzing the levels of autophagy substrate protein p62 – another widely used autophagy marker, which is degraded by autophagy.[31-32] ML-9 induced p62 accumulation in LNCaP cells in a dose-dependent manner, once again supporting the inhibitory role for ML-9 in autophagy (Figure 2c).

Interestingly, we found that ML-9 blocked serum-starvation-induced autophagic flux. Addition of CQ to LNCaP cells treated with ML-9+starvation caused no further increase in LC3-II and p62 levels (Figure 2d). Thus, these results demonstrate that ML-9 arrests basal and induced autophagic flux.

ML-9 affects maturation of autophagosomes and increases lysosomal pH. Next, to confirm these results we utilized a tandem mCherry-GFP reporter fluorescence assay designed to monitor autophagic flux (see Materials and Methods). [32] LNCaP cells were transiently transfected with mCherry-GFP-LC3B and treated with normal, serum-starved, or ML-9 (30 μM) containing medium for 12h. The colocalization of GFP and mCherry signals was analyzed. Under starvation conditions both the number of puncta and fraction of red-only puncta increased, indicating that fusion with acidic endosomes/lysosomes occurs rapidly after autophagosome formation. In contrast, ML-9 treatment caused a dramatic decrease in the proportion of red-only puncta, thus the vast majority of the puncta were double positive mCherry+/GFP+ (Figure 3a). Thus, the accumulation of enlarged double positive mCherry+/GFP+ LC3-puncta (as it follows from confocal microscopy) together with the accumulation of autolysosomes (as it goes from TEM) following ML-9 treatment suggest that ML-9 inhibits autophagic flux mainly through the increase in endosomal/lysosomal pH and impairment of proteolytic degradation of autophagy substrates inside the autolysosomes, but not through the prevention of fusion between autophagosomes and endosomes/lysosomes.

To test this assumption we utilized acridine orange, an acidotropic dye which is widely used as an indicator of acidification. LNCaP cells stained with acridine orange exhibited green fluorescence with a few red puncta in basal medium. Serum-starvation induced accumulation of red puncta, indicating an increase in autolysosomes number. In contrast, ML-9-treated cells showed an

accumulation of enlarged vesicles emitting in both green and red, indicating increase in intraorganellar pH (Figure 3b). This effect of ML-9 could be explained by its weak base properties (pKa=8.04, calculated using Marvin software by ChemAxon). Thus, it seems that ML-9 behaves like a lysosomotropic agent which is accumulated in the lysosomes and leads to an increase in lysosomal pH. We confirmed these results by assessing Lamp1 immunofluorescence in LNCaP cells transfected with mCherry-GFP-LC3B. ML-9 visibly increased the size of Lamp1-positive vesicles/puncta (Supplementary Figure S6a). In addition, ML-9 induced accumulation of mCherry-GFP-LC3B puncta, which were largely colocalized with Lamp1-positive vesicles/puncta (Supplementary Figure S6b).
Altogether these data suggest that ML-9 represents a "two-in-one" compound which stimulates autophagosome formation (by downregulating Akt/mTOR pathway) and inhibits their degradation (by acting like a lysosomotropic agent and increasing lysosomal pH).

ML-9 stimulates autophagy via both Vps34-dependent and -independent mechanisms.
Next, we assessed the levels of another important autophagy protein Beclin1 upon treatment with ML-9. Western blot analysis showed that ML-9 induced an increase in Beclin1 levels (Figure 4a). To test if Vps34-Beclin1-complex is important in ML-9-induced accumulation of autophagic vacuoles, we treated LNCaP cells with ML-9 or serum-starved medium, followed by the addition of 100 nM wortmannin (an inhibitor of Vps34) for 1,5 h. Wortmannin effectively reduced the LC3-II levels in LNCaP cells treated with low concentrations of ML-9 (up to 20 μM). In contrast, wortmannin showed no significant effect when cells were treated with higher concentrations of ML-9 (Figure 4b). This could be due to the inability of wortmannin to influence autophagosome degradation. To test this possibility, we used another Vps34 inhibitor 3-MA and pretreated our cells with 5mM 3-MA for 1h before treatments with full, serum-starved or ML-9 containing medium for 6h in the continuous presence of 3-MA. 3-MA effectively reduced LC3-II levels in basal and serum-starved conditions as well as in LNCaP cells treated with 20 μM ML-9. In contrast, 30 μM ML-9 induced LC3-II accumulation even following pretreatment with 3-MA (Figure 4c). In line with this, ML-9 (30 μM) was effective in increasing the number of eGFP-LC3 positive puncta in LNCaP-eGFP-LC3 cells pretreated with wortmannin (Figure 4d). Overall, these results suggest that ML-9 stimulates autophagy via both Vps34-dependent and -independent mechanisms.

ML-9 induces prostate cancer cell death.
Considering the previously reported data, suggesting that combination of Akt inhibitors with late stage autophagy inhibitors promotes cancer cell death,[14-16] we next assessed the effect of ML-9 on prostate cancer cell viability. MTS and trypan blue staining assays revealed that ML-9 reduced the viability of LNCaP cells in a concentration-dependent manner. Moreover, this cell-killing effect of ML-9 was strikingly potentiated in a serum-free medium (Figures 5a and b). Next, we assessed if ML-9 induce apoptosis in LNCaP cells. First, we checked if ML-9 could induce cleavage of PARP. Treatment of LNCaP cells with ML-9 (30 μM) for 24 h resulted in the appearance of a cleaved PARP 89-kD fragment characteristic of apoptosis. PARP cleavage was significantly higher when cells were treated with ML-9 in serum-starved conditions (Figure 5c). These results indicate that ML-9 induces apoptosis in LNCaP cells. To confirm this we determined the level of apoptosis by Annexin V/Propidium iodide double staining. Treatment with ML-9 (30 μM) for 24 h significantly increased the percentage of Annexin V-positive LNCaP cells and serum-free medium greatly potentiated this effect (Figure 5d).
In addition to LNCaP cells ML-9 effectively reduced the viability of PC-3, DU-145 as well as HEK-293 cells, although these cell lines exhibited greater resistance to ML-9-induced cell death compared to LNCaP cells, with DU-145 being the most resistant (Supplementary Figure S7). At high concentrations (50 μM – 100 μM) ML-9 induced pronounced rounding up and detachment of LNCaP cells within the first 2 h of treatment. Again, this effect was intensified by the removal of serum from medium (data not shown).
Of note, after 4 hours of treatment with ML-9 (30 μM -50 μM) we detected vacuolization of the majority of LNCaP cells under the light microscope (Supplementary Figure S8). This effect correlates well with the weak base

properties of ML-9 and the accumulation of autophagic vacuoles (as revealed by TEM and fluorescence microscopy).
Thus, we conclude that ML-9 as a monotherapy effectively induces prostate cancer cell death associated with the accumulation of autophagic vacuoles.

ML-9 accelerates cell death in combination with chemotherapy. Given that ML-9 effectively induces cell death in prostate cancer cells, our next step was to see if ML-9 could be useful as an adjuvant to anti-cancer chemotherapy. LNCaP, PC3 and DU-145 cells were treated with docetaxel (5 nM), ML-9 (30 μM) or combination of docetaxel+ML-9 for 48 h. As shown in Figure 5e, cell viability was significantly reduced when docetaxel was combined with ML-9 compared to either drug alone. These results suggest that ML-9 could potentially be considered as an adjuvant to existing anti-cancer chemotherapy.

ML-9 modulates Ca^{2+} homeostasis and stimulates autophagy in a calcium-dependent manner. Previous reports demonstrated that ML-9 could influence cellular calcium homeostasis.[20, 33-35] As calcium was shown to be an important autophagy regulator, we next investigated if calcium-related mechanisms are involved in ML-9-induced autophagy. First, we checked whether ML-9 influences cytosolic calcium levels in LNCaP cells using a Ca^{2+} ionophore ionomycin and an inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) thapsigargin (TG). Application of 2 μM ionomycin in the absence of extracellular Ca^{2+} caused a transient increase in cytosolic calcium which was significantly less when cells were pretreated with 30 μM ML-9 for 6 h (Figure 6a). Consistent with this, ML-9 treatment significantly reduced both TG-induced calcium release and store operated calcium entry (SOCE) (Figure 6b), suggesting that ML-9 reduces both calcium content of intracellular calcium stores and SOCE in LNCaP cells.
It is well known that depletion of ER calcium content can trigger ER stress.[36-37] ER stress has been linked to both autophagy and cell death.[38-39] Given that ML-9 induces Ca^{2+} release from intracellular stores and slows down its refilling by the inhibition of SOCE, we hypothesized that in prolonged treatments ML-9 could induce ER stress. To test this hypothesis, we assessed the GRP78 (a widely used ER stress marker) level and phosphorylation of PERK, a protein kinase representing a major sensor of unfolded protein response. Treatment with ML-9 (50 μM) induced an increase in both the GRP78 level and the phosphorylation level of PERK, suggesting ER stress induction (Figure 6c), which could potentially contribute to ML-9-induced autophagy and cell death.
It is known that Ca^{2+} mobilizing agents, such as ionomycin and thapsigargin, regulate autophagy in a calcium-dependent manner.[40] We therefore tested if ML-9-induced autophagy is calcium-dependent using an intracellular calcium chelator BAPTA/AM. Addition of 20 μM BAPTA/AM together with 30 μM ML-9 to LNCaP cells for 3 hours completely abolished the ML-9-induced increase in LC3-II levels (Figure 6d) indicating that Ca^{2+} is required for ML-9-induced autophagosome formation.
Recent reports demonstrated that the inhibitory effect of ML-9 on SOCE is attributable to its inhibition of Stim1 redistribution.[20] We therefore tested whether the effect of ML-9 on autophagy is mediated by the inhibition of Stim1. Silencing of Stim1 by siRNA did not induce accumulation of LC3-II in LNCaP cells (Supplementary Figure. S9a). In line with this result, treatment of LNCaP cells with BTP2, another inhibitor of SOCE, was without effect on LC3-II (Supplementary Figure S9b). These results indicate that ML-9 exerts its effects on autophagy independently of Stim1 and SOCE inhibition.

Discussion

In this study we provide evidence that ML-9, a widely used inhibitor of Akt kinase, MLCK and stromal interaction molecule 1 (STIM1) represents the “two-in-one” compound which stimulates autophagosome formation and inhibits their degradation. We also demonstrate that cytosolic calcium is essential for ML-9-induced autophagosome formation, however neither STIM1 nor SOCE are necessary. Further, we show that ML-9 as a monotherapy effectively induces

prostate cancer cell death associated with the accumulation of autophagic vacuoles. In addition, ML-9 enhances the anticancer activity of docetaxel, suggesting its potential application as an adjuvant to existing anticancer chemotherapy. We also demonstrate that ML-9 significantly reduces ER calcium content and induces ER-stress, which could contribute to its cytotoxicity. Altogether our results revealed the complex effect of ML-9 on autophagy and indentified ML-9 as an attractive tool for targeting autophagy in cancer therapy through dual inhibition of both the Akt pathway and autophagy.

Recently Zhang L. et al. performed high-throughput image-based screening for small-molecule regulators of autophagy and among others identified ML-9 as a compound that increases GFP-LC3 vesicles in human glioblastoma H4 cells.[24] In another study ML-7, a structurally related analog of ML-9, was shown to induce accumulation of vesicle-like structures in Schwann cells.[41]

The evidence presented here clearly demonstrates that the reported increase in GFP-LC3 vesicles by ML-9 is in fact the consequence of both the stimulation of autophagosome formation and inhibition of their degradation. To reach this conclusion, we used a number of approaches including LC3 protein detection, electron microscopy for direct visualization of autophagic process, fluorescence and confocal microscopy for GFP-LC3+ and mCherry-GFP-LC3+ puncta analysis as well as endogenous autophagy substrate (p62) degradation analysis.

We demonstrated that ML-9 stimulates autophagy through inhibition of mTOR kinase. Several mechanisms may contribute to the inhibition of mTOR by ML-9. First, as mTOR acts downstream of Akt and Akt is known to positively regulate mTOR, inhibition of Akt by ML-9 could consequently induce mTOR inhibition and activation of autophagy. Second mechanism is based on the fact that during autophagy the nutrients generated by degradation of cargo in the autolysosomes stimulate mTOR, representing a feedback regulatory loop. Accordingly, ML-9-induced lysosomal dysfunction leads to a decrease in autophagic flux thereby decreasing nutrients availability and promoting mTOR inhibition.

Intriguingly, we discovered that ML-9 being an autophagy activator inhibits autophagy in the late stages. This effect of ML-9 could be attributed to its weak base properties. We propose that ML-9 as a lipophilic weak base enters the cell by simple diffusion. At neutral pH ML-9 is in its non-protonated form. When ML-9 enters acidic vacuoles (such as lysosomes and amphisomes) it becomes positively charged through protonation and trapped in these vacuoles. Then the increased osmotic pressure in these vacuoles stimulates water influx and vacuoles enlargement. All these events result in the increased intravacuolar pH, subsequent inhibition of lysosomal enzymes and block in degradation of the autolysosomal content. Based on the immuno-TEM and immunofluorescence analysis we concluded that the majority of the vacuoles represent autolysosomes favoring the hypothesis that ML-9 does not prevent fusion between autophagosomes and lysosomes.

Surprisingly, the early stage autophagy inhibitors 3-MA and wortmannin did not prevent accumulation of autophagic vacuoles induced by high doses of ML-9 (>30 μM). This result could indicate that ML-9 could at once induce both canonical and non-canonical autophagy via different pathways.

A number of studies reported the modulation of cellular Ca^{2+} homeostasis by ML-9.[20, 34-35] ML-9 has been shown to inhibit redistribution of STIM1 and it is widely used as a SOCE inhibitor.[20] However, our data demonstrate that STIM1 and SOCE do not contribute to ML-9-induced autophagy. In contrast, ML-9 induces increase in GRP78 level and PERK activation (suggesting ER-stress induction) presumably through the decrease in ER calcium content. Interestingly, ER-stress has been shown to negatively regulate Akt/mTOR pathway and as such stimulate autophagy.[42] Of note, it was proposed that ML-9 promotes calcium release through the IP3 receptor.[33] The accumulated data suggested a complex role for IP3R in autophagy regulation.[43-45] Thus, IP3R-pathway as well as PERK-pathway[46-47] could potentially contribute to both ML-9-induced autophagy and cell death. In addition, we demonstrated that cytosolic calcium is essential factor for ML-9-induced autophagy, as its removal by chelation blocks autophagy at an early stage prior to autophagosome formation. A graphic model for ML-9's mode of action is represented on Figure 7.

Recently, it was proposed that dual inhibition of Akt kinase and autophagy represents a prospective strategy in anticancer therapy.[14-16] Indeed, one of the most important functions of Akt kinase is cell survival. This kinase is often upregulated in cancer and promotes cell proliferation, cell growth and resistance to apoptosis. Thus, inhibiting Akt kinase appears to be an effective approach in cancer treatment. Similarly, autophagy is thought to be predominantly a cell-survival mechanism. Elevated autophagy is often detected in cancer cells in response to radiation and chemotherapy.[13, 48-49] Furthermore, autophagy seems to contribute to the therapeutic resistance of some cancers. Thus, autophagy inhibition also seems to represent a promising therapeutic strategy in the treatment of cancer. The combination of these two approaches shows additive efficacy in anticancer therapy.

Our results suggest that ML-9 represents a dual inhibitor of both Akt pathway and autophagy and thus is potentially interesting for cancer treatment. Indeed, ML-9 as a monotherapy effectively induces prostate cancer cell death as well as enhances the anticancer activity of docetaxel, indicating its potential application as an adjuvant to existing anticancer chemotherapy. These data are in line with the previously published studies showing that ML-9 and its structurally related analog ML-7 promote apoptotic cell death in a variety of cell lines, although these effects have been linked to MLCK inhibition.[21-23] Moreover, ML-7 was proposed as a promising candidate for treating cancer.[22] Interestingly, we found that cell-killing effect of ML-9 was strikingly potentiated in a serum-free medium. This result can be explained by the dual inhibition of androgen receptor (AR) and Akt/mTOR signaling pathways in these conditions. Indeed, serum-free medium mimics steroid-deprived conditions and thus inhibits androgen receptor (AR) signaling pathway. It should be noted, that interaction between AR and Akt/mTOR pathways has been reported to be involved in tumorigenesis.[25, 50] Moreover the use of Akt/mTOR inhibitors in combination with AR antagonists has been demonstrated to improve anticancer efficacy.[51-52] Thus, combining inhibitors of Akt, AR and autophagy could potentially constitute a novel strategy in prostate cancer therapy.

Overall our data suggest that ML-9 represents an attractive tool for targeting autophagy in cancer through dual inhibition of AKT pathway and autophagy. Further, the chemical structure of ML-9 could serve as a “template” for the synthesis of improved structurally related and more selective compounds, which could potentially be used for cancer treatment.

Materials and Methods

Antibodies and reagents. Rabbit anti-LC3B (L7543), mouse anti-betaActin (A5441) were from Sigma. Guinea pig anti-p62 (GP62-C) was from PROGEN Biotechnik GmbH. Rabbit anti-mTOR (2972S), rabbit anti-p-mTOR(2971S) were from Cell Signaling. Rabbit anti-p-Akt, rabbit-anti-p-PERK (sc-32577), mouse-anti-Lamp2 (sc-18822) were from Santa Cruz Biotechnology.

ML-9 (105637) was from Enzo Life Sciences. Acridine orange (A-3568) was from Life Technologies. Bafilomycin A1 (1334) was from Tocris. Chloroquine (C6628), Wortmannin (W1628), trypan blue solution (T8154) were from Sigma.

Constructs. The pDest-mCherry-eGFP-LC3B plasmid was kindly provided by Prof. Terje Johansen (Institute of Medical Biology, University of Tromso, Tromso, Norway). The pcDNA3.1(-)-GFP-LC3 plasmid was a kind gift from Prof. Geert Bultynck (KU Leuven, Leuven, Belgium).

Cell culture and transfection. Prostate cancer cell lines LNCaP, PC3 and DU-145 were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium (31870, Gibco-Life Technologies) supplemented with 5 mM L-glutamine (25030, Gibco) and 10% fetal bovine serum (F7524, Sigma). HEK-293 cells were cultured in Dulbecco's minimal essential medium DMEM+GlutaMAX (31966, Invitrogen, Life Technologies Inc.) supplemented with 10% fetal bovine serum (F7524, Sigma). Pancreatic cancer cell lines ASPC1 and BxPC3 were purchased from the ATCC and cultured in RPMI 1640 medium (31870, Gibco-Life Technologies)

supplemented with 5 mM L-glutamine (25030, Gibco) and 10% FCS (PAA Gold).
LNCaP cells stably expressing eGFP-LC3 (LNCaP-eGFP-LC3) were generated by stable transfection with eGFP-LC3 plasmid using Xtremegene HP transfection reagent (Qiagen) and selection with 500 µg/mL G418 (Sigma-Aldrich).
LNCaP cells were transiently transfected with mCherry-eGFP-LC3B construct using Nucleofector technology (Lonza) as described by the manufacturer. Briefly, 1 million of cells were transfected with 2 µg of mCherry-eGFP-LC3B plasmid and seeded on tissue culture dishes with cover glass bottom (FluoroDish, FD35, World Presicion Instruments, Inc.). Two days after plating cells were used for treatments and subsequent confocal imaging. Tandem mCherry-GFP reporter fluorescence assay is based on the use of the pH-sensitive fluorescent tag consisting of a tandem fusion of the red, acid-insensitive mCherry and the acid-sensitive GFP.[32] Under neutral pH both mCherry and GFP fluoresce and colocalize indicating an autophagosome which is not fused with acidic lysosome. Alternativelly, colocalization of mCherry and GFP signals could point on an amphisome or autolysosome with decreased acidification and/or impaired proteolytic degradation. In contrast, mCherry signal without GFP corresponds to an amphisome or autolysosome with physiologically acidic interior.[32]
LNCaP cells were transfected with 40 nM of siRNA against STIM1 (Eurogentec) using 6 µl Hyperfect transfection reagent (Qiagen Inc.), following the manufacturer's instructions.

Cell viability. Cells were seeded at 10000 cells/well on 96-well plates in normal medium. The cells were treated either with DMSO or the indicated concentrations of ML-9 for up to 72 h in full or serum-starved media. Cell viability was monitored using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega), on the basis of the cellular conversion of the colorimetric reagent MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] into soluble formazan by dehydrogenase enzymes found only in metabolically active cells. Following treatment the cells were incubated with reagent solution and absorbance was recorded at 490 nm wavelength using an ELISA plate reader (Molecular Devices).
The cell viability was also assessed by trypan blue exclusion assay. After treatment with either DMSO or ML-9, the cells were collected by trypsin-EDTA, incubated with trypan blue and the viable cells as well as dead (blue) cells were counted.

Apoptosis assays. The level of apoptosis was determined by Alexa Fluor® 488 Annexin V/Propidium iodide double staining. At the end of the treatments, both floating and attached cells were collected by trypsinization, centrifuged, washed with PBS and stained with Alexa Fluor® 488 Annexin V and Propidium iodide according to the manufacturer instructions (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Life Technologies). Cells were examined by fluorescence microscopy on Zeiss Axio Imager A1 microscope. The percentage of Alexa Fluor® 488 Annexin V-positive cells was determined by counting at least 500 cells in random fields.

Electron microscopy. Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for at least 30 minutes at 4°C. After fixation, the specimens were thoroughly washed in 0.1 M cacodylate buffer and then postfixed with 1% osmium tetroxide in the same buffer for 1 hour at room temperature, stained with 2% uranyl acetate in distilled water for 15 minutes, dehydrated in graded acetonitrile, and embedded in Epon. Ultrathin sections (80 to 90 nm thick) were cut on a Leica UC7, transferred on 150-mesh grids and contrasted with 2% uranyl acetate solution and Reynolds lead citrate solution. The electron micrographs were taken with a Hitachi H600 transmission electron microscope at 75 kV accelerating voltage.
For immuno-electron microscopy, the cells were fixed overnight at 4°C in 8% paraformaldehyde and 0.05 % glutaraldehyde in PBS buffer, thoroughly washed in the same buffer and infused in sucrose 2.3 M containing 20% polyvinyl pyrrolidone 10000 in phosphate buffer 0.1 M. The pellets were mounted on microtome supports and rapidly frozen in melting nitrogen. Ultrathin sections of about 90–100 nm were obtained using a Leica UC7 cryoultramicrotome equipped with a FC7 device.

Sections were first incubated in blocking medium (0.05 M glycine, 5% fish gelatine in 0.1 M PBS buffer) for 30 min. The grids were incubated with the antibody anti-LAMP2 for 1 hour at 37°C or overnight at 4°C. After washing, sections were incubated at room temperature for 30 min in the secondary gold conjugates (18 nm, Jackson Immuno Research) diluted in the same buffer. Following a final thorough wash in PBS alone, the grids were fixed in 2% glutaraldehyde for 10 min at room temperature and washed in water. After staining with 0.5% uranyl acetate in 1.5% methyl cellulose, sections were observed on a Hitachi H600 transmission electron microscope at 75 kV accelerating voltage.

Western blotting. Cells were washed with cold PBS and lysed in ice-cold buffer containing: 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1% Sodium deoxycholate, 10 mM PO4Na2/K buffer, a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail PhosSTOP (Roche). The lysates were centrifuged at 15,000×g at 4°C for 15 minutes to remove cell debris and supernatant protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology). 30 µg of total protein were subjected to SDS-PAGE followed by transfer to PVDF membranes using the Trans-Blot® SD semi-dry transfer cell (Bio-Rad). The membranes were blocked in a 5% fat-free milk containing TNT buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were next incubated overnight at 4°C with primary antibodies, and then for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase. After washing, the membranes were processed for chemiluminescence detection using Luminata Western HRP substrate (Milipore). Image J software was employed for quantitative analysis.

Immunocytochemistry and fluorescence microscopy. LNCaP-GFP-LC3 cells were grown on glass coverslips. Following treatments cells were rinsed with PBS, fixed with 4% paraformaldehyde-1X PBS for 15 min. After three washes with PBS the slides were mounted with Mowiol® on glass slides and subjected to subsequent fluorescence analysis using Zeiss Axiovert microscope.

Acridine orange staining. LNCaP cells were seeded on tissue culture dishes with cover glass bottom (FluoroDish, FD35, World Presicion Instruments, Inc.). Two days after plating cells were treated with normal, serum-starved or ML-9 (30 µM) containing medium for 12 h. At the end of treatments acridine orange was added to the cells (1 µg/ml final concentration) for 15 min in 37°C. Then the cells were washed 2 times with appropriate medium and subjected to confocal imaging. Upon excitation by blue light acridine orange emits at 525 nm (green). Due to its weak base properties acridine orange accumulates in acidic organelles, such as lysosomes and autolysosomes, where it precipitates and emits at around 650 nm (red). Thus healthy acidic vesicles appear as red puncta in green cytoplasm. When the pH inside the acidic organelles increases, acridine orange fluorescence switches from red to green.

Confocal Microscopy. Live cell images were obtained using confocal laser scanning microscope (LSM 700, Carl Zeiss MicroImaging GmbH) with a Plan Apochromat 40×/1.3 numerical aperture oil immersion objective and equipped with a CO_2 and thermocontrolled chamber. The images were analyzed in Zeiss LSM Image Browser software and prepared for publication in Adobe Photoshop.

Calcium imaging. Ratiometric dye Fura-2/AM was used as a Ca^{2+} indicator. LNCaP cells were loaded with 2 µM Fura-2/AM for 45 min at 37°C and 5%CO2 in RPMI medium and subsequently washed three times with external solution containing (in mM): 140 NaCl, 5KCl, 1 MgCl2, 2 CaCl2, 5 Glucose, 10 Hepes (pH 7.4). The coverslip was then transferred in a perfusion chamber on the stage of Nikon Eclipse Ti microscope. Fluorescence was alternatively excited at 340 and 380 nm with a monochromator (Polychrome IV, TILL Photonics Gmbh) and captured at 510 nm by a QImaging CCD camera (QImaging). Acquisition and analysis was performed with the MetaFluor 7.7.5.0 software (Molecular Devices Corp.).

Statistical analysis. Data were analyzed using Origin 7.0 (Microcal Software). Statistical analysis was performed using Student's t-test, and $p<0.05$ was considered as significant. Asterisks denote: * - $p<0.05$, ** - $p<0.01$ and *** - $p<0,001$.

Acknowledgements
We thank Prof. Terje Johansen for the pDest-mCherry-eGFP-LC3B plasmid, Prof. Geert Bultynck for the pcDNA3.1(-)-GFP-LC3 plasmid and Prof. Cristophe Biot (University Lille 1) for the valuable discussions. We acknowledge financial support from the INSERM, la Ligue Nationale Contre le Cancer, le Ministere de l'Education Nationale, the Region Nord/Pas-de-Calais. Artem Kondratskyi was supported by fellowship from FRM (Fondation de Recherche Medicale). Maya Yassine was a recipient of a PhD scholarship from Erasmus Mundus. Kateryna Kondratska was an IonTrac Project fellow.

Supplementary information is available at Cell Death & Disease website.

Conflict of interest
The authors declare no conflict of interest.

References

1. Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. *J Clin Oncol* 2010 Feb 20; 28(6): 1075-1083.

2. McCubrey JA, Steelman LS, Kempf CR, Chappell WH, Abrams SL, Stivala F*, et al.* Therapeutic resistance resulting from mutations in Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR signaling pathways. *J Cell Physiol* 2011 Nov; 226(11): 2762-2781.

3. Cheng CK, Fan QW, Weiss WA. PI3K signaling in glioma--animal models and therapeutic challenges. *Brain Pathol* 2009 Jan; 19(1): 112-120.

4. Fan QW, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D*, et al.* A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 2006 May; 9(5): 341-349.

5. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. *J Cell Sci* 2005 Jan 1; 118(Pt 1): 7-18.

6. Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW*, et al.* Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev* 2010 Oct; 90(4): 1383-1435.

7. Shen S, Kepp O, Kroemer G. The end of autophagic cell death? *Autophagy* 2012 Jan; 8(1): 1-3.

8. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med* 2013 Feb 14; 368(7): 651-662.

9. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H*, et al.* Pancreatic cancers require autophagy for tumor growth. *Genes Dev* 2011 Apr 1; 25(7): 717-729.

10. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. *Mol Cancer Ther* 2011 Sep; 10(9): 1533-1541.

11. Guo XL, Li D, Hu F, Song JR, Zhang SS, Deng WJ*, et al.* Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells. *Cancer Lett* 2012 Jul 28; 320(2): 171-179.

12. Selvakumaran M, Amaravadi RK, Vasilevskaya IA, O'Dwyer PJ. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. *Clin Cancer Res* 2013 Jun 1; 19(11): 2995-3007.

13. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nature reviews Cancer* 2012 Jun; 12(6): 401-410.

14. Degtyarev M, De Maziere A, Orr C, Lin J, Lee BB, Tien JY*, et al.* Akt inhibition promotes autophagy and sensitizes PTEN-null

tumors to lysosomotropic agents. *J Cell Biol* 2008 Oct 6; 183(1): 101-116.

15. Lamoureux F, Thomas C, Crafter C, Kumano M, Zhang F, Davies BR*, et al.* Blocked autophagy using lysosomotropic agents sensitizes resistant prostate tumor cells to the novel Akt inhibitor AZD5363. *Clin Cancer Res* 2013 Feb 15; 19(4): 833-844.

16. Lamoureux F, Zoubeidi A. Dual inhibition of autophagy and the AKT pathway in prostate cancer. *Autophagy* 2013 May 6; 9(7).

17. Garcia BG, Wei Y, Moron JA, Lin RZ, Javitch JA, Galli A. Akt is essential for insulin modulation of amphetamine-induced human dopamine transporter cell-surface redistribution. *Mol Pharmacol* 2005 Jul; 68(1): 102-109.

18. Smith U, Carvalho E, Mosialou E, Beguinot F, Formisano P, Rondinone C. PKB inhibition prevents the stimulatory effect of insulin on glucose transport and protein translocation but not the antilipolytic effect in rat adipocytes. *Biochem Biophys Res Commun* 2000 Feb 16; 268(2): 315-320.

19. Saitoh M, Ishikawa T, Matsushima S, Naka M, Hidaka H. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *J Biol Chem* 1987 Jun 5; 262(16): 7796-7801.

20. Smyth JT, Dehaven WI, Bird GS, Putney JW, Jr. Ca2+-store-dependent and -independent reversal of Stim1 localization and function. *J Cell Sci* 2008 Mar 15; 121(Pt 6): 762-772.

21. Connell LE, Helfman DM. Myosin light chain kinase plays a role in the regulation of epithelial cell survival. *J Cell Sci* 2006 Jun 1; 119(Pt 11): 2269-2281.

22. Gu LZ, Hu WY, Antic N, Mehta R, Turner JR, de Lanerolle P. Inhibiting myosin light chain kinase retards the growth of mammary and prostate cancer cells. *Eur J Cancer* 2006 May; 42(7): 948-957.

23. Kaneko K, Satoh K, Masamune A, Satoh A, Shimosegawa T. Myosin light chain kinase inhibitors can block invasion and adhesion of human pancreatic cancer cell lines. *Pancreas* 2002 Jan; 24(1): 34-41.

24. Zhang L, Yu J, Pan H, Hu P, Hao Y, Cai W*, et al.* Small molecule regulators of autophagy identified by an image-based high-throughput screen. *Proc Natl Acad Sci U S A* 2007 Nov 27; 104(48): 19023-19028.

25. Bitting RL, Armstrong AJ. Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. *Endocr Relat Cancer* 2013 Jun; 20(3): R83-99.

26. Majumder PK, Sellers WR. Akt-regulated pathways in prostate cancer. *Oncogene* 2005 Nov 14; 24(50): 7465-7474.

27. Morgan TM, Koreckij TD, Corey E. Targeted therapy for advanced prostate cancer: inhibition of the PI3K/Akt/mTOR pathway. *Curr Cancer Drug Targets* 2009 Mar; 9(2): 237-249.

28. Pourmand G, Ziaee AA, Abedi AR, Mehrsai A, Alavi HA, Ahmadi A*, et al.* Role of PTEN gene in progression of prostate cancer. *Urol J* 2007 Spring; 4(2): 95-100.

29. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K*, et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2012 Apr; 8(4): 445-544.

30. Ouyang DY, Xu LH, He XH, Zhang YT, Zeng LH, Cai JY*, et al.* Autophagy is differentially induced in prostate cancer LNCaP, DU145 and PC-3 cells via distinct splicing profiles of ATG5. *Autophagy* 2013 Jan; 9(1): 20-32.

31. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A*, et al.* p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 2005 Nov 21; 171(4): 603-614.

32. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H*, et al.*

p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 2007 Aug 17; 282(33): 24131-24145.

33. Norwood N, Moore TM, Dean DA, Bhattacharjee R, Li M, Stevens T. Store-operated calcium entry and increased endothelial cell permeability. *Am J Physiol Lung Cell Mol Physiol* 2000 Nov; 279(5): L815-824.

34. Tran QK, Watanabe H, Le HY, Pan L, Seto M, Takeuchi K*, et al.* Myosin light chain kinase regulates capacitative ca(2+) entry in human monocytes/macrophages. *Arterioscler Thromb Vasc Biol* 2001 Apr; 21(4): 509-515.

35. Watanabe H, Takahashi R, Zhang XX, Kakizawa H, Hayashi H, Ohno R. Inhibition of agonist-induced Ca2+ entry in endothelial cells by myosin light-chain kinase inhibitor. *Biochem Biophys Res Commun* 1996 Aug 23; 225(3): 777-784.

36. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 1999 May 15; 13(10): 1211-1233.

37. Ma Y, Hendershot LM. The unfolding tale of the unfolded protein response. *Cell* 2001 Dec 28; 107(7): 827-830.

38. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S*, et al.* Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* 2006 Dec; 26(24): 9220-9231.

39. Yorimitsu T, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* 2006 Oct 6; 281(40): 30299-30304.

40. Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T*, et al.* Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. *Mol Cell* 2007 Jan 26; 25(2): 193-205.

41. Leitman EM, Tewari A, Horn M, Urbanski M, Damanakis E, Einheber S*, et al.* MLCK regulates Schwann cell cytoskeletal organization, differentiation and myelination. *J Cell Sci* 2011 Nov 15; 124(Pt 22): 3784-3796.

42. Qin L, Wang Z, Tao L, Wang Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. *Autophagy* 2010 Feb; 6(2): 239-247.

43. Decuypere JP, Welkenhuyzen K, Luyten T, Ponsaerts R, Dewaele M, Molgo J*, et al.* Ins(1,4,5)P3 receptor-mediated Ca2+ signaling and autophagy induction are interrelated. *Autophagy* 2011 Dec; 7(12): 1472-1489.

44. Parys JB, Decuypere JP, Bultynck G. Role of the inositol 1,4,5-trisphosphate receptor/Ca2+-release channel in autophagy. *Cell Commun Signal* 2012; 10(1): 17.

45. Wang SH, Shih YL, Ko WC, Wei YH, Shih CM. Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway. *Cell Mol Life Sci* 2008 Nov; 65(22): 3640-3652.

