Laboratory of Cell Physiology
Institute of Neuroscience
Catholic University of Louvain
Brussels
Belgium

Novel insights into the development of hormone resistance phenotype of prostate cancer cells.

Benoît Boutin

Promoter: Pr. Philippe Gailly
Co-promoter: Pr. Nicolas Tajeddine

Chairwoman: Pr. Sonia Brichard
Jury members:
Pr. Pedro Buc Calderon
Pr. Pierre Courtoy
Pr. Jean-Christophe Jonas
Pr. Jan Parys
Pr. Natalia Prevarskaya

UCL
Université catholique de Louvain

PhD thesis in biomedical sciences

Remerciements

Avant tout, je désire remercier le professeur Philippe Gailly pour m'avoir accueilli dans son équipe et m'avoir permis de réaliser ce travail de thèse à ses côtés. Je souhaite le remercier pour sa disponibilité et tout ce qu'il s'est efforcé de me transmettre au cours de ces dernières années.

Je voudrais également remercier mon co-promoteur, le professeur Nicolas Tajeddine, de m'avoir fait bénéficier de ses nombreuses idées et de sa maîtrise de la littérature, ainsi que le professeur Jean Lebacq pour ses conseils divers et fort appréciables.

Je souhaite remercier les membres de mon jury, le professeur Sonia Brichard, la présidente de ce jury, le professeur Pierre Courtoy, le professeur Jean-Christophe Jonas et le professeur Pedro Buc Calderon pour le temps qu'ils m'ont consacré au cours de cette thèse et pour leurs conseils avisés. Merci également au professeur Jan Parys et au professeur Natalia Prevarskaya d'avoir accepté de prendre part à ce jury chargé d'examiner ma thèse.

Je tiens à remercier Didier Vertommen, Patrick Vandersmissen, Giovanni Monaco et Geert Bultynck pour leur aide précieuse dans la réalisation de nos projets de recherche.

Je voudrais également saluer tous les membres de l'équipe FYCL que j'ai pu côtoyer sur ces dernières années et remercier plus particulièrement Nadège à qui je dois beaucoup, Anneleos et Ludivine, sans lesquelles la pizza ou les bières du vendredi soir auraient perdu toute saveur (enfin, celles que mon fragile estomac pouvait supporter…), le porte-bonheur Xavier, le sémillant Olivier mais aussi Marie et Monique pour leurs coups de main répétés, Nicolas, François, Noémie, Sophie, Hafsa, Fabienne, Pierre, Clélia et le professeur Nicole Morel. Travailler en leur compagnie a bien souvent été un réconfort face aux obstacles et aux difficultés rencontrés au cours de ces années de recherche.

Enfin, je voudrais remercier mes parents ainsi que Cécile, Catherine et Christine pour leur indéfectible soutien et leur compréhension.

A toutes et à tous, merci.

Table of contents

List of abbreviations

AD : androgen deprivation
ADP : adenosine diphosphate
Akt : akt serine/threonine kinase
AIF : apoptosis-inducing factor
Ambra1 : activating molecule in Beclin-1-regulating autophagy protein 1
AMP : adenosine monophosphate
AMPK : 5' adenosine monophosphate-activated protein kinase
AND : other androgenic steroidal precursor
ANT : adenine nucleotide transporter
APAF-1 : apoptotic peptidase activating factor 1
AR : androgen receptor
ARE : androgen response element
ATP : adenosine triphosphate
ATF4 : activating transcription factor 4
ATF6 : activating transcription factor 6
Atg : autophagy-related gene
ATP : adenosine triphosphate
Bad : Bcl-2-associated death promoter
Bak : Bcl-2 homologous antagonist killer
BAPTA-AM : 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid - acetomethyl
Bax : Bcl-2-associated X protein
Bcl-2 : B-cell lymphoma-2
Bcl-w : Bcl-2-like protein 2
Bcl-xL : B-cell lymphoma-extra large
BH : Bcl-2 homology domain
BI-1 : Bax inhibitor-1
Bid : BH3 interacting-domain death agonist
Bif-1 : Bax-interacting factor-1
Bik : Bcl-2 interacting killer
Bim : Bcl-2 interacting protein
BiP : binding immunoglobulin protein (= GRP78)
BRCA1 : breast cancer 1
BPH : benign prostatic hyperplasia
CAD : caspase-activated DNase
CALHM1 : calcium homeostasis modulator 1
CaMKII : Ca^{2+}/Calmodulin dependent kinase II
CAMKK : Ca^{2+}/Calmodulin dependent protein kinase kinase
cAMP : cyclic adenosine monophosphate
CDK : cyclin-dependent kinase
CDKN2A : cyclin-dependent kinase inhibitor 2A
CHOP : CCAAT/-enhancer-binding protein homologous protein
CMA : chaperone-mediated autophagy
DAG : diacylglycerol
DAPK-1 : death-associated protein kinase 1
DARPP-32 : dopamine and cAMP-regulated phosphoprotein of 32 kDa
DHEA : dehydroepiandrosterone
DHT : dihydrotestosterone

DIABLO : direct inhibitor of apoptosis/binding protein with low pI (= SMAC)
DISC : death-inducing signaling complex
DRAM : damage-regulated autophagy modulator
$\Delta\Psi_m$: mitochondrial transmembrane potential
4EBP-1 : eukaryotic translation initiation factor 4E binding protein-1
eEF-2 kinase : kinase of the eukaryotic elongation factor 2
eIF2α : eukaryotic initiation factor 2α
eIF4E : eukaryotic initiation factor 4E
ER : endoplasmic reticulum
ERAD : ER-associated degradation
Erk : extracellular signal-regulated kinase
FADD : Fas-associated protein with death domain
FoxA1 : A-class of Forkhead box factor
GABARAP : Gamma-aminobutyric acid A receptor-associated protein
GATA2 : GATA binding protein 2
GATE-16 : Golgi-associated ATPase Enhancer of 16 kDa
GnRH : gonadotropin-releasing hormone
GPCR : G protein-coupled receptor
GRP75 : 75 kDa glucose regulation protein
GRP78 : 78 kDa glucose regulation protein (= BiP)
GTPase : guanosine triphosphate hydrolase
HDAC : histone deacetylase
HRPCa : hormone-refractory prostate cancer
HTRA2 : high temperature requirement protein A2
IAP : inhibitors of apoptosis proteins
IICR : IP3-induced Ca^{2+} release
IP3 : inositol trisphosphate
IP3R : inositol trisphosphate receptor
IRBIT : IP3R-binding protein released by IP3
IRE1 : inositol requiring enzyme 1
ITPR : inositol trisphosphate receptor gene
Jnk1 : c-Jun N-terminal kinase
LAMP-2 : lysosomal-associated membrane protein 2
LC3 : microtubule-associated protein light chain 3
MAM : mitochondria-associated membranes
MAP1B : microtubule-associated protein 1B
MAPK : mitogen-activated protein kinase
Mcl-1 : induced myeloid leukemia cell differentiation protein
MCU : mitochondrial Ca^{2+} uniporter
MED1 : mediator complex subunit 1
MOMP : mitochondrial outer membrane permeabilization
mTORC1 : mammalian target of rapamycin complex 1
NAADP : nicotinic acid adenine dinucleotide phosphate
NCoR : nuclear receptor corepressor
NCX : $Na+/Ca^{2+}$ exchanger
Nix : Bcl2/adenovirus E18 interacting protein 3-like
NOXA : Phorbol-12-myristate-13-acetate-induced protein 1
NBR1 : neighbour of BRCA1 gene
ORAI : calcium release-activated calcium modulator
$p14^{ARF}$: alternative reading frame protein product of CDKN2A locus