46. Avivar-Valderas A, Salas E, Bobrovnikova-Marjon E, Diehl JA, Nagi C, Debnath J*, et al.* PERK integrates autophagy and oxidative stress responses to promote survival during extracellular matrix detachment. *Mol Cell Biol* 2011 Sep; 31(17): 3616-3629.

47. Verfaillie T, Salazar M, Velasco G, Agostinis P. Linking ER Stress to Autophagy: Potential Implications for Cancer Therapy. *Int J Cell Biol* 2010; 2010: 930509.

48. Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. *International journal of oncology* 2005 May; 26(5): 1401-1410.

49. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nature reviews Cancer* 2005 Sep; 5(9): 726-734.

50. Schayowitz A, Sabnis G, Goloubeva O, Njar VC, Brodie AM. Prolonging hormone sensitivity in prostate cancer xenografts through dual inhibition of AR and mTOR. *Br J Cancer* 2010 Sep 28; 103(7): 1001-1007.

51. Festuccia C, Gravina GL, Angelucci A, Millimaggi D, Muzi P, Vicentini C*, et al.* Additive antitumor effects of the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa), and the nonsteroidal antiandrogen, bicalutamide (Casodex), in prostate cancer cells in vitro. *Int J Cancer* 2005 Jul 1; 115(4): 630-640.

52. Schayowitz A, Sabnis G, Njar VC, Brodie AM. Synergistic effect of a novel antiandrogen, VN/124-1, and signal transduction inhibitors in prostate cancer progression to hormone independence in vitro. *Mol Cancer Ther* 2008 Jan; 7(1): 121-132.

Titles and legends to figures

Figure 1. ML-9 stimulates autophagy by downregulating Akt/mTOR pathway in prostate cancer cells. (a) ML-9 inhibits Akt. To assess Akt phosphorylation LNCaP cells were treated with full or 30 μM ML-9-containing medium for 12 h. Densitometric quantitation for normalized p-Akt and total Akt relative to Actin is shown. Values represent means±sem. n=3. (b) ML-9 inhibits mTOR. LNCaP cells were incubated in full medium, serum-starved medium or full medium with indicated concentrations of ML-9 for 12 h. The expression levels of p-mTOR, mTOR and beta-Actin were analyzed. Densitometric quantitation for normalized p-mTOR and mTOR relative to Actin is shown. Values represent means±sem. n=3. (c) ML-9 increases LC3-II levels in LNCaP cells in a dose-dependent manner. LNCaP cells were incubated in full medium, serum-starved medium or full medium with indicated concentrations of ML-9 for 6 h. Densitometric quantitation for LC3-II/(LC3-II+LC3-I) ratio is shown. Values represent means±sem. n=3. (d) ML-9 increases LC3-II levels in LNCaP cells in a time-dependent manner. LNCaP cells were untreated or treated with 30 μM ML-9 for 2, 6, 12 and 24 hours. Densitometric quantitation for LC3-II/(LC3-II+LC3-I) ratio is shown. Values represent means±sem. n=4. (e) LNCaP cells stably expressing eGFP-LC3 (green) were incubated in full, serum-starved or 30 μM ML-9-containing media for 6h. Quantitation shown on the right represents means±S.D. GFP positive puncta per cell (n = 50) from three independent experiments. (f) TEM images of LNCaP cells untreated and treated with 30 μM ML-9 for 12 h showing autophagosome (black arrow) and autolysosomes/amphisomes (white arrows). N: nucleus.

Figure 2. ML-9 blocks autophagic flux. (a) Chloroquine does not increase LC3-II levels induced by elevated concentrations of ML-9. LNCaP cells were incubated in full, serum-starved or ML-9-containing media for 6 h in the absence or presence of 50 μM chloroquine for the last hour. Densitometric quantitation showing autophagic flux (change in LC-II levels induced by chloroquine) is represented. Values represent means±sem. n=3. (b) Chloroquine fails to increase the number of eGFP positive puncta following ML-9 treatment. eGFP-LC3 expressing LNCaP cells were treated with full, serum-starved or 30 μM ML-9-containing media for 6 h in the absence or presence of 50 μM chloroquine for the last hour. Quantitation shown on the right represents means±S.D. GFP positive puncta per cell (n = 50) from three independent experiments. (c) ML-9 induces p62 accumulation. LNCaP cells were treated with full, serum-starved or ML-9 containing media for 12 h. Densitometric quantitation for normalized p62 relative to Actin is shown. Values represent means±sem. n=3. (d) ML-9 blocks starvation induced autophagic flux. LNCaP cells were treated with full, serum-starved, 30 μM ML-9-containing full medium or 30 μM ML-9-containing serum-starved medium for 6 h in the absence or presence of 50 μM chloroquine for the last hour. Densitometric quantitation showing autophagic flux (change in LC-II levels induced by chloroquine) as well as normalized p62 relative to Actin is shown. Values represent means±sem. n=3.

Figure 3. ML-9 affects maturation of autophagosomes and increases lysosomal pH. (a) LNCaP cells were transiently transfected with mCherry-GFP-LC3B and treated with full, serum-starved, or ML-9 (30 μM) containing medium for 12 h. The colocalization of GFP and mCherry signals was analyzed. Quantitation shown on the right represents means±S.D. mCherry positive/GFP negative puncta per cell (n = 20). (b) ML-9 decreases lysosomal acidification. LNCaP cells were treated with full, serum-starved, 30 μM ML-9-containing full medium or 30 μM ML-9-containing serum-starved medium for12 h, stained with acridine orange and analyzed using confocal microscopy.

Figure 4. ML-9 stimulates autophagy via both Vps34-dependent and -independent mechanisms. (a) ML-9 increase Beclin1 levels in LNCaP cells. LNCaP cells were incubated in full medium, serum-starved medium or full medium with indicated concentrations of ML-9 for 12 h. Densitometric quantitation for normalized Beclin1 relative to Actin is shown. Values represent means±sem. n=3. (b) Wortmannin effectively reduces LC3-II levels

induced by ML-9 at low concentrations but is almost ineffective in reducing LC3-II levels induced by ML-9 at high concentrations. LNCaP cells were incubated in full medium, serum-starved medium or full medium with indicated concentrations of ML-9 for 6 h in the absence or presence of 100 nM wortmannin for the last 1,5 hours. Densitometric quantitation for normalized LC3-II relative to Actin is shown. Values represent means±sem. n=3. (c) 3-MA does not preclude ML-9 induced LC3-II accumulation. LNCaP cells were incubated in full medium, serum-starved medium, 20 μM ML-9-containing medium or 30 μM ML-9-containing medium for 6 h. Alternatively, LNCaP cells were pretreated with 5mM 3-MA for 1 h followed by the 6 h treatment with full medium, serum-starved medium, 20 μM ML-9-containing medium or 30 μM ML-9-containing medium in the continuous presence of 5 mM 3-MA. Densitometric quantitation for normalized LC3-II relative to Actin is shown. Values represent means±sem. n=3. (d) eGFP-LC3 expressing LNCaP cells were treated with full, serum-starved or 30 μM ML-9-containing media for 6 h in the absence or presence of 100 nM wortmannin. Quantitation shown on the right represents means±S.D. GFP positive puncta per cell (n = 30).

Figure 5. ML-9 induces prostate cancer cell death. (a) ML-9 reduced cell viability in a concentration-dependent manner. LNCaP cells, plated on 96-well plate, were treated with different doses of ML-9 in full or serum-starved media for 24 h. Cell viability was monitored using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS assay). Following treatment the cells were incubated with reagent solution and absorbance was recorded at 490 nm wavelength using an ELISA plate reader. Values represent means±sem. n=3. (b) Cell viability was assessed by trypan blue exclusion assay. LNCaP cells were treated with different concentrations of ML-9 in full or serum-starved media for 24 h, the cells were collected by trypsin-EDTA, incubated with trypan blue and viable cells as well as dead (blue) cells were counted using haemocytometer. Values represent means±sem. +++ - p<0,001. n=4. (c) ML-9 induces PARP cleavage. LNCaP cells were treated with full, serum-starved, 30 μM ML-9-containing full medium or 30 μM ML-9-containing serum-starved medium for 24 h. (d) ML-9 induces apoptosis in LNCaP cells. LNCaP cells were treated as in (c). At the end of the treatment cells were collected, stained with Annexin V/PI and subjected to fluorescence microscopy analysis. Data is represented as means±sem. n=4. (e) LNCaP, PC3 and DU145 cells, plated on 96-well plate, were treated with docetaxel (5 nM), ML-9 (30 μM) or combination of docetaxel+ML-9 for 48 h. Cell viability was monitored using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS assay).

Figure 6. ML-9 modulates Ca^{2+} homeostasis and stimulates autophagy in a calcium-dependent manner. (a) LNCaP cells were pretreated with full medium or 30 μM ML-9-containing medium for 6 h, loaded with Fura2/AM probe and subjected to calcium imaging experiment. 2 μM ionomycin-induced transients in calcium-free extracellular medium were analyzed. (b) ML-9 reduces both calcium content of intracellular calcium stores and SOCE in LNCaP cells. LNCaP cells were treated as in (a). 2 μM TG-induced transients in calcium-free extracellular medium as well as SOCE were analyzed. (c) ML-9 induces ER-stress. LNCaP cells were treated with full medium, 1 μM TG-containing medium or 50 μM ML-9-containing medium for 24 h. The levels of p-PERK and GRP78 were analyzed. Densitometric quantitation for normalized p-PERK and GRP78 relative to Actin is shown. Values represent means±sem. n=3. (d) Ca^{2+} is required for ML-9-induced autophagy. LNCaP cells were treated with full, serum-starved, 30 μM ML-9-containing full media in the absence or presence of 20 μM BAPTA/AM for 3h. Densitometric quantitation for normalized LC3-II relative to Actin is shown. Values represent means±sem. n=3.

Figure 7. A model for ML-9's mode of action. ML-9 as a lipophilic weak base enters the cell by simple diffusion. ML-9 downregulates Akt/mTOR pathway and as such stimulates autophagosome formation. ML-9 induces calcium release from ER and provokes ER stress. ER stress is a well known autophagy inducer. At neutral pH ML-9 is in its

non-protonated form. When ML-9 enters acidic vacuoles (such as lysosomes, late endosomes, amphisomes and autolysosomes) it becomes positively charged through protonation and trapped in these vacuoles. Then the increased osmotic pressure in these vacuoles stimulates water influx and vacuoles enlargement. All these events result in the increased intravacuolar pH, subsequent inhibition of lysosomal enzymes and block in degradation of the autolysosomal content as well as accumulation of autophagic vacuoles.

Figure 1

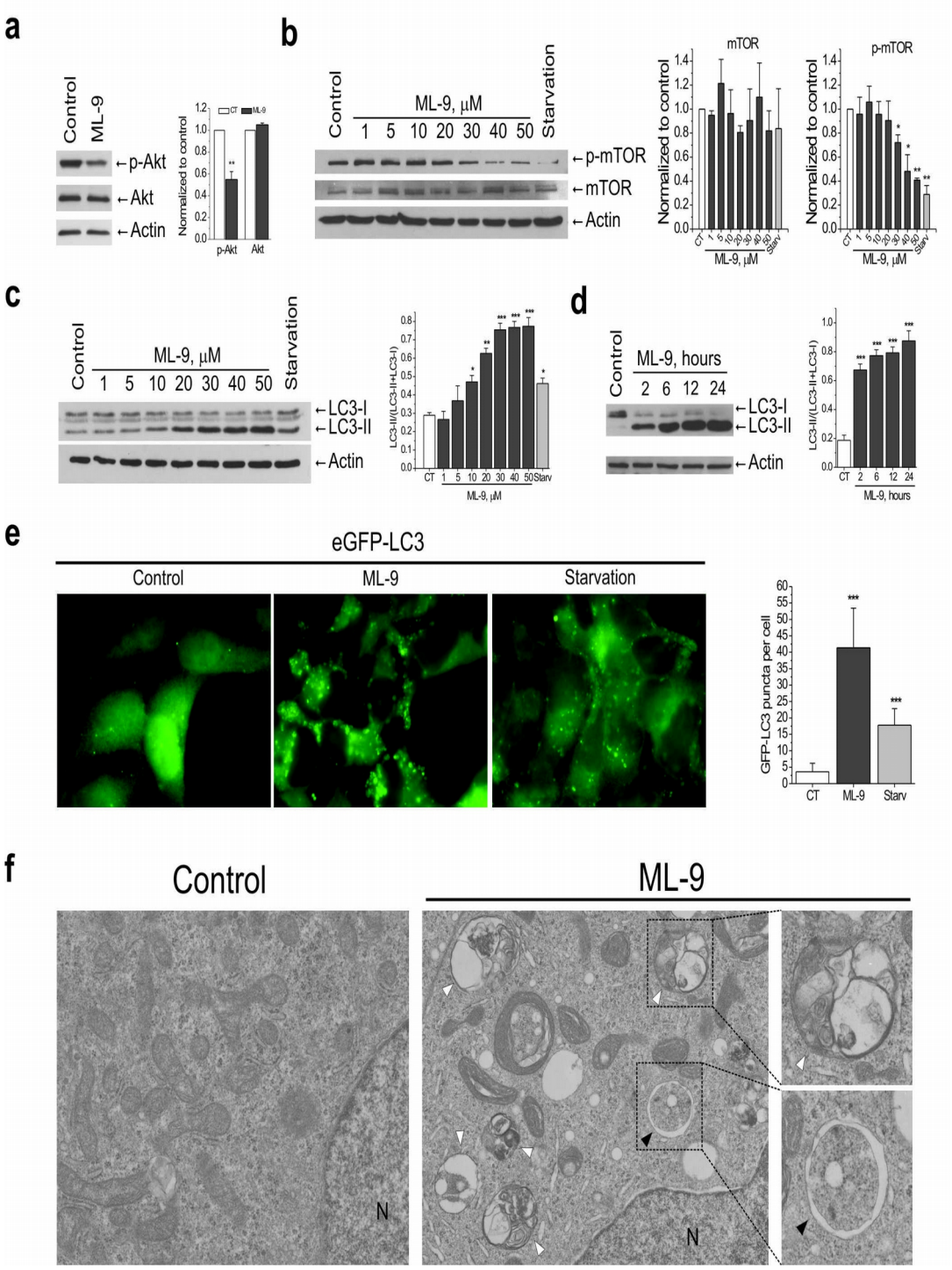


Figure 2

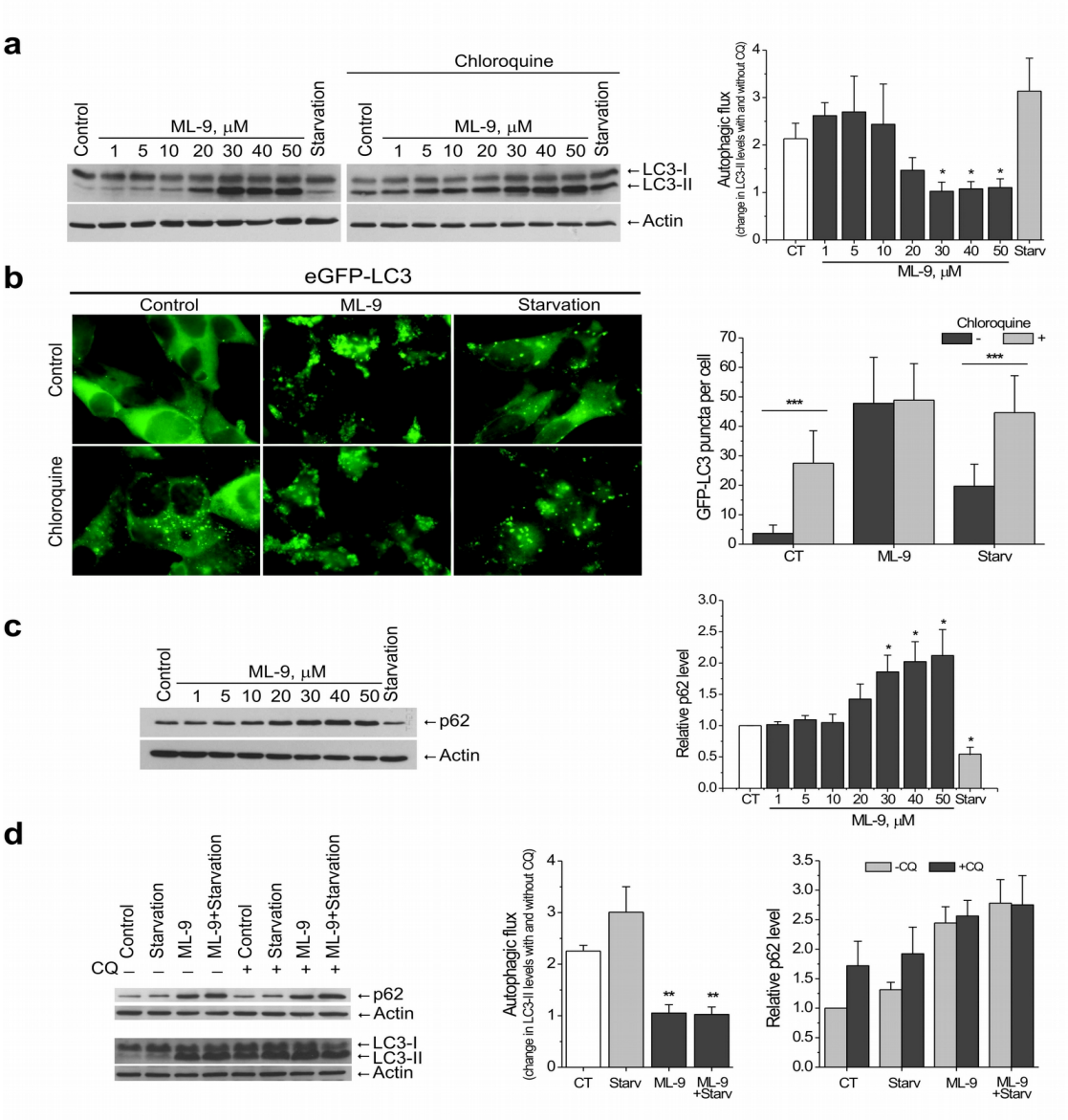


Figure 3

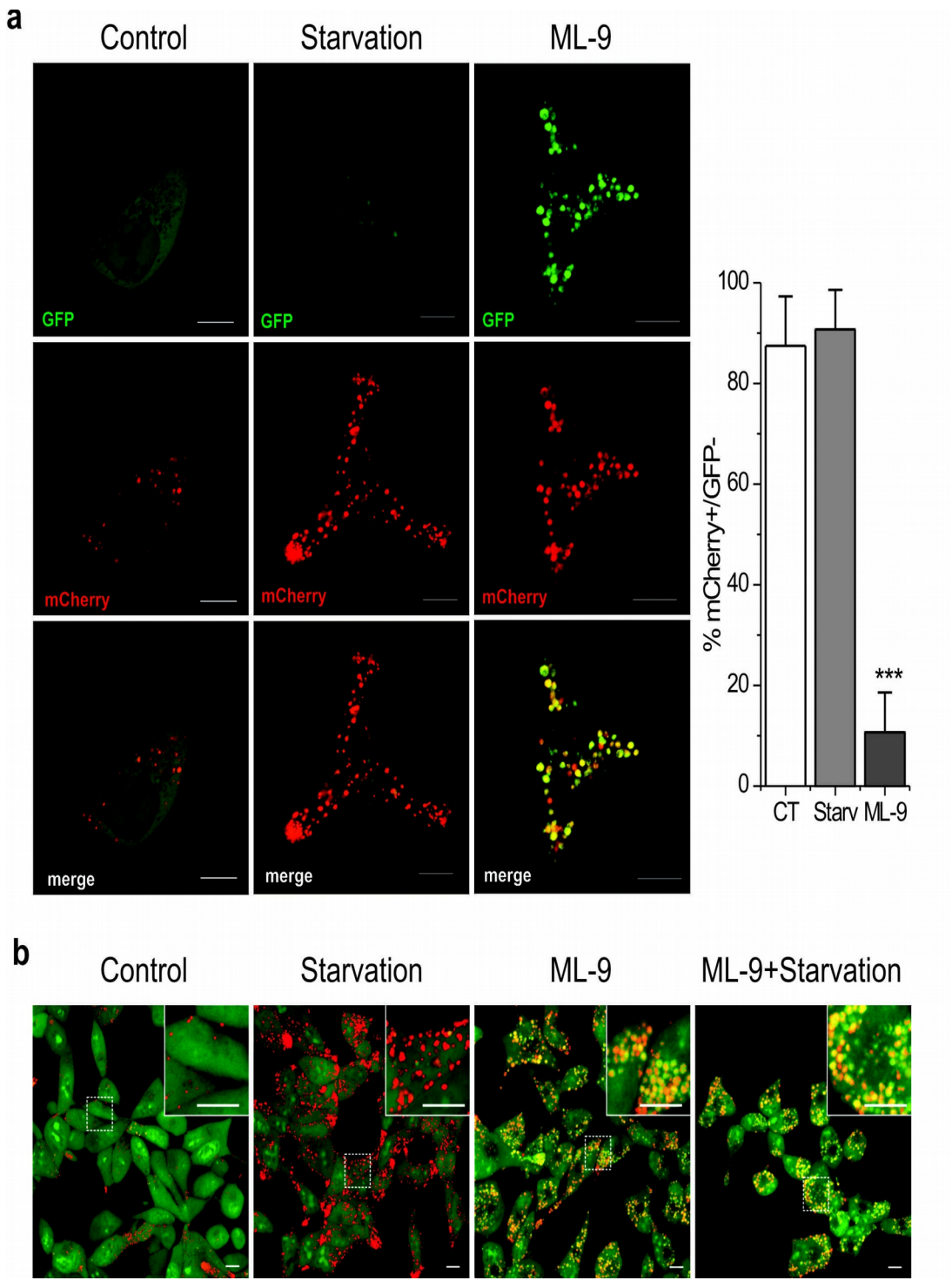


Figure 4

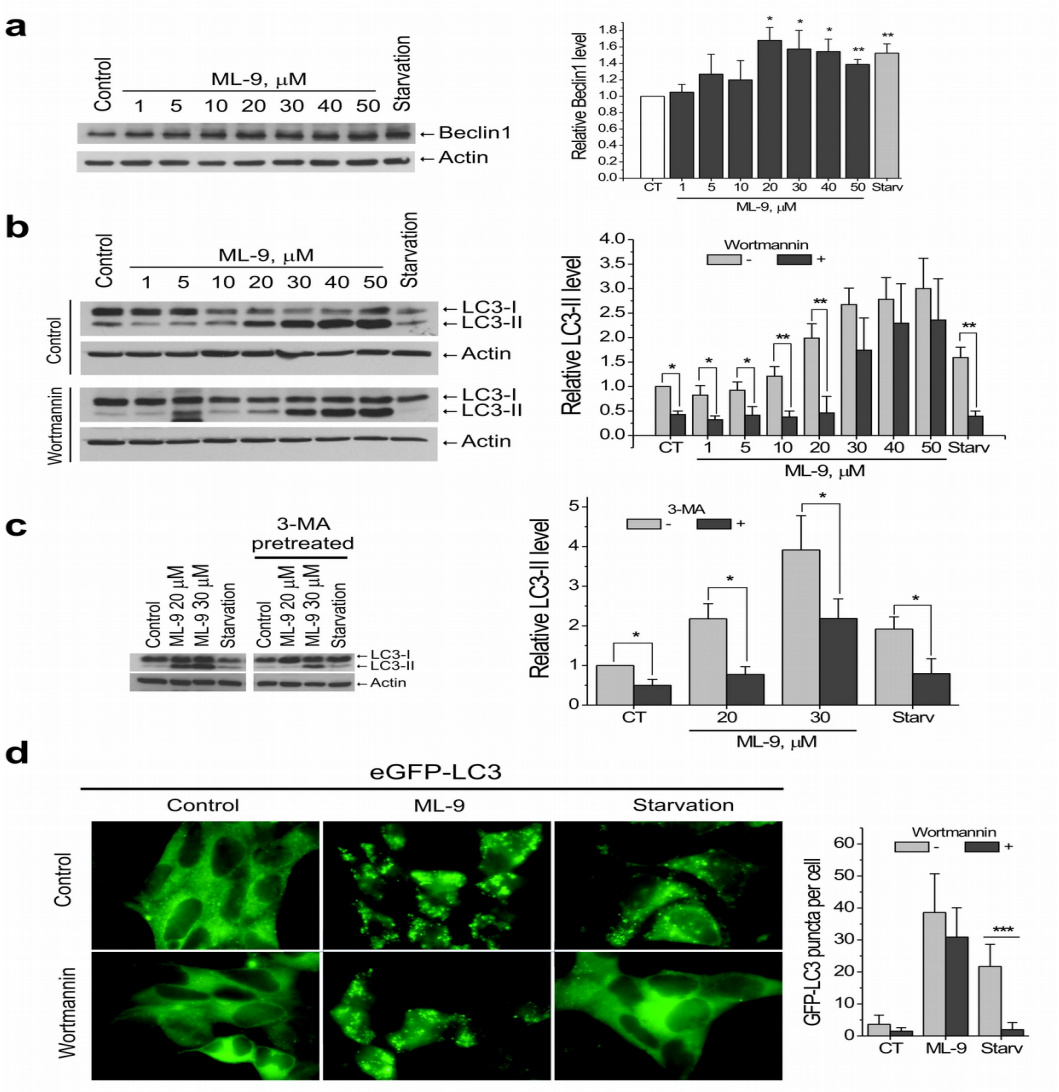


Figure 5

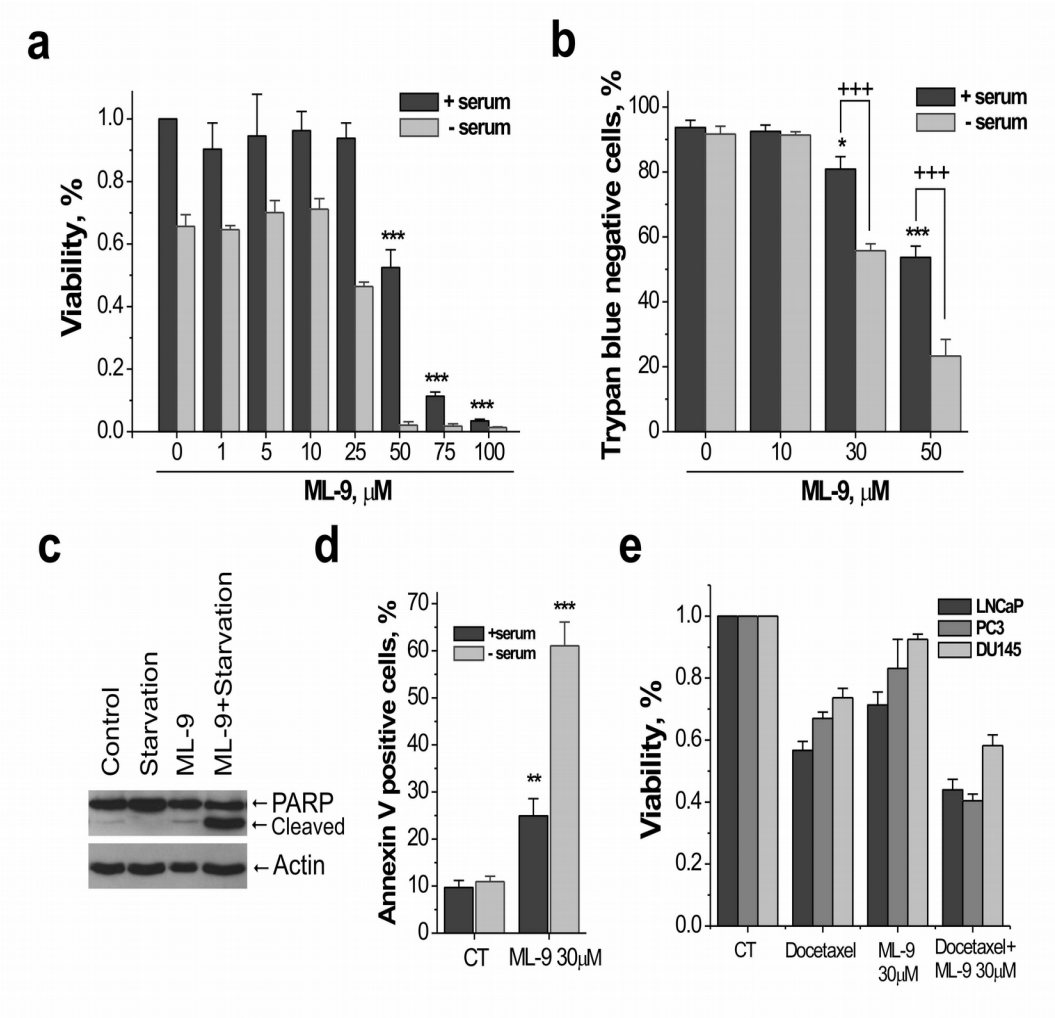


Figure 6

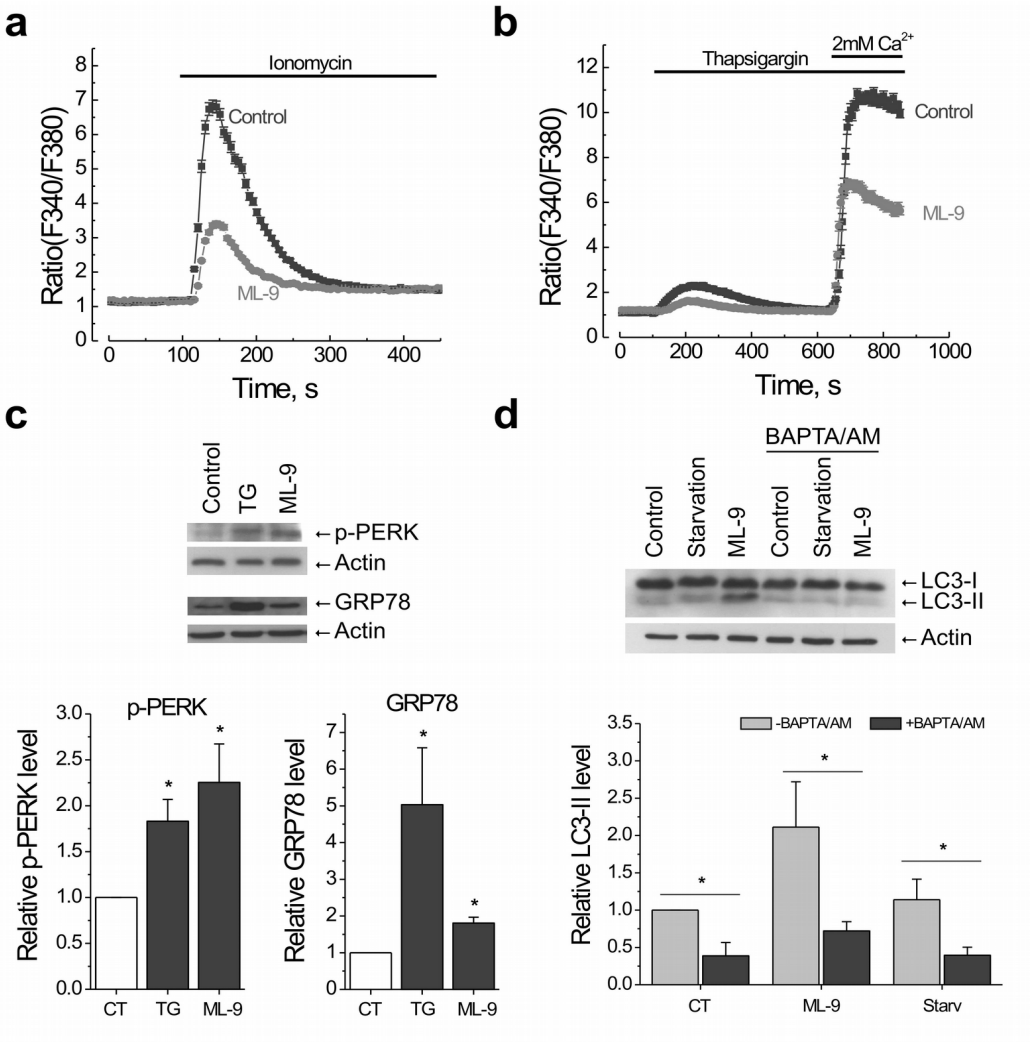
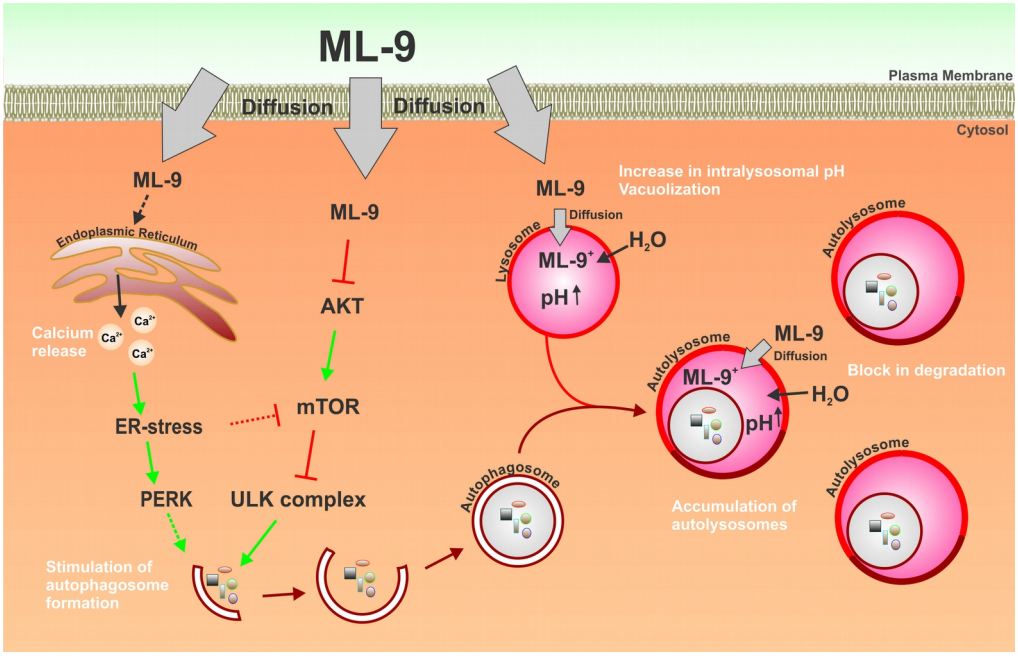
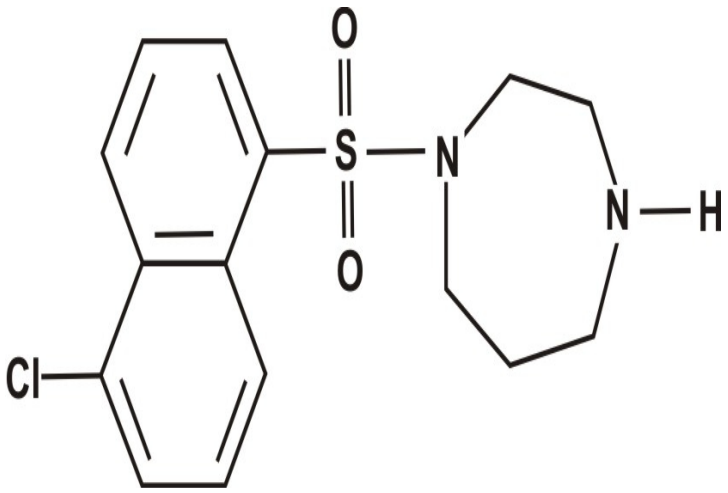


Figure 7



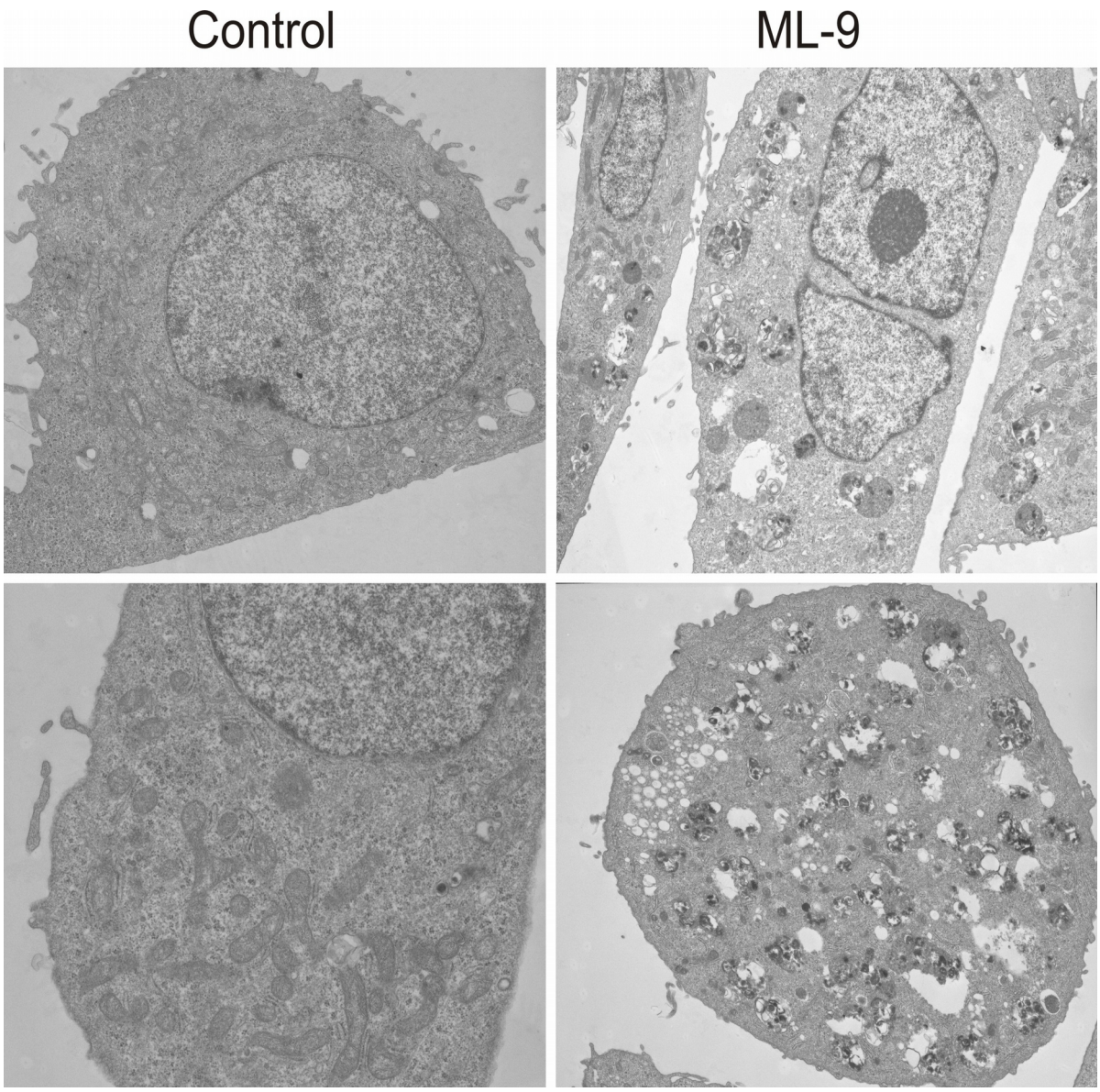
SUPPLEMENTARY FIGURES

Supplementary Figure 1



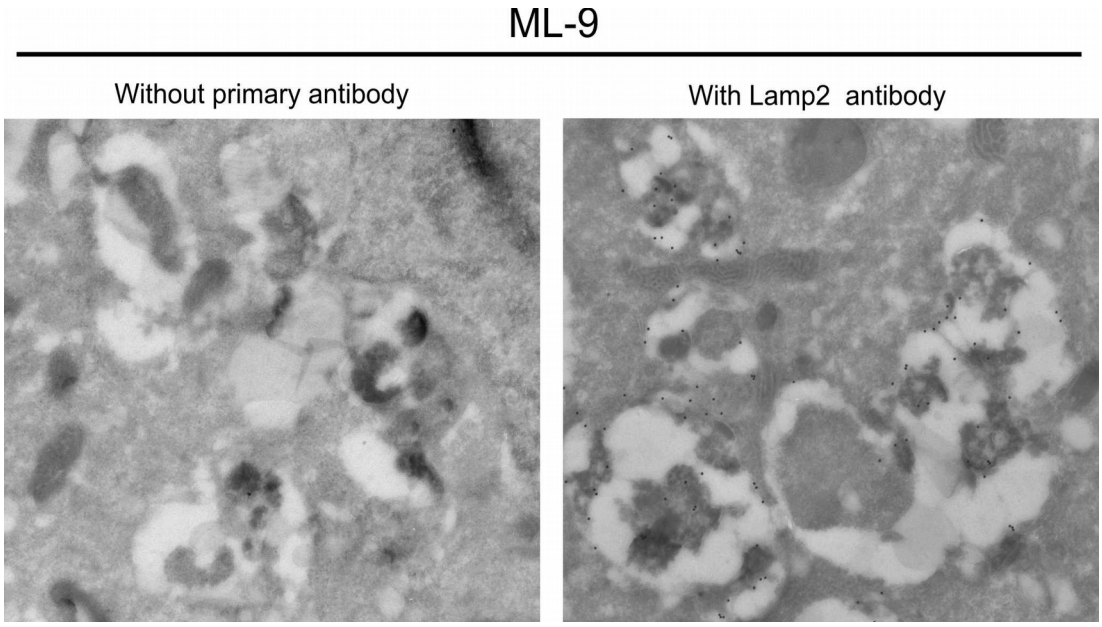
Supplementary Figure 1. Chemical structure of ML-9.

Supplementary Figure 2



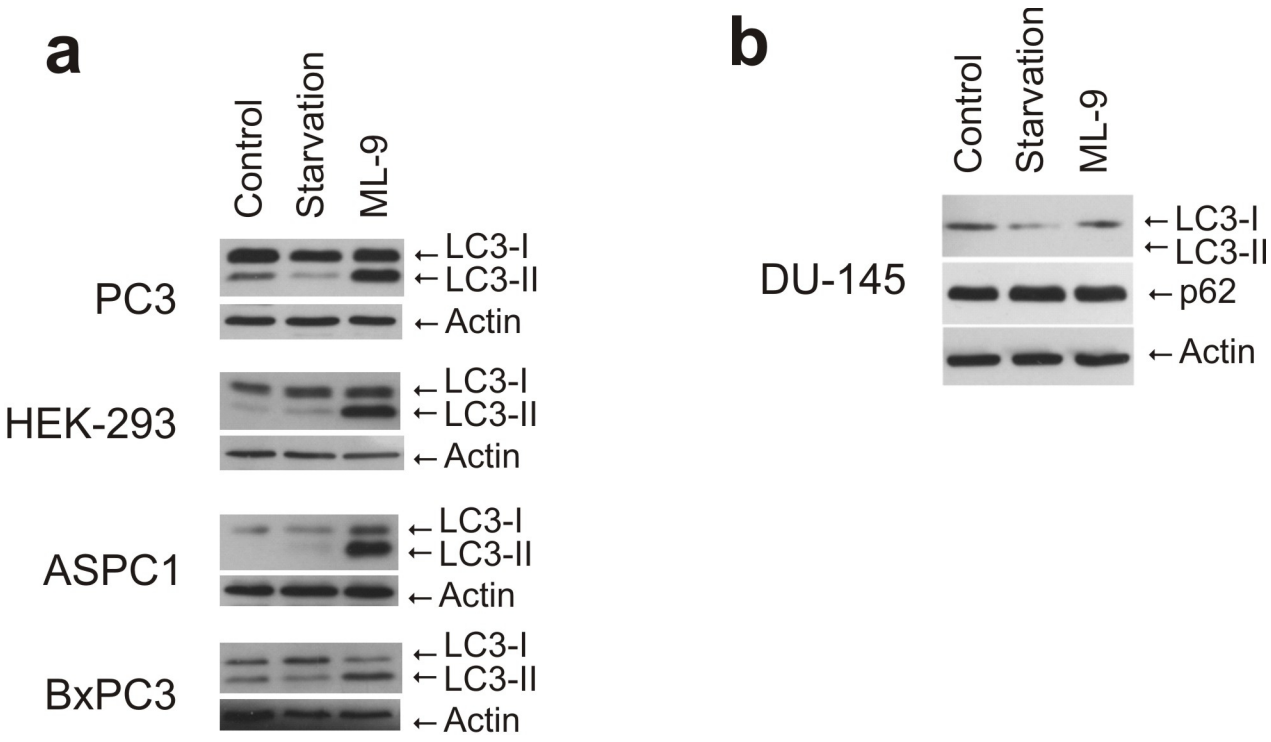
Supplementary Figure 2. ML-9 induces accumulation of autophagic vacuoles. TEM images of LNCaP cells untreated and treated with 30 μM ML-9 for 12 h. The numerous autophagosomes and autolysosomes/amphisomes are clearly visible upon ML-9 treatment. Accumulation of multi-lamellar membrane structures, small vesicles, cytoplasmic material and organelles at different stages of degradation is evident suggesting induction of phospholipodosis.

Supplementary Figure 3



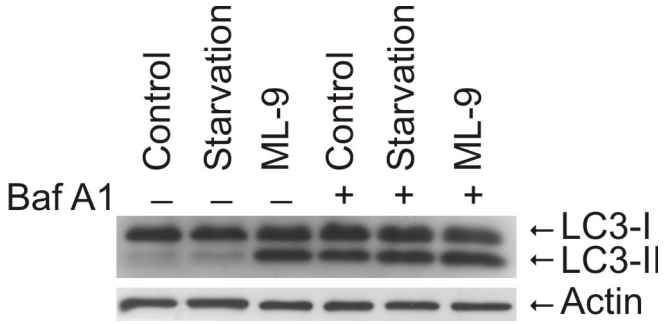
Supplementary Figure 3. ML-9 induces accumulation of Lamp2-positive autophagic vacuoles. To the right - immuno-TEM image of LNCaP cell treated with 30 μM ML-9 for 12 h shows the presence of immunogold-labeled lysosomal protein Lamp2 in large vesicles with degraded content. To the left – control of specificity (secondary antibodies without primary antibodies).

Supplementary Figure 4



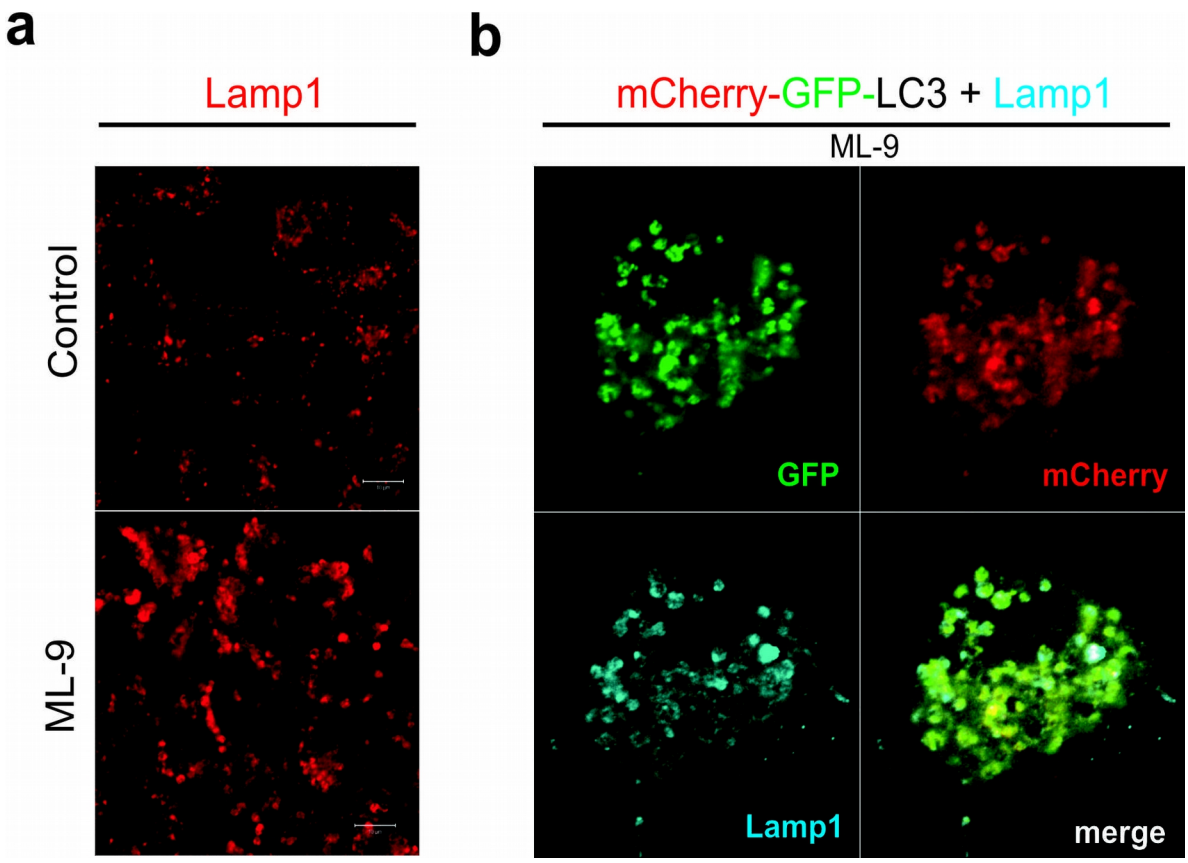
Supplementary Figure 4. (a) ML-9 caused an increase in LC3-II levels in a range of cell lines. PC-3, HEK-293, ASPC1 and BxPC3 cells were treated with full, serum-starved or 30 μM ML-9-containing media for 12 h. Increase in LC3-II levels is obvious in all the cell lines. (b) ML-9 failed to increase LC3-II and p62 levels in DU145 cells. DU145 cells were treated with full, serum-starved or 30 μM ML-9-containing media for 12 h.

Supplementary Figure 5



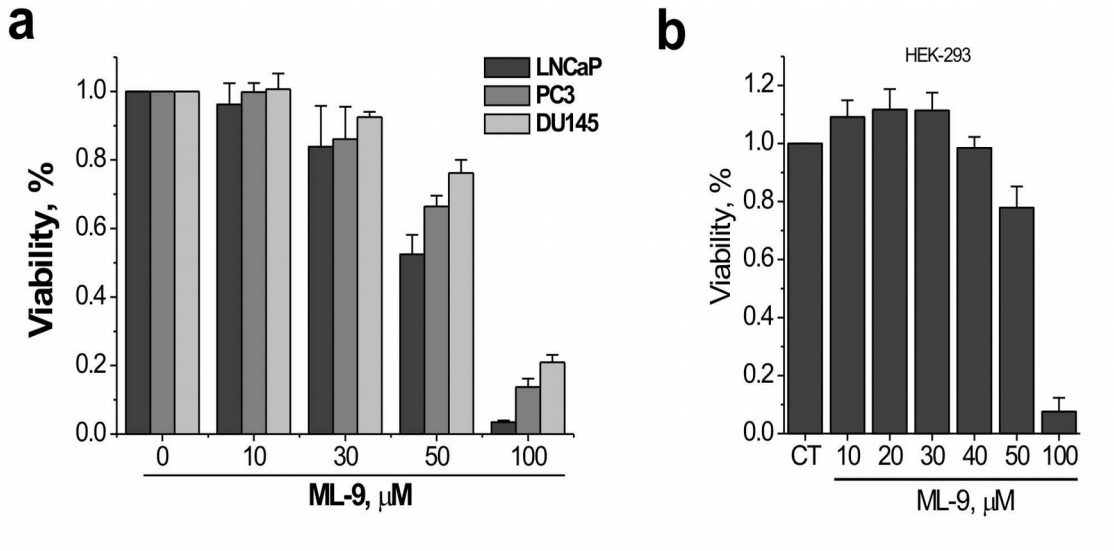
Supplementary Figure 5. ML-9 blocks autophagic flux. Bafilomycin A1 (100 nM) does not increase LC3-II levels induced by elevated concentrations of ML-9. LNCaP cells were incubated in full, serum-starved or 30 μM ML-9-containing media for 6 h in the absence or presence of 100 nM bafilimycin A1 for the last hour.

Supplementary Figure 6



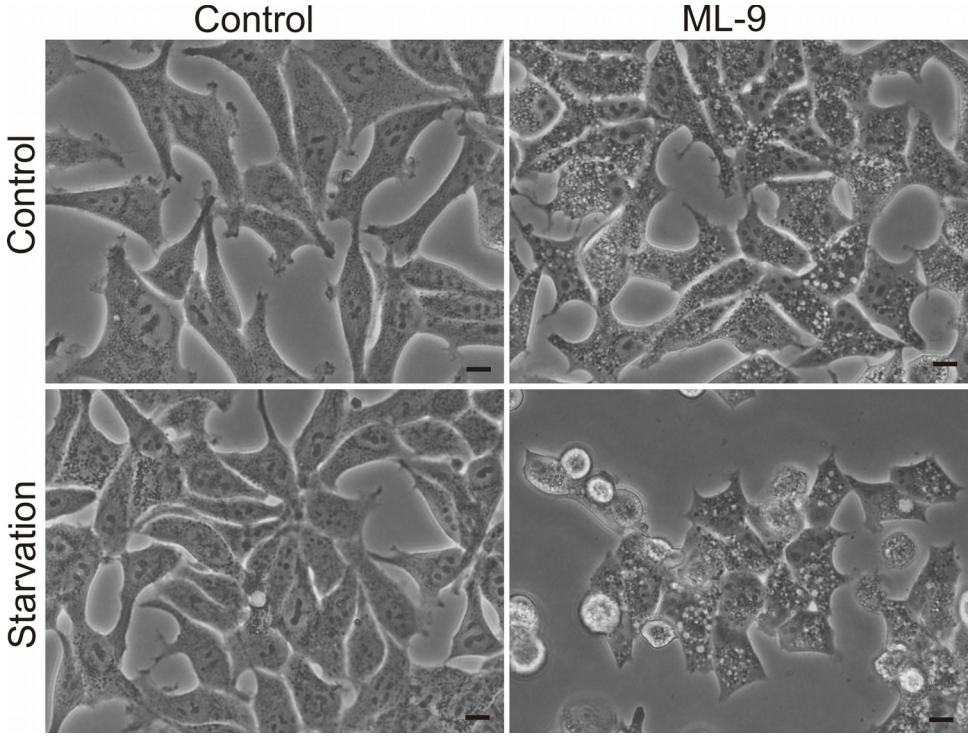
Supplementary Figure 6. ML-9 affects maturation of autophagosomes and induces lysosome swelling. (a) ML-9 visibly increased the size of Lamp1-positive vesicles/puncta. LNCaP cells were treated with full medium or ML-9 (30 μM) containing medium for 12 h. (b) ML-9 induces accumulation of mCherry-GFP-LC3B puncta, which are largely colocalized with Lamp1-positive vesicles/puncta. LNCaP cells were transiently transfected with mCherry-GFP-LC3B and treated with ML-9 (30 μM) containing medium for 12 h.

Supplementary Figure 7



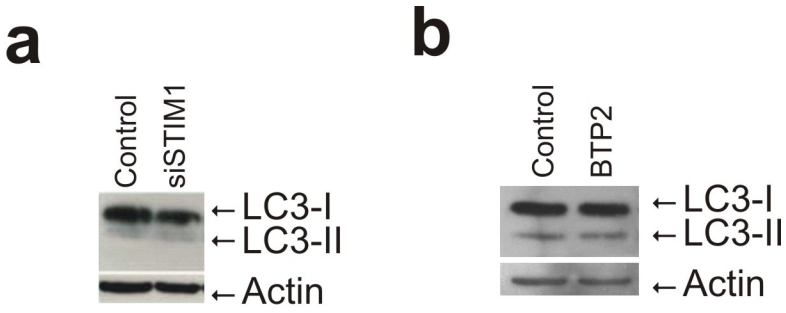
Supplementary Figure 7. ML-9 induces cell death in normal and cancer cells. (a) ML-9 reduced cell viability in a concentration-dependent manner. LNCaP, PC3 and DU145 cells, plated on 96-well plate, were treated with different doses of ML-9 in full media for 24 h. Cell viability was monitored using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS assay). Following treatment the cells were incubated with reagent solution and absorbance was recorded at 490 nm wavelength using an ELISA plate reader. Values represent means±sem. n=3. (b) ML-9 reduced HEK-293 cell viability in a concentration-dependent manner. HEK-293 cells were treated as in (a). Cell viability was monitored as in (a). Values represent means±sem. n=3.

Supplementary Figure 8



Supplementary Figure 8. ML-9 induces vacuolization. LNCaP cells were treated with 30 μM ML-9 in full or serum-starved media for 12 h. Scale bars represent 20 μm.

Supplementary Figure 9



Supplementary Figure 9. ML-9 stimulates autophagy independently of Stim1 and SOCE inhibition. (a) LNCaP cells were untransfected or transfected with siSTIM1 and the LC3-II levels were analyzed. n=3. (b) LNCaP cells were treated with full medium or 10 μM BTP2-containing medium for 6h and the LC3-II levels were analyzed.

Article 2 is accepted in BBA *Molecular Cell Research journal* (2014)

The contribution of Yassine to the article:

Weternblots and their results section.

Orai1 and STIM1 mediate SOCE and contribute to apoptotic resistance of pancreatic adenocarcinoma

Kateryna Kondratska1§, Artem Kondratskyi1§, Maya Yassine1, Loic Lemonnier1, Gilbert Lepage1, Angela Morabito1, Roman Skryma1, Natalia Prevarskaya1*

1 Inserm, U-1003, Equipe labellisée par la Ligue Nationale contre le cancer. Laboratory of Excellence, Ion Channels Science and

Therapeutics, Université Lille 1, Villeneuve d'Ascq, France.

§These authors contributed equally to this work

*Correspondence to: Professor Natalia Prevarskaya; Laboratory of Cell Physiology INSERM U 1003; Bat. SN 3, UFR de Biologie

Université Lille 1, 59655 Villeneuve d'Asq cedex, France ; Tel. : +(33) 3 20 33 60 18 ; Fax. : +(33) 3 20 43 40 66; E-mail:

Natacha.Prevarskaya@univ-lille1.fr

Abstract

The store-operated calcium channels (SOCs) represent one of the major calcium-entry pathways in non-excitable cells. SOCs and in particular their major components ORAI1 and STIM1 have been shown to be implicated in a number of physiological and pathological processes such as apoptosis, proliferation and invasion. Here, we demonstrate that ORAI1 and STIM1 mediate store-operated calcium entry (SOCE) in pancreatic adenocarcinoma cell lines. We show that both ORAI1 and STIM1 play pro-survival antiapoptotic role in pancreatic adenocarcinoma cell lines, as siRNA mediated knockdown of ORAI1 and/or STIM1 increase apoptosis induced by chemotherapy drugs 5-fluorouracil (5-FU) or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line via upregulation of ORAI1 and STIM1. Altogether, our results reveal the novel calcium-dependent mechanism of action of the chemotherapy drugs 5-FU and gemcitabine and emphasize the antiapoptotic role of ORAI1 and STIM1 in pancreatic adenocarcinoma cells.

Keywords:calcium,SOCE,pancreatic adenocarcinoma,apoptosis,chemotherapy, resistance

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; SOCE, store operated calcium entry; SOCs, store-operated calcium channels; STIM1, stromal interaction molecule 1; 5-FU, 5-fluorouracil; TG, thapsigargin; SERCA, sarco/endoplasmic reticulum Ca2+ ATPase; siRNA, small interfering RNA.

frontiers in PHYSIOLOGY

Review is accepted in *Frontiers in Physiology* (2013)

The intellectual contribution of Yassine:

- Role of calcium in autophagy

- Calcium permeable channels in control of autophagy

- Conclusions

& contributed to the potential new candidates for autophagy regulation in cancer

Calcium-permeable ion channels in control of autophagy and cancer

Running title: Calcium channels, autophagy and cancer

Maya Yassine[§], Artem Kondratskyi§, Kateryna Kondratska, Roman Skryma, Christian Slomianny, Natalia Prevarskaya*

Inserm, U-1003, Equipe labellisée par la Ligue Nationale contre le cancer. Laboratory of Excellence, Ion Channels Science and Therapeutics, Université Lille 1, Villeneuve d'Ascq, France.

[§]These authors contributed equally to this work

Correspondence:
Professor Natalia Prevarskaya
Laboratory of Cell Physiology
INSERM U 1003
Bat. SN 3, UFR de Biologie
Université Lille 1,
59655 Villeneuve d'Asq cedex,
France
Natacha.Prevarskaya@univ-lille1.fr

Autophagy, or cellular self-eating, is a tightly regulated cellular pathway the main purpose of which is lysosomal degradation and subsequent recycling of cytoplasmic material to maintain normal cellular homeostasis. Defects in autophagy are linked to a variety of pathological states, including cancer. Cancer is the disease associated with abnormal tissue growth following an alteration in such fundamental cellular processes as apoptosis, proliferation, differentiation, migration and autophagy. The role of autophagy in cancer is complex, as it can promote both tumor prevention and survival/treatment resistance. It's now clear, that modulation of autophagy has a great potential in cancer diagnosis and treatment. Recent findings identified intracellular calcium as an important regulator of both basal and induced autophagy. Calcium is a ubiquitous secondary messenger which regulates plethora of physiological and pathological processes such as aging, neurodegeneration and cancer. The role of calcium and calcium-permeable channels in cancer is well established, whereas the information about molecular nature of channels regulating autophagy and the mechanisms of this regulation is still limited. Here we review existing mechanisms of autophagy regulation by calcium and calcium-permeable ion channels. Furthermore, we will also discuss some calcium-permeable channels as the potential new candidates for autophagy regulation. Finally we will propose the possible link between calcium permeable channels, autophagy and cancer progression and therapeutic response.

Keywords: calcium, autophagy, TRP, ion channels, cancer

Autophagy

Autophagy is a cellular catabolic process for the degradation and recycling of protein aggregates, long-lived proteins and damaged organelles to maintain cellular homeostasis (Ravikumar et al., 2010;Chen and Klionsky, 2011). Normally, autophagy occurs under basal conditions but it can be stimulated in response to different types of cellular stress, such as nutrient starvation, hypoxia, endoplasmic reticulum (ER) stress, oxidative stress, mitochondrial damage as well as treatment with some pharmacological agents (Kroemer et al., 2010). To date, three types of autophagy have been described, including macroautophagy, microautophagy, and chaperone-mediated autophagy (Klionsky, 2005). These types differ in their functions and regulatory mechanisms. During macroautophagy, further mentioned as autophagy, cytoplasmic components are engulfed by double membrane structures termed autophagosomes that mature by fusing first with late endosomes or directly with lysosomes to form autolysosomes. Finally, the content of autolysosome together with its inner membrane is degraded by lysosomal hydrolases to be reused in the cell (Ravikumar et al., 2010;Chen and Klionsky, 2011).

The process of autophagosome formation consists of several stages, namely initiation, elongation and maturation and fusion. At present, around 30 autophagy-related genes are identified, and most of them have mammalian orthologs (Ravikumar et al., 2010). These genes are implicated in different stages of autophagy. Thus, the autophagosome formation requires the activity of the class III phosphatidylinositol 3-kinase (PI3K), Vps34. Mammalian Atg18 homolog WIPI-1 binds to PI3P, the product of Vps34 activity, and is recruited to autophagosomal membrane. Vps34 is a part of a protein complex containing Beclin1/Atg6, p150/Vps15 and Atg14/barkor proteins (He and Levine, 2010). Another complex implicated in the initiation stage of autophagosome formation is the focal adhesion kinase family interacting protein FIP200-ULK1/2/Atg1-Atg13 complex, where Atg13, ULK1 and ULK2 proteins have been shown to be direct targets of the mammalian target of rapamycin (mTOR) (Mizushima, 2010). Under nutrient-rich conditions, mTOR is associated with this complex, whereas under starvation conditions inhibition of mTOR causes its dissociation from the complex and leads to activation of ULK1/2, subsequent phosphorylation of FIP200 and autophagy induction (Mizushima, 2010;Kim et al., 2011). The elongation stage requires cleavage of the microtubule-associated protein 1 light chain 3 (Atg8/LC3) by Atg4, resulting in the formation of cytosolic LC3-I protein, which is conjugated to phosphatidylethanolamine (PE) to form membrane bound LC3-II (Tanida et al., 2004). Another stage is the formation of Atg5–Atg12–Atg16L1 protein complex which facilitates LC3-I conjugation to PE and determines the sites of LC3 lipidation. Thus, LC3-II is specifically targeted to the autophagosome membrane and remains associated with autophagosome even after fusion with the lysosomes (Geng and Klionsky, 2008;Ravikumar et al., 2010). This peculiarity makes it a useful marker for autophagy research (Klionsky et al., 2012).

Autophagy in Cancer

Autophagy is thought to be predominantly a cell-survival mechanism. Under basal conditions, autophagy helps to maintain cellular homeostasis through the elimination of damaged organelles and protein aggregates, whereas in stress autophagy provides nutrients from macromolecules, produces energy, removes potentially dangerous elements thus assures cytoprotective response to support cell survival (Mizushima et al., 2008;Ravikumar et al., 2010). Deregulation of autophagy is known to affect many physiological processes and has been implicated in a number of diseases, such as neurodegenerative disorders and cancer (Choi et al., 2013). In cancer, current evidence indicate, that autophagy may act as a tumor suppressor, in particular at the early stages of tumor initiation (White, 2012). Indeed, a number of autophagy related proteins, including Beclin1 (Liang et al., 1999;Yue et al., 2003), ATG5 (Yousefi et al., 2006;Takamura et al., 2011), ATG4c (Marino et al., 2007), and ATG7 (Takamura et al., 2011) as well as some accessory proteins (Bif1, UVRAG) (Liang et al., 2007a;Takahashi et al., 2007) have been shown to be tumor suppressors. In addition,

several oncosuppressor proteins, such as DAPK (Inbal et al., 2002), PTEN (Arico et al., 2001), TSC1/2, p53 (Feng et al., 2005) and LKB1 (Liang et al., 2007b) have been recently shown to stimulate autophagic process, while a number of oncoproteins, like Bcl-2 (Pattingre et al., 2005), AKT and class I PI3K (Arico et al., 2001) negatively regulate autophagy (Rubinsztein et al., 2012). One of the possible mechanisms for antitumorigenic functions of autophagy is based on its cytoprotective role. More specifically, inhibition of autophagy will favor DNA damage, genomic instability and thus tumorigenesis through the accumulation of protein aggregates, damaged mitochondria and reactive oxygen species (ROS) (White, 2012).

In contrast, in established tumors, autophagy being a primarily survival mechanism can support cancer cell survival in harsh conditions, characterized by nutrient deficiency and hypoxia (Degenhardt et al., 2006;White, 2012). Indeed, accumulated data suggest that autophagy inhibition by genetic or chemical means facilitates apoptotic cell death and inhibits tumor cells growth in several cancers (Amaravadi et al., 2007;Takamura et al., 2011;Yang et al., 2011a;Yang et al., 2011b). In addition, elevated autophagy is often detected in cancer cells in response to radiation and chemotherapy (Ito et al., 2005;Kondo et al., 2005;White, 2012). Furthermore, autophagy seems to contribute to the therapeutic resistance of some cancers, as inhibition of autophagy has been shown to sensitize tumor cells to chemotherapy treatments (Guo et al., 2012;Selvakumaran et al., 2013). Therefore, autophagy inhibition as an adjuvant to chemotherapy represents a promising strategy in the treatment of some cancers (Amaravadi et al., 2011;White, 2012). Indeed, more than 20 clinical trials are currently evaluating the efficacy of chloroquine and hydroxychloroquine (autophagy inhibitors) in treatment of different cancers either in monotherapy or in combination with other anticancer agents. The preliminary results of many of these trials show apparent antitumour activity(Yang et al., 2011b;Kimura et al., 2013). In addition to chloroquine and hydroxychloroquine another autophagy inhibitors, including 3-Methyladenine, bafilomycin A1 and pepstatin A have been shown to enhance the antitumour efficacy of chemotherapeutic drugs both *in vitro* and *in vivo* (Hsu et al., 2009;Li et al., 2010;Cheong et al., 2012;Lamoureux and Zoubeidi, 2013). However, it should be noted that all these autophagy inhibitors are not specific and can modulate other cellular processes, such as endocytosis, lysosomal function etc. Hence unexpected side effects could occur when treating patients with these drugs. Therefore, more specific and potent autophagy inhibitors are clearly needed.

Thus, figuring out whether to stimulate or inhibit autophagy in each particular case will provide a powerful approach to treat cancer.

Calcium, Ca^{2+}-permeable ion channels and cancer

Changes in the cytosolic free Ca^{2+} concentration, play a central role in many fundamental cellular processes including muscle contraction, transmitter release, cell proliferation, differentiation, gene transcription and cell death (Berridge et al., 2000). Giving that Ca^{2+} controls so many vital processes, disturbance of the Ca^{2+} homeostasis regulatory mechanisms leads to a vast variety of severe pathologies, including cancer. Indeed, the role of Ca^{2+} is well established in many cell signaling pathways involved in carcinogenesis (Monteith et al., 2007;Prevarskaya et al., 2011;Monteith et al., 2012).

Increase in cytosolic calcium can occur as a result of Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular sources. Both Ca^{2+} influx and Ca^{2+} release are tightly controlled by numerous regulatory systems that provide the specific spatial and temporal characteristics of an intracellular calcium signal that are required for sustaining certain cellular functions (Berridge et al., 2000).

Mitochondrial, ER, lysosomal and cytosolic calcium levels are regulated by calcium permeable ion channels localized either on the membranes of the intracellular organelles or on the plasma membrane (Berridge et al., 2003;Rizzuto et al., 2012). The calcium permeable channels, including families of transient receptor potential (TRP) channels, store-operated channels (SOCs), voltage-gated calcium channels, two-pore channels, mitochondrial permeability transition pore (MPTP), mitochondrial calcium uniporter

(MCU), IP3 and ryanodine receptors and others contribute to changes in $[Ca^{2+}]i$ by providing Ca^{2+} entry pathways, by modulating the driving force for the Ca^{2+} entry, and also by providing intracellular pathways for Ca^{2+} uptake/release into/from cellular organelles (Berridge et al., 2003;Pedersen et al., 2005;Bernardi and von Stockum, 2012;Rizzuto et al., 2012).