$p16^{INK4a}$: cyclin-dependent kinase inhibitor 2A (inhibitor of cyclin-dependent kinase 4)
$p21^{Cip1}$: cyclin-dependent kinase inhibitor 1A (CDK-interacting protein 1)
$p27^{Kip1}$: cyclin-dependent kinase inhibitor 1B
PAS : pre-autophagosomal structure
PARP1 : poly(ADP-ribose) polymerase-1
PCa : prostate cancer
PDK1 : phosphoinositide-dependent kinase-1
PERK : protein kinase RNA-like endoplasmic reticulum kinase
PI3K : phosphoinositide 3-kinase
PIP : phosphatidylinositol-3-phosphate
PIP2 : phosphatidylinositol-4,5-bisphosphate
PIP3 : phosphatidylinositol-3,4,5-trisphosphate
PKA : cyclic AMP-dependent protein kinase
PKB : protein kinase B
PKC : protein kinase C
PKG : cyclic GMP-dependent protein kinase
PLC : phospholipase C
PMCA : plasma-membrane Ca^{2+}-ATPase
PML : promyelocytic leukemia protein
PP1 : protein phosphatase 1
PRAS40 : proline-rich Akt substrate of 40 kDa
PSA : prostate specific antigen
p70S6K : p70 ribosomal protein S6 kinaseF
PTEN : phosphatase and tensin homolog
PTPC : permeability transition pore complexe
PUMA : p53 upregulated modulator of apoptosis
Rab7 : Ras-related GTP-binging protein 7
Raf-1 : rapidly accelerated fibrosarcoma kinase-1
Raptor : regulatory-associated protein of mTOR
Rb : retinoblastoma tumor suppressor protein
Rheb : Ras homolog enriched in brain
RIPK : receptor-interacting protein kinase
ROC : receptor-operated channel
ROS : reactive oxygen species
RTK : receptor tyrosine kinase
RyR : ryanodine receptor
SA-βgal : senescence-associated β-galactosidase
SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA : sarco/endoplasmic reticulum Ca^{2+} ATPase
shRNA : small hairpin ribonucleic acid
siRNA : small interfering ribonucleic acid
Skp2 : S-phase kinase-associated protein 2
SMAC : second mitochondria-derived activator of caspases (DIABLO)
SMOC : second-messenger-operated channel
SOC : store-operated channel
SOCE : store-operated calcium entry
SQTM1 : sequestosome-1
SRC : steroid receptor coactivator
STIM1 : stromal interaction molecule-1
TAT : transactivator of transcription

TGFβ1: transforming growth factor beta-1
TNFα : tumor necrosis factor α
TRAIL : tumor-necrosis-factor related apoptosis inducing ligand
TRP : transient receptor potential
TAF1 and 2 : TRP channel-associated factors 1 and 2
tBid : truncated Bid
TKO : triple knock out
TM : transmembrane
TNFα : tumor necrosis factor α
TNM : Tumor/Node/Metastasis
TRAIL : TNF-related apoptosis-induced ligand
TSC1 : tuberous sclerosis complex-1
TSC2 : tuberous sclerosis complex-2
Ulk-1 : Unc-51-like kinase-1
UPR : unfolded protein response
UPS : ubiquitin-proteasome system
UVRAG : UV radiation resistance-associated gene
VDAC : voltage-dependent anion channel
VIP : vasoactive intestinal peptide
VOC : voltage-operated channel
ZIP9 : zinc-regulated transporters and iron-regulated transporter-like protein 9

Introduction

1 - The prostate cancer

The prostate is an exocrine gland of the male reproductive system located in the pelvis just below the bladder and traversed by the urethra and the ejaculatory ducts (Fig. 1). It has the dimension of a chestnut and weighs approximately 20 grams in adulthood. Its main function is to secrete a part of the seminal fluid, a constituent of the sperm. It also contributes to the proper functioning of ejaculation. The prostate is composed of tubulo-alveolar glands and smooth muscle cells surrounded by a fibro-elastic capsule. The integrity of this prostatic capsule is a crucial element to consider in prostate cancer (PCa) diagnosis. Cancers that are fully enclosed by this capsule are called localized, and those whose cancer cells go beyond the capsule are at least locally advanced (classified as T3 in the TNM staging system). The vast majority of cases of PCa develop from the glandular epithelium and therefore are adenocarcinomas [1].

PCa is the most frequent cancer of men above the age of 50 years in the European Union (Fig. 2). More than 340,000 cases and 70,000 deaths have been reported in 2012 in Europe [2]. PCa development can be monitored by the measurement of serum prostate specific antigen (PSA) levels, a peptidase whose expression is regulated by the androgen receptor (AR). Despite radical prostatectomy or radiation therapy as primary treatment, rising PSA values are still observed in approximately 15-30% of patients and are related to an advanced or metastatic cancer requiring systemic therapies [3]. Like normal prostate cells, PCa cells depend on androgens for their survival and growth. Charles Huggins was awarded the 1966 Nobel Prize for discovering that removal of androgens triggers their death [4]. Thus, reference treatment of advanced PCa relies on pharmacological or surgical androgen deprivation (AD) therapy [5].