Thus, modulation of calcium permeable ion channel's expression/function affects intracellular Ca^{2+} concentrations and consequently calcium dependent processes, such as proliferation, apoptosis and autophagy (Flourakis and Prevarskaya, 2009;Decuypere et al., 2011a;Dubois et al., 2013). Indeed, defects in Ca^{2+} channels expression/function are involved in a number of pathologies, including tumorigenesis, since increased expression of Ca^{2+} channels could lead to elevated cytosolic Ca^{2+} levels and promotion of Ca^{2+}-dependent proliferative pathways (Nilius, 2007;Prevarskaya et al., 2010). As an example, several members of the TRP family of ion channels, namely TRPC1, TRPC3, TRPC6, TRPV1, TRPV6, TRPM1, TRPM4, TRPM5, TRPM7 and TRPM8, show altered expression in cancer cells (Shapovalov et al., 2011). The involvement of SOCs, MPTP, MCU, IP3 receptors and ryanodine receptors in the regulation of cell death has also been described (Hajnoczky et al., 2000;Boehning et al., 2004;Flourakis et al., 2010;Wong et al., 2012b;Bernardi, 2013;Curry et al., 2013;Dubois et al., 2013;Qiu et al., 2013).

Role of Ca^{2+} in Autophagy

Recent findings identified intracellular calcium as a key regulator of both basal (Cardenas et al., 2010) and induced (Hoyer-Hansen et al., 2007) autophagy. The complex role for Ca^{2+} in autophagy regulation has become obvious since 1993, when the first report linking autophagy and intracellularly sequestered calcium was published (Gordon et al., 1993). Indeed, Gordon et al. demonstrated that decrease as well as increase in cytosolic Ca^{2+} levels inhibited autophagy in rat hepatocytes (Gordon et al., 1993). And till now, the data on the mechanisms by which calcium controls autophagy remain rather controversial. Several groups reported inhibitory actions of calcium on autophagy, while another proposed mechanisms for calcium to activate autophagy (Decuypere et al., 2011a;Cardenas and Foskett, 2012;Parys et al., 2012). Indeed, Hoyer-Hansen and colleagues provided evidence that a rise in the free cytosolic calcium is a potent inducer of macroautophagy (Hoyer-Hansen et al., 2007). They demonstrated, that Ca^{2+} mobilizing agents, namely vitamin D3, thapsigargin, ATP and ionomycin, stimulate autophagy via a signaling pathway involving Ca^{2+} -activated kinase CAMKK-beta, which directly activates AMPK to inhibit mTOR (Hoyer-Hansen et al., 2007). Recently, this pathway was shown to be required for amyloid-beta peptide induced autophagosome formation (Son et al., 2012). Ca^{2+}/CAMKK-beta/AMPK pathway, although mTOR-independent, has been found to be involved in the leucine-rich repeat kinase-2 (LRRK2) induced autophagy (Gomez-Suaga et al., 2012). Authors proposed the mechanism in which LRRK2 activates NAADP receptors, in particular TPC2, leading to Ca^{2+} mobilization from acidic stores, that in turn stimulates Ca^{2+}-induced Ca^{2+} release from ER and subsequent CAMKK-beta/AMPK pathway activation (Gomez-Suaga et al., 2012). In all the cases discussed above, buffering of cytosolic Ca^{2+} with BAPTA-AM effectively inhibited autophagosomes accumulation, confirming the role of Ca^{2+}. Another evidence supporting stimulatory effect of Ca^{2+} on autophagy is that exogenously introduced calcium in the form of calcium phosphate precipitates induces macroautophagy, which is Beclin1, Atg5 and PI3K class III dependent (Gao et al., 2008). This effect could be antagonized by extra- or intracellular calcium chelation.

In line with activatory role of calcium in autophagy, Sakaki et al. showed that Ca^{2+}-dependent activation of protein kinase Ctheta is required for ER-stress induced autophagy but not for starvation induced autophagy (Sakaki et al., 2008).

In another studies calcium/calmodulin dependent death associated protein kinase (DAPK) was shown to positively regulate autophagy in a Beclin1 dependent manner. Thus, DAPK phosphorylates Beclin1, thereby promoting its dissociation from Bcl-X_L and Bcl-2 inhibitory proteins (Zalckvar et al., 2009a;Zalckvar et al., 2009b).

On the contrary, Khan et al suggested that basal autophagic flux may be negatively

regulated by IP3R-dependent Ca^{2+} release from the ER (Khan and Joseph, 2010). The authors proposed a mechanism in which cytosolic Ca^{2+} elevation acts to maintain an elevated mTORC1 activity through AMPK independent pathway (Khan and Joseph, 2010). Furthermore, amino acids were shown to induce an increase in $[Ca^{2+}]i$, supposedly through the influx of extracellular Ca^{2+}, which acts to enhance the binding of Ca^{2+}/CaM to hVps34, resulting in mTOR activation (Gulati et al., 2008). This pathway could also lead to autophagy inhibition.

Thus, calcium is likely to have different regulatory effects on autophagy, depending on spatial and temporal parameters of Ca^{2+} signaling, nutrient and growth factor availability, as well as pathology (cancer, neurodegenerative disorders, inflammation etc.) (Decuypere et al., 2011a).

Calcium permeable channels in the control of autophagy

Modulation of calcium permeable channels expression/function affects intracellular Ca^{2+} concentrations and, consequently calcium dependent processes, such as proliferation, apoptosis and autophagy. The role of calcium-permeable channels for proliferation and apoptosis is largely recognized (Monteith et al., 2007;Flourakis and Prevarskaya, 2009;Prevarskaya et al., 2010;Dubois et al., 2013), whereas the information about molecular nature of channels regulating autophagy and the mechanisms of this regulation is still limited.

Hereafter, we will provide an overview of the literature on this subject and discuss the possible involvement of calcium permeable ion channels in the regulation of autophagy.

Most reports considering calcium permeable channels as autophagy regulators focused on the inositol trisphosphate receptor (InsP3R), the main intracellular Ca^{2+} release channel (Parys et al., 2012). Together these reports suggested a complex role for IP3R, since both stimulatory as well as inhibitory functions for IP3R towards autophagy have been described.

Thus, in one paper it was suggested that cadmium (Cd^{2+}) induces autophagy through elevation of cytosolic calcium via IP3R and subsequent extracellular signal-regulated kinase (ERK) activation (Wang et al., 2008). As a proof for this, the authors showed that 2-aminoethoxydiphenil borate (2-APB), a blocker of IP3R, suppressed, while knockdown of calcineurin, a putative IP3R inhibitor, increased Cd^{2+}-induced autophagy (Wang et al., 2008). However, 2-APB was shown to modulate a number of TRP channels, SERCA pump and store-operated channels (Peppiatt et al., 2003;Clapham, 2007) and as to calcineurin, its role in the IP3R regulation is debated at present (Bultynck et al., 2003).

In another study, IP3R was shown to be required for differentiation factor DIF-induced autophagic cell death in *Dictyostelium discoideum* (Lam et al., 2008). Through random insertional mutagenesis, the authors showed that inactivation of the *iplA* gene, the only gene encoding an IP3R in this organism, prevented autophagic cell death (Lam et al., 2008).

The stimulatory role of IP3R on starvation-induced autophagy has been recently shown (Decuypere et al., 2011b). The authors showed that the Ca^{2+} chelator BAPTA-AM as well as the IP3R inhibitor xestospongin B abolished starvation induced increase in LC3 lipidation and GFP-LC3-puncta formation. Moreover, starvation lead to IP3R sensitization through increased Beclin1 binding to the IP3R (Decuypere et al., 2011b).

To date, most reports on IP3R-dependent regulation of autophagy suggest inhibitory role for IP3R towards autophagy (Parys et al., 2012).

Thus, lithium (Li^{+}) was found to induce mTOR independent autophagy through inhibition of inositol monophosphatase and further decrease in IP3 levels (Sarkar et al., 2005). Consistently, another study demonstrated that IP3R inhibitor xestospongin (XeB) or IP3R knockdown induced autophagy in HeLa cells (Criollo et al., 2007). One of the mechanisms, by which XeB and starvation induce autophagy was proposed by Vicencio et al. (Vicencio et al., 2009). The authors suggested that xestospongin B and nutrient starvation disrupt a molecular complex formed by the IP3R, Beclin 1 and Bcl-2, and presented evidence that the IP3R represses autophagy through Bcl-2-mediated binding of Beclin 1, thus suggesting Ca^{2+} independent mechanism (Vicencio et al., 2009).

As three IP3R isoforms exist, some groups studied the impact of IP3R on autophagy in the triple IP3R-deficient DT40 cells (Cardenas et

al., 2010;Khan and Joseph, 2010). These cells demonstrate higher basal autophagy levels, compared to wild-type. Interestingly, expression of IP3R3, but not of ryanodine receptor type 2, rescued elevated autophagy in these cells (Cardenas et al., 2010). In contrast, expression of Ca^{2+} impermeable mutant D2550A-IP3R3 failed to suppress constitutive autophagy, suggesting a necessity of the Ca^{2+}-release activity for IP3R. The authors proposed the mechanism in which constitutive IP3R mediated Ca^{2+} release and uptake of this Ca^{2+} by mitochondria is fundamentally required to maintain mitochondrial bioenergetics and ATP production in resting cells thereby suppressing autophagy. Absence of this Ca^{2+} transfer results in inhibition of pyruvate dehydrogenase and activation of AMPK, which activates prosurvival macroautophagy in mTOR independent manner (Cardenas et al., 2010).

Along with IP3R some other calcium permeable channels were shown to be involved in autophagy regulation. Among them, TRPML1, also known as mucolipin-1, ubiquitously expressed TRP channel primarily localized to the late endosomal and lysosomal compartments (Zeevi et al., 2009;Cheng et al., 2010). Direct patch-clamp of enlarged lysosomes revealed that TRPML1 is a Ca^{2+} permeable channel (Dong et al., 2008). The main physiological function of TRPML1 channel is considered to serve as a late endosomal/lysosomal Ca^{2+} release channel. Loss-of-function mutations in the human TRPML1 gene results in mucolipidosis type IV, a neurodegenerative lysosomal storage disorder characterized by mental retardation and retinal degeneration (Bach, 2001;Altarescu et al., 2002). Fibroblasts from mucolipidosis type IV patients exhibit enlarged vacuoles with accumulated lipids and acid mucopolysacharides, suggesting the role for TRPML1 in trafficking of proteins and lipids(Riedel et al., 1985;Goldin et al., 1999;Slaugenhaupt et al., 1999). Loss of TRPML1 has been shown to be accompanied by impairment in the lysosomal pH, accumulation of autophagosomes and abnormal mitochondria, accumulation and aggregation of p62 and ubiquitin proteins, all of which suggested a defective autophagy (Jennings et al., 2006;Soyombo et al., 2006;Vergarajauregui et al., 2008;Curcio-Morelli et al., 2010). Indeed, several studies have proposed TRPML1 as autophagy regulator (Vergarajauregui et al., 2008;Venugopal et al., 2009;Curcio-Morelli et al., 2010;Wong et al., 2012a;Venkatachalam et al., 2013). Vergarajauregui et al. showed that accumulation of autophagosomes in TRPML1-deficient fibroblasts obtained from mucolipidosis type IV patients was due to increased Beclin-1 dependent autophagosome formation and delayed fusion of autophagosomes with late endosomes/lysosomes. The authors claimed that TRPML1 is necessary for efficient fusion of both autophagosomes and late endosomes with lysosomes although it is not clear if the Ca^{2+}-channel function of the TRPML1 is essential here (Vergarajauregui et al., 2008). In another study, group of S. Slaugenhaupt showed that chaperone-mediated autophagy (CMA) is impaired in mucolipidosis type IV fibroblasts (Venugopal et al., 2009). The authors showed that TRPML1 directly interacts with Hsc70 and Hsp40, members of molecular chaperone complex required for CMA, and hypothesized that this interaction may be required for intralysosomal Hsc70 to facilitate the translocation of CMA substrate proteins across the lysosomal membrane. The authors also speculated that TRPML1 channel activity is required for CMA (Venugopal et al., 2009). In 2010 same group investigated macroautophagy in neurons isolated from cerebellum of $TRPML1^{-/-}$ mouse embryos (Curcio-Morelli et al., 2010). These cells displayed higher levels of basal autophagy markers compared to wild-type ones. In addition, LC3-II clearance was affected in these cells, suggesting impairment of lysosomal function. However, the link between observed defects in autophagy and functionality of TRPML1 as a Ca^{2+} permeable channel is missing. Recently, Wong et al showed that Drosophila TRPML is required for TORC1 activation (Wong et al., 2012a). Authors demonstrated defects in amphisomes/lysosomes fusion and elevated late endosomal/lysosomal Ca^{2+} levels in flies lacking TRPML1. Authors also showed decreased TORC1 activity and increased induction of autophagy in $TRPML1^{-}$ mutants. Moreover, authors suggested that TORC1 regulates the subcellular localization of TRPML1. Thus, this study points out to

TRPML1 as a Ca^{2+} channel present in amphisomes which releases luminal Ca^{2+} to facilitate Ca^{2+}-dependent fusion of amphisomes with lysosomes (Wong et al., 2012a).

In addition to TRPML1, another member of mucolipin family, TRPML3 has been shown to be involved in autophagy regulation. In contrast to TRPML1, TRPML3 exhibits more restrictive tissue distribution, and is primarily localized to early as well as late endosomes/lysosomes and less to the plasma membrane (Zeevi et al., 2009;Cheng et al., 2010).

It has been shown that overexpression of TRPML3 leads to increased autophagy in HeLa cells (Kim et al., 2009). Moreover, TRPML3 is recruited to autophagosomes upon induction of autophagy. Additionally, expression of dominant negative mutant TRPML3 (D458K) or knockdown of endogenous TRPML3 by siRNA reduces autophagy. Thus, it has been proposed that TRPML3 provides Ca^{2+} that is required for fusion and fission events in autophagy (Kim et al., 2009). Further, heteromultimerization of TRPML channels was shown to affect autophagy (Zeevi et al., 2010).

Also TRPV1 was proposed to regulate autophagy in thymocytes (Farfariello et al., 2012). The authors showed, that capsaicin, an activator of TRPV1, induce Beclin-1 dependent accumulation of LC3-II protein. This effect can be antagonized by capsazepine, a blocker of TRPV1 and compound C, an AMPK inhibitor, suggesting AMPK involvement. The authors proposed that capsaicin induced autophagy is calcium dependent, as cotreatment with EDTA markedly reduced LC3-II accumulation. Moreover, it was shown that capsaicin induces accumulation of ATG4C and triggers its oxidation in a ROS-dependent manner, thus regulating LC3 lipidation levels (Farfariello et al., 2012). However, capsaicin was shown to have TRPV1-independent effects, such as inhibitition of voltage-gated calcium channels (Hagenacker et al., 2005), cancer cell growth inhibition and apoptosis induction (Mori et al., 2006;Chow et al., 2007). Additionally, upon prolonged exposure to capsaicin, TRPV1 desensitization occurs and its activity decreases (Caterina et al., 1997). Thus, additional experiments using more specific agonists and antagonists as well as siRNA knockdown are needed to confirm the role of TRPV1 in autophagy regulation. It would be interesting as well to compare the effect of capsaicin on autophagy in TRPV1-expressing and TRPV1-null cells.

Some ion channels, which do not belong to the family of TRP channels, were also proposed to regulate autophagy. Williams et al. found that L-type calcium channels antagonists, namely verapamil, loperamide, nimodipine, nitrendipine and amiodarone induce mTOR-independent autophagy (Williams et al., 2008). Conversely, the L-type Ca^{2+} channel agonist (±)-BAY K 8644, that increases cytosolic Ca^{2+} levels, inhibits autophagy. Authors demonstrated that elevated cytosolic Ca^{2+}, presumably due to activity of L-type calcium channels on the plasma membrane, can activate calpains, a family of Ca^{2+}-dependent cysteine proteases, which cleave and activate the a-subunit of heterotrimeric G proteins $G_s\alpha$. $G_s\alpha$ activation, in turn, increases adenylyl cyclase activity leading to increase in cAMP levels. Next, elevated intracellular cAMP levels negatively regulate autophagy by promoting IP3 production via cAMP-Epac-Rap2B-PLC-ε pathway. Finally, IP3, via IP3R influence cytosolic Ca^{2+} levels, which can again activate calpains, thus creating a potential positive feedback loop for autophagy inhibition (Williams et al., 2008). Again, it is important to mention, that although several different L-type calcium channel inhibitors as well as agonist were used in the study, the data showing the effect of siRNA mediated knockdown of L-type calcium channels and/or channel-dead mutants on autophagy are missing. It would be interesting as well to check the effect of these inhibitors on "negative control" cells lacking L-type calcium channels. In addition, verapamil is known to passively diffuse into the lysosome, where it becomes protonated and could cause an increase in lysosomal pH (Lemieux et al., 2004). This could lead to the inhibition of lysosome function and thus block fusion with the autophagosome.

Two-pore channels (TPC) has been also proposed to regulate autophagy (Pereira et al., 2011;Gomez-Suaga et al., 2012). Mammalian TPC family comprise two members TPC1 and TPC2, widely expressed in humans and localized intracellularly on endolysosomes,

with TPC2 being specifically targeted to lysosomes. Several groups proposed TPC as a mediator of endolysosomal calcium release in response to the elevation of the second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) (Calcraft et al., 2009;Galione et al., 2009). Recently Pereira et al. demonstrated that NAADP stimulates autophagy via TPCs in rat astrocytes (Pereira et al., 2011). The authors showed that NAADP mediated increase in the number of LC3-GFP puncta was reduced in cells, transfected with dominant negative TPC2 L265P construct, suggesting the importance of TPC2 for autophagy (Pereira et al., 2011). TPC2 channel has been also proposed to be involved in leucine-rich repeat kinase-2 (LRRK2) induced autophagy (Gomez-Suaga et al., 2012).

The mitochondrial calcium uniporter (MCU) that was recently identified as a channel responsible for mitochondrial Ca^{2+} uptake (Baughman et al., 2011;De Stefani et al., 2011) has been demonstrated to have the role in autophagy regulation. Indeed, Cardenas et al. showed that the uniporter inhibitor Ru360 inhibited cell O_2 consumption rate, activated AMPK, and induced autophagy (Cardenas et al., 2010). In line with these data, MCUR1(mitochondrial calcium uniporter regulator 1) was shown to regulate autophagy (Mallilankaraman et al., 2012). MCUR1 represents an integral membrane protein that is required for MCU-dependent mitochondrial Ca^{2+} uptake. Knockdown of MCUR1 in HeLa and HEK293T cells reduced cell O_2 consumption rate, activated AMPK, and induced macroautophagy (Mallilankaraman et al., 2012). Importantly, stable knockdown of MCU in HeLa cells elicited essentially the same effects, confirming the regulatory role for MCU towards autophagy (Mallilankaraman et al., 2012).

Mitochondrial permeability transition pore (MPTP) has been also suggested to be implicated in autophagy regulation. Elmore et al. proposed that mitochondrial permeability transition (MPT) initiates autophagy in rat hepatocytes. Although the mechanism by which the MPT signals autophagic sequestration was not investigated in this work, the authors hypothesized that factors released from the mitochondrial intermembrane space as a consequence of MPT could stimulate autophagy (Elmore et al., 2001). It is not clear if the Ca^{2+}-release channel function of the MPTP is essential here as well. A functional MPTP was also shown to be required for starvation-induced mitochondrial autophagy (Carreira et al., 2010). The authors demonstrated that starvation induced mitochondrial depolarization in cardiac cells. This depolarization was prevented by cyclosporin A (MPT inhibitor). Further, the authors showed that cyclophilin D a component of the MPTP, is required for mitochondrial removal by starvation-induced autophagy. Interestingly, cardiomyocytes from cyclophilin D deficient mice failed to upregulate autophagy in response to nutrient deprivation, suggesting that MPTP is essential here (Carreira et al., 2010). Again the role of calcium and the importance of calcium permeability for MPTP in the regulation of autophagy were not assessed in this study.

Potential new candidates for autophagy regulation in cancer treatment

Aside from ion channels, described above, all the other calcium permeable channels could potentially be involved in autophagy regulation, as they contribute to the changes in cytosolic calcium levels. Here, we will provide several hypotheses for autophagy regulation by some of the calcium permeable channels that have not been shown to be directly involved in autophagy regulation. Further, we will propose the possible link between calcium permeable channels, autophagy and cancer progression and therapeutic response. Considering both physiological roles as well as cellular localization we selected several calcium permeable channels, which in our opinion could have an impact on autophagy.

TRPML2 channel, a member of the mucolipin family, has been shown to localize to late and recycling endosomes as well as lysosomes (Zeevi et al., 2009;Cheng et al., 2010). Recent study claimed that TRPML2 does not appear to play a role in starvation-induced autophagy (Zeevi et al., 2010). However, TRPML2 knockdown was demonstrated to induce lysosomal inclusions accumulation in HEK cells (Zeevi et al., 2009). This fact along with the endolysosomal distribution of TRPML2 indicates the potential role of TRPML2 in the regulation of basal as well as other types of autophagy.

TRPM2 is known as a chanzyme, combining two functions: as an ion channel and an enzyme, since the C-terminal of TRPM2 contains enzymatically active adenosine diphosphoribose (ADPR) hydrolase domain (Sumoza-Toledo and Penner, 2011). TRPM2 has been shown to be activated and regulated by variety of stimuli including ADPR, H_2O_2, NAADP, pH and cytosolic calcium. It is involved in numerous physiological processes, such as production of cytokines, insulin secretion, oxidative stress, apoptosis (Jiang et al., 2010). TRPM2 functions as a Ca^{2+} permeable channel on the cell surface, but recently TRPM2 has been shown to be also localized intracellularly on the late endosomal and lysosomal membranes where it functions as a lysosomal Ca^{2+} release channel (Lange et al., 2009). Thus it can possibly affect autophagy in the same manner as TRPML and TPC channels. Interesting, that both Ca^{2+}-entry and Ca^{2+}-release channel functions of TRPM2 were shown to be important in H_2O_2-induced beta-cell death (Lange et al., 2009). Further, H_2O_2 is a known activator of autophagy (Chen et al., 2008). Thus, potentially TRPM2 could be involved in H_2O_2-induced autophagy.

Another interesting candidate is the cold receptor TRPM8, which is found in sensory neurons, where it constitutes the principal detector of cold (<~28ºC) (Bautista et al., 2007). In addition to its role as plasmalemmal Ca^{2+} channel, TRPM8 could function as intracellular Ca^{2+}-release channel on the ER membrane (Zhang and Barritt, 2004;Thebault et al., 2005). Initially, TRPM8 was cloned from the human prostate as prostate-specific gene, which is upregulated in malignant tissues (Tsavaler et al., 2001). The role of TRPM8 in cancer was extensively studied in recent years, and published data suggest, that TRPM8 could be involved in proliferation, differentiation and apoptosis in cancer cells (Zhang and Barritt, 2004;Thebault et al., 2005). Given the localization of TRPM8 on ER, it would be interesting to study the possible autophagy regulation by TRPM8-mediated Ca^{2+} release from the ER. The potential mechanisms could be the same as for IP3 receptor.

It is worth to note, that TRPV1 channel, discussed above, has also been found to be expressed intracellularly at the endoplasmic reticulum and trans-Golgi network (Turner et al., 2003), so apart from the autophagy modulating mechanism provided above, TRPV1 could also be involved in another autophagy related signaling pathways.

TRPP2, the product of the gene mutated in autosomal dominant polycystic kidney disease (ADPKD), is another possible candidate for autophagy regulation. It is widely expressed, with a highest level in the kidney, and primarily localized in cilia, where it seems to function as a mechanosensor involved in the nodal ciliary movement (Delmas et al., 2004). TRPP2 might function as a plasma membrane calcium-permeable channel (when interacting with TRPP1) or as a calcium release channel located in the endoplasmic reticulum (Hanaoka et al., 2000;Cahalan, 2002;Koulen et al., 2002). Interesting, TRPP2 was shown to function as a calcium-activated intracellular calcium release channel – property reminiscent of IP3 receptors and ryanodine receptors (Koulen et al., 2002). Thus, potentially TRPP2 could regulate autophagy in a way similar to IP3R.

Interesting, although ryanodine receptor (RyR) constitutes the major cellular mediator of calcium-induced calcium release, the data on its role in autophagy is very scarce. As it was mentioned above, overexpression of ryanodine receptor type 2 in triple IP3R-deficient DT40 cells was without effect on constitutive autophagy (Cardenas et al., 2010). Despite this, we believe that more experiments are required to unravel the role of RyR in autophagy regulation.

The next candidate, TRPV2, is a Ca^{2+} permeable nonselective cationic channel, which has been found to be activated by noxious heat (>50ºC), growth factors (i.e. IGF) and stretch (Caterina et al., 1999;Kanzaki et al., 1999;Muraki et al., 2003). It was shown that insulin induced translocation and insertion of TRPV2 into the plasma membrane in a PI3K-dependent manner (Aoyagi et al., 2010). Recent studies revealed the role for TRPV2 in promoting prostate cancer migration and progression to androgen resistance (Monet et al., 2010). Interestingly, Saito et al. demonstrated the function of 2-APB activated and Rutenium Red-inhibited calcium-permeable ion channel in early endosomes (Saito et al., 2007). The authors reported that this channel has similar pharmacology to that of TRPV2. As early endosomes, and fusion of

autophagosomes with functional early endosomes have been shown to be essential for autophagy (Razi et al., 2009), we hypothesize that TRPV2, which apparently forms early endosomal Ca^{2+} release channel, could be involved in autophagy where it may regulate fusion between autophagosomes and early endosomes. Function on the plasma membrane as well as dependence on PI3K suggest possible complex role in autophagy regulation.

ORAI1 (the calcium release-activated calcium channel protein 1) which constitutes a major molecular component of store-operated calcium (SOC) channels (Hewavitharana et al., 2007) also represents an attractive candidate for autophagy regulation. Recently, Abdelmohsen et al. reported that microRNA miR-519 stimulates autophagy through the downregulation of ORAI1 and ATP2C1 proteins, increase in the cytosolic Ca^{2+} levels, activation of Ca^{2+}-activated calmodulin kinase II (CaMKII) as well as glycogen synthase kinase 3β (GSK3 β) and subsequent p21 upregulation (Abdelmohsen et al., 2012). However, the direct link between autophagy stimulation and functionality of ORAI1 as a Ca^{2+} permeable channel is missing. Thus, additional experiments using specific agonists and antagonists as well as siRNA knockdown of ORAI1 are needed to confirm its role in autophagy regulation.

A subset of other channels, found to be localized both on plasma membrane and intracellular vesicles (i.e. recycling endosomes), including TRPC3, TRPV6 and TRPV5, could also potentially modulate autophagy, although at present it's not clear if these channels are functional intracellularly (Dong et al., 2010;Toro et al., 2011). A graphic overview of the calcium-related mechanisms of autophagy regulation is presented in Fig. 1.

Thus, available data strongly suggests that calcium permeable channels represent good candidates for autophagy regulation. Given that both autophagy and calcium-permeable ion channels have a role in cancer, this can be highly valuable in order to achieve specific outcomes in anti-cancer therapy. Ion channels could provide some advantages, when targeting autophagy *in vivo* for cancer treatment. Indeed, most ion channels are localized on cell surface, thus they can be subjected to antibody-based targeting, that can be particularly useful in the case of channel upregulation in cancer. Moreover, anti-channel antibodies could be used as carriers for radionuclides, toxic molecules or nanoparticles, which can themselves affect autophagy and as such influence cell fate.

The growing number of studies pointing on the fact that inflammation increases the incidence of cancer (Mantovani et al., 2008). Autophagy has been linked to both cancer and inflammation, and is often defective in the inflammatory conditions (White et al., 2010). Among them, Crohn's disease and pancreatitis have been associated with an increased risk of colorectal and pancreatic cancers, respectively (Freeman, 2008;Raimondi et al., 2010). Accordingly, these pathological states are characterized by the accumulation of damaged organelles and poly-ubiquitinilated protein aggregates, ROS production and DNA damage, the factors that create a cancer-promoting environment (Gukovsky et al., 2012;Nguyen et al., 2013). Hence, this implies that functional autophagy stimulation, to eliminate dangerous garbage, may constitute an effective approach to cancer prevention. Interestingly, a number of calcium-permeable ion channels, including TRPV1, TRPV4, TRPA1 and TRPM8, were shown to be regulated by inflammatory mediators (Nilius et al., 2007;Kochukov et al., 2009;Zhang et al., 2012). Thus, these channels can represent potential targets to stimulate autophagy in inflammatory conditions in order to avert tumorigenesis initiation. Additionally, lysosomal dysfunctions have been reported in pancreatitis (Gukovsky et al., 2012), therefore it could be interesting to consider lysosomal ion channels as well.

On the other hand, in existing tumors autophagy may favor survival and progression. Thus, the possible anticancer therapy should be focused on autophagy inhibition. In this case, considering calcium-permeable channels as a potential tool to target autophagy could also be useful. More specifically, as we discussed above, a number of calcium-permeable ion channels exhibit altered expression in cancer cells. For instance, TRPM8 is upregulated in androgen-dependent prostate cancer cells (Zhang and Barritt, 2004;Thebault et al., 2005). This could possibly influence intracellular calcium levels and consequently autophagy. Hence, targeting TRPM8 as well as another channels overexpressed in cancers could provide an additional control over

autophagy, particularly during chemotherapy, and as such contribute to cancer treatment.

Conclusions

Calcium permeable ion channels have emerged as important regulators of autophagy and the effect of such regulation most likely depends on Ca^{2+} signals in a spatially restricted subcellular domains. Apparently, such regulation can represent a fundamental mechanism of fine tuning the autophagy. However, the data concerning this subject is very limited, thus further studies are needed to understand the variety of mechanisms, by which calcium channels can influence autophagy.

Accumulated data proves that both calcium-permeable ion channels and autophagy are implicated in cancer initiation and progression as well as chemotherapy resistance. Paradoxically, autophagy has opposite roles in cancer, with both tumorigenesis suppressor action, in particular at the early stages of tumor initiation and cancer promotion effect resulting in tumor cell survival, chemotherapy resistance and cancer progression. Thus, it is important to unravel autophagy regulating pathways to most effectively target autophagy to cure cancer. Identification of the connections between calcium channels and autophagy could define a new strategy in cancer treatment, and identify useful tools and biomarkers for the elaboration of effective anti-cancer therapies. Moreover, as malfunction of autophagy has been linked to a wide range of human pathologies including liver disease, neurodegeneration, Crohn's disease and cancer, uncovering novel mechanisms of autophagy regulation by calcium permeable ion channels could have a broad impact on the "Autophagy" field and contribute to the developing of autophagy as a potential clinical approach to cure diseases.

Acknowledgments

The authors acknowledge financial support from the INSERM, la Ligue Nationale Contre le Cancer, le Ministere de l'Education Nationale, the Region Nord/Pas-de-Calais. A. Kondratskyi was supported by fellowship from FRM (Fondation de Recherche Medicale). M. Yassine was a recipient of a PhD scholarship from Erasmus Mundus. K. Kondratska is an IonTrac Project fellow.