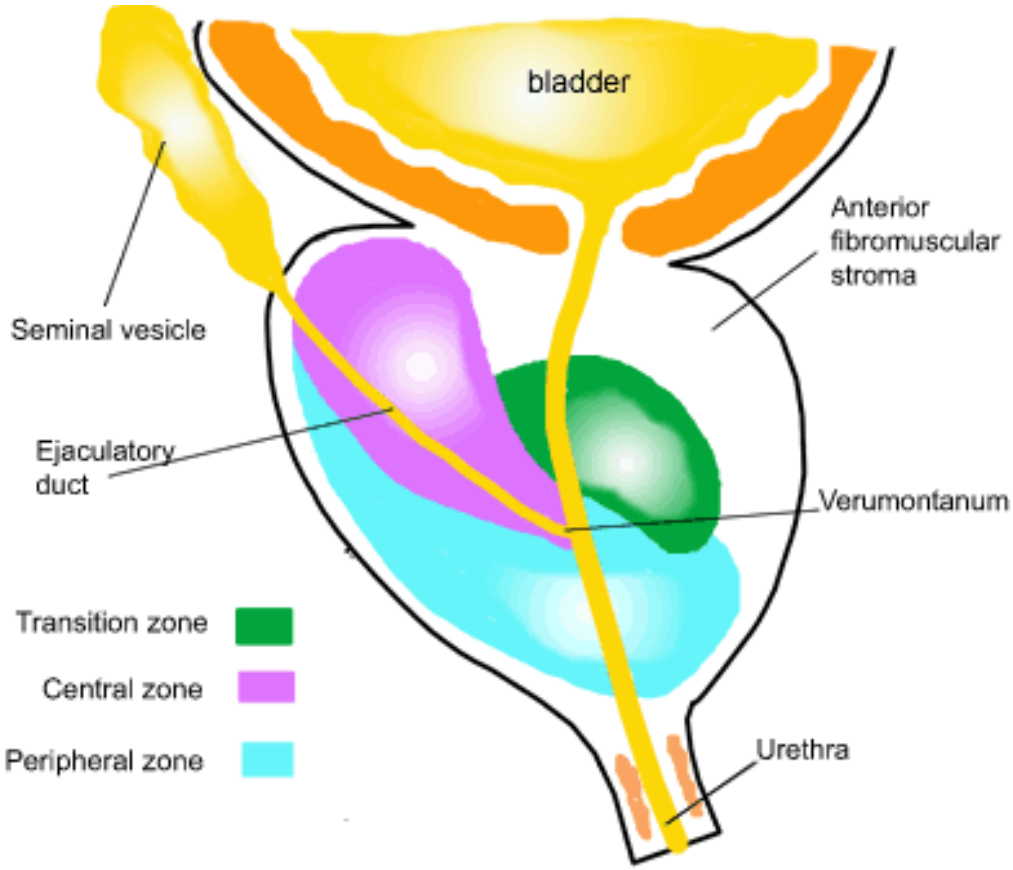

Fig. 1: The prostate gland and its different zones. From [6]
Under the bladder, the prostate is traversed by the urethra and the ejaculatory ducts. It comprises four different zones: the transition zone, the central zone, the peripheral zone and the anterior fibromuscular stroma. The transition zone, in contact with the urethra, is the place where benign prostatic hyperplasia (BPH) develops. BPH is encountered in the majority of men after 50 years and may result into an increase in volume of a portion of prostate but does not always require treatment. The peripheral zone, easily palpable on rectal examination, is the place where cancer frequently (but not exclusively) develops [1].

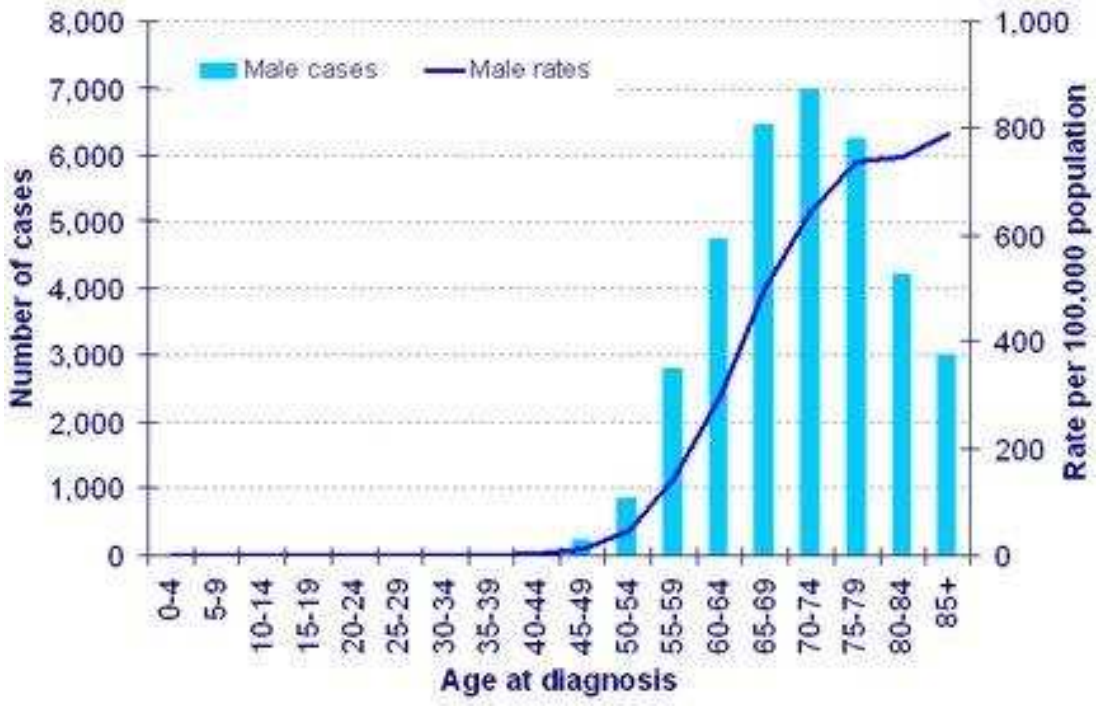

Fig. 2: Number of new cases and age specific incidence rates of PCa in the United Kingdom in 2006. From [7]
PCa is the most common cancer among men in developed countries. PCa risk is strongly related to age.

2 - The androgen receptor

In prostatic cells, testosterone is converted by the 5α-reductase to dihydrotestosterone (DHT) that has a greater affinity for the AR [8]. DHT binds to AR which functions as an intracellular transcriptional factor. It comprises an N-terminal modulatory domain, a DNA-binding domain and a ligand-binding domain. In its unbound conformation, AR, mainly located in the cytoplasm, is associated with a complex of heat shock proteins. The androgen binding releases AR from the complex and leads to its homodimerization and its translocation to the nucleus. Translocated AR binds to androgen response element (ARE) sequences on the promoter/enhancer regions of AR target genes. It recruits coregulators (including coactivators and corepressors) and the transcriptional machinery to allow the expression of androgen-responsive genes [9]. In PCa cells, AR regulates the expression of numerous genes involved in transcription, splicing, ribosomal biogenesis, cell growth and bioenergetics processes [10].
Besides, steroid hormones can activate multiple signaling cascades within minutes (Fig. 3). These rapid androgen effects do not require the nuclear translocation of AR but rather depend on non-genomic activities of AR located in the cytoplasm and/or tethered to the plasma

membrane [11, 12]. They have especially been observed in PCa cells, in which AR splice variants without DNA-binding domain and primarily associated to the membrane were also identified [13]. In these cells, androgens stimulate the phosphoinositide 3-kinase (PI3K), the kinase Raf-1 and the protein kinase C (PKC), which results in activation of Erk-1 and -2 (extracellular signal-regulated kinases-1 and -2 also called mitogen-activated protein kinases or MAPKs) [14]. The activation of the MAPK pathway also depends on an association of AR with oestradiol receptor β and Src kinase and promotes cell proliferation [15]. Furthermore, androgens have been shown to activate the prosurvival PI3K/Akt pathway by enhancing an AR binding to p85, the regulatory subunit of class Ia PI3K, in the PCa-derived LNCaP cell model [16] and in vas deferens epithelial cells [17]. An interaction of Akt with AR located into lipid rafts microdomains is also stimulated by androgens and prevented by the AR antagonist bicalutamide in LNCaP cells [18]. Besides, androgens can induce a rapid Ca^{2+} influx via L-type calcium channels in the same cell line. The intracellular Ca^{2+} increase does not appear to be mediated by classical AR, but rather through a G protein-coupled receptor (GPCR) or a receptor associated with a GPCR [19]. Indeed, the androgen non-genomic activities seem also related to unconventional receptors localized on the plasma membrane and which cannot be blocked by AR antagonists. The stimulation of these membrane-bound receptors seems to result in anti-tumor responses. Non-internalizable testosterone-BSA conjugates activate signaling cascades leading to a reorganisation of the actin cytoskeleton, the cell growth suppression and the apoptotic regression of PCa cells [20, 21]. The exact molecular identity of these unconventional receptors had long been remained elusive. Recently, such a membrane receptor was discovered in the ZIP9 zinc transporter subfamily. Its activation by testosterone induces zinc influx and triggers apoptosis in prostate and breast cancer cells [22].