References

Abdelmohsen, K., Srikantan, S., Tominaga, K., Kang, M.J., Yaniv, Y., Martindale, J.L., Yang, X., Park, S.S., Becker, K.G., Subramanian, M., Maudsley, S., Lal, A., and Gorospe, M. (2012). Growth inhibition by miR-519 via multiple p21-inducing pathways. *Mol Cell Biol* 32**,** 2530-2548.doi:10.1128/MCB.00510-12

Altarescu, G., Sun, M., Moore, D.F., Smith, J.A., Wiggs, E.A., Solomon, B.I., Patronas, N.J., Frei, K.P., Gupta, S., Kaneski, C.R., Quarrell, O.W., Slaugenhaupt, S.A., Goldin, E., and Schiffmann, R. (2002). The neurogenetics of mucolipidosis type IV. *Neurology* 59**,** 306-313

Amaravadi, R.K., Lippincott-Schwartz, J., Yin, X.M., Weiss, W.A., Takebe, N., Timmer, W., Dipaola, R.S., Lotze, M.T., and White, E. (2011). Principles and current strategies for targeting autophagy for cancer treatment. *Clin Cancer Res* 17**,** 654-666.doi:10.1158/1078-0432.CCR-10-2634

Amaravadi, R.K., Yu, D., Lum, J.J., Bui, T., Christophorou, M.A., Evan, G.I., Thomas-Tikhonenko, A., and Thompson, C.B. (2007). Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* 117**,** 326-336.doi:10.1172/JCI28833

Aoyagi, K., Ohara-Imaizumi, M., Nishiwaki, C., Nakamichi, Y., and Nagamatsu, S. (2010). Insulin/phosphoinositide 3-kinase pathway accelerates the glucose-induced first-phase insulin secretion through TrpV2 recruitment in pancreatic beta-cells. *Biochem J* 432**,** 375-386.doi:10.1042/BJ20100864

Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P.F., Meijer, A.J., Codogno, P., and Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 276**,** 35243-35246.doi:10.1074/jbc.C100319200

Bach, G. (2001). Mucolipidosis type IV. *Mol.Genet.Metab* 73**,** 197-

203.doi:10.1006/mgme.2001.3195 [doi];S1096-7192(01)93195-6 [pii]

Baughman, J.M., Perocchi, F., Girgis, H.S., Plovanich, M., Belcher-Timme, C.A., Sancak, Y., Bao, X.R., Strittmatter, L., Goldberger, O., Bogorad, R.L., Koteliansky, V., and Mootha, V.K. (2011). Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* 476**,** 341-345.doi:10.1038/nature10234

Bautista, D.M., Siemens, J., Glazer, J.M., Tsuruda, P.R., Basbaum, A.I., Stucky, C.L., Jordt, S.E., and Julius, D. (2007). The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* 448**,** 204-208.doi:10.1038/nature05910

Bernardi, P. (2013). The mitochondrial permeability transition pore: a mystery solved? *Front Physiol* 4**,** 95.doi:10.3389/fphys.2013.00095

Bernardi, P., and Von Stockum, S. (2012). The permeability transition pore as a Ca(2+) release channel: new answers to an old question. *Cell Calcium* 52**,** 22-27.doi:10.1016/j.ceca.2012.03.004

Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4**,** 517-529.doi:10.1038/nrm1155

Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1**,** 11-21.doi:10.1038/35036035

Boehning, D., Patterson, R.L., and Snyder, S.H. (2004). Apoptosis and calcium: new roles for cytochrome c and inositol 1,4,5-trisphosphate. *Cell Cycle* 3**,** 252-254

Bultynck, G., Vermassen, E., Szlufcik, K., De Smet, P., Fissore, R.A., Callewaert, G., Missiaen, L., De Smedt, H., and Parys, J.B. (2003). Calcineurin and intracellular Ca2+-release channels: regulation or association? *Biochem Biophys Res Commun* 311**,** 1181-1193

Cahalan, M.D. (2002). The ins and outs of polycystin-2 as a calcium release channel. *Nat Cell Biol* 4**,** E56-57.doi:10.1038/ncb0302-e56

Calcraft, P.J., Ruas, M., Pan, Z., Cheng, X., Arredouani, A., Hao, X., Tang, J., Rietdorf, K., Teboul, L., Chuang, K.T., Lin, P., Xiao, R., Wang, C., Zhu, Y., Lin, Y., Wyatt, C.N., Parrington, J., Ma, J., Evans, A.M., Galione, A., and Zhu, M.X. (2009). NAADP mobilizes calcium from acidic organelles through two-pore channels. *Nature* 459**,** 596-600.doi:10.1038/nature08030

Cardenas, C., and Foskett, J.K. (2012). Mitochondrial Ca(2+) signals in autophagy. *Cell Calcium* 52**,** 44-51.doi:10.1016/j.ceca.2012.03.001

Cardenas, C., Miller, R.A., Smith, I., Bui, T., Molgo, J., Muller, M., Vais, H., Cheung, K.H., Yang, J., Parker, I., Thompson, C.B., Birnbaum, M.J., Hallows, K.R., and Foskett, J.K. (2010). Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. *Cell* 142**,** 270-283.doi:10.1016/j.cell.2010.06.007

Carreira, R.S., Lee, Y., Ghochani, M., Gustafsson, A.B., and Gottlieb, R.A. (2010). Cyclophilin D is required for mitochondrial removal by autophagy in cardiac cells. *Autophagy* 6**,** 462-472.doi:10.4161/auto.6.4.11553

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., and Julius, D. (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398**,** 436-441.doi:10.1038/18906

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389**,** 816-824.doi:10.1038/39807

Chen, Y., and Klionsky, D.J. (2011). The regulation of autophagy - unanswered questions. *J Cell Sci* 124**,** 161-170.doi:10.1242/jcs.064576

Chen, Y., Mcmillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2008). Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Differ* 15**,** 171-182.doi:10.1038/sj.cdd.4402233

Cheng, X., Shen, D., Samie, M., and Xu, H. (2010). Mucolipins: Intracellular TRPML1-3 channels. *FEBS Lett* 584**,** 2013-2021.doi:10.1016/j.febslet.2009.12.056

Cheong, H., Lu, C., Lindsten, T., and Thompson, C.B. (2012). Therapeutic targets in cancer cell metabolism and autophagy. *Nat Biotechnol* 30**,** 671-678.doi:10.1038/nbt.2285

Choi, A.M., Ryter, S.W., and Levine, B. (2013). Autophagy in human health and disease. *N Engl J Med* 368**,** 651-662.doi:10.1056/NEJMra1205406

Chow, J., Norng, M., Zhang, J., and Chai, J. (2007). TRPV6 mediates capsaicin-induced apoptosis in gastric cancer cells--Mechanisms behind a possible new "hot" cancer treatment. *Biochim Biophys Acta* 1773**,** 565-576.doi:10.1016/j.bbamcr.2007.01.001

Clapham, D.E. (2007). SnapShot: mammalian TRP channels. *Cell* 129, 220.e1-220.e2.doi:10.1016/j.cell.2007.03.034

Criollo, A., Maiuri, M.C., Tasdemir, E., Vitale, I., Fiebig, A.A., Andrews, D., Molgo, J., Diaz, J., Lavandero, S., Harper, F., Pierron, G., Di Stefano, D., Rizzuto, R., Szabadkai, G., and Kroemer, G. (2007). Regulation of autophagy by the inositol trisphosphate receptor. *Cell Death Differ* 14**,** 1029-1039.doi:10.1038/sj.cdd.4402099

Curcio-Morelli, C., Charles, F.A., Micsenyi, M.C., Cao, Y., Venugopal, B., Browning, M.F., Dobrenis, K., Cotman, S.L., Walkley, S.U., and Slaugenhaupt, S.A. (2010). Macroautophagy is defective in mucolipin-1-deficient mouse neurons. *Neurobiol.Dis.* 40**,** 370-377.doi:S0969-9961(10)00206-8 [pii];10.1016/j.nbd.2010.06.010 [doi]

Curry, M.C., Peters, A.A., Kenny, P.A., Roberts-Thomson, S.J., and Monteith, G.R. (2013). Mitochondrial calcium uniporter silencing potentiates caspase-independent cell death in MDA-MB-231 breast cancer cells. *Biochem Biophys Res Commun* 434**,** 695-700.doi:10.1016/j.bbrc.2013.04.015

De Stefani, D., Raffaello, A., Teardo, E., Szabo, I., and Rizzuto, R. (2011). A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature* 476**,** 336-340.doi:10.1038/nature10230

Decuypere, J.P., Bultynck, G., and Parys, J.B. (2011a). A dual role for Ca(2+) in autophagy regulation. *Cell Calcium* 50**,** 242-250.doi:10.1016/j.ceca.2011.04.001

Decuypere, J.P., Welkenhuyzen, K., Luyten, T., Ponsaerts, R., Dewaele, M., Molgo, J., Agostinis, P., Missiaen, L., De Smedt, H., Parys, J.B., and Bultynck, G. (2011b). Ins(1,4,5)P3 receptor-mediated Ca2+ signaling and autophagy induction are interrelated. *Autophagy* 7**,** 1472-1489

Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gelinas, C., Fan, Y., Nelson, D.A., Jin, S., and White, E. (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 10**,** 51-64.doi:10.1016/j.ccr.2006.06.001

Delmas, P., Padilla, F., Osorio, N., Coste, B., Raoux, M., and Crest, M. (2004). Polycystins, calcium signaling, and human diseases. *Biochem Biophys Res Commun* 322**,** 1374-1383.doi:10.1016/j.bbrc.2004.08.044

Dong, X.P., Cheng, X., Mills, E., Delling, M., Wang, F., Kurz, T., and Xu, H. (2008). The type IV mucolipidosis-associated protein TRPML1 is an endolysosomal iron release channel. *Nature* 455**,** 992-996.doi:10.1038/nature07311

Dong, X.P., Wang, X., and Xu, H. (2010). TRP channels of intracellular membranes. *J Neurochem* 113**,** 313-328.doi:10.1111/j.1471-4159.2010.06626.x

Dubois, C., Vanden Abeele, F., and Prevarskaya, N. (2013). Targeting apoptosis by the remodelling of calcium-transporting proteins in cancerogenesis. *FEBS J*.doi:10.1111/febs.12246

Elmore, S.P., Qian, T., Grissom, S.F., and Lemasters, J.J. (2001). The mitochondrial permeability transition

initiates autophagy in rat hepatocytes. *FASEB J* 15**,** 2286-2287.doi:10.1096/fj.01-0206fje

Farfariello, V., Amantini, C., and Santoni, G. (2012). Transient receptor potential vanilloid 1 activation induces autophagy in thymocytes through ROS-regulated AMPK and Atg4C pathways. *J Leukoc Biol* 92**,** 421-431.doi:10.1189/jlb.0312123

Feng, Z., Zhang, H., Levine, A.J., and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A* 102**,** 8204-8209.doi:10.1073/pnas.0502857102

Flourakis, M., Lehen'kyi, V., Beck, B., Raphael, M., Vandenberghe, M., Abeele, F.V., Roudbaraki, M., Lepage, G., Mauroy, B., Romanin, C., Shuba, Y., Skryma, R., and Prevarskaya, N. (2010). Orai1 contributes to the establishment of an apoptosis-resistant phenotype in prostate cancer cells. *Cell Death Dis* 1**,** e75.doi:10.1038/cddis.2010.52

Flourakis, M., and Prevarskaya, N. (2009). Insights into Ca2+ homeostasis of advanced prostate cancer cells. *Biochim Biophys Acta* 1793**,** 1105-1109.doi:10.1016/j.bbamcr.2009.01.009

Freeman, H.J. (2008). Colorectal cancer risk in Crohn's disease. *World J Gastroenterol* 14**,** 1810-1811

Galione, A., Evans, A.M., Ma, J., Parrington, J., Arredouani, A., Cheng, X., and Zhu, M.X. (2009). The acid test: the discovery of two-pore channels (TPCs) as NAADP-gated endolysosomal Ca(2+) release channels. *Pflugers Arch* 458**,** 869-876.doi:10.1007/s00424-009-0682-y

Gao, W., Ding, W.X., Stolz, D.B., and Yin, X.M. (2008). Induction of macroautophagy by exogenously introduced calcium. *Autophagy* 4**,** 754-761

Geng, J., and Klionsky, D.J. (2008). The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO Rep* 9**,** 859-864.doi:10.1038/embor.2008.163

Goldin, E., Cooney, A., Kaneski, C.R., Brady, R.O., and Schiffmann, R. (1999). Mucolipidosis IV consists of one complementation group. *Proc.Natl.Acad.Sci.U.S.A* 96**,** 8562-8566

Gomez-Suaga, P., Luzon-Toro, B., Churamani, D., Zhang, L., Bloor-Young, D., Patel, S., Woodman, P.G., Churchill, G.C., and Hilfiker, S. (2012). Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. *Hum Mol Genet* 21**,** 511-525.doi:10.1093/hmg/ddr481

Gordon, P.B., Holen, I., Fosse, M., Rotnes, J.S., and Seglen, P.O. (1993). Dependence of hepatocytic autophagy on intracellularly sequestered calcium. *J Biol Chem* 268**,** 26107-26112

Gukovsky, I., Pandol, S.J., Mareninova, O.A., Shalbueva, N., Jia, W., and Gukovskaya, A.S. (2012). Impaired autophagy and organellar dysfunction in pancreatitis. *J Gastroenterol Hepatol* 27 Suppl 2**,** 27-32.doi:10.1111/j.1440-1746.2011.07004.x

Gulati, P., Gaspers, L.D., Dann, S.G., Joaquin, M., Nobukuni, T., Natt, F., Kozma, S.C., Thomas, A.P., and Thomas, G. (2008). Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. *Cell Metab* 7**,** 456-465.doi:10.1016/j.cmet.2008.03.002

Guo, X.L., Li, D., Hu, F., Song, J.R., Zhang, S.S., Deng, W.J., Sun, K., Zhao, Q.D., Xie, X.Q., Song, Y.J., Wu, M.C., and Wei, L.X. (2012). Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells. *Cancer Lett* 320**,** 171-179.doi:10.1016/j.canlet.2012.03.002

Hagenacker, T., Splettstoesser, F., Greffrath, W., Treede, R.D., and Busselberg, D. (2005). Capsaicin differentially modulates voltage-activated calcium channel currents in dorsal root ganglion neurones of rats. *Brain Res* 1062**,** 74-85.doi:10.1016/j.brainres.2005.09.033

Hajnoczky, G., Csordas, G., Madesh, M., and Pacher, P. (2000). Control of apoptosis by IP(3) and ryanodine receptor driven

calcium signals. *Cell Calcium* 28**,** 349-363.doi:10.1054/ceca.2000.0169

Hanaoka, K., Qian, F., Boletta, A., Bhunia, A.K., Piontek, K., Tsiokas, L., Sukhatme, V.P., Guggino, W.B., and Germino, G.G. (2000). Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. *Nature* 408**,** 990-994.doi:10.1038/35050128

He, C., and Levine, B. (2010). The Beclin 1 interactome. *Curr Opin Cell Biol* 22**,** 140-149.doi:10.1016/j.ceb.2010.01.001

Hewavitharana, T., Deng, X., Soboloff, J., and Gill, D.L. (2007). Role of STIM and Orai proteins in the store-operated calcium signaling pathway. *Cell Calcium* 42**,** 173-182.doi:10.1016/j.ceca.2007.03.009

Hoyer-Hansen, M., Bastholm, L., Szyniarowski, P., Campanella, M., Szabadkai, G., Farkas, T., Bianchi, K., Fehrenbacher, N., Elling, F., Rizzuto, R., Mathiasen, I.S., and Jaattela, M. (2007). Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. *Mol Cell* 25**,** 193-205.doi:10.1016/j.molcel.2006.12.009

Hsu, K.F., Wu, C.L., Huang, S.C., Wu, C.M., Hsiao, J.R., Yo, Y.T., Chen, Y.H., Shiau, A.L., and Chou, C.Y. (2009). Cathepsin L mediates resveratrol-induced autophagy and apoptotic cell death in cervical cancer cells. *Autophagy* 5**,** 451-460.doi:7666 [pii]

Inbal, B., Bialik, S., Sabanay, I., Shani, G., and Kimchi, A. (2002). DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* 157**,** 455-468.doi:10.1083/jcb.200109094

Ito, H., Daido, S., Kanzawa, T., Kondo, S., and Kondo, Y. (2005). Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. *Int J Oncol* 26**,** 1401-1410

Jennings, J.J., Jr., Zhu, J.H., Rbaibi, Y., Luo, X., Chu, C.T., and Kiselyov, K. (2006). Mitochondrial aberrations in mucolipidosis Type IV. *J.Biol.Chem.* 281**,** 39041-39050.doi:M607982200 [pii];10.1074/jbc.M607982200 [doi]

Jiang, L.H., Yang, W., Zou, J., and Beech, D.J. (2010). TRPM2 channel properties, functions and therapeutic potentials. *Expert Opin Ther Targets* 14**,** 973-988.doi:10.1517/14728222.2010.510135

Kanzaki, M., Zhang, Y.Q., Mashima, H., Li, L., Shibata, H., and Kojima, I. (1999). Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. *Nat Cell Biol* 1**,** 165-170.doi:10.1038/11086

Khan, M.T., and Joseph, S.K. (2010). Role of inositol trisphosphate receptors in autophagy in DT40 cells. *J Biol Chem* 285**,** 16912-16920.doi:10.1074/jbc.M110.114207

Kim, H.J., Soyombo, A.A., Tjon-Kon-Sang, S., So, I., and Muallem, S. (2009). The Ca(2+) channel TRPML3 regulates membrane trafficking and autophagy. *Traffic* 10**,** 1157-1167.doi:10.1111/j.1600-0854.2009.00924.x

Kim, J., Kundu, M., Viollet, B., and Guan, K.L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* 13**,** 132-141.doi:10.1038/ncb2152

Kimura, T., Takabatake, Y., Takahashi, A., and Isaka, Y. (2013). Chloroquine in cancer therapy: a double-edged sword of autophagy. *Cancer Res* 73**,** 3-7.doi:10.1158/0008-5472.CAN-12-2464

Klionsky, D.J. (2005). The molecular machinery of autophagy: unanswered questions. *J Cell Sci* 118**,** 7-18.doi:10.1242/jcs.01620

Klionsky, D.J., Abdalla, F.C., Abeliovich, H., Abraham, R.T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J.A., Ahn, H.J., Ait-Mohamed, O., Ait-Si-Ali, S., Akematsu, T., Akira, S., Al-Younes, H.M., Al-Zeer, M.A., Albert, M.L., Albin, R.L., Alegre-Abarrategui, J., Aleo, M.F., Alirezaei, M., Almasan, A., Almonte-Becerril, M., Amano, A., Amaravadi, R., Amarnath, S., Amer, A.O., Andrieu-Abadie, N., Anantharam, V., Ann, D.K.,

Anoopkumar-Dukie, S., Aoki, H., Apostolova, N., Arancia, G., Aris, J.P., Asanuma, K., Asare, N.Y., Ashida, H., Askanas, V., Askew, D.S., Auberger, P., Baba, M., Backues, S.K., Baehrecke, E.H., Bahr, B.A., Bai, X.Y., Bailly, Y., Baiocchi, R., Baldini, G., Balduini, W., Ballabio, A., Bamber, B.A., Bampton, E.T., Banhegyi, G., Bartholomew, C.R., Bassham, D.C., Bast, R.C., Jr., Batoko, H., Bay, B.H., Beau, I., Bechet, D.M., Begley, T.J., Behl, C., Behrends, C., Bekri, S., Bellaire, B., Bendall, L.J., Benetti, L., Berliocchi, L., Bernardi, H., Bernassola, F., Besteiro, S., Bhatia-Kissova, I., Bi, X., Biard-Piechaczyk, M., Blum, J.S., Boise, L.H., Bonaldo, P., Boone, D.L., Bornhauser, B.C., Bortoluci, K.R., Bossis, I., Bost, F., Bourquin, J.P., Boya, P., Boyer-Guittaut, M., Bozhkov, P.V., Brady, N.R., Brancolini, C., Brech, A., Brenman, J.E., Brennand, A., Bresnick, E.H., Brest, P., Bridges, D., Bristol, M.L., Brookes, P.S., Brown, E.J., Brumell, J.H., et al. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 8**,** 445-544

Kochukov, M.Y., Mcnearney, T.A., Yin, H., Zhang, L., Ma, F., Ponomareva, L., Abshire, S., and Westlund, K.N. (2009). Tumor necrosis factor-alpha (TNF-alpha) enhances functional thermal and chemical responses of TRP cation channels in human synoviocytes. *Mol Pain* 5**,** 49.doi:10.1186/1744-8069-5-49

Kondo, Y., Kanzawa, T., Sawaya, R., and Kondo, S. (2005). The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 5**,** 726-734.doi:10.1038/nrc1692

Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B.E., and Somlo, S. (2002). Polycystin-2 is an intracellular calcium release channel. *Nat Cell Biol* 4**,** 191-197.doi:10.1038/ncb754

Kroemer, G., Marino, G., and Levine, B. (2010). Autophagy and the integrated stress response. *Mol Cell* 40**,** 280-293.doi:10.1016/j.molcel.2010.09.023

Lam, D., Kosta, A., Luciani, M.F., and Golstein, P. (2008). The inositol 1,4,5-trisphosphate receptor is required to signal autophagic cell death. *Mol Biol Cell* 19**,** 691-700.doi:10.1091/mbc.E07-08-0823

Lamoureux, F., and Zoubeidi, A. (2013). Dual inhibition of autophagy and the AKT pathway in prostate cancer. *Autophagy* 9.doi:24921 [pii]

Lange, I., Yamamoto, S., Partida-Sanchez, S., Mori, Y., Fleig, A., and Penner, R. (2009). TRPM2 functions as a lysosomal Ca2+-release channel in beta cells. *Sci Signal* 2**,** ra23.doi:10.1126/scisignal.2000278

Lemieux, B., Percival, M.D., and Falgueyret, J.P. (2004). Quantitation of the lysosomotropic character of cationic amphiphilic drugs using the fluorescent basic amine Red DND-99. *Anal Biochem* 327**,** 247-251.doi:10.1016/j.ab.2004.01.010

Li, J., Hou, N., Faried, A., Tsutsumi, S., and Kuwano, H. (2010). Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. *Eur J Cancer* 46**,** 1900-1909.doi:10.1016/j.ejca.2010.02.021

Liang, C., Feng, P., Ku, B., Oh, B.H., and Jung, J.U. (2007a). UVRAG: a new player in autophagy and tumor cell growth. *Autophagy* 3**,** 69-71

Liang, J., Shao, S.H., Xu, Z.X., Hennessy, B., Ding, Z., Larrea, M., Kondo, S., Dumont, D.J., Gutterman, J.U., Walker, C.L., Slingerland, J.M., and Mills, G.B. (2007b). The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol* 9**,** 218-224.doi:10.1038/ncb1537

Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 402**,** 672-676.doi:10.1038/45257

Mallilankaraman, K., Cardenas, C., Doonan, P.J., Chandramoorthy, H.C., Irrinki, K.M., Golenar, T., Csordas, G., Madireddi, P., Yang, J., Muller, M.,

Miller, R., Kolesar, J.E., Molgo, J., Kaufman, B., Hajnoczky, G., Foskett, J.K., and Madesh, M. (2012). MCUR1 is an essential component of mitochondrial Ca2+ uptake that regulates cellular metabolism. *Nat Cell Biol* 14**,** 1336-1343.doi:10.1038/ncb2622

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. *Nature* 454**,** 436-444.doi:10.1038/nature07205

Marino, G., Salvador-Montoliu, N., Fueyo, A., Knecht, E., Mizushima, N., and Lopez-Otin, C. (2007). Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J Biol Chem* 282**,** 18573-18583.doi:10.1074/jbc.M701194200

Mizushima, N. (2010). The role of the Atg1/ULK1 complex in autophagy regulation. *Curr Opin Cell Biol* 22**,** 132-139.doi:10.1016/j.ceb.2009.12.004

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. *Nature* 451**,** 1069-1075.doi:10.1038/nature06639

Monet, M., Lehen'kyi, V., Gackiere, F., Firlej, V., Vandenberghe, M., Roudbaraki, M., Gkika, D., Pourtier, A., Bidaux, G., Slomianny, C., Delcourt, P., Rassendren, F., Bergerat, J.P., Ceraline, J., Cabon, F., Humez, S., and Prevarskaya, N. (2010). Role of cationic channel TRPV2 in promoting prostate cancer migration and progression to androgen resistance. *Cancer Res* 70**,** 1225-1235.doi:10.1158/0008-5472.CAN-09-2205

Monteith, G.R., Davis, F.M., and Roberts-Thomson, S.J. (2012). Calcium channels and pumps in cancer: changes and consequences. *J Biol Chem* 287**,** 31666-31673.doi:10.1074/jbc.R112.343061

Monteith, G.R., Mcandrew, D., Faddy, H.M., and Roberts-Thomson, S.J. (2007). Calcium and cancer: targeting Ca2+ transport. *Nat Rev Cancer* 7**,** 519-530.doi:10.1038/nrc2171

Mori, A., Lehmann, S., O'kelly, J., Kumagai, T., Desmond, J.C., Pervan, M., Mcbride, W.H., Kizaki, M., and Koeffler, H.P. (2006). Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res* 66**,** 3222-3229.doi:10.1158/0008-5472.CAN-05-0087

Muraki, K., Iwata, Y., Katanosaka, Y., Ito, T., Ohya, S., Shigekawa, M., and Imaizumi, Y. (2003). TRPV2 is a component of osmotically sensitive cation channels in murine aortic myocytes. *Circ Res* 93**,** 829-838.doi:10.1161/01.RES.0000097263.10220.0C

Nguyen, H.T., Lapaquette, P., Bringer, M.A., and Darfeuille-Michaud, A. (2013). Autophagy and Crohn's Disease. *J Innate Immun*.doi:10.1159/000345129

Nilius, B. (2007). TRP channels in disease. *Biochim Biophys Acta* 1772**,** 805-812.doi:10.1016/j.bbadis.2007.02.002

Nilius, B., Owsianik, G., Voets, T., and Peters, J.A. (2007). Transient receptor potential cation channels in disease. *Physiol Rev* 87**,** 165-217.doi:10.1152/physrev.00021.2006

Parys, J.B., Decuypere, J.P., and Bultynck, G. (2012). Role of the inositol 1,4,5-trisphosphate receptor/Ca2+-release channel in autophagy. *Cell Commun Signal* 10**,** 17.doi:10.1186/1478-811X-10-17

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122**,** 927-939.doi:10.1016/j.cell.2005.07.002

Pedersen, S.F., Owsianik, G., and Nilius, B. (2005). TRP channels: an overview. *Cell Calcium* 38**,** 233-252.doi:10.1016/j.ceca.2005.06.028

Peppiatt, C.M., Collins, T.J., Mackenzie, L., Conway, S.J., Holmes, A.B., Bootman, M.D., Berridge, M.J., Seo, J.T., and Roderick, H.L. (2003). 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-

dependent and slowly reversible action on store-operated calcium entry channels. *Cell Calcium* 34**,** 97-108

Pereira, G.J., Hirata, H., Fimia, G.M., Do Carmo, L.G., Bincoletto, C., Han, S.W., Stilhano, R.S., Ureshino, R.P., Bloor-Young, D., Churchill, G., Piacentini, M., Patel, S., and Smaili, S.S. (2011). Nicotinic acid adenine dinucleotide phosphate (NAADP) regulates autophagy in cultured astrocytes. *J Biol Chem* 286**,** 27875-27881.doi:10.1074/jbc.C110.216580

Prevarskaya, N., Skryma, R., and Shuba, Y. (2010). Ion channels and the hallmarks of cancer. *Trends Mol Med* 16**,** 107-121.doi:10.1016/j.molmed.2010.01.005

Prevarskaya, N., Skryma, R., and Shuba, Y. (2011). Calcium in tumour metastasis: new roles for known actors. *Nat Rev Cancer* 11**,** 609-618.doi:10.1038/nrc3105

Qiu, J., Tan, Y.W., Hagenston, A.M., Martel, M.A., Kneisel, N., Skehel, P.A., Wyllie, D.J., Bading, H., and Hardingham, G.E. (2013). Mitochondrial calcium uniporter Mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. *Nat Commun* 4**,** 2034.doi:10.1038/ncomms3034

Raimondi, S., Lowenfels, A.B., Morselli-Labate, A.M., Maisonneuve, P., and Pezzilli, R. (2010). Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. *Best Pract Res Clin Gastroenterol* 24**,** 349-358.doi:10.1016/j.bpg.2010.02.007

Ravikumar, B., Sarkar, S., Davies, J.E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z.W., Jimenez-Sanchez, M., Korolchuk, V.I., Lichtenberg, M., Luo, S., Massey, D.C., Menzies, F.M., Moreau, K., Narayanan, U., Renna, M., Siddiqi, F.H., Underwood, B.R., Winslow, A.R., and Rubinsztein, D.C. (2010). Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev* 90**,** 1383-1435.doi:10.1152/physrev.00030.2009

Razi, M., Chan, E.Y., and Tooze, S.A. (2009). Early endosomes and endosomal coatomer are required for autophagy. *J Cell Biol* 185**,** 305-321.doi:10.1083/jcb.200810098

Riedel, K.G., Zwaan, J., Kenyon, K.R., Kolodny, E.H., Hanninen, L., and Albert, D.M. (1985). Ocular abnormalities in mucolipidosis IV. *Am.J.Ophthalmol.* 99**,** 125-136

Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. *Nat Rev Mol Cell Biol* 13**,** 566-578.doi:10.1038/nrm3412

Rubinsztein, D.C., Codogno, P., and Levine, B. (2012). Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat Rev Drug Discov* 11**,** 709-730.doi:10.1038/nrd3802

Saito, M., Hanson, P.I., and Schlesinger, P. (2007). Luminal chloride-dependent activation of endosome calcium channels: patch clamp study of enlarged endosomes. *J Biol Chem* 282**,** 27327-27333.doi:10.1074/jbc.M702557200

Sakaki, K., Wu, J., and Kaufman, R.J. (2008). Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum. *J Biol Chem* 283**,** 15370-15380.doi:10.1074/jbc.M710209200

Sarkar, S., Floto, R.A., Berger, Z., Imarisio, S., Cordenier, A., Pasco, M., Cook, L.J., and Rubinsztein, D.C. (2005). Lithium induces autophagy by inhibiting inositol monophosphatase. *J Cell Biol* 170**,** 1101-1111.doi:10.1083/jcb.200504035

Selvakumaran, M., Amaravadi, R.K., Vasilevskaya, I.A., and O'dwyer, P.J. (2013). Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. *Clin Cancer Res* 19**,** 2995-3007.doi:10.1158/1078-0432.CCR-12-1542

Shapovalov, G., Lehen'kyi, V., Skryma, R., and Prevarskaya, N. (2011). TRP channels in cell survival and cell death in normal and transformed cells. *Cell Calcium* 50**,** 295-302.doi:10.1016/j.ceca.2011.05.006

Slaugenhaupt, S.A., Acierno, J.S., Jr., Helbling, L.A., Bove, C., Goldin, E., Bach, G., Schiffmann, R., and Gusella, J.F. (1999). Mapping of the mucolipidosis type IV gene to chromosome 19p and definition of founder haplotypes. *Am.J.Hum.Genet.* 65**,** 773-778.doi:S0002-9297(07)62330-1 [pii]

Son, S.M., Jung, E.S., Shin, H.J., Byun, J., and Mook-Jung, I. (2012). Abeta-induced formation of autophagosomes is mediated by RAGE-CaMKKbeta-AMPK signaling. *Neurobiol Aging* 33**,** 1006 e1011-1023.doi:10.1016/j.neurobiolaging.2011.09.039

Soyombo, A.A., Tjon-Kon-Sang, S., Rbaibi, Y., Bashllari, E., Bisceglia, J., Muallem, S., and Kiselyov, K. (2006). TRP-ML1 regulates lysosomal pH and acidic lysosomal lipid hydrolytic activity. *J.Biol.Chem.* 281**,** 7294-7301.doi:M508211200 [pii];10.1074/jbc.M508211200 [doi]

Sumoza-Toledo, A., and Penner, R. (2011). TRPM2: a multifunctional ion channel for calcium signalling. *J Physiol* 589**,** 1515-1525.doi:10.1113/jphysiol.2010.201855

Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H.D., Sun, M., Sato, Y., Liang, C., Jung, J.U., Cheng, J.Q., Mule, J.J., Pledger, W.J., and Wang, H.G. (2007). Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* 9**,** 1142-1151.doi:10.1038/ncb1634

Takamura, A., Komatsu, M., Hara, T., Sakamoto, A., Kishi, C., Waguri, S., Eishi, Y., Hino, O., Tanaka, K., and Mizushima, N. (2011). Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* 25**,** 795-800.doi:10.1101/gad.2016211

Tanida, I., Ueno, T., and Kominami, E. (2004). LC3 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* 36**,** 2503-2518.doi:10.1016/j.biocel.2004.05.009

Thebault, S., Lemonnier, L., Bidaux, G., Flourakis, M., Bavencoffe, A., Gordienko, D., Roudbaraki, M., Delcourt, P., Panchin, Y., Shuba, Y., Skryma, R., and Prevarskaya, N. (2005). Novel role of cold/menthol-sensitive transient receptor potential melastatine family member 8 (TRPM8) in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells. *J Biol Chem* 280**,** 39423-39435.doi:10.1074/jbc.M503544200

Toro, C.A., Arias, L.A., and Brauchi, S. (2011). Sub-cellular distribution and translocation of TRP channels. *Curr Pharm Biotechnol* 12**,** 12-23

Tsavaler, L., Shapero, M.H., Morkowski, S., and Laus, R. (2001). Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. *Cancer Res* 61**,** 3760-3769

Turner, H., Fleig, A., Stokes, A., Kinet, J.P., and Penner, R. (2003). Discrimination of intracellular calcium store subcompartments using TRPV1 (transient receptor potential channel, vanilloid subfamily member 1) release channel activity. *Biochem J* 371**,** 341-350.doi:10.1042/BJ20021381

Venkatachalam, K., Wong, C.O., and Montell, C. (2013). Feast or famine: role of TRPML in preventing cellular amino acid starvation. *Autophagy.* 9**,** 98-100.doi:22260 [pii];10.4161/auto.22260 [doi]

Venugopal, B., Mesires, N.T., Kennedy, J.C., Curcio-Morelli, C., Laplante, J.M., Dice, J.F., and Slaugenhaupt, S.A. (2009). Chaperone-mediated autophagy is defective in mucolipidosis type IV. *J.Cell Physiol* 219**,** 344-353.doi:10.1002/jcp.21676 [doi]

Vergarajauregui, S., Connelly, P.S., Daniels, M.P., and Puertollano, R. (2008). Autophagic dysfunction in mucolipidosis type IV patients. *Hum.Mol.Genet.* 17**,** 2723-2737.doi:ddn174 [pii];10.1093/hmg/ddn174 [doi]

Vicencio, J.M., Ortiz, C., Criollo, A., Jones, A.W., Kepp, O., Galluzzi, L., Joza, N., Vitale, I., Morselli, E., Tailler, M.,

Castedo, M., Maiuri, M.C., Molgo, J., Szabadkai, G., Lavandero, S., and Kroemer, G. (2009). The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1. *Cell Death Differ* 16**,** 1006-1017.doi:10.1038/cdd.2009.34

Wang, S.H., Shih, Y.L., Ko, W.C., Wei, Y.H., and Shih, C.M. (2008). Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway. *Cell Mol Life Sci* 65**,** 3640-3652.doi:10.1007/s00018-008-8383-9

White, E. (2012). Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer* 12**,** 401-410.doi:10.1038/nrc3262

White, E., Karp, C., Strohecker, A.M., Guo, Y., and Mathew, R. (2010). Role of autophagy in suppression of inflammation and cancer. *Curr Opin Cell Biol* 22**,** 212-217.doi:10.1016/j.ceb.2009.12.008

Williams, A., Sarkar, S., Cuddon, P., Ttofi, E.K., Saiki, S., Siddiqi, F.H., Jahreiss, L., Fleming, A., Pask, D., Goldsmith, P., O'kane, C.J., Floto, R.A., and Rubinsztein, D.C. (2008). Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nat Chem Biol* 4**,** 295-305.doi:10.1038/nchembio.79

Wong, C.O., Li, R., Montell, C., and Venkatachalam, K. (2012a). Drosophila TRPML is required for TORC1 activation. *Curr.Biol.* 22**,** 1616-1621.doi:S0960-9822(12)00731-2 [pii];10.1016/j.cub.2012.06.055 [doi]

Wong, R., Steenbergen, C., and Murphy, E. (2012b). Mitochondrial permeability transition pore and calcium handling. *Methods Mol Biol* 810**,** 235-242.doi:10.1007/978-1-61779-382-0_15

Yang, S., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., Bause, A., Li, Y., Stommel, J.M., Dell'antonio, G., Mautner, J., Tonon, G., Haigis, M., Shirihai, O.S., Doglioni, C., Bardeesy, N., and Kimmelman, A.C. (2011a). Pancreatic cancers require autophagy for tumor growth. *Genes Dev* 25**,** 717-729.doi:10.1101/gad.2016111

Yang, Z.J., Chee, C.E., Huang, S., and Sinicrope, F.A. (2011b). The role of autophagy in cancer: therapeutic implications. *Mol Cancer Ther* 10**,** 1533-1541.doi:10.1158/1535-7163.MCT-11-0047

Yousefi, S., Perozzo, R., Schmid, I., Ziemiecki, A., Schaffner, T., Scapozza, L., Brunner, T., and Simon, H.U. (2006). Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol* 8**,** 1124-1132.doi:10.1038/ncb1482

Yue, Z., Jin, S., Yang, C., Levine, A.J., and Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A* 100**,** 15077-15082.doi:10.1073/pnas.2436255100

Zalckvar, E., Berissi, H., Eisenstein, M., and Kimchi, A. (2009a). Phosphorylation of Beclin 1 by DAP-kinase promotes autophagy by weakening its interactions with Bcl-2 and Bcl-XL. *Autophagy* 5**,** 720-722

Zalckvar, E., Berissi, H., Mizrachy, L., Idelchuk, Y., Koren, I., Eisenstein, M., Sabanay, H., Pinkas-Kramarski, R., and Kimchi, A. (2009b). DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. *EMBO Rep* 10**,** 285-292.doi:10.1038/embor.2008.246

Zeevi, D.A., Frumkin, A., Offen-Glasner, V., Kogot-Levin, A., and Bach, G. (2009). A potentially dynamic lysosomal role for the endogenous TRPML proteins. *J Pathol* 219**,** 153-162.doi:10.1002/path.2587

Zeevi, D.A., Lev, S., Frumkin, A., Minke, B., and Bach, G. (2010). Heteromultimeric TRPML channel assemblies play a crucial role in the regulation of cell viability models and starvation-induced autophagy. *J Cell Sci* 123**,** 3112-3124.doi:10.1242/jcs.067330

Zhang, L., and Barritt, G.J. (2004). Evidence that TRPM8 is an androgen-dependent Ca2+ channel required for the survival of prostate cancer cells. *Cancer Res*

64**,** 8365-8373.doi:10.1158/0008-5472.CAN-04-2146

Zhang, X., Mak, S., Li, L., Parra, A., Denlinger, B., Belmonte, C., and Mcnaughton, P.A. (2012). Direct inhibition of the cold-activated TRPM8 ion channel by Galphaq. *Nat Cell Biol* 14**,** 851-858.doi:10.1038/ncb2529

Figure 1 | Calcium and calcium-permeable channels in the control of autophagy.
Inhibitory and stimulatory actions of Ca^{2+} on autophagy as well as calcium-permeable channels that could be potentially involved in autophagy regulation are depicted.
Ca^{2+} mobilizing agents, such as vitamin D3, thapsigargin and ionomycin, lead to increase in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) concentration and subsequent activation of CAMKK-beta, followed by AMPK-dependent mTOR inhibition and autophagy stimulation. ER-stress induced elevation of $[Ca^{2+}]_{cyt}$ levels activate PKCQ which stimulates autophagy. Additionally, increased $[Ca^{2+}]_{cyt}$ induce activation of DAPK, which phosphorylates Beclin1, thereby promoting its dissociation from Bcl-X_L and Bcl-2 inhibitory proteins, and thus stimulate autophagy. Cadmium induces autophagy through elevation of cytosolic Ca^{2+} via IP3R and subsequent ERK activation. In contrast, constitutive IP3R mediated Ca^{2+} release to mitochondria maintains ATP production and AMPK inhibition, thereby suppressing autophagy. The inhibition of IMPase by Li^{+} causes a decrease in IP3 levels and autophagy induction. Further, IP3R-dependent Ca^{2+} release from the ER as well as amino acids-induced increase in $[Ca^{2+}]_{cyt}$ maintain an elevated mTORC1 activity, thus inhibiting autophagy. In addition, IP3R represses autophagy through Bcl-2-mediated binding of Beclin1. Also, increased Ca^{2+} influx through L-type calcium channels on the plasma membrane activates calpains and consequently Gsa/Adenylyl cyclase/cAMP/Epac/Rap2B/PLC-ε pathway which negatively regulate autophagy by promoting IP3 production, IP3R activation and Ca^{2+} release.