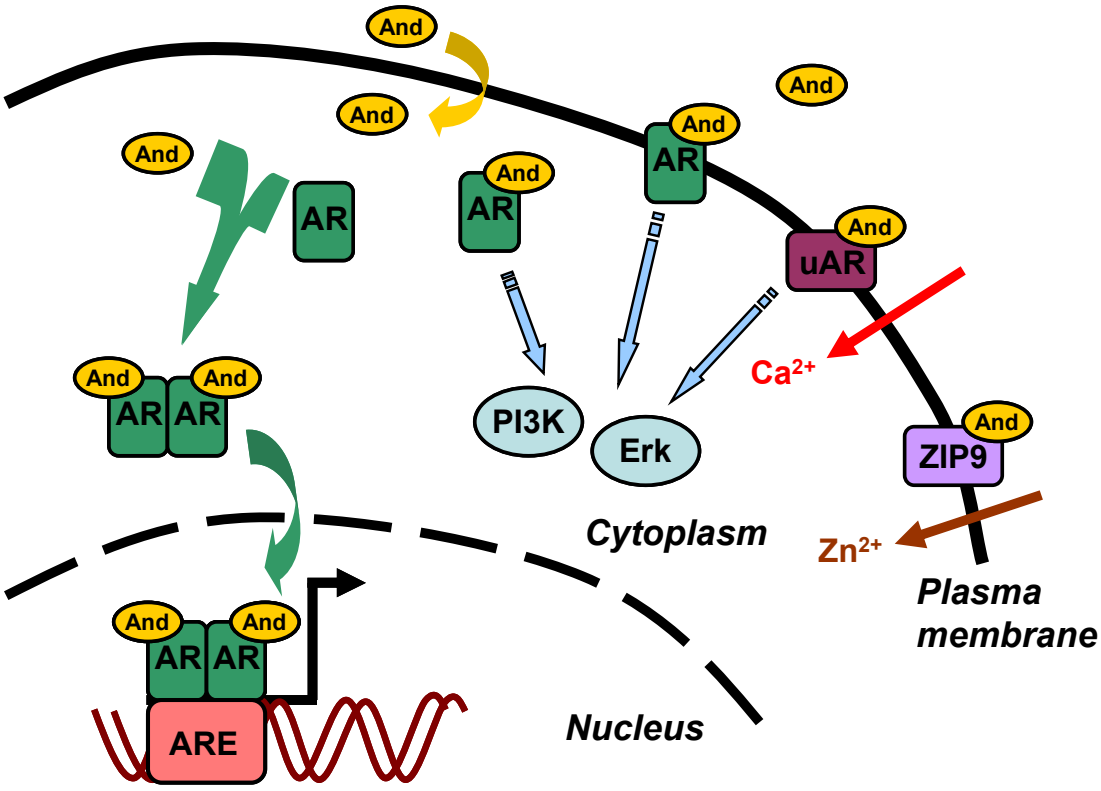

Fig. 3: Genomic and non-genomic AR activities in PCa.
Androgens (And) can cross the plasma membrane and interact with cytoplasmic androgen receptor (AR). This leads to the dimerization of AR and its translocation to the nucleus. Translocated AR recognizes androgen response element (ARE) sequences on the promoter/enhancer regions of AR target genes and promotes their expression. Besides, androgens can have rapid non-genomics effects. Activated AR in the cytoplasm and/or at the plasma membrane can interact with several signaling molecules and trigger multiple signaling cascades including the PI3K and MAPK/Erk pathways, thereby stimulating the cell growth. Oppositely, activation of membrane-bound unconventional ARs (uARs) can elevate $[Ca^{2+}]_c$ and modulate the activity of several signaling pathways, which results in the PCa regression. The stimulation by androgens of ZIP9 protein, one of these uARs, leads to Zn^{2+} entry and cell death.

The genomic and non-genomic activities of AR are therefore multiple in PCa cells, regulating cell survival as well as cell growth. Despite the anti-tumoral effects of the activation of some types of membrane AR, the suppression of androgenic stimulation seems the best strategy to counter the development of advanced PCa.

3 - The androgen deprivation therapy

Strategies of AD therapy are diverse and can be combined. They include orchiectomy (which irreversibly reduces the level of androgen in the blood), the use of AR antagonists such as flutamide, bicalutamide and enzalutamide (which compete with androgens for binding to the AR) and the use of GnRH antagonists as well as GnRH agonists.

GnRH antagonists compete with GnRH for binding to its receptor in the pituitary gland, which limits the secretion of luteinizing hormone and consequently of androgen. GnRH agonists have a longer biological half-life than normal GnRH and their continued presence at high levels initially results in an excessive production of luteinizing hormone by the pituitary gland and in turn of testosterone by the testicles. However, chronic exposure to GnRH agonists eventually leads to a downregulation of GnRH receptors with subsequent suppression of luteinizing hormone secretion and testosterone production. A castrate testosterone level is usually achieved within 2 to 4 weeks after injection of GnRH agonists [23].

4 - Mechanisms of hormone resistance in PCa

The blockade of AR activities results in tumor regression. However, despite this initial efficacy of AD, most PCa will adapt to low testosterone environment becoming hormone-refractory (HRPCa) [24]. Until recently, HRPCa progression was considered as a necessarily lethal event, mainly characterised by the development of bone metastases and their complications. Since 2004, two drugs have been improving the care of patients: docetaxel, an anti-microtubule agent, resulting in prolonged survival by about three months, and zoledronic acid, a bone-protecting bisphophonate, which delays the onset of skeletal-related events [25]. However, benefits of this additional treatment remain modest. A better understanding of the phenomenon of androgen independence is necessary to ameliorate the management of HRPCa. Some mechanisms explaining the progression to HRPCa have already been identified (Fig. 4).

AR keeps playing a major role in the cancer growth despite androgen depletion. Mutations or amplifications of the AR have been observed, thereby increasing its sensitivity to androgen or permitting its activation by other ligands including estrogens, corticosteroids and progesterone. Moreover, members of the family of steroid receptor coactivators (SRC) are often overexpressed. The activities of numerous AR coactivators such as MED1, FoxA1 and GATA2 are stimulated whereas those of the nuclear receptor corepressor (NCoR) are reduced [26]. Besides, growth factors, via their binding with receptor tyrosine kinases, can activate PI3K/Akt or MAPK/Erk signaling pathways leading to AR activation independently of steroid interaction. Interleukins and neuropeptides have also been noticed to produce the same effect [27]. Thereby, transcriptional activities of the AR can be stimulated by its phosphorylation despite the absence of ligand [28].

Even in the absence of constitutive activation of AR-dependent pathways, PCa cells can acquire the ability to synthesize androgen from cholesterol which limits the benefits of androgen level reduction in cancerous tissues despite castration. This makes necessary the use of steroids synthesis inhibitors, which prevent PCa cells as well as testicles and adrenal glands from producing androgens by inhibiting the family of cytochrome P450 enzymes [29].

However, the AR pathway is sometimes bypassed, other signaling pathways being deregulated. Stimulation of the insulin-like growth factor pathway and overexpression of prosurvival chaperone proteins in response to androgen ablation are involved in androgen independence [30, 31]. The loss of phosphatase PTEN (phosphatase and tensin homolog) often occurs during the progression of PCa, thereby stimulating pro-survival PI3K/Akt pathway [32]. Upregulation of anti-apoptotic molecules such as Bcl-2 and Bcl-xL has also been reported to participate to androgen resistance of PCa [33, 34]. Besides, mutations of p53 tumor suppressor often observed in HRPCa cells contribute to their growth in the absence of androgen [35].

Moreover, a neuroendocrine differentiation is favored. Indeed, neuroendocrine cells become more prevalent after long-term AD. They present dense core granules in their cytoplasm together with extensions of neuron-like processes, are nonmitotic and do not express the AR. However, they produce various neuropeptides such as chromogranin A and neurotensin, which have been shown to stimulate proliferation of the surrounding androgen-independent cancer cells [36].