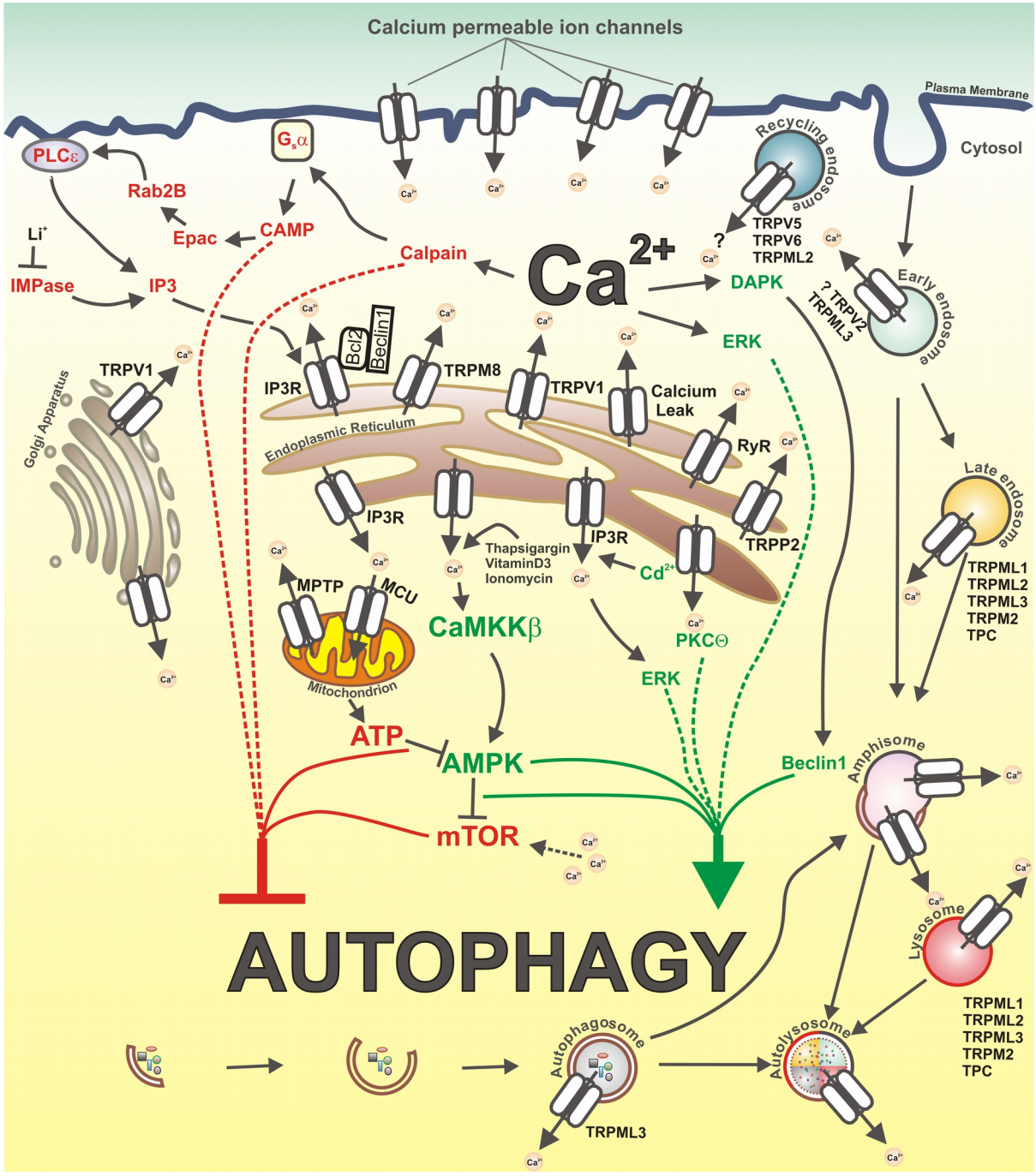


Figure 1

As proposed earlier, the possibility of involvement of calcium permeable channels in autophagy regulation and in order to understand the calcium dependent mechanisms of autophagy regulation in treating prostate cancer, several experiments were done and more experiments are in progress right now to further verify the following results so as to be published.

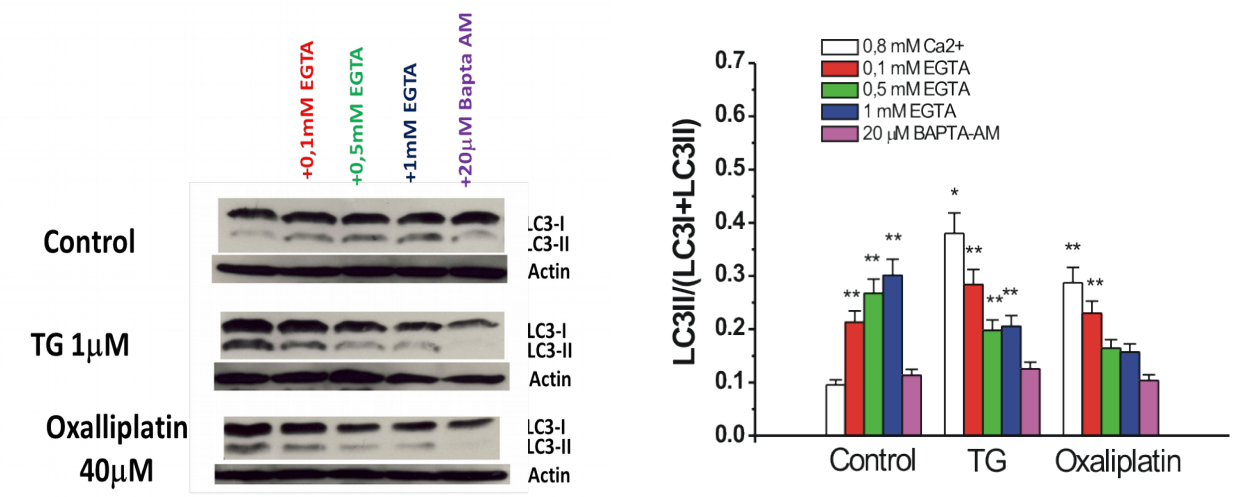


Figure 1: Intracellular calcium regulates autophagy in prostate cancer cells in basal and induced conditions depending on type of autophagy inducer and spatial parameters of calcium signaling. LNCaP cells were left untreated or treated with TG of 1µM and oxaliplatin of 40 µM for 6 hours in RPMI medium. When indicated calcium chelators (EGTA of 0.1, 0.5, 1mM and BAPTA-AM of 20 µM) were added.

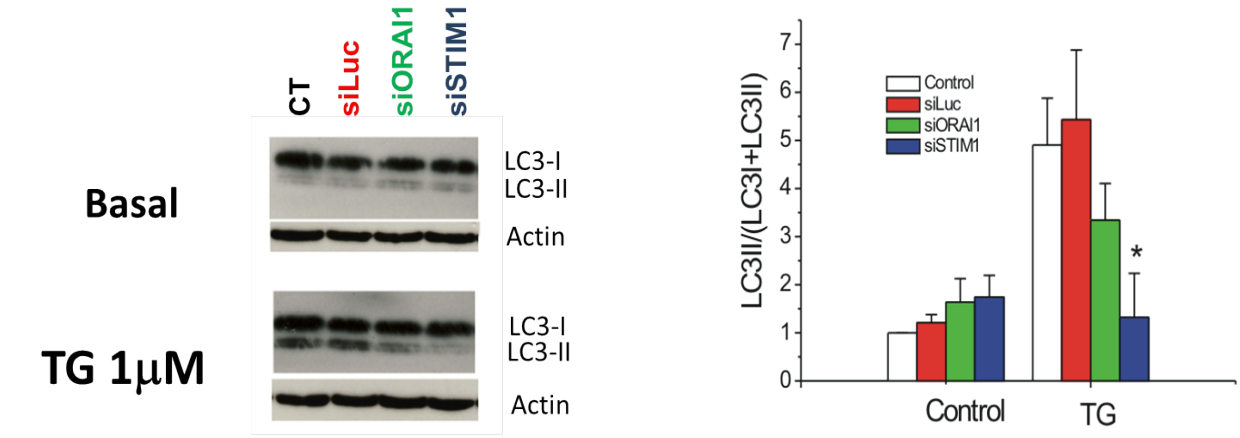


Figure 2: SOC channels in basal and induced autophagy. STIM1 is essential for TG induced autophagy and suppression of Orai1 decreases TG induced LC3 accumulation.
LNCaP cells were left untreated or treated with siLuc, siOrai1 or STIM1 for 48 hours.When indicated TG of 1µM was added for 6 hrs.

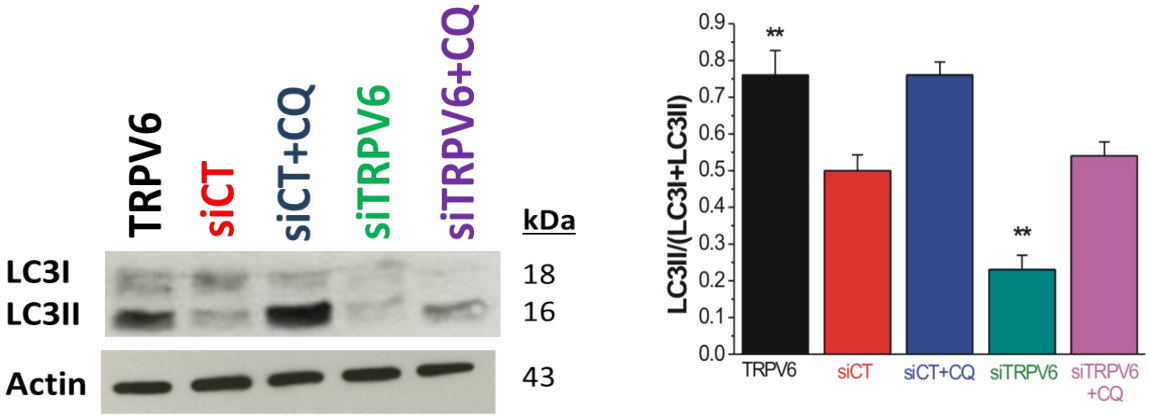


Figure 3: TRPV6, a selective calcium channel, is essential for basal autophagy in LNCaP cells.
LNCaP cells were left untreated or treated with siLuc, siTRPV6 for 48 hours or were transfected with YFP-TRPV6 plasmid. Chloroquine of 20µM was added for 2 hours.

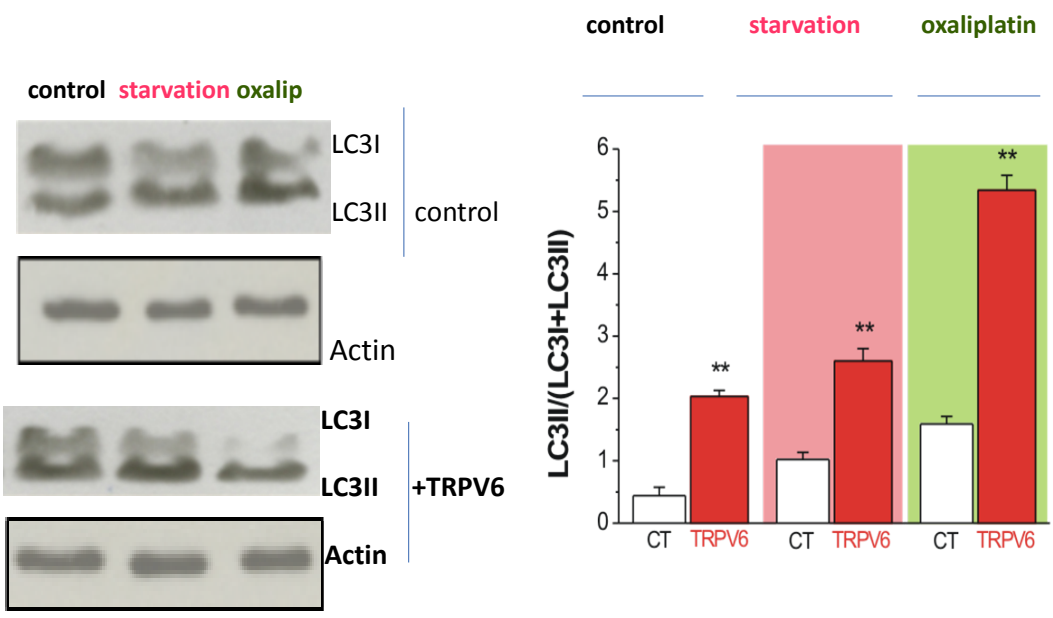


Figure 4: TRPV6 overexpression increases both basal and induced autophagy.
LNCaP cells were left transfected or untransfected with YFP-TRPV6 plasmid. Cells were treated witth serum free RPMI medium or with oxaliplatin 40µM for 6 hours.

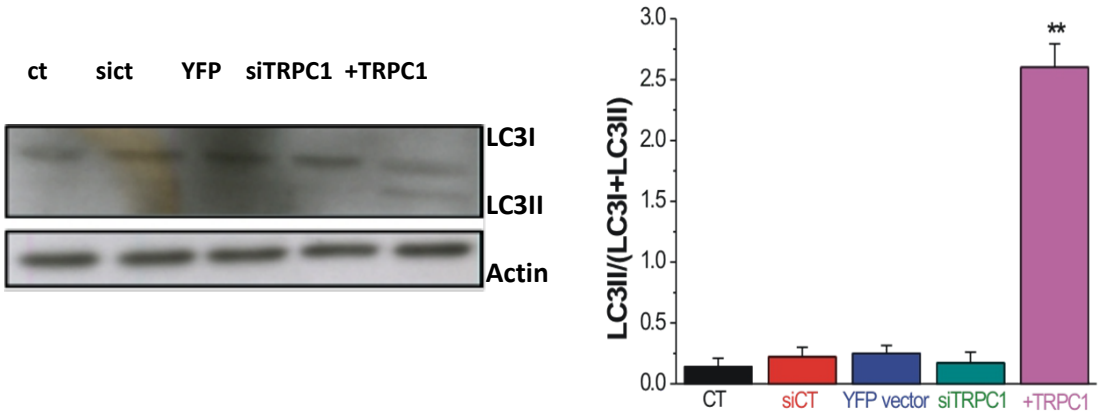


Figure 5: TRPC1 increases basal autophagy. It increases autophagosomes formation.
LNCaP cells were left untreated or treated with siLuc or siTRPC1 for 48 hours or were transfected with YFP-TRPC1 plasmid.

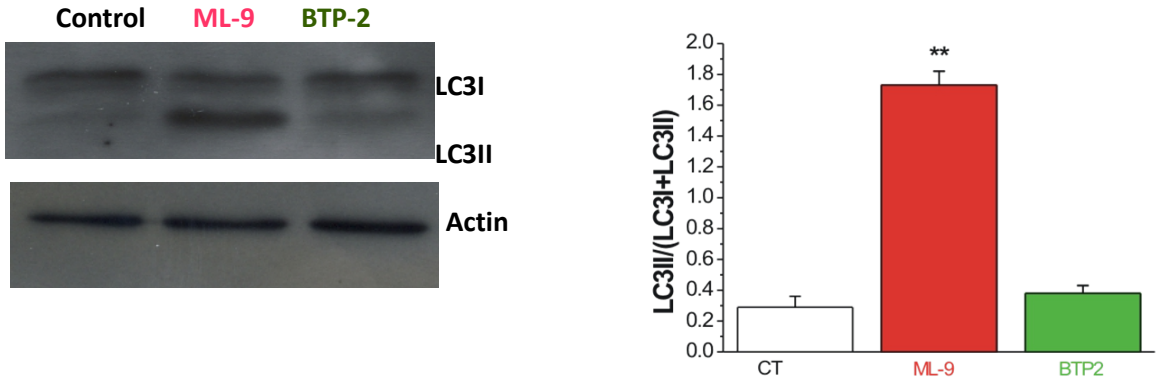
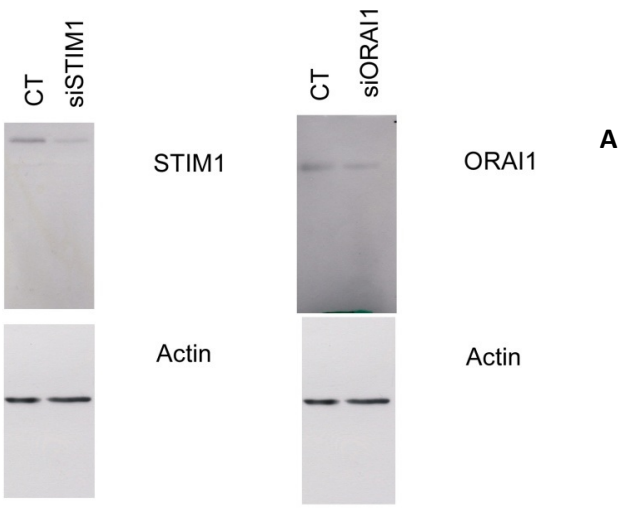
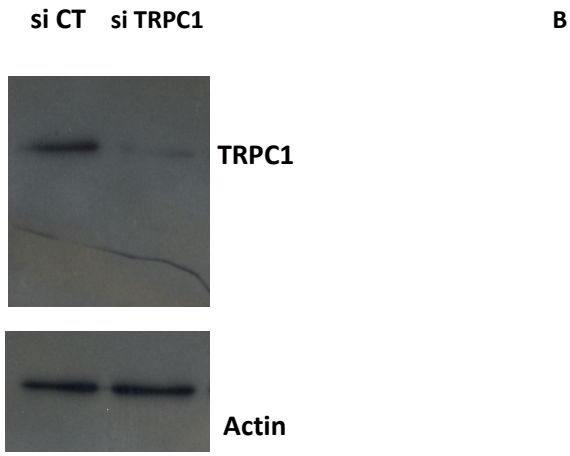


Figure 6: SOC inhibitors have little or no effect on autophagy except for ML-9. It induces autophagosomes formation.
LNCaP cells were left untreated or treated with ML-9 of 30 µM or BTP-2 of 1µM for 6 hours.

Supplementary Figures





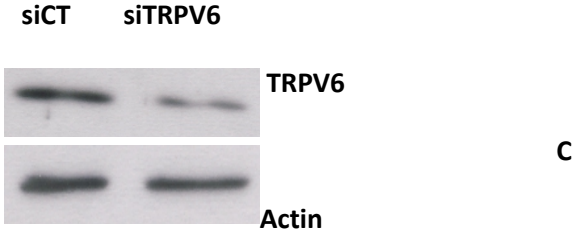


Figure1: LNCaP cells were transfected with siLuc, siSTIM1, siOrai1 (A), siTRPC1 (B) and si TRPV6 (C) for 48 hours. Effeciency of transfection was tested by westernblots against the following antibodies (STIM1 at 84 kDa, Orai1 at 55 kDa, TRPV6 at 95 kDa, TRPC1 at 80 kDa).

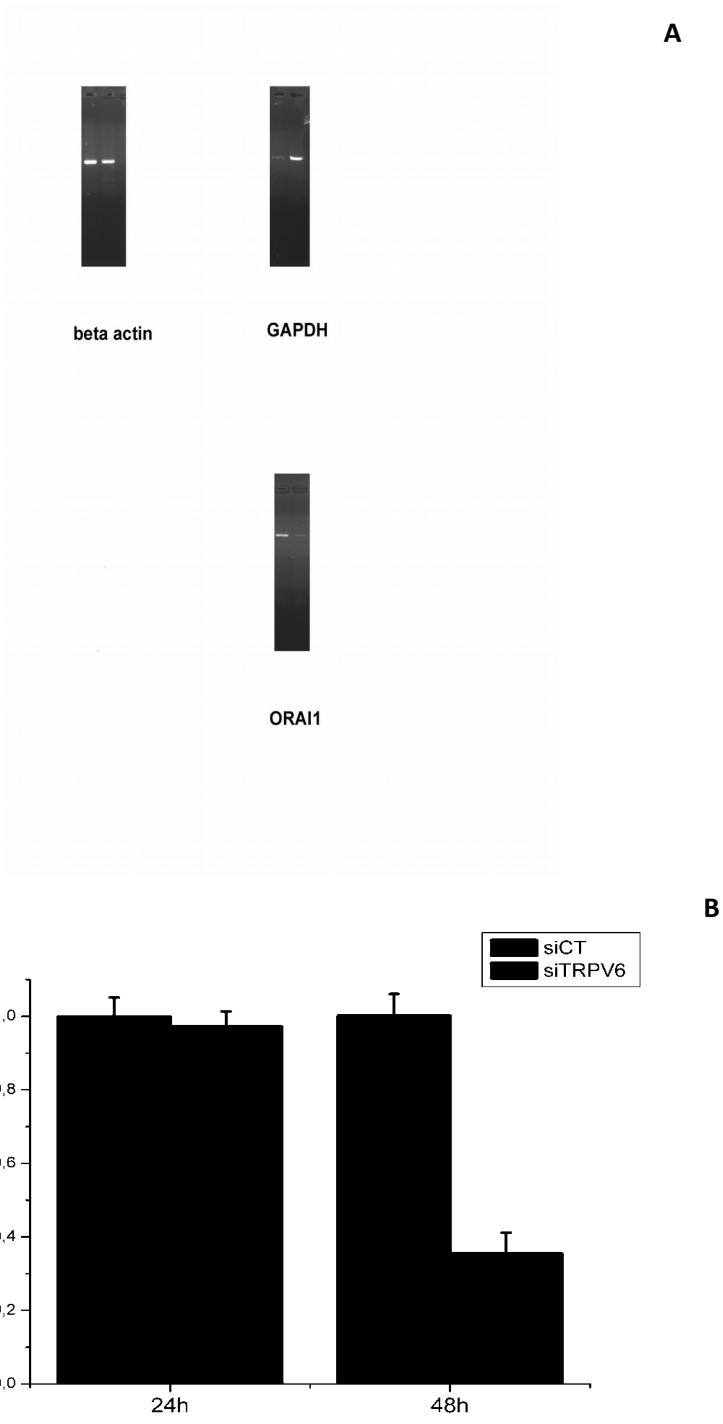


Figure2: RT-PCR was done to verify the efficiency of Orai1 transfection (A)

Q-PCR was done to verify the efficiency ot TRPV6 transfection (B)

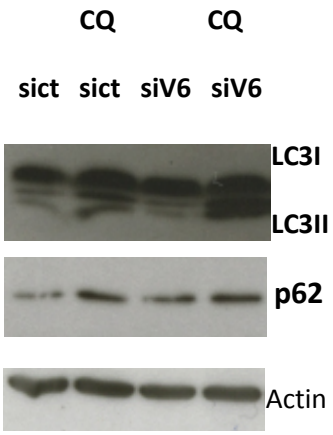


Figure 3: TRPV6 is essential for basal autophagy. It increases LC3 accumulation and decreases p62 levels.
LNCaP cells were transfected with siLuc or siTRPV6 (siV6) for 48 hours.

Discussion

As shown in unpublished results section, TRPC1, TRPV6, Orai1 and stim1 channels have a potential role in regulating autophagy that needs to be further investigated concerning the role of these channels in autophagy regulation and functionality as calcium permeable channels. Aside from the calcium permeable channels that were mentioned in introduction as in literature showing a role in autophagy regulation, we assume that other channels could potentially be involved in autophagy regulation since they contribute to the changes in cytosolic calcium levels. Therefore, we propose a link between calcium permeable channels, autophagy and cancer progression and therapeutic response. Due to cellular localization and physiological roles, we selected several calcium permeable channels, which in our opinion could have an impact on autophagy.

TRPML2 channel, a member of the mucolipin family, has been shown to localize to late and recycling endosomes as well as lysosomes (Zeevi et al., 2009; Cheng et al., 2010). Recent study claimed that TRPML2 does not appear to play a role in starvation-induced autophagy (Zeevi et al., 2010). However, TRPML2 knockdown was demonstrated to induce lysosomal inclusions accumulation in HEK cells (Zeevi et al., 2009). This fact along with the endolysosomal distribution of TRPML2 indicates the potential role of TRPML2 in the regulation of basal as well as other types of autophagy.

TRPM2 is known as a chanzyme, combining two functions: of an ion channel and an enzyme, since the C-terminal of TRPM2 contains enzymatically active adenosine diphosphoribose (ADPR) hydrolase domain (Sumoza-Toledo and Penner, 2011). TRPM2 has been shown to be activated and regulated by variety of stimuli including ADPR, H2O2, NAADP, pH, and cytosolic calcium. It is involved in numerous physiological processes, such as production of cytokines, insulin secretion, oxidative stress, apoptosis (Jiang et al., 2010). TRPM2 functions as a Ca2+ permeable channel on the cell surface, but recently TRPM2 has been shown to be also localized intracellularly on the late endosomal and lysosomal membranes where it functions as a lysosomal Ca2+ release channel (Lange et al., 2009). Thus, it can possibly affect autophagy in the same manner as TRPML and TPC channels. Interestingly that both Ca2+ entry and Ca2+ release channel functions of TRPM2 were shown to be important in H2O2-induced beta-cell death (Lange et al., 2009). Further, H2O2 is a

known activator of autophagy (Chen et al., 2008). Thus, potentially TRPM2 could be involved in H2O2-induced autophagy.

Another interesting candidate is the cold receptor TRPM8, which is found in sensory neurons, where it constitutes the principal detector of cold (<~28°C) (Bautista et al., 2007). In addition to its role as plasmalemmal Ca2+ channel, TRPM8 could function as intracellular Ca2+ release channel on the ER membrane (Zhang and Barritt, 2004; Thebault et al., 2005). Initially, TRPM8 was cloned from the human prostate as prostate-specific gene, which is upregulated in malignant tissues (Tsavaler et al., 2001). The role of TRPM8 in cancer was extensively studied in recent years, and published data suggest that TRPM8 could be involved in proliferation, differentiation and apoptosis in cancer cells (Zhang and Barritt, 2004; Thebault et al., 2005). Given the localization of TRPM8 on ER, it would be interesting to study the possible autophagy regulation by TRPM8-mediated Ca2+ release from the ER. The potential mechanisms could be the same as for IP3 receptor.

It is worth to note that TRPV1 channel, discussed above, has also been found to be expressed intracellularly at the ER and trans-Golgi network (Turner et al., 2003), so apart from the autophagy modulating mechanism provided above, TRPV1 could also be involved in another autophagy related signaling pathways.

TRPP2, the product of the gene mutated in autosomal dominant polycystic kidney disease, is another possible candidate for autophagy regulation. It is widely expressed, with a highest level in the kidney, and primarily localized in cilia, where it seems to function as a mechanosensor involved in the nodal ciliary movement (Delmas et al., 2004). TRPP2 might function as a plasma membrane calcium-permeable channel (when interacting with TRPP1) or as a calcium release channel located in the ER (Hanaoka et al., 2000; Cahalan, 2002; Koulen et al., 2002). Interesting, TRPP2 was shown to function as a calcium-activated intracellular calcium release channel—property reminiscent of IP3 receptors and ryanodine receptors (Koulen et al., 2002). Thus, potentially TRPP2 could regulate autophagy in a way similar to IP3R.

Interesting, although RyR constitutes the major cellular mediator of calcium-induced calcium release, the data on its role in autophagy is very scarce. As it was mentioned above, overexpression of ryanodine receptor type 2 in triple IP3R-deficient DT40 cells was without

effect on constitutive autophagy (Cardenas et al., 2010). Despite this, we believe that more experiments are required to unravel the role of RyR in autophagy regulation.

The next candidate, TRPV2, is a Ca2+ permeable non-selective cationic channel, which has been found to be activated by noxious heat (>50°C), growth factors (i.e., IGF) and stretch (Caterina et al., 1999; Kanzaki et al., 1999; Muraki et al., 2003). It was shown that insulin induced translocation and insertion of TRPV2 into the plasma membrane in a PI3K-dependent manner (Aoyagi et al., 2010). Recent studies revealed the role for TRPV2 in promoting prostate cancer migration and progression to androgen resistance (Monet et al., 2010). Interestingly, Saito et al. demonstrated the function of 2-APB-activated and Rutenium Red-inhibited calcium-permeable ion channel in early endosomes (Saito et al., 2007). The authors reported that this channel has similar pharmacology to that of TRPV2. As early endosomes, and fusion of autophagosomes with functional early endosomes have been shown to be essential for autophagy (Razi et al., 2009), we hypothesize that TRPV2, which apparently forms early endosomal Ca2+ release channel, could be involved in autophagy where it may regulate fusion between autophagosomes and early endosomes. Function on the plasma membrane as well as dependence on PI3K suggest possible complex role in autophagy regulation.

Also, ORAI1 which constitutes a major molecular component of SOC channels (Hewavitharana et al., 2007) also represents an attractive candidate for autophagy regulation. Recently, Abdelmohsen et al. reported that microRNA miR-519 stimulates autophagy through the downregulation of ORAI1 and ATP2C1 proteins, increase in the cytosolic Ca2+ levels, activation of Ca2+ activated calmodulin kinase II as well as glycogen synthase kinase 3β and subsequent p21 upregulation (Abdelmohsen et al., 2012). However, the direct link between autophagy stimulation and functionality of ORAI1 as a Ca2+ permeable channel is missing. Thus, additional experiments using specific agonists and antagonists as well as siRNA knockdown of ORAI1 are needed to confirm its role in autophagy regulation.

Our findings identified intracellular calcium as an important regulator of autophagy. All of the above assures the importance of autophagy regulation for cancer treatment. However, autophagy seems to contribute to the therapeutic resistance of some cancers and

it is elevated upon introduction of chemotherapy. Thus, modulation of autophagy has a great potential in cancer diagnosis and treatment. Now, we will be discussing the work done on ML-9, new autophagy modulator, as an attractive tool for targeting autophagy in cancer therapy.

In this work, we provide evidence that ML-9, a widely used inhibitor of Akt kinase, MLCK and STIM1 represents the “two-in-one” compound which stimulates autophagosome formation (by downregulating Akt/mtor pathway) and inhibits their degradation (by acting like a lysosomotropic agent and increasing lysosomal pH). We also demonstrate that cytosolic calcium is essential for ML-9-induced autophagosome formation, however neither STIM1 nor SOCE are necessary. Further, we show that ML-9 as a monotherapy effectively induces prostate cancer cell death associated with the accumulation of autophagic vacuoles. In addition, ML-9 enhances the anticancer activity of docetaxel, suggesting its potential application as an adjuvant to existing anticancer chemotherapy. We also demonstrate that ML-9 significantly reduces ER calcium content and induces ER-stress, as revealed by PERK activation, which could contribute to its cytotoxicity.

Recently Zhang L. et al. performed high-throughput image-based screening for small molecule regulators of autophagy and among others identified ML-9 as a compound that increases GFP-LC3 vesicles in human glioblastoma H4 cells (Zhang et al., 2007). In another study ML-7, a structurally related analog of ML-9, was shown to induce accumulation of vesicle-like structures in Schwann cells (Leitman et al, 2011).

The evidence presented in our study clearly demonstrates that the reported increase in GFP-LC3 vesicles by ML-9 is in fact the consequence of both the stimulation of autophagosome formation and inhibition of their degradation. To reach this conclusion, we used a number of approaches including LC3 protein detection, electron microscopy for direct visualization of autophagic process, fluorescence and confocal microscopy for GFP-LC3+ and mCherry-GFP-LC3+ puncta analysis as well as endogenous autophagy substrate (p62) degradation analysis.

We demonstrated that ML-9 stimulates autophagy through inhibition of mtor kinase. Several mechanisms may contribute to the inhibition of mtor by ML-9. First, as mtor acts downstream of Akt and Akt is known to positively regulate mtor, inhibition of Akt by ML-9

could consequently induce mtor inhibition and activation of autophagy. Second mechanism is based on the fact that during autophagy the nutrients generated by degradation of cargo in the autolysosomes stimulate mtor, representing a feedback regulatory loop. Accordingly, ML-9- induced lysosomal dysfunction leads to a decrease in autophagic flux thereby decreasing nutrients availability and promoting mtor inhibition. Third, one cannot exclude the possibility of the direct interaction of ML-9 with mtor.

Intriguingly, we discovered the dual role of ML-9 in autophagy regulation. Namely, ML-9 being an autophagy activator inhibits autophagy in the late stages. This effect of ML-9 could be attributed to its weak base properties (the predicted pKa value is 8.04 as calculated using Marvin software by ChemAxon). We propose that ML-9 as a lipophilic weak base enters the cell by simple diffusion. At neutral pH ML-9 is in its non-protonated form. When ML-9 enters acidic vacuoles (such as lysosomes, late endosomes, amphisomes and autolysosomes) it becomes positively charged through protonation and trapped in these vacuoles. Then the increased osmotic pressure in these vacuoles stimulates water influx and vacuoles enlargement. All these events result in the increased intravacuolar pH, subsequent inhibition of lysosomal enzymes and block in degradation of the autolysosomal content. Based on the immuno-TEM analysis we concluded that the majority of the vacuoles represent autolysosomes favoring the hypothesis that ML-9 does not prevent fusion between autophagosomes and lysosomes.