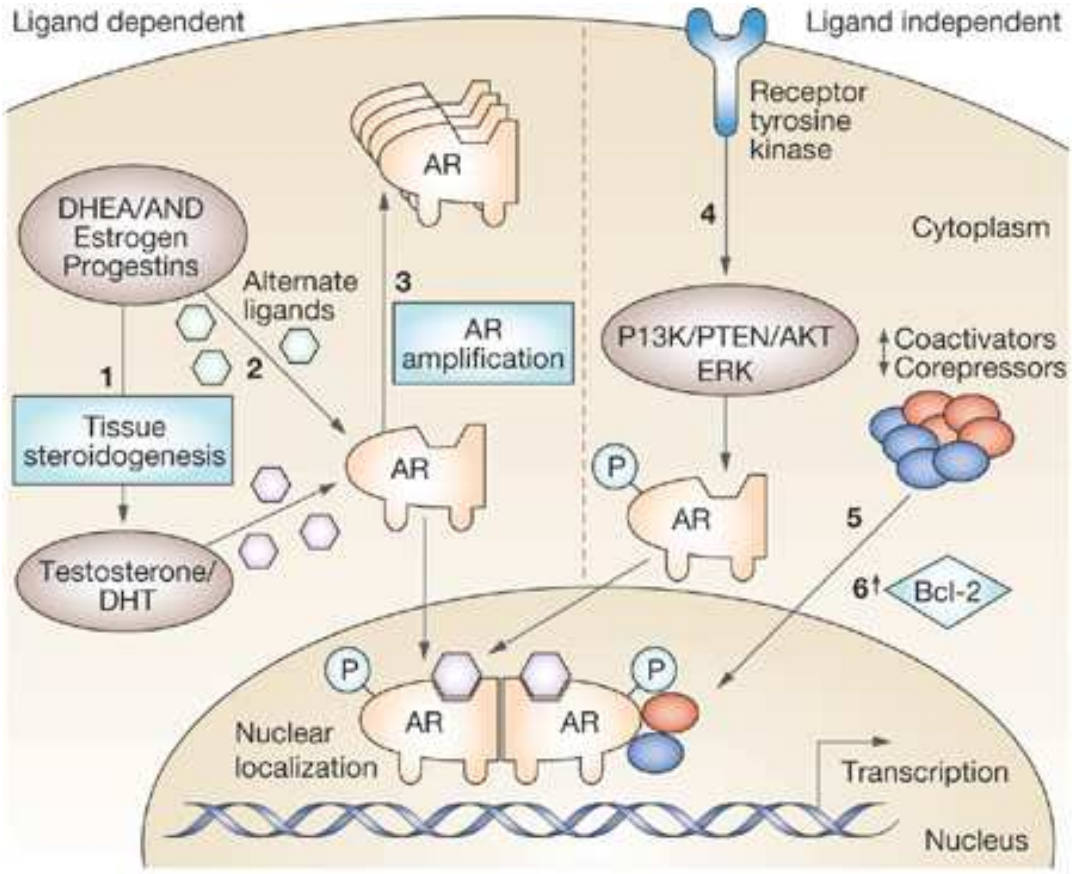

Fig. 4: Mechanisms of hormone resistance and AR deregulation in PCa. From [37]
Ligand-dependent and ligand-independent mechanisms involved in HRPCa progression can be distinguished : (**1**) acquisition by the PCa cells of the ability to synthesize androgen from steroidal precursors ; (**2**) mutations of the AR increasing its sensitivity to androgen or allowing its activation by alternate ligands ; (**3**) amplification of the AR, enhancing its activity ; (**4**) ligand-independent activation of AR through cross-talk with other signaling pathways ; (**5**) stimulation of the AR coactivators and inhibition of the AR corepressors enhancing its transcriptional activity ; (**6**) deregulation of other signaling pathways and overexpression of anti-apoptotic molecules, such as Bcl-2. (Abbreviations: AKT, akt serine/threonine kinase; AND, other androgenic steroidal precursors; AR, androgen receptor; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; ERK, extracellular signal-regulated kinase; P, phosphorylated residues; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; Bcl-2, B-cell lymphoma 2.)

5 - Cell growth and AD therapy

Tumor regression induced after AD is dependent on apoptosis in a subset of PCa cells but also on cell cycle arrest in others [38].

Progression within the cell cycle is allowed by the ordered activation of cyclin-dependent kinases (CDKs) combined to their associated cyclins, the latter controlling the catalytic activity of kinases [39]. AD leads to the reduced activity of CDK2 and 4 and the hypophosphorylation of their downstream substrate, the retinoblastoma tumor suppressor protein (Rb), resulting in G_0-G_1 cell cycle arrest. The inhibition of CDK2 and 4 is related to a decrease of cyclins A and D levels, to an overexpression of $p27^{Kip1}$, a CDK2 inhibitor, and to a downexpression of $p21^{Cip1}$ [40, 41]. Originally identified as CDK inhibitor, $p21^{Cip1}$, an androgen-responsive gene product [42], also participates to the formation of CDK4-cyclin D complexes [43, 44].

AR therefore governs the G_1-S transition (Fig. 5). Androgens indirectly control cyclins D levels by promoting the expression of numerous genes favoring nutrient availability that activates the energy sensor mTOR (mammalian target of rapamycin) and stimulates the translation of cyclins D proteins [45]. Activation of AMPK (5' adenosine monophosphate-activated protein kinase) by androgens and subsequent enhancement of mitochondrial function seem also participate in the stimulation of cell growth [46].

It can be noted that while AD activates Rb by suppressing its phosphorylation, thereby enabling it to repress the expression of genes required for S-phase entry [47], Rb expression is often altered in advanced PCa [48]. Rb inhibition induces cell proliferation in the absence of androgenic stimulation [49], suggesting that Rb deficiency may participate to the development of hormone resistance phenotype.

Unfortunately, long-term AD results in the outgrowth of AD-resistant clones from the initially growth arrested population [50]. Surprisingly, androgens induce G1 cell cycle arrest in such castration-resistant PCa cells through the accumulation of $p27^{Kip1}$, the inhibition of CDK2 activity and the lowered expression of cyclin A and S-phase kinase-associated protein 2 (Skp2) required for $p27^{Kip1}$ degradation [51, 52]. Interestingly, an intermittent AD (a succession of AD periods and off-therapy intervals) may delay the definitive acquisition of an androgen-independent growth [53, 54].

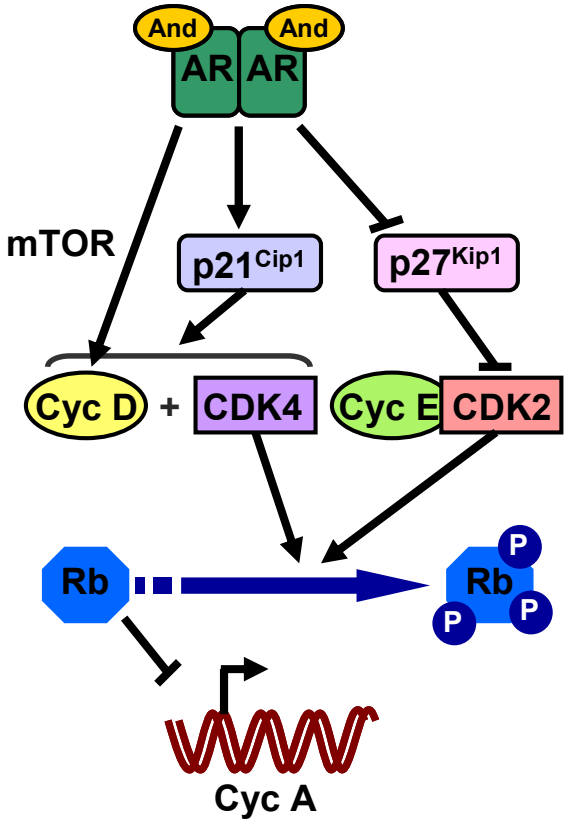

Fig. 5: The regulation of cell cycle by AR. Adapted from [41]
Activated AR induces the accumulation of cyclins D through mTOR stimulation, which results in CDK4 activation. Besides, androgen-induced $p21^{Cip1}$ expression and $p27^{Kip1}$ degradation improve the activity of cyclin D/CDK4 and cyclin E/CDK2 complexes, phosphorylating the retinoblastoma tumor suppressor protein Rb. Phosphorylated Rb is inactivated and cannot impede the expression of genes required for G_1-S transition including cyclin A, which allows the progression within the cell cycle.

Furthermore, high concentrations of testosterone or DHT (low nanomolar) also repress cell proliferation of androgen-sensitive PCa cells [55, 56]. In fact, androgen-stimulated response appears to be biphasic. At low levels, increasing androgen concentration promotes a dose-dependent cell growth whereas at higher concentrations, proliferation is inhibited. The optimal androgen level varies depending on the cellular model. These data, the anti-tumoral effects of membrane AR activation (see above) and the fact that high serum testosterone levels do not seem to increase the risk of PCa as well as the tumor aggressivity [57] have changed minds on the role of androgens in PCa development. Without questioning the interest of AD to block the PCa progression, testosterone therapies are now considered. Clinical trials have even been conducted [58-60].

6 - Cellular senescence and AD therapy

Cell cycle arrest encountered after AD was recently investigated in more detail and senescence characteristics were identified [61-63]. Cellular senescence is an irreversible arrest of cell growth triggered in response to diverse stimuli including genomic and epigenomic damages, oncogenic stimulation and telomere shortening (reviewed in [64]). Senescence restricts the replicative life span of cells and impedes uncontrolled cell division. In addition of its definitive growth arrest, a senescent cell is characterised by an increase in volume and, if adherent, a flattened morphology. Moreover, several markers allow its detection such as the senescence-associated β-galactosidase (SA-βgal), the $p16^{INK4a}$ tumor suppressor protein and a persistent DNA damage response signaling. Besides, a senescent cell, which remains metabolically active, secretes various molecules including cytokines, growth factors and proteases to modulate the proliferation of the surrounding cells, to promote inflammation and to prepare the tissue for repair.

A subset of PCa cells submitted to AD presents *in vitro* and *in vivo* senescence-associated changes, among which a stable cell cycle arrest, a modification of their phenotype, the expression of molecular markers of senescence and the secretion of cytokines and proteases [61, 62]. A senescent arrest is seen as a mechanism of tumor suppression. However, AD-induced senescence in PCa cells seems to contribute to their survival and to promote the clonal expansion of a senescence-resistant androgen-refractory subpopulation through the secretion of paracrine factors [65].

It can be noticed that the cell cycle arrest triggered by high concentrations of androgen is also a form of cellular senescence [66]. The latter is rapidly induced through the stimulation of the PI3K/Akt/mTOR pathway by the high levels of AR agonists.

Cellular senescence therefore participates to the failure of AD therapy. However, it seems that other mechanisms, in addition to those previously described, could be provided to explain the development of hormone resistance phenotype. Indeed, numerous studies established a participation of autophagy in apoptosis resistance in many cancers [67] but its role in HRPCa progression remains unrecognized.

7 - Autophagy

7.1 - Three distinct autophagic processes

The word “autophagy”, derived from the Greek meaning “eating of self”, was first introduced in 1963 by the Nobel Prize laureate Christian de Duve who described lysosomal degradation of intracellular components in rat liver perfused with glucagon [68]. The concept of autophagy has been rediscovered in the mid-1990s and now refers to three different processes (Fig. 6): macro-autophagy, micro-autophagy and chaperone-mediated autophagy, all of which converge to a common degradation phase mediated by lysosomes [69]. Macro-autophagy involves the formation of double- or multiple-membrane vesicles, named autophagosomes, which sequester cytosolic components such as proteins, mitochondria, endoplasmic reticulum (ER) or Golgi membranes. These autophagosomes fuse with lysosomes to form autolysosomes, which degrade their contents by hydrolysis. In micro-autophagy, portions of the cytoplasm are directly engulfed by the lysosomes through invaginations of the lysosomal membrane. By contrast, in chaperone-mediated autophagy, proteins are translocated one-by-one across the lysosomal membrane thanks to their association with chaperone proteins that are recognized by the lysosomal membrane receptor Lamp-2 (lysosomal-associated membrane protein 2) [70].

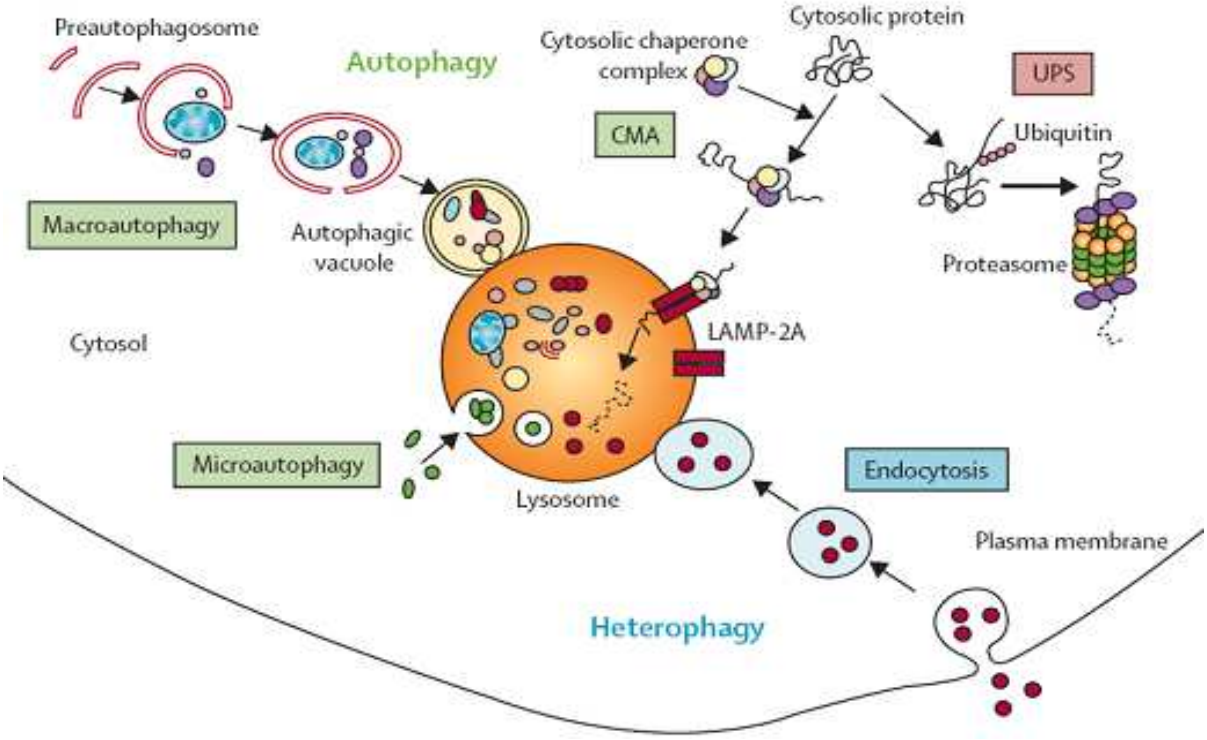

Fig. 6: Proteolytic systems in mammalian cells. From [71]
Proteins or organelles are brought to lysosomes from the extracellular environment (heterophagy) or from inside the cell (autophagy). A well-known heterophagy form is endocytosis. Three different types of autophagic mechanisms are distinguished in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Besides, an alternative, lysosomal-independent, pathway for degradation of intracellular proteins is well described: the ubiquitin-proteasome system (UPS).