Surprisingly, the early stage autophagy inhibitors 3-MA and wortmannin, although being effective at low sub-toxic ML-9 concentrations (1 μM - 20 μM), did not prevent accumulation of autophagic vacuoles induced by higher doses of ML-9 (>30 μM). This result could indicate that ML-9 could at once induce both canonical and non-canonical autophagy. Although a clear differentiation between these two processes could constitute a challenge, potentially this situation can exist, as with increasing the concentration of ML-9 it may nonspecifically affect more and more targets which could stimulate diverse autophahic pathways. A number of studies reported the modulation of cellular Ca2+ homeostasis by ML-9 (Smyth et al., 2008, Tran et al., 2001, Watanabe et al., 1996). ML-9 has been shown to inhibit redistribution of STIM1, and thus it is widely used as a SOCE inhibitor (Smyth et al., 2008). However, our data demonstrate that STIM1 and SOCE do not contribute to ML-9

induced autophagy. In contrast, ML- 9 induces PERK activation (suggesting ER-stress induction) presumably through the decrease in ER calcium content. ER-stress has been linked to both autophagy activation and cell death (Ogata et al., 2006, Hoyer-Hansen et al., 2007). Interestingly, ER-stress has been shown to negatively regulate Akt/mtor pathway and as such stimulate autophagy (Qin et al., 2010). Of note, it was proposed that ML-9 promotes calcium release through the IP3 receptor (Norwood et al., 2000). The accumulated data suggested a complex role for IP3R in autophagy regulation, since both stimulatory as well as inhibitory functions for IP3R towards autophagy have been described (Decuypere et al., 2011, Wang et al., 2008). Thus, IP3R-pathway as well as PERK-pathway could potentially contribute to both ML-9 induced autophagy and cell death (Avivar-Valderas et al., 2011, Verfaillie et al., 2010). In addition, we demonstrated that cytosolic calcium is essential factor for ML-9-induced autophagy, as its removal by chelation blocks autophagy at an early stage prior to autophagosome formation.

Recently, it was proposed that dual inhibition of Akt kinase and autophagy represents a prospective strategy in anticancer therapy (Degtyarev et al., 2008, Lamoureux et al., 2013). Indeed, one of the most important functions of Akt kinase is cell survival. This kinase is often upregulated in cancer and promotes cell proliferation, cell growth and resistance to apoptosis. Thus, inhibiting Akt kinase appears to be an effective approach in cancer treatment. Similarly, autophagy is thought to be predominantly a cell survival mechanism. Elevated autophagy is often detected in cancer cells in response to radiation and chemotherapy (White et al., 2012, Ito et al., 2005, Kondo et al., 2005). Furthermore, autophagy seems to contribute to the therapeutic resistance of some cancers. Thus, autophagy inhibition also seems to represent a promising therapeutic strategy in the treatment of cancer. The combination of these two approaches shows additive efficacy in anticancer therapy.

Our results suggest that ML-9 represents a dual inhibitor of both Akt pathway and autophagy and thus is potentially interesting for cancer treatment. Indeed, ML-9 as a monotherapy effectively induces prostate cancer cell death as well as enhances the anticancer activity of docetaxel, indicating its potential application as an adjuvant to existing anticancer chemotherapy. These data are in line with the previously published studies showing that ML-9 and its structurally related analog ML-7 promote apoptotic cell death in a

variety of cell lines and these effects have been linked to MLCK inhibition (Connell et al., 2006, kaneko et al., 2002). Moreover, ML-7 was proposed as a promising candidate for treating cancer (Gu et al., 2006). Interestingly, we found that cell-killing effect of ML-9 was strikingly potentiated in a serum-free medium. This result can be explained by the dual inhibition of androgen receptor (AR) and Akt/mtor signaling pathways in these conditions. Indeed, serum-free medium mimics steroid-deprived conditions and thus inhibits AR signaling pathway. It should be noted, that interaction between AR and Akt/mtor pathways has been reported to be involved in tumorigenesis (Bitting et al., 2013, Schayowitz et al., 2010). Moreover the use of Akt/mtor inhibitors in combination with AR antagonists has been demonstrated improve anticancer efficacy (Festuccia et al., 2005, Schayowitz et al., 2008). Thus, combining inhibitors of Akt, AR and autophagy could potentially constitute a novel strategy in prostate cancer therapy.

In conclusion, we have proposed some new calcium permeable channels as potential regulators of autophagy. We have showed that cytosolic calcium is essential for ML-9-induced autophagosome formation, however independently from STIM1 and SOCE. Finally, revealed ML-9 as a novel autophagy modulator in cancer treatment.

Perspectives

Available data strongly suggests that calcium permeable channels represent good candidates for autophagy regulation. Given that both autophagy and calcium-permeable ion channels have a role in cancer this can be highly valuable in order to achieve specific outcomes in anti-cancer therapy. Ion channels could provide some advantages, when targeting autophagy in vivo for cancer treatment. Indeed, most ion channels are localized on cell surface, thus they can be subjected to antibody-based targeting that can be particularly useful in the case of channel upregulation in cancer. Moreover, anti-channel antibodies could be used as carriers for radionuclides, toxic molecules or nanoparticles, which can themselves, affect autophagy and as such influence cell fate.

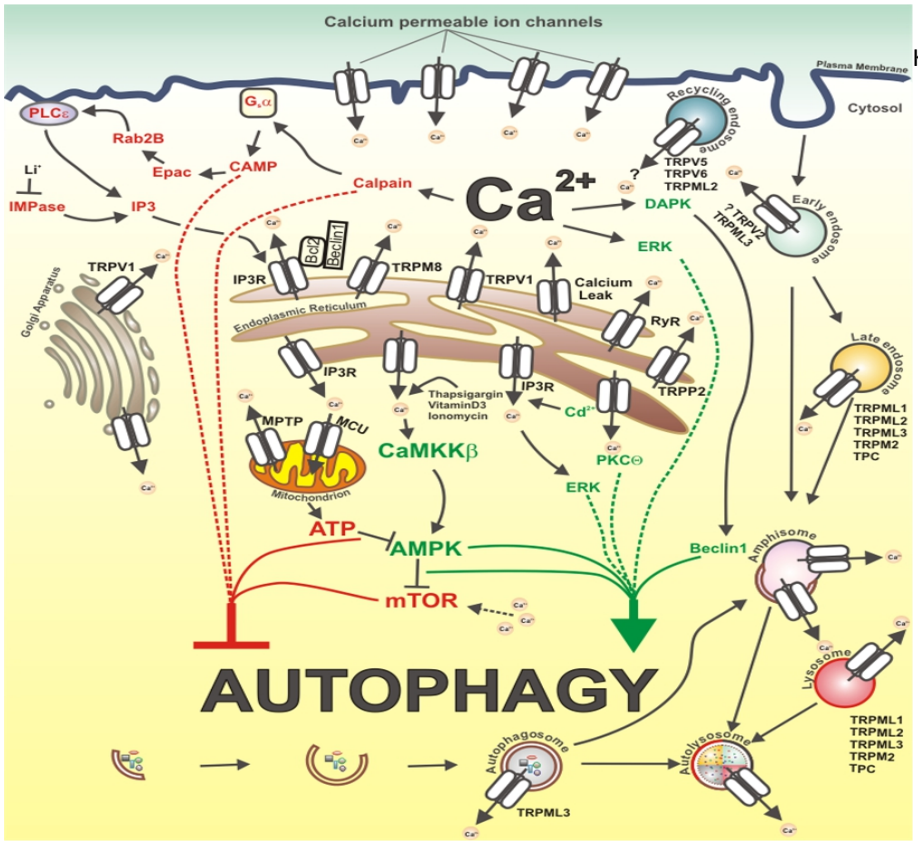
The growing number of studies pointing on the fact that inflammation increases the incidence of cancer (Mantovani et al., 2008). Autophagy has been linked to both cancer and inflammation, and is often defective in the inflammatory conditions (White et al., 2010). Among them, Crohn's disease and pancreatitis have been associated with an increased risk of colorectal and pancreatic cancers, respectively (Freeman, 2008; Raimondi et al., 2010). Accordingly, these pathological states are characterized by the accumulation of damaged organelles and poly-ubiquitinilated protein aggregates, ROS production and DNA damage, the factors that create a cancer-promoting environment (Gukovsky et al., 2012; Nguyen et al., 2013). Hence, this implies that functional autophagy stimulation, to eliminate dangerous garbage, may constitute an effective approach to cancer prevention. Interestingly, a number of calcium-permeable ion channels, including TRPV1, TRPV4, TRPA1, and TRPM8, were shown to be regulated by inflammatory mediators (Nilius et al., 2007; Kochukov et al., 2009; Zhang et al., 2012). Thus, these channels can represent potential targets to stimulate autophagy in inflammatory conditions in order to avert tumorigenesis initiation. Additionally, lysosomal dysfunctions have been reported in pancreatitis (Gukovsky et al., 2012), therefore it could be interesting to consider lysosomal ion channels as well.

On the other hand, in existing tumors autophagy may favor survival and progression. Thus, the possible anticancer therapy should be focused on autophagy inhibition. In this case, considering calcium-permeable channels as a potential tool to target autophagy could also be useful. More specifically, as we discussed above, a number of calcium-permeable ion channels exhibit altered expression in cancer cells. For instance, TRPM8 is upregulated in

androgen-dependent prostate cancer cells (Zhang and Barritt, 2004; Thebault et al., 2005). This could possibly influence intracellular calcium levels and consequently autophagy. Hence, targeting TRPM8 as well as another channels overexpressed in cancers could provide an additional control over autophagy, particularly during chemotherapy, and as such contribute to cancer treatment.

Concerning cancer targeted therapy, high-throughput screening of chemical libraries to identify small-molecule inhibitors of autophagy is ongoing. Biomarkers to measure autophagy modulation during treatment should be an important component of drug development efforts. Although important efforts have been made, several key issues remain unresolved, including how autophagy is exactly regulated in tumor cells, the interplay between autophagy and apoptosis, and the specific mechanism by which autophagy confers treatment resistance and the role of calcium permeable channels in autophagy regulation are questions to be answered.

Perspectives:



Kondratski et Yassine, 2013

Conclusion

Calcium-permeable ion channels have emerged as important regulators of autophagy and the effect of such regulation most likely depends on calcium signals in a spatially restricted subcellular domains. Apparently, such regulation can represent a fundamental mechanism of fine tuning the autophagy. However, the data concerning this subject is very limited, thus further studies are needed to understand the variety of mechanisms, by which calcium channels can influence autophagy and in this work we have proposed some calcium permeable channels as potential candidates for autophagy regulation. In addition, we have identified a novel autophagy modulator for cancer treatment.

Accumulated data proves that both calcium-permeable ion channels and autophagy are implicated in cancer initiation and progression as well as chemotherapy resistance. Paradoxically, autophagy has opposite roles in cancer, with both tumorigenesis suppressor action, in particular at the early stages of tumor initiation and cancer promotion effect resulting in tumor cell survival, chemotherapy resistance and cancer progression. Thus, it is important to unravel autophagy regulating pathways to most effectively target autophagy to cure cancer. Identification of the connections between calcium channels and autophagy could define a new strategy in cancer treatment, and identify useful tools and biomarkers for the elaboration of effective anti-cancer therapies. Moreover, as malfunction of autophagy has been linked to a wide range of human pathologies including liver disease, neurodegeneration, Crohn's disease and cancer, uncovering novel mechanisms of autophagy regulation by calcium permeable ion channels could have a broad impact on the Autophagy field and contribute to the developing of autophagy as a potential clinical approach to cure diseases.

Abundant preclinical evidence indicates that stress induced autophagy in tumor cells is predominantly cytoprotective and that inhibition of autophagy can enhance cell death by diverse anticancer therapies. These data establish autophagy as a novel therapeutic target whose modulation presents new opportunities for cancer treatment and overall, our results suggest that ML-9 represents an attractive tool for targeting autophagy in cancer through dual inhibition of AKT and autophagy pathways. Further, the chemical structure of ML-9 could serve as a “template” for the synthesis of structurally improved related and more selective compounds, which could potentially be used for cancer treatment.

Finally, autophagy maintains nutrient and energy homeostasis and defends mammalian cells against microbial attack and many diseases. The activation of autophagy, however, is not without potential risks. Autophagy may help keep alive those cells that should die, such as chemotherapy-treated tumor cells, or, if present in excess kill cells that should live. At the end, it could be just a matter of balance in Calcium-the life and death signal.

Annexes

Solutions pour extraction de protéines

Tampon RIPA :

- Trion X100 : 1 %
- Sodium Déoxycholate : 1 %
- Tampon Phosphate (PO4 Na2/K) : 10 Mm
- pH : 7,2

Solutions pour immunofluorescence

Tampon citrate :

- Acide citrique 0,1 M
- Citrate de soude 0,1 M
- pH 6

Solution de PBS :

- NaC1 : 8 g /L
- KC1 : 0,2 g/L
- Na2HPO4 : 1,44 g/L
- KH2PO4 : 0,24 g/L
- pH : 7,4

Para-Formaldéhyde (PFA) :

- Tampon phosphate (PO4 Na2/K) 200 Mm
- PFA : 10 %

Solution de saturation pour immunofluorescence :

- PBS
- Sérum d'ane : 5 %
- BSA : 0,2 %
- Triton X100 : 0,2 %
- pH 7,4

Solution pour électrophorèse

Tampon TBE :

- Tris HCl : 108 g/L
- Acide Borique : 55 g/L
- EDTA : 9,3 g/L
- pH : 8,3

Tampon TAE :

- Tris HCl : 108 g/L
- Acide Acétique : 55 g/L
- EDTA : 9,3 g/L
- pH

Solution (run-SDS) pour électrophorese SDS-PAGE :

- Tris : 25 Mm
- Glycine : 192 Mm
- SDS : 0,1 %
- pH : 8,3 – 8,5

Solution de transfert :

- Tris : 48 Mm
- Glycine : 39 Mm
- SDS : 0,037 %
- Méthanol : 10 %

Tampon de charge et marqueurs de poids moléculaire

Tampon de charge pour protéin (Tampon de laemelli) :

- Tris pH 6,8 / 62,5 mM
- SDS : 2 %
- Saccharose : 10 %
- Bleu de Bromophénol : 0,001 %
- DTT : 100 mM

Tampon de charge pour ADN :

- 50 cl de bleu concentré (0,25% de bleu de Bromophenol + 0,25 de xyléne cyanate dans H2O).
- 950 cl d'un mix Tris-HCl 10mM finale (pH 8) + 40 % glycérol.

Marqueur de poids moléculaire pour ADN /ARN :

- Tampon << bleu >> de charge : 15 %
- Marqueur << 1 Kb >> : 10 %

Autres solutions

Tampon TE (TRIS-EDTA) :

- Tris HCl : 1,21 g/L

- EDTA : 0,37 g/L
- pH : 7,9

HBSS:

- Nacl : 140 mM
- KCl / 5 mM
- CaCl2 : 2 mM
- MgCl2 : 2
- Na2PO4 : 0,3 mM
- KH2PO4 : 0,4 Mm
- NaHCO3 : 4mM
- Glucose : 5 mM
- HEPES : 10 mM (pH 7,3, osmolalité 330mosmol/l)

Bibliography

Abdelmohsen, K., Srikantan, S., Tominaga, K., Kang, M.J., Yaniv, Y., Martindale, J.L., Yang, X., Park, S.S., Becker, K.G., Subramanian, M., Maudsley, S., Lal, A., and Gorospe, M. (2012). Growth inhibition by miR-519 via multiple p21-inducing pathways. Mol Cell Biol 32, 2530-2548.doi:10.1128/MCB.00510-12

Abdullaev, I. F., Bisaillon, J. M., Potier, M., Gonzalez, J. C., Motiani, R. K., Trebak, M. (2008)Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. Circ Res. 103(11):1289-99.

Adams, J.M., and Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 26, 1324–1337.

Altarescu, G., Sun, M., Moore, D.F., Smith, J.A., Wiggs, E.A., Solomon, B.I., Patronas, N.J., Frei, K.P., Gupta, S., Kaneski, C.R., Quarrell, O.W., Slaugenhaupt, S.A., Goldin, E., and Schiffmann, R. (2002). The neurogenetics of mucolipidosis type IV. Neurology 59, 306-313

Amaravadi, R.K., Lippincott-Schwartz, J., Yin, X.M., Weiss, W.A., Takebe, N., Timmer, W., Dipaola, R.S., Lotze, M.T., and White, E. (2011). Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res 17, 654-666.doi:10.1158/1078-0432.CCR-10-2634

Amaravadi, R.K., Yu, D., Lum, J.J., Bui, T., Christophorou, M.A., Evan, G.I., Thomas-Tikhonenko, A., and Thompson, C.B. (2007). Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 117, 326-336.doi:10.1172/JCI28833

Aoyagi, K., Ohara-Imaizumi, M., Nishiwaki, C., Nakamichi, Y., and Nagamatsu, S. (2010). Insulin/phosphoinositide 3-kinase pathway accelerates the glucose-induced first-phase insulin secretion through TrpV2 recruitment in pancreatic beta-cells. Biochem J 432, 375-386.doi:10.1042/BJ20100864

Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P.F., Meijer, A.J., Codogno, P., and Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem 276, 35243-35246.doi:10.1074/jbc.C100319200

Axe, E. L., Walker, S. A., Manifava, M., Chandra, P., Roderick, H. L., Habermann, A., Griffiths, G., Ktistakis, N. T. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol. 182(4):685-701.

Bach,G.(2001).Mucolipidosis type IV. Mol.Genet.Metab 73, 197-203.doi:10.1006/mgme.2001.3195 [doi];S1096-7192(01)93195-6 [pii]

Baeriswyl, V., and Christofori, G. (2009). The angiogenic switch in carcinogenesis. Semin. Cancer Biol. 19, 329–337.

Barth, S., Glick, D. and Macleod, K. F. (2010). Autophagy: assays and artifacts. Journal of Pathology. 221(2): 117–124.

Baughman, J.M., Perocchi, F., Girgis, H.S., Plovanich, M., Belcher-Timme, C.A., Sancak, Y., Bao, X.R., Strittmatter, L., Goldberger, O., Bogorad, R.L., Koteliansky, V., and Mootha, V.K. (2011). Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature 476, 341-345.doi:10.1038/nature10234

Bautista, D.M., Siemens, J., Glazer, J.M., Tsuruda, P.R., Basbaum, A.I., Stucky, C.L., Jordt, S.E., and Julius, D. (2007). The menthol receptor TRPM8 is the principal detector of environmental cold. Nature 448, 204-208.doi:10.1038/nature05910

Bernardi, P. (2013). The mitochondrial permeability transition pore: a mystery solved? Front Physiol 4, 95.doi:10.3389/fphys.2013.00095

Bernardi, P., and Von Stockum, S. (2012). The permeability transition pore as a Ca(2+) release channel: new answers to an old question. Cell Calcium 52, 22-27.doi:10.1016/j.ceca.2012.03.004

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. Nature. 361(6410):315-25.

Berridge, M. J., Bootman, M. D., Lipp, P. (1998). Calcium-a life and death signal. Nature. 395(6703):645-8.

Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1, 11-21.doi:10.1038/35036035

Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4, 517-529.doi:10.1038/nrm1155

Berridge, M. (2004). Conformational Coupling: A physiological calcium entry mechanism. Science's stke. 243:1-4

Berridge, M. (2006). Calcium microdomains: Organization and function. Cell Calcium. 40: 405-412

Berridge, M. J. (2012). Calcium signalling remodelling and disease. Biochem Soc Trans. 40(2):297-309.

Berliocchi, L. Bano, D. Nicoter, P. (2005).Ca2+ signals and death programmes in neurons Philos. Trans. R. Soc. Lond. B: Biol. Sci. 360:2255-2258

Berna-Erro, A. Braun, R. Kraft, C. Kleinschnitz, M., K. Schuhmann, D. Stegner, T. Wultsch, J.Eilers, S.G. Meuth, G. (2009). STIM2 regulates capacitive Ca2+ entry in neurons and plays a key role in hypoxic neuronal cell death. Sci. Signal. 2: 67.

Bernardi,P. Rasola, A. (2007). Calcium and cell death: the mitochondrial connection. Subcell. Biochem. 45:481–505

Bidaux, G., Roudbaraki, M., Merle, C., Crépin, A., Delcourt, P., Slomianny, C., Thebault, S., Bonnal, J. L., Benahmed, M., Cabon, F., Mauroy, B., Prevarskaya, N. (2005). Evidence for specific TRPM8 expression in human prostate secretory epithelial cells: functional androgen receptor requirement. Endocr Relat Cancer. 12(2):367-82

Bidaux, G., Flourakis, M., Thebault, S., Zholos, A., Beck, B., Gkika, D., Roudbaraki, M., Bonnal, J. L., Mauroy, B., Shuba, Y., Skryma, R., Prevarskaya, N. (2007). Prostate cell differentiation status determines transient receptor potential melastatin member 8 channel subcellular localization and function. J Clin Invest. 117(6):1647-57.B

Boehning, D., Patterson, R.L., and Snyder, S.H. (2004). Apoptosis and calcium: new roles for cytochrome c and inositol 1,4,5-trisphosphate. Cell Cycle 3, 252-254

Bolanz, K. A., Hediger, M. A., Landowski, C. P. (2008). The role of TRPV6 in breast carcinogenesis. Mol Cancer Ther. 7(2):271-9.

Boya, P., González-Polo, R. A., Casares, N., Perfettini, J. L., Dessen, P., Larochette, N., Métivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P., Kroemer, G. (2005).Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 25(3):1025-40

Bultynck, G., Vermassen, E., Szlufcik, K., De Smet, P., Fissore, R.A., Callewaert, G., Missiaen, L., De Smedt, H., and Parys, J.B. (2003). Calcineurin and intracellular Ca2+-release channels: regulation or association? Biochem Biophys Res Commun 311, 1181- 1193

Burkhart, D.L., and Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat. Rev. Cancer 8, 671–682.

Cahalan, M.D. (2002). The ins and outs of polycystin-2 as a calcium release channel. Nat Cell Biol 4, E56-57.doi:10.1038/ncb0302-e56

Calcraft, P.J., Ruas, M., Pan, Z., Cheng, X., Arredouani, A., Hao, X., Tang, J., Rietdorf, K., Teboul, L.,
Chuang, K.T., Lin, P., Xiao, R., Wang, C., Zhu, Y., Lin, Y., Wyatt, C.N., Parrington, J., Ma, J., Evans, A.M., Galione, A., and Zhu, M.X. (2009). NAADP mobilizes calcium from acidic organelles through two-pore channels. Nature 459, 596-600.doi:10.1038/nature08030

Capiod, T. (2011). Cell proliferation, calcium influx and calcium channels. Biochimie. 93(12):2075-9.

Castro-Obregon, S. (2010). The discovery of lysosomes and autophagy. Nature Education. 3(9):49.

Charbel, C., Toussaint, F., Ledoux, J. (2013). Les dynamiques calciques : Côté Lumineux de la signalisation endothéliale or Calcium dynamics: Bright Side of endothelial signalling. Medicine Sciences Amerique MSA . 2(2):1-15

Cardenas, C., and Foskett, J.K. (2012). Mitochondrial Ca(2+) signals in autophagy. Cell Calcium 52, 44-51.doi:10.1016/j.ceca.2012.03.001

Cardenas, C., Miller, R.A., Smith, I., Bui, T., Molgo, J., Muller, M., Vais, H., Cheung, K.H., Yang, J., Parker, I., Thompson, C.B., Birnbaum, M.J., Hallows, K.R., and Foskett, J.K. (2010). Essential regulation

of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. Cell 142, 270-283.doi:10.1016/j.cell.2010.06.007

Carreira, R.S., Lee, Y., Ghochani, M., Gustafsson, A.B., and Gottlieb, R.A. (2010). Cyclophilin D is required for mitochondrial removal by autophagy in cardiac cells. Autophagy 6, 462-472.doi:10.4161/auto.6.4.11553

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., and Julius, D. (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398, 436-441.doi:10.1038/18906

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389, 816-824.doi:10.1038/39807

Chen, Y., Mcmillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2008). Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. Cell Death Differ 15, 171-182.doi:10.1038/sj.cdd.4402233

Chen, Y., Azad, M. B. and Gibson, S. B. (2010). Methods for detecting autophagy and determining autophagy-induced cell death. Can J Physiol Pharmacol. 88(3):285-95.

Chen, Y., and Klionsky, D.J. (2011). The regulation of autophagy - unanswered questions. J Cell Sci 124, 161-170.doi:10.1242/jcs.064576

Chen, Y. F., Chen, Y. T., Chiu, W. T. and Shen, M. R. (2013). Remodeling of calcium signaling in tumor Progression. Journal of Biomedical Science. 20(1): 23.

Cheng, X., Shen, D., Samie, M., and Xu, H. (2010). Mucolipins: Intracellular TRPML1-3 channels. FEBS Lett 584, 2013-2021.doi:10.1016/j.febslet.2009.12.056

Cheong, H., Lu, C., Lindsten, T., and Thompson, C.B. (2012). Therapeutic targets in cancer cell metabolism and autophagy. Nat Biotechnol 30, 671-678.doi:10.1038/nbt.2285

Cho, R.W., and Clarke, M.F. (2008). Recent advances in cancer stem cells. Curr. Opin. Genet. Dev. 18, 1–6.

Choi, A.M., Ryter, S.W., and Levine, B. (2013). Autophagy in human health and disease. N Engl J Med 368, 651-662.doi:10.1056/NEJMra1205406

Chow, J., Norng, M., Zhang, J., and Chai, J. (2007). TRPV6 mediates capsaicin-induced apoptosis in gastric cancer cells--Mechanisms behind a possible new "hot" cancer treatment. Biochim Biophys Acta 1773, 565-576.doi:10.1016/j.bbamcr.2007.01.001

Clapham, D. E., Runnels, L.W., Strübing, C. (2001). The TRP ion channel family. Nat Rev Neurosci. 2(6):387-96.

Clapham, D. E. (2007). Calcium signaling. Cell. 131(6):1047-58.

Clapham, D.E. (2007). SnapShot: mammalian TRP channels. Cell 129, 220.e1-220.e2.doi:10.1016/j.cell.2007.03.034

Criollo, A., Maiuri, M.C., Tasdemir, E., Vitale, I., Fiebig, A.A., Andrews, D., Molgo, J., Diaz, J., Lavandero, S., Harper, F., Pierron, G., Di Stefano, D., Rizzuto, R., Szabadkai, G., and Kroemer, G. (2007). Regulation of autophagy by the inositol trisphosphate receptor. Cell Death Differ 14, 1029-1039.doi:10.1038/sj.cdd.4402099

Collado, M., and Serrano, M. (2010). Senescence in tumours: evidence from mice and humans. Nat. Rev. Cancer 10, 51–57.

Connolly, C. N., Wafford, K. A. (2004). The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. Biochem. Soc. Trans. 32 (Pt3): 529-34.83.

Curcio-Morelli, C., Charles, F.A., Micsenyi, M.C., Cao, Y., Venugopal, B., Browning, M.F., Dobrenis, K., Cotman, S.L., Walkley, S.U., and Slaugenhaupt, S.A. (2010). Macroautophagy is defective in mucolipin-1-deficient mouse neurons. Neurobiol.Dis. 40, 370-377.doi:S0969-9961(10)00206-8 [pii];10.1016/j.nbd.2010.06.010 [doi]

Curry, M.C., Peters, A.A., Kenny, P.A., Roberts-Thomson, S.J., and Monteith, G.R. (2013). Mitochondrial calcium uniporter silencing potentiates caspase-independent cell death in MDA-MB-231 breast cancer cells. Biochem Biophys Res Commun 434, 695-700.doi:10.1016/j.bbrc.2013.04.015

Darszon, A., Nishigaki, T., Beltran, C., Treviño, C. L. (2011). Calcium channels in the development, maturation, and function of spermatozoa. Physiol Rev. 91(4):1305-55.

Davies, M.A., and Samuels, Y. (2010). Analysis of the genome to personalize therapy for melanoma. Oncogene 29, 5545–5555.

De Stefani, D., Raffaello, A., Teardo, E., Szabo, I., and Rizzuto, R. (2011). A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476, 336-340.doi:10.1038/nature10230

Decuypere, J.P., Bultynck, G., and Parys, J.B. (2011a). A dual role for Ca(2+) in autophagy regulation. Cell Calcium 50, 242-250.doi:10.1016/j.ceca.2011.04.001

Decuypere, J.P., Welkenhuyzen, K., Luyten, T., Ponsaerts, R., Dewaele, M., Molgo, J., Agostinis, P., Missiaen, L., De Smedt, H., Parys, J.B., and Bultynck, G. (2011b). Ins(1,4,5)P3 receptor-mediated Ca2+ signaling and autophagy induction are interrelated. Autophagy 7, 1472-1489

Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gelinas, C., Fan, Y., Nelson, D.A., Jin, S., and White, E. (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10, 51-64.doi:10.1016/j.ccr.2006.06.001

Delmas, P., Padilla, F., Osorio, N., Coste, B., Raoux, M., and Crest, M. (2004). Polycystins, calcium signaling, and human diseases. Biochem Biophys Res Commun 322, 1374-1383.doi:10.1016/j.bbrc.2004.08.044

Dong, X.P., Cheng, X., Mills, E., Delling, M., Wang, F., Kurz, T., and Xu, H. (2008). The type IV mucolipidosis-associated protein TRPML1 is an endolysosomal iron release channel. Nature 455, 992-996.doi:10.1038/nature07311

Dong, X.P., Wang, X., and Xu, H. (2010). TRP channels of intracellular membranes. J Neurochem 113, 313-328.doi:10.1111/j.1471-4159.2010.06626.x

Dubois, C., Vanden Abeele, F., and Prevarskaya, N. (2013). Targeting apoptosis by the remodelling of calcium-transporting proteins in cancerogenesis. FEBS J.doi:10.1111/febs.12246

Elmore, S.P., Qian, T., Grissom, S.F., and Lemasters, J.J. (2001). The mitochondrial permeability transition initiates autophagy in rat hepatocytes. FASEB J 15, 2286-2287.doi:10.1096/fj.01-0206fje

Farfariello, V., Amantini, C., and Santoni, G. (2012). Transient receptor potential vanilloid 1 activation induces autophagy in thymocytes through ROS-regulated AMPK and Atg4C pathways. J Leukoc Biol 92, 421-431.doi:10.1189/jlb.0312123

Feng, Z., Zhang, H., Levine, A.J., and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. Proc Natl Acad Sci U S A 102, 8204-8209.doi:10.1073/pnas.0502857102

Flourakis, M., Lehen'kyi, V., Beck, B., Raphael, M., Vandenberghe, M., Abeele, F.V., Roudbaraki, M., Lepage, G., Mauroy, B., Romanin, C., Shuba, Y., Skryma, R., and Prevarskaya, N. (2010). Orai1 contributes to the establishment of an apoptosis-resistant phenotype in prostate cancer cells. Cell Death Dis 1, e75.doi:10.1038/cddis.2010.52

Flourakis, M., and Prevarskaya, N. (2009). Insights into Ca2+ homeostasis of advanced prostate cancer cells. Biochim Biophys Acta 1793, 1105-1109.doi:10.1016/j.bbamcr.2009.01.009

Foskett, J., White,C., Cheung, K., Mak, D. (2007). Inositol triphosphate receptor calcium release channel. Physiol Rev.2007 Apr;87(2):593-658.

Freeman, H.J. (2008). Colorectal cancer risk in Crohn's disease. World J Gastroenterol 14, 1810-1811

Galione, A., Evans, A.M., Ma, J., Parrington, J., Arredouani, A., Cheng, X., and Zhu, M.X. (2009). The acid test: the discovery of two-pore channels (TPCs) as NAADP-gated endolysosomal Ca(2+) release channels. Pflugers Arch 458, 869-876.doi:10.1007/s00424-009-0682-y

Gackière, F., Warnier, M., Katsogiannou, M., Derouiche, S., Delcourt, P., Dewailly, E., Slomianny, C., Humez, S., Prevarskaya, N., Roudbaraki, M., Mariot, P. (2013). Functional coupling between large-conductance potassium channels and Cav3.2 voltage-dependent calcium channels participates in prostate cancer cell growth. Biol Open. 2(9):941-51.

Gao, W., Ding, W.X., Stolz, D.B., and Yin, X.M. (2008). Induction of macroautophagy by exogenously introduced calcium. Autophagy 4, 754-761

Geng, J., and Klionsky, D.J. (2008). The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep 9, 859-864.doi:10.1038/embor.2008.163

Gkika, D., Flourakis, M., Lemonnier, L., Prevarskaya, N. (2010). PSA reduces prostate cancer cell motility by stimulating TRPM8 activity and plasma membrane expression. Oncogene. 29(32):4611-6.

Goldin, E., Cooney, A., Kaneski, C.R., Brady, R.O., and Schiffmann, R. (1999). Mucolipidosis IV consists of one complementation group. Proc.Natl.Acad.Sci.U.S.A 96, 8562-8566

Gomez-Suaga, P., Luzon-Toro, B., Churamani, D., Zhang, L., Bloor-Young, D., Patel, S., Woodman, P.G., Churchill, G.C., and Hilfiker, S. (2012). Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. Hum Mol Genet 21, 511-525.doi:10.1093/hmg/ddr481

Goodison, W. V., Frisardi, V., Kehoe, P. G. (2012). Calcium channel blockers and Alzheimer's disease: potential relevance in treatment strategies of metabolic syndrome. J Alzheimers Dis. 30 Suppl 2:S269-82.

Gordon, P.B., Holen, I., Fosse, M., Rotnes, J.S., and Seglen, P.O. (1993). Dependence of hepatocytic autophagy on intracellularly sequestered calcium. J Biol Chem 268, 26107-26112

Gukovsky, I., Pandol, S.J., Mareninova, O.A., Shalbueva, N., Jia, W., and Gukovskaya, A.S. (2012). Impaired autophagy and organellar dysfunction in pancreatitis. J Gastroenterol Hepatol 27 Suppl 2, 27-32.doi:10.1111/j.1440-1746.2011.07004.x

Gulati, P., Gaspers, L.D., Dann, S.G., Joaquin, M., Nobukuni, T., Natt, F., Kozma, S.C., Thomas, A.P., and Thomas, G. (2008). Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. Cell Metab 7, 456-465.doi:10.1016/j.cmet.2008.03.002

Guo, X.L., Li, D., Hu, F., Song, J.R., Zhang, S.S., Deng, W.J., Sun, K., Zhao, Q.D., Xie, X.Q., Song, Y.J., Wu, M.C., and Wei, L.X. (2012). Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells. Cancer Lett 320, 171-179.doi:10.1016/j.canlet.2012.03.002

Hagenacker, T., Splettstoesser, F., Greffrath, W., Treede, R.D., and Busselberg, D. (2005). Capsaicin differentially modulates voltage-activated calcium channel currents in dorsal root ganglion neurones of rats. Brain Res 1062, 74-85.doi:10.1016/j.brainres.2005.09.033

Hajnoczky, G., Csordas, G., Madesh, M., and Pacher, P. (2000). Control of apoptosis by IP(3) and ryanodine receptor driven calcium signals. Cell Calcium 28, 349-363.doi:10.1054/ceca.2000.0169

Hall, M. N. (2008). mTOR-what does it do? Transplant Proc. 40(10 Suppl):S5-8.

Hanahan, D., Weinberg, R.A. (2000). The hallmarks of cancer. Cell. 100(1):57-70.

Hanahan, D., Weinberg, A.(2011). The hallmarks of cancer: Next generation. Cell. 144:646- 674.

Hanaoka, K., Qian, F., Boletta, A., Bhunia, A.K., Piontek, K., Tsiokas, L., Sukhatme, V.P., Guggino, W.B., and Germino, G.G. (2000). Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. Nature 408, 990-994.doi:10.1038/35050128

Hao, F., Tan, M., Xu, X., Han, J., Miller, D. D., Tigyi, G., Cui, M. Z. (2007). Lysophosphatidic acid induces prostate cancer PC3 cell migration via activation of LPA(1), p42 and p38alpha. Biochim Biophys Acta. 1771(7):883-92.