7.2 - The autophagy machinery

Molecular mechanisms underlying autophagy have been extensively reviewed in [72-74]. The physiological importance of macro-autophagy being well established as its role in many diseases, autophagy is often reduced to the concept of macro-autophagy. Indeed, macro-autophagy (henceforth simply referred to as "autophagy") is more often observed and most characterized. Autophagy was initially identified as a mechanism triggered by the deficiency in nutrients or "starvation". The major player in energy-sensing is the complex mTORC1 (mammalian target of rapamycin complex 1), which is activated downstream of Akt, PI3-kinase and growth factor receptors (Fig. 7) and inhibits autophagy when nutrients are available [75]. In response to amino acid sufficiency, mTORC1 is associated to the lysosome surface that contains the GTPase Rheb (Ras homolog enriched in brain), required for its activation [76]. Under starvation, mTORC1 is detached from the lysosome and its kinase activity is rapidly shut down allowing autophagy induction to supply cells with metabolic substrates. Thereby, mTORC1 is considered as the major negative regulator of autophagy. Its artificial inhibition by rapamycin promotes autophagy [77]. Nevertheless, autophagy is a finely regulated process involving numerous other signaling pathways like p53 or MAPK pathways. Today, autophagy is recognized as a cellular function answering diverse stimuli including hypoxia, reactive oxygen species (ROS), absence of growth factor, DNA damage and viral or bacterial infection. However, autophagy is also known to be active at basal levels in most cell types where it plays a housekeeping role eliminating unfolded proteins and supernumerary or damaged organelles [74].

Identification of more than 30 AuTophaGy-related (Atg) genes has been made in yeast *Saccharomyces cerevisiae*. Many of them are conserved in other eukaryotes as mammals, thus emphasizing the significance of autophagy through evolution [78]. Among these Atg genes, a subset is required for the formation of an isolation membrane. The latter, also named phagophore or preautophagosome, is generated at the ER-mitochondria contact site in mammalian cells [79]. Membrane extensions of the ER from which these nascent organelles are formed have been described [80, 81]. Nevertheless, the lipid bilayer of phagophores could have other origins including the Golgi apparatus [82], the outer mitochondrial membrane [83], the plasma membrane [84] and possibly the nuclear envelope [85]. Besides, one cannot completely rule out *de novo* membrane formation from cytosolic lipids.

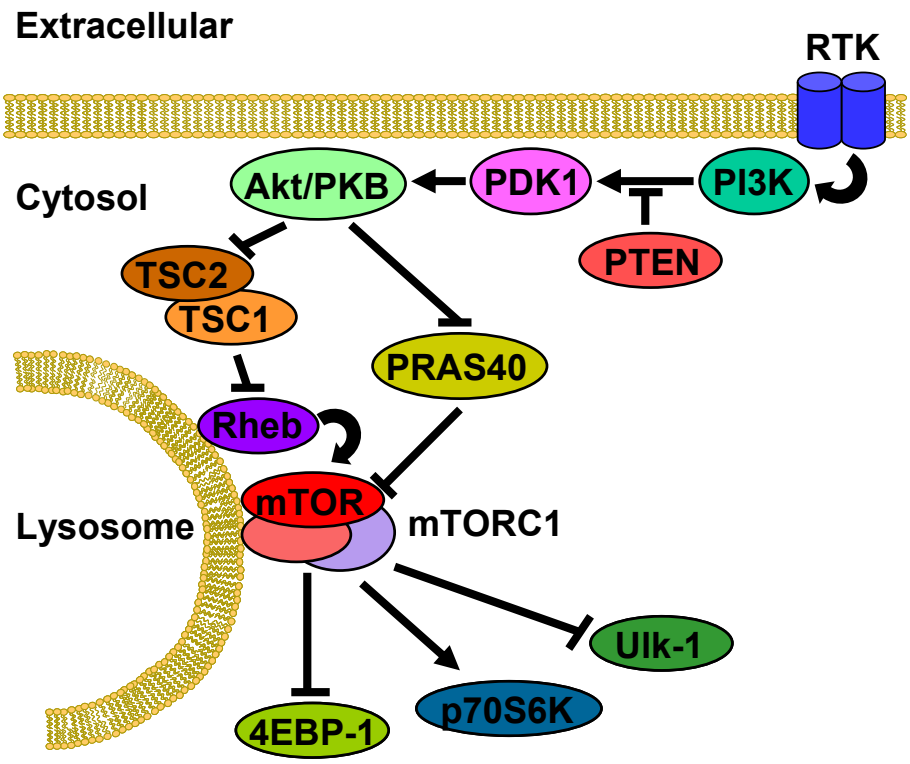

Fig. 7: The PI3K/Akt/mTOR pathway.
The PI3K/Akt/mTOR pathway regulates various cellular functions including proliferation, survival, metabolism, autophagy and protein synthesis. Growth factors, cytokines and hormones, by binding receptor tyrosine kinases (RTKs), cytokine receptors, or G protein–coupled receptors (GPCRs), activate class I PI-3 kinase, which generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the plasma membrane. PIP3 recruits Akt to the membrane where it can be phosphorylated and stimulated by the phosphoinositide-dependent kinase-1 (PDK1). The phosphatase, PTEN, can negatively regulate the activation of Akt by catalysing the dephosphorylation of PIP3. Activated Akt, also known as protein kinase B (PKB), can phosphorylate a large number of substrates including tuberous sclerosis protein 2 (TSC2). Phosphorylation of TSC2 within the TSC1-TSC2 complex blocks its ability to inactivate Rheb (Ras homolog enriched in brain), thereby allowing Rheb to stimulate mTOR complex 1 (mTORC1) at the lysosome surface where they are both recruited in response to amino acid sufficiency. The full activation of mTORC1 therefore depends on the presence of both growth factors and amino acids. Besides, Akt promotes mTORC1 activation by phosphorylating PRAS40 (proline-rich Akt substrate of 40 kDa), an mTORC1 inhibitor protein. Activated mTORC1 can in turn phosphorylate various proteins including the Ulk-1 complex (Unc-51-like kinase 1), the eukaryotic translation initiation factor 4E binding protein 1 (4EBP-1) and the p70 ribosomal protein S6 kinase (p70S6K). The phosphorylation of 4EBP-1 releases the translation initiation factor eIF4E while p70S6K phosphorylates and activates the S6 protein of the 40S ribosomal subunit, therefore allowing the protein synthesis. In contrast, the phosphorylation of Ulk-1 complex represses the catabolic process of autophagy [86].