He, C., and Levine, B. (2010). The Beclin 1 interactome. Curr Opin Cell Biol 22, 140-149.doi:10.1016/j.ceb.2010.01.001

Hewavitharana, T., Deng, X., Soboloff, J., and Gill, D.L. (2007). Role of STIM and Orai proteins in the store-operated calcium signaling pathway. Cell Calcium 42, 173-182.doi:10.1016/j.ceca.2007.03.009

Higo,T. Hamada, C. Hisatsune, N. Nukina, T. Hashikawa, M. Hattori, T. Nakamura, K. (2010). Mechanism of ER stress-induced brain damage by IP3 receptor. Neuron. 68:865-878

Hilgemann, D. W., Yaradanakul, A., Wang, Y., Fuster, D. (2006). Molecular control of cardiac sodium homeostasis in health and disease. J Cardiovasc Electrophysiol. 1:S47-S56.Hippert, M. M., O'Toole, P. S. and Thorburn, A. (2006). Autophagy in Cancer: Good, Bad, or Both?. Cancer Res. 66(19):9349-51.

Hoyer-Hansen, M., Bastholm, L., Szyniarowski, P., Campanella, M., Szabadkai, G., Farkas, T., Bianchi, K., Fehrenbacher, N., Elling, F., Rizzuto, R., Mathiasen, I.S., and Jaattela, M. (2007). Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol Cell 25, 193-205.doi:10.1016/j.molcel.2006.12.009

Hsu, K.F., Wu, C.L., Huang, S.C., Wu, C.M., Hsiao, J.R., Yo, Y.T., Chen, Y.H., Shiau, A.L., and Chou, C.Y. (2009). Cathepsin L mediates resveratrol-induced autophagy and apoptotic cell death in cervical cancer cells. Autophagy 5, 451-460.doi:7666 [pii]

Huang, J. K., Chou, C. T., Chang, H. T., Shu, S. S., Kuo, C. C., Tsai, J. Y., Liao, W. C., Wang, J. L., Inbal, B., Bialik, S., Sabanay, I., Shani, G., and Kimchi, A. (2002). DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. J Cell Biol 157, 455-468.doi:10.1083/jcb.200109094

Ito, H., Daido, S., Kanzawa, T., Kondo, S., and Kondo, Y. (2005). Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. Int J Oncol 26, 1401-1410

Izquierdo, J. H., Bonilla-Abadía, F., Cañas, C. A., Tobón, G. J. (2013). Calcium, channels, intracellular signaling and autoimmunity. Reumatol Clin. pii: S1699-258X (13) 00158-7.

Jennings, J.J., Jr., Zhu, J.H., Rbaibi, Y., Luo, X., Chu, C.T., and Kiselyov, K. (2006). Mitochondrial aberrations in mucolipidosis Type IV. J.Biol.Chem. 281, 39041-39050.doi:M607982200 [pii];10.1074/jbc.M607982200 [doi]

Jiang, B.H., and Liu, L.Z. (2009). PI3K/PTEN signaling in angiogenesis and tumorigenesis. Adv. Cancer Res. 102, 19–65.

Jiang, L.H., Yang, W., Zou, J., and Beech, D.J. (2010). TRPM2 channel properties, functions and therapeutic potentials. Expert Opin Ther Targets 14, 973-988.doi:10.1517/14728222.2010.510135

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19(21):5720-8.

Kanzaki, M., Zhang, Y.Q., Mashima, H., Li, L., Shibata, H., and Kojima, I. (1999). Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. Nat Cell Biol 1, 165-170.doi:10.1038/11086

Khan, M.T., and Joseph, S.K. (2010). Role of inositol trisphosphate receptors in autophagy in DT40 cells. J Biol Chem 285, 16912-16920.doi:10.1074/jbc.M110.114207

Kim, H.J., Soyombo, A.A., Tjon-Kon-Sang, S., So, I., and Muallem, S. (2009). The Ca(2+) channel TRPML3 regulates membrane trafficking and autophagy. Traffic 10, 1157-1167.doi:10.1111/j.1600-0854.2009.00924.x

Kim, J., Kundu, M., Viollet, B., and Guan, K.L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13, 132-141.doi:10.1038/ncb2152

Kimura, T., Takabatake, Y., Takahashi, A., and Isaka, Y. (2013). Chloroquine in cancer therapy: a double-edged sword of autophagy. Cancer Res 73, 3-7.doi:10.1158/0008-5472.CAN-12-2464

Klionsky, D.J. (2005). The molecular machinery of autophagy: unanswered questions. J Cell Sci 118, 7-18.doi:10.1242/jcs.01620

Klionsky, D. J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Molecular cell biology. 8 (11):931-7.

Klionsky, D.J., Abdalla, F.C., Abeliovich, H., Abraham, R.T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J.A., Ahn, H.J., Ait-Mohamed, O., Ait-Si-Ali, S., Akematsu, T., Akira, S., Al-Younes, H.M., Al-Zeer, M.A., Albert, M.L., Albin, R.L., Alegre-Abarrategui, J., Aleo, M.F., Alirezaei, M., Almasan, A., Almonte-Becerril, M., Amano, A.,

Amaravadi, R., Amarnath, S., Amer, A.O., Andrieu-Abadie, N., Anantharam, V., Ann, D.K., Anoopkumar-Dukie, S., Aoki, H., Apostolova, N., Arancia, G., Aris, J.P., Asanuma, K., Asare, N.Y., Ashida, H., Askanas, V., Askew, D.S., Auberger, P., Baba, M., Backues, S.K., Baehrecke, E.H., Bahr, B.A., Bai, X.Y., Bailly, Y., Baiocchi, R., Baldini, G., Balduini, W., Ballabio, A., Bamber, B.A., Bampton, E.T., Banhegyi, G., Bartholomew, C.R., Bassham, D.C., Bast, R.C., Jr., Batoko, H., Bay, B.H., Beau, I., Bechet, D.M., Begley, T.J., Behl, C., Behrends, C., Bekri, S., Bellaire, B., Bendall, L.J., Benetti, L., Berliocchi, L., Bernardi, H., Bernassola, F., Besteiro, S., Bhatia-Kissova, I., Bi, X., Biard-Piechaczyk, M., Blum, J.S., Boise, L.H., Bonaldo, P., Boone, D.L., Bornhauser, B.C., Bortoluci, K.R., Bossis, I., Bost, F., Bourquin, J.P., Boya, P., Boyer-Guittaut, M., Bozhkov, P.V., Brady, N.R., Brancolini, C., Brech, A., Brenman, J.E., Brennand, A., Bresnick, E.H., Brest, P., Bridges, D., Bristol, M.L., Brookes, P.S., Brown, E.J., Brumell, J.H., et al. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8, 445-544

Kochukov, M.Y., Mcnearney, T.A., Yin, H., Zhang, L., Ma, F., Ponomareva, L., Abshire, S., and Westlund, K.N. (2009). Tumor necrosis factor-alpha (TNF-alpha) enhances functional thermal and chemical responses of TRP cation channels in human synoviocytes. Mol Pain 5, 49.doi:10.1186/1744-8069-5-49

Kondo, Y., Kanzawa, T., Sawaya, R., and Kondo, S. (2005). The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 5, 726-734.doi:10.1038/nrc1692

Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B.E., and Somlo, S. (2002). Polycystin-2 is an intracellular calcium release channel. Nat Cell Biol 4, 191-197.doi:10.1038/ncb754

Kreuzaler, P., Watson, C. J. (2012). Killing a cancer: what are the alternatives? Nat Rev Cancer. 12(6):411-24

Kroemer, G., Levine, B. (2008). Autophagy in the Pathogenesis of Disease . Cell journal. 132(1):27-42.

Kroemer, G., Marino, G., and Levine, B. (2010). Autophagy and the integrated stress response. Mol Cell 40, 280-293.doi:10.1016/j.molcel.2010.09.023

Kroemer, G., Morselli, E. Galluzzi, L.(2011) Oncosuppressive functions of autophagy. Antioxid Redox Signal.14(11):2251-69

Kummerow, C. Junker, K. Kruse, H. Rieger, A. Quintana, M. (2009). The immunological synapse controls local and global calcium signals in T lymphocytes. Immunol. Rev. 231:132-147.

Lam, D., Kosta, A., Luciani, M.F., and Golstein, P. (2008). The inositol 1,4,5-trisphosphate receptor is required to signal autophagic cell death. Mol Biol Cell 19, 691-700.doi:10.1091/mbc.E07-08-0823

Lamoureux, F., and Zoubeidi, A. (2013). Dual inhibition of autophagy and the AKT pathway in prostate cancer. Autophagy 9.doi:24921 [pii]

Lange, I., Yamamoto, S., Partida-Sanchez, S., Mori, Y., Fleig, A., and Penner, R. (2009). TRPM2 functions as a lysosomal Ca2+-release channel in beta cells. Sci Signal 2, ra23.doi:10.1126/scisignal.2000278

Lehen'kyi, V., Rapha¨el, M. and Prevarskaya, N. (2012). The role of the TRPV6 channel in cancer. The Journal of Physiology. 590(Pt 6):1369-76.

Lehen'kyi, V., Flourakis, M., Skryma, R., Prevarskaya, N. (2007). TRPV6 channel controls prostate cancer cell proliferation via Ca(2+)/NFAT-dependent pathways. Oncogene. 26(52):7380-5.

Levine, B., Abrams, J. (2008). p53: The Janus of autophagy? Nat Cell Biol. 10(6):637-9.

Lee, K., P. Yuan, J., P. Hong, J., H et al. (2010). An endoplasmic reticulum/plasma membrane junction: STIM1/Orai1/TRPCs.FEBS.584:2022-2027

Lee, H. C., (2011). Cyclic ADP-ribose and NAADP: fraternal twin messengers for calcium signaling. Life Science. 54(8):699-711.

Lemieux, B., Percival, M.D., and Falgueyret, J.P. (2004). Quantitation of the lysosomotropic character of cationic amphiphilic drugs using the fluorescent basic amine Red DND-99. Anal Biochem 327, 247-251.doi:10.1016/j.ab.2004.01.010

Li, J., Hou, N., Faried, A., Tsutsumi, S., and Kuwano, H. (2010). Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. Eur J Cancer 46, 1900-1909.doi:10.1016/j.ejca.2010.02.021

Liang, C., Feng, P., Ku, B., Oh, B.H., and Jung, J.U. (2007a). UVRAG: a new player in autophagy and tumor cell growth. Autophagy 3, 69-71

Liang, J., Shao, S.H., Xu, Z.X., Hennessy, B., Ding, Z., Larrea, M., Kondo, S., Dumont, D.J., Gutterman, J.U., Walker, C.L., Slingerland, J.M., and Mills, G.B. (2007b). The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 9, 218-224.doi:10.1038/ncb1537

Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402, 672-676.doi:10.1038/45257

Lilja, H., Schröder, F. H., Hugosson, J., Roobol, M. J., Tammela, T. L., Ciatto, S., Nelen, V., Kwiatkowski, M., Lujan, M., Zappa, M., Denis, L. J., Recker, F., Páez, A., Määttänen, L., Bangma, C. H., Aus, G., Carlsson, S., Villers, A., Rebillard, X., van der Kwast, T., Kujala, P. M., Blijenberg, B. G., Stenman, U. H., Huber, A., Taari, K., Hakama, M., Moss, S. M., de Koning, H. J., Auvinen, A. (2012). Prostate-cancer mortality at 11 years of follow-up. N Engl J Med. 366(11):981-90.

Lin, K. L., Lu, Y. C., Chen, I. S., Liu, S. I., Ho, C. M., Jan, C. R. (2011). Effect of thapsigargin on Ca^{2}+ fluxes and viability in human prostate cancer cells. J Recept Signal Transduct Res. 31(3):247-55.

Liou, J., Kim, M.L., Heo, W. D., Ferrell, J.E., Meyer T. (2005). STIM is a Ca21 sensor essential for Ca21–store–depletion–triggered Ca21 influx. Curr Biol 15:1235–1241.

Mallilankaraman, K., Cardenas, C., Doonan, P.J., Chandramoorthy, H.C., Irrinki, K.M., Golenar, T., Csordas, G., Madireddi, P., Yang, J., Muller, M., Miller, R., Kolesar, J.E., Molgo, J., Kaufman, B., Hajnoczky, G., Foskett, J.K., and Madesh, M. (2012). MCUR1 is an essential component of mitochondrial Ca2+ uptake that regulates cellular metabolism. Nat Cell Biol 14, 1336-1343.doi:10.1038/ncb2622

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. Nature 454, 436-444.doi:10.1038/nature07205

Marino, G., Salvador-Montoliu, N., Fueyo, A., Knecht, E., Mizushima, N., and Lopez-Otin, C. (2007). Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem 282, 18573-18583.doi:10.1074/jbc.M701194200

Mariot, P., Vanoverberghe, K., Lalevee, N., Rossier, M. F., Prevarskaya, N. (2002). Overexpression of an alpha 1H (Cav3.2) T-type calcium channel during neuroendocrine differentiation of human prostate cancer cells. J Biol Chem. 277(13):10824-33

Martens, S., Rusten, T. E., Kraft, C. (2013). Autophagy at sea. Autophagy. 9(9):1286-91.

Mathew, R., White, E. (2011). Autophagy, stress, and cancer metabolism: what doesn't kill you makes you stronger. Cold Spring Harb Symp Quant Biol. 76:389-96.

Mekahli, D., Bultynck, G., Parys, J. B., De Smedt, H., Missiaen, L. (2011). Endoplasmic-reticulum calcium depletion and disease. Cold Spring Harb Perspect Biol. 3(6).

Mizushima, N. (2010). The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol 22, 132-139.doi:10.1016/j.ceb.2009.12.004

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature 451, 1069-1075.doi:10.1038/nature06639

Monet, M., Lehen'kyi, V., Gackiere, F., Firlej, V., Vandenberghe, M., Roudbaraki, M., Gkika, D., Pourtier, A., Bidaux, G., Slomianny, C., Delcourt, P., Rassendren, F., Bergerat, J.P., Ceraline, J., Cabon, F., Humez, S., and Prevarskaya, N. (2010). Role of cationic channel TRPV2 in promoting prostate cancer migration and progression to androgen resistance. Cancer Res 70, 1225-1235.doi:10.1158/0008-5472.CAN-09-2205

Monteith, G.R., Davis, F.M., and Roberts-Thomson, S.J. (2012). Calcium channels and pumps in cancer: changes and consequences. J Biol Chem 287, 31666-31673.doi:10.1074/jbc.R112.343061

Monteith, G.R., Mcandrew, D., Faddy, H.M., and Roberts-Thomson, S.J. (2007). Calcium and cancer: targeting Ca2+ transport. Nat Rev Cancer 7, 519-530.doi:10.1038/nrc2171

Mori, A., Lehmann, S., O'kelly, J., Kumagai, T., Desmond, J.C., Pervan, M., Mcbride, W.H., Kizaki, M., and Koeffler, H.P. (2006). Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. Cancer Res 66, 3222-3229.doi:10.1158/0008-5472.CAN-05-0087

Muraki, K., Iwata, Y., Katanosaka, Y., Ito, T., Ohya, S., Shigekawa, M., and Imaizumi, Y. (2003). TRPV2 is a component of osmotically sensitive cation channels in murine aortic myocytes. Circ Res 93, 829-838.doi:10.1161/01.RES.0000097263.10220.0C

Negrini, S., Gorgoulis, V.G., and Halazonetis, T.D. (2010). Genomic instabilityan evolving hallmark of cancer. Nat. Rev. Mol. Cell Biol. 11, 220–228.

Nguyen, H.T., Lapaquette, P., Bringer, M.A., and Darfeuille-Michaud, A. (2013). Autophagy and Crohn's Disease. J Innate Immun.doi:10.1159/000345129

Nilius, B. (2007). TRP channels in disease. Biochim Biophys Acta 1772, 805-812.doi:10.1016/j.bbadis.2007.02.002

Nilius, B., Owsianik, G., Voets, T., and Peters, J.A. (2007). Transient receptor potential cation channels in disease. Physiol Rev 87, 165-217.doi:10.1152/physrev.00021.2006

Parys, J.B., Decuypere, J.P., and Bultynck, G. (2012). Role of the inositol 1,4,5-trisphosphate receptor/Ca2+-release channel in autophagy. Cell Commun Signal 10, 17.doi:10.1186/1478-811X-10-17

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927-939.doi:10.1016/j.cell.2005.07.002

Pedersen, S.F., Owsianik, G., and Nilius, B. (2005). TRP channels: an overview. Cell Calcium 38, 233-252.doi:10.1016/j.ceca.2005.06.028

Pedersen, S. F., Stock, C. (2013). Ion channels and transporters in cancer: pathophysiology, regulation, and clinical potential. Cancer Res. 73(6):1658-61.

Peppiatt, C.M., Collins, T.J., Mackenzie, L., Conway, S.J., Holmes, A.B., Bootman, M.D., Berridge, M.J., Seo, J.T., and Roderick, H.L. (2003). 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. Cell Calcium 34, 97-108

Pereira, G.J., Hirata, H., Fimia, G.M., Do Carmo, L.G., Bincoletto, C., Han, S.W., Stilhano, R.S., Ureshino, R.P., Bloor-Young, D., Churchill, G., Piacentini, M., Patel, S., and Smaili, S.S. (2011). Nicotinic acid adenine dinucleotide phosphate (NAADP) regulates autophagy in cultured astrocytes. J Biol Chem 286, 27875-27881.doi:10.1074/jbc.C110.216580

Perez,P. J., Ramos-Franco J., Fill, M., Mignery, G.A.(1997). Identification and functional reconstitution of the type 2 inositol 1,4,5-trisphosphate receptor from ventricular cardiac myocytes.J Biol Chem. 272:23961–23969.

Petersen, O.,H. Tepikin A., V et al. (2009).Fatty acids, alcohol and fatty acid ethyl esters: toxic Ca2+ signal generation and pancreatitis. Cell Calcium, 45: 634-642.

Prevarskaya, N., Zhang, L., Barritt, G. (2007). TRP channels in cancer. Biochim Biophys Acta. 1772(8):937-46

Prevarskaya, N., Skryma, R., and Shuba, Y. (2010). Ion channels and the hallmarks of cancer. Trends Mol Med 16, 107-121.doi:10.1016/j.molmed.2010.01.005

Prevarskaya, N., Skryma, R., and Shuba, Y. (2011). Calcium in tumour metastasis: new roles for known actors. Nat Rev Cancer 11, 609-618.doi:10.1038/nrc3105

Pochynyuk, O., Zaika, O., O'Neil, R. G., Mamenko, M. (2013). Novel insights into TRPV4 function in the kidney. Pflugers Arch. 465(2):177-86.

Qian, B.Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. Cell 141, 39–51.

Qiu, J., Tan, Y.W., Hagenston, A.M., Martel, M.A., Kneisel, N., Skehel, P.A., Wyllie, D.J., Bading, H., and Hardingham, G.E. (2013). Mitochondrial calcium uniporter Mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. Nat Commun 4, 2034.doi:10.1038/ncomms3034

Qu, X., Zhongju, Zou., Qihua, Sun., et al. (2007). Autophagy Gene-Dependent Clearance of Apoptotic Cells during Embryonic Development. Cell. 931-946.

Rah, S.Y., Mushtaq, M. Nam, T.S., Kim, S.H., Kim, U.H. (2010). Generation of cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate by CD38 for Ca2+ signaling in interleukin-8-treated lymphokine-activated killer cells. J Biol Chem.285(28):21877-88.

Rabinowitz, J. D., White, E. (2010). Autophagy Metabolism. Science. 330(6009):1344-8

Raimondi, S., Lowenfels, A.B., Morselli-Labate, A.M., Maisonneuve, P., and Pezzilli, R. (2010). Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. Best Pract Res Clin Gastroenterol 24, 349-358.doi:10.1016/j.bpg.2010.02.007

Ravikumar, B., Vacher, C., Berger, Z., Davies, J. E., Luo, S., Oroz, L. G., Scaravilli, F., Easton, D. F., Duden, R., O'Kane, C. J., Rubinsztein, D. C. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 36(6):585-95.

Ravikumar, B., Futter, M., Jahreiss, L., Korolchuk, V. I., Lichtenberg, M., Luo, S., Massey, D. C., Menzies, F. M., Narayanan, U., Renna, M., Jimenez-Sanchez, M., Sarkar, S., Underwood, B., Winslow, A. and Rubinsztein, D. C. (2009). Mammalian macroautophagy at a glance. Journal of Cell Science. 122(Pt 11):1707-11.

Ravikumar, B., Sarkar, S., Davies, J.E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z.W., Jimenez-Sanchez, M., Korolchuk, V.I., Lichtenberg, M., Luo, S., Massey, D.C., Menzies, F.M., Moreau, K., Narayanan, U., Renna, M., Siddiqi, F.H., Underwood, B.R., Winslow, A.R., and Rubinsztein, D.C. (2010). Regulation of mammalian autophagy in physiology and pathophysiology. Physiol Rev 90, 1383-1435.doi:10.1152/physrev.00030.2009

Razi, M., Chan, E.Y., and Tooze, S.A. (2009). Early endosomes and endosomal coatomer are required for autophagy. J Cell Biol 185, 305-321.doi:10.1083/jcb.200810098

Riedel, K.G., Zwaan, J., Kenyon, K.R., Kolodny, E.H., Hanninen, L., and Albert, D.M. (1985). Ocular abnormalities in mucolipidosis IV. Am.J.Ophthalmol. 99, 125-136

Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol 13, 566-578.doi:10.1038/nrm3412

Roderick, HL., Cook SJ. (2008). Calcium signalling checkpoints in cancer: Remodelling calcium for cancer cell proliferation and survival. Nat Rev Cancer. 8(5):361-75.

Rubinsztein, D.C., Codogno, P., and Levine, B. (2012). Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov 11, 709-730.doi:10.1038/nrd3802

Saito, M., Hanson, P.I., and Schlesinger, P. (2007). Luminal chloride-dependent activation of endosome calcium channels: patch clamp study of enlarged endosomes. J Biol Chem 282, 27327-27333.doi:10.1074/jbc.M702557200

Sakaki, K., Wu, J., and Kaufman, R.J. (2008). Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum. J Biol Chem 283, 15370-15380.doi:10.1074/jbc.M710209200

Sarkar, S., Floto, R.A., Berger, Z., Imarisio, S., Cordenier, A., Pasco, M., Cook, L.J., and Rubinsztein, D.C. (2005). Lithium induces autophagy by inhibiting inositol monophosphatase. J Cell Biol 170, 1101-1111.doi:10.1083/jcb.200504035

Selvakumaran, M., Amaravadi, R.K., Vasilevskaya, I.A., and O'dwyer, P.J. (2013). Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. Clin Cancer Res 19, 2995-3007.doi:10.1158/1078-0432.CCR-12-1542

Shapovalov, G., Lehen'kyi, V., Skryma, R., and Prevarskaya, N. (2011). TRP channels in cell survival and cell death in normal and transformed cells. Cell Calcium 50, 295-302.doi:10.1016/j.ceca.2011.05.006

Shapovalov, G., Skryma, R., Prevarskaya, N. (2013). Calcium channels and prostate cancer. Recent Pat Anticancer Drug Discov. 8(1):18-26

Siekmann,I.,Wagner, L.,Yule, D.,Crampin, E.,Sneyd, J., (2012). A kinetic model for type I and II IP3R accounting for mode changes .Biophys journal. 103(4):658-68

Singh, A., and Settleman, J. (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 29, 4741–4751.

Slaugenhaupt, S.A., Acierno, J.S., Jr., Helbling, L.A., Bove, C., Goldin, E., Bach, G., Schiffmann, R., and Gusella, J.F. (1999). Mapping of the mucolipidosis type IV gene to chromosome 19p and definition of founder haplotypes. Am.J.Hum.Genet. 65, 773-778.doi:S0002-9297(07)62330-1 [pii]

Soboloff, J., Rothberg, B. S., Madesh, M., Gill, D. L. (2012). STIM proteins: dynamic calcium signal transducers. Nat Rev Mol Cell Biol. 13(9):549-65.

Son, S.M., Jung, E.S., Shin, H.J., Byun, J., and Mook-Jung, I. (2012). Abeta-induced formation of autophagosomes is mediated by RAGE-CaMKKbeta-AMPK signaling. Neurobiol Aging 33, 1006 e1011-1023.doi:10.1016/j.neurobiolaging.2011.09.039

Sou, Y. S., Waguri, S., Iwata, J., Ueno, T., Fujimura, T., Hara, T., Sawada, N., Yamada, A., Mizushima, N., Uchiyama, Y., Kominami, E., Tanaka, K., Komatsu, M. (2008). The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. Mol Biol Cell. 19(11):4762-75.

Soyombo, A.A., Tjon-Kon-Sang, S., Rbaibi, Y., Bashllari, E., Bisceglia, J., Muallem, S., and Kiselyov, K. (2006). TRP-ML1 regulates lysosomal pH and acidic lysosomal lipid hydrolytic activity. J.Biol.Chem. 281, 7294-7301.doi:M508211200 [pii];10.1074/jbc.M508211200 [doi]

Strehler, E. E., Treiman, M. (2004). Calcium pumps of plasma membrane and cell interior. Curr Mol Med. 4(3):323-35.

Sudarsanam, S., and Johnson, D.E. (2010). Functional consequences of mTOR inhibition. Curr. Opin. Drug Discov. Devel. 13, 31–40.

Sumoza-Toledo, A., and Penner, R. (2011). TRPM2: a multifunctional ion channel for calcium signalling. J Physiol 589, 1515-1525.doi:10.1113/jphysiol.2010.201855

Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H.D., Sun, M., Sato, Y., Liang, C., Jung, J.U., Cheng, J.Q., Mule, J.J., Pledger, W.J., and Wang, H.G. (2007). Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat Cell Biol 9, 1142-1151.doi:10.1038/ncb1634

Takamura, A., Komatsu, M., Hara, T., Sakamoto, A., Kishi, C., Waguri, S., Eishi, Y., Hino, O., Tanaka, K., and Mizushima, N. (2011). Autophagy-deficient mice develop multiple liver tumors. Genes Dev 25, 795-800.doi:10.1101/gad.2016211

Talmadge, J.E., and Fidler, I.J. (2010). AACR centennial series: the biology of cancer metastasis: historical perspective. Cancer Res. 70, 5649–5669.

Tanida, I., Ueno, T., and Kominami, E. (2004). LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 36, 2503-2518.doi:10.1016/j.biocel.2004.05.009

Tanida, I., Minematsu-Ikeguchi, N., Ueno, T., Kominami, E. (2005). Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy. 1(2):84-91

Taylor, C. W., Genazzani, A. A., Morris, S. A. (1999). Expression of inositol trisphosphate receptors.Cell Calcium.26:237–251.

Thebault, S., Lemonnier, L., Bidaux, G., Flourakis, M., Bavencoffe, A., Gordienko, D., Roudbaraki, M., Delcourt, P., Panchin, Y., Shuba, Y., Skryma, R., and Prevarskaya, N. (2005). Novel role of cold/menthol-sensitive transient receptor potential melastatine family member 8 (TRPM8) in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells. J Biol Chem 280, 39423-39435.doi:10.1074/jbc.M503544200

Thebault, S., Flourakis, M., Vanoverberghe, K., Vandermoere , F., Roudbaraki, M., Lehen'kyi, V., Slomianny, C., Beck, B., Mariot, P., Bonnal, J. L., Mauroy, B., Shuba, Y., Capiod, T., Skryma, R., Prevarskaya, N. (2006). Differential role of transient receptor potential channels in Ca2+ entry and proliferation of prostate cancer epithelial cells. Cancer Res. 66(4):2038-47.

Toro, C.A., Arias, L.A., and Brauchi, S. (2011). Sub-cellular distribution and translocation of TRP channels. Curr Pharm Biotechnol 12, 12-23

Tsavaler, L., Shapero, M.H., Morkowski, S., and Laus, R. (2001). Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. Cancer Res 61, 3760-3769

Tsukamoto, S., Kuma, A., Murakami, M., Kishi, C., Yamamoto, A., Mizushima. N. (2008). Autophagy is essential for preimplantation development of mouse embryos. Science. 321(5885):117-20.

Turner, H., Fleig, A., Stokes, A., Kinet, J.P., and Penner, R. (2003). Discrimination of intracellular calcium store subcompartments using TRPV1 (transient receptor potential channel, vanilloid subfamily member 1) release channel activity. Biochem J 371, 341-350.doi:10.1042/BJ20021381.

Van der Vaart, A., Mari, M., Reggiori, F. (2008). A picky eater: exploring the mechanisms of selective autophagy in human pathologies. Traffic. 9(3):281-9.

Venkatachalam, K., Wong, C.O., and Montell, C. (2013). Feast or famine: role of TRPML in preventing cellular amino acid starvation. Autophagy. 9, 98-100.doi:22260 [pii];10.4161/auto.22260 [doi]

Venugopal, B., Mesires, N.T., Kennedy, J.C., Curcio-Morelli, C., Laplante, J.M., Dice, J.F., and Slaugenhaupt, S.A. (2009). Chaperone-mediated autophagy is defective in mucolipidosis type IV. J.Cell Physiol 219, 344-353.doi:10.1002/jcp.21676 [doi]

Vergarajauregui, S., Connelly, P.S., Daniels, M.P., and Puertollano, R. (2008). Autophagic dysfunction in mucolipidosistype IV patients. Hum.Mol.Genet. 17, 2723-2737.doi:ddn174 [pii];10.1093/hmg/ddn174 [doi]

Vicencio, J.M., Ortiz, C., Criollo, A., Jones, A.W., Kepp, O., Galluzzi, L., Joza, N., Vitale, I., Morselli, E., Tailler, M., Castedo, M., Maiuri, M.C., Molgo, J., Szabadkai, G., Lavandero, S., and Kroemer, G. (2009). The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1. Cell Death Differ 16, 1006-1017.doi:10.1038/cdd.2009.34

Wang, S.H., Shih, Y.L., Ko, W.C., Wei, Y.H., and Shih, C.M. (2008). Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway. Cell Mol Life Sci 65, 3640-3652.doi:10.1007/s00018-008-8383-9

Wertz, I.E., and Dixit, V.M. (2010). Regulation of death receptor signaling by the ubiquitin system. Cell Death Differ. 17, 14–24.

White, E. (2012). Deconvoluting the context-dependent role for autophagy in cancer. Nat Rev Cancer 12, 401-410.doi:10.1038/nrc3262

White, E., Karp, C., Strohecker, A.M., Guo, Y., and Mathew, R. (2010). Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol 22, 212-217.doi:10.1016/j.ceb.2009.12.008

Williams, A., Sarkar, S., Cuddon, P., Ttofi, E.K., Saiki, S., Siddiqi, F.H., Jahreiss, L., Fleming, A., Pask, D., Goldsmith, P., O'kane, C.J., Floto, R.A., and Rubinsztein, D.C. (2008). Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol 4, 295-305.doi:10.1038/nchembio.79

Wong, C.O., Li, R., Montell, C., and Venkatachalam, K. (2012a). Drosophila TRPML is required for TORC1 activation. Curr.Biol. 22, 1616-1621.doi:S0960-9822(12)00731-2 [pii];10.1016/j.cub.2012.06.055 [doi]

Wong, R., Steenbergen, C., and Murphy, E. (2012b). Mitochondrial permeability transition pore and calcium handling. Methods Mol Biol 810, 235-242.doi:10.1007/978-1-61779-382-0_15

Yang, Z. H., Wang, X. H., Wang, H. P., Hu, L. Q. (2003). Effects of TRPM8 on the proliferation and motility of prostate cancer PC-3 cells. Asian J Androl. 11(2):157-65.

Yang, S., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., Bause, A., Li, Y., Stommel, J.M., Dell'antonio, G., Mautner, J., Tonon, G., Haigis, M., Shirihai, O.S., Doglioni, C., Bardeesy, N., and Kimmelman, A.C. (2011a). Pancreatic cancers require autophagy for tumor growth. Genes Dev 25, 717-729.doi:10.1101/gad.2016111

Yang, Z.J., Chee, C.E., Huang, S., and Sinicrope, F.A. (2011b). The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther 10, 1533-1541.doi:10.1158/1535-7163.MCT-11-0047.

Yoshimori, T. Mizushima, N. (2007). How to interpret LC3 blotting. Autophagy. 3(6):542-555.

Yousefi, S., Perozzo, R., Schmid, I., Ziemiecki, A., Schaffner, T., Scapozza, L., Brunner, T., and Simon, H.U. (2006). Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol 8, 1124-1132.doi:10.1038/ncb1482

Yuan, T.L., and Cantley, L.C. (2008). PI3K pathway alterations in cancer: variations on a theme. Oncogene 27, 5497–5510

Yue, L., Peng, J. B., Hediger, M. A., Clapham, D. E. (2001). CaT1 manifests the pore properties of the calcium-release-activated calcium channel. Nature. 410(6829):705-9

Yue, Z., Jin, S., Yang, C., Levine, A.J., and Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A 100, 15077-15082.doi:10.1073/pnas.2436255100

Zalckvar, E., Berissi, H., Eisenstein, M., and Kimchi, A. (2009a). Phosphorylation of Beclin 1 by DAP-kinase promotes autophagy by weakening its interactions with Bcl-2 and Bcl-XL. Autophagy 5, 720-722

Zalckvar, E., Berissi, H., Mizrachy, L., Idelchuk, Y., Koren, I., Eisenstein, M., Sabanay, H., Pinkas-Kramarski, R., and Kimchi, A. (2009b). DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep 10, 285-292.doi:10.1038/embor.2008.246

Zeevi, D.A., Frumkin, A., Offen-Glasner, V., Kogot-Levin, A., and Bach, G. (2009). A potentially dynamic lysosomal role for the endogenous TRPML proteins. J Pathol 219, 153-162.doi:10.1002/path.2587

Zeevi, D.A., Lev, S., Frumkin, A., Minke, B., and Bach, G. (2010). Heteromultimeric TRPML channel assemblies play a crucial role in the regulation of cell viability models and starvation-induced autophagy. J Cell Sci 123, 3112-3124.doi:10.1242/jcs.067330

Zeng, X., Sikka, S. C., Huang, L., Sun, C., Xu, C., Jia, D., Abdel-Mageed, A. B., Pottle, J. E., Taylor, J. T., Li, M. (2010). Novel role for the transient receptor potential channel TRPM2 in prostate cancer cell proliferation. Prostate Cancer Prostatic Dis. 13(2):195-201.

Zhang, L., and Barritt, G.J. (2004). Evidence that TRPM8 is an androgen-dependent Ca2+ channel required for the survival of prostate cancer cells. Cancer Res 64, 8365-8373.doi:10.1158/0008-5472.CAN-04-2146

Zhang, X., Mak, S., Li, L., Parra, A., Denlinger, B., Belmonte, C., and Mcnaughton, P.A. (2012). Direct inhibition of the cold-activated TRPM8 ion channel by Galphaq. Nat Cell Biol 14, 851-858.doi:10.1038/ncb2529

Zhineng, W., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., Bause, A., Li, Y., Stommel, J. M., Dell'antonio G., et al. (2011). Pancreatic cancers require autophagy for tumor growth. Genes Dev. 25:717-29.