The mammalian homologue of Atg1, Unc-51-like kinase 1 (Ulk-1), in a complex with other Atg proteins including Atg13, regulates the phagophore formation. Ulk-1 activity is itself negatively regulated by mTORC1 that phosphorylates Ulk-1 and Atg13 under nutrient-rich conditions. After starvation or rapamycin inhibition, mTORC1 is inactivated and dephosphorylated Ulk-1 becomes enzymatically active [87]. In contrast, AMPK stimulates the Ulk-1 complex by phosphorylating distinct sites in response to decreases in intracellular ATP (Fig. 8) [88]. Ulk-1 plays a pivotal role in regulating the localization and the activity of Atg proteins such as Atg9 essential for the initiation of autophagy [89]. Besides, Ulk-1 can maintain the autophagy activation by inhibiting mTORC1 in a positive-feedback loop [90, 91]. Surprisingly, Ulk-1 also exerts a negative-feedback by phosphorylating AMPK and inhibiting its activity, highlighting the acute regulation of the catabolic process [92].

The class III PI-3 kinase is involved in several vesicular trafficking pathways and participates in the autophagosome formation when complexed to Beclin-1, the mammalian homologue of Atg6, and other regulatory proteins such as Atg14 [93]. Class III PI3K/Beclin-1 produces phosphatidylinositol-3-phosphate (PIP), therefore recruiting PIP-binding proteins to the site of phagophore formation [80, 94] and stabilizing Ulk-1 nearby [95]. The presence of the complex near the site is itself controlled by the Ulk-1-mediated phosphorylation of one of its associated regulatory proteins, Ambra1 [96]. Besides, Ulk-1 and AMPK stimulate the kinase activity of pro-autophagic class III PI3K/Beclin-1 complex by phosphorylating Beclin-1 [97, 98] whereas mTORC1 represses it by phosphorylating Atg14 [99]. Another regulatory protein of the complex is Bcl-2, which disrupts the interaction of Beclin-1 with class III PI3K. Thereby, the interaction between Beclin-1 and Bcl-2 (and Bcl-xL) at the ER inhibits autophagy [100, 101]. This dual role of Bcl-2, anti-apoptotic as anti-autophagic, emphasizes the close relationship between these two cellular functions (see below).

The concerted actions of Atg proteins eventually lead to the expansion and conversion of the phagophore into an autophagosome. Two ubiquitin-like conjugation systems have been reported (Fig. 9). The first one allows the binding of the ubiquitin-like protein, Atg12, with Atg5. Conjugated Atg5-Atg12 further associate with Atg16 and interact with the phagophore membrane, where they participate to the insertion of another conjugated Atg protein, the LC3-II [102]. Indeed, the second ubiquitin-like conjugation system refers to the processing of microtubule-associated protein, light chain 3 (LC3), a mammalian homologue of Atg8. Upon induction of autophagy, cytosolic pro-LC3 is cleaved by the protease Atg4 to generate LC3-I. The carboxyterminal glycine exposed by the cleavage then becomes covalently linked to phosphatidylethanolamine, so as to produce processed LC3-II. The latter is integrated on both

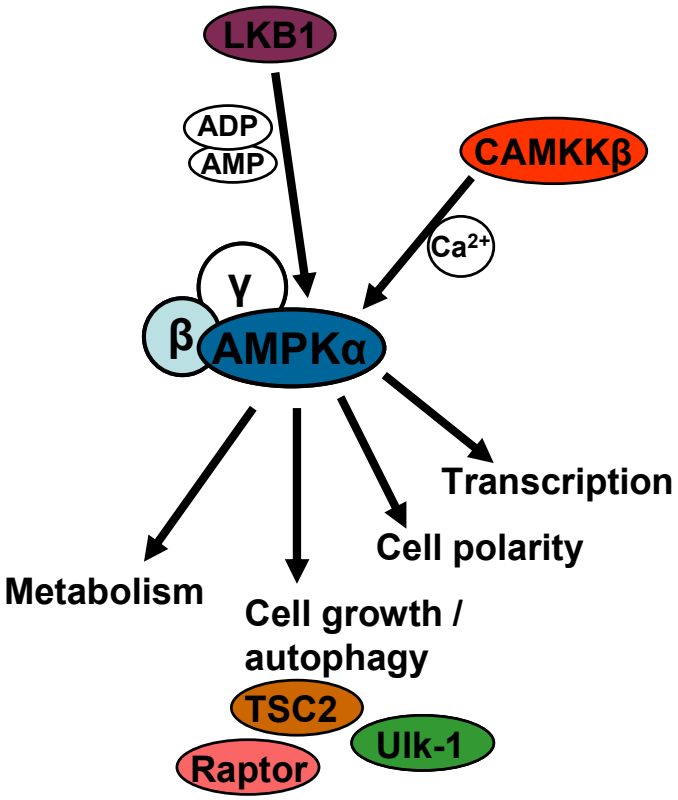

Fig. 8: The AMPK signaling pathway. Adapted from [103]
AMPK (5' adenosine monophosphate-activated protein kinase) is a central regulator of cellular metabolism which is stimulated when adenosine triphosphate (ATP) production is insufficient and adenosine monophosphate (AMP) and adenosine diphosphate (ADP) levels are increased. AMPK is composed of a catalytic subunit (α) and two regulatory subunits (β and γ). Its phosphorylation on Thr172 by LKB1 (liver kinase B1) and conformational changes induced by its binding to ADP leads to its activation. Besides, an intracellular Ca^{2+} elevation can also stimulate AMPK through CaMKKβ (Ca^{2+}/calmodulin-dependent kinase kinase β). AMPK controls cell growth, autophagy, metabolism and cell polarity by phosphorylating various substrates such as TSC2 (tuberous sclerosis protein), Raptor (regulatory-associated protein of mTOR), Ulk-1 (Unc-51-like kinase 1) and transcriptional regulators. The phosphorylation of TSC2 and Raptor by AMPK results in mTOR complex 1 inhibition and participates, in addition to the phosphorylation of Ulk-1 and class III PI3K/Beclin 1 complexes, in the stimulation of autophagy by AMPK.

internal and external membranes of the crescent phagophore. The other Atg8 homologues which have been described (GABARAP, GATE-16) are also modified by the same mechanism [104]. LC3-II is involved in the autophagosomes closure [105] and can link "adaptors" molecules to target defined structures such as protein aggregates or organelles to the autophagic machinery. Among the adaptor molecules, the best-characterized ones are p62 (also named sequestosome1, SQTM1) and NBR1 (neighbour of BRCA1 gene 1), both recognizing ubiquitinated proteins, thus complementing the proteasome in the degradation of oligomeric and aggregated proteins [106]. The binding affinity of p62 to ubiquitinated proteins can be upregulated by ULK1 in response to an accumulation of protein aggregates [107]. Furthermore, LC3-II can specifically recognize the mitochondrial protein Nix, a member of the Bcl-2 family of apoptotic regulators, to allow the degradation of damaged mitochondria, thereby demonstrating that autophagy is more than a nonspecific self-degradation process [108].

Detection of LC3-I to LC3-II conversion by immunoblotting, LC3 punctate relocalization by fluorescence, together with electron microscopy for autophagosome observation, are the main methods for monitoring autophagy. Conversion of cytosolic LC3-I to autophagosomal LC3-II can be readily followed in immunoblotting, LC3-II migrating faster than LC3-I in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). LC3-II/actin and LC3-II/LC3-I ratios can be measured and are correlated with the number of autophagosomes. However, LC3-II life-time is relatively short, since autophagosomes get rapidly degraded. This interferes with autophagy monitoring by LC3-II/LC3-I measurement. To circumvent this inconvenience, lysosomal degradation can be prevented by protease inhibitors, such as E64d and pepstatin A, or inhibitors of the vacuolar H^+-ATPase, such as bafilomycin A1. This procedure allows to determine total influx of processed LC3-II and thereby the cumulative autophagic activity. It has long been advised to exclusively normalize the levels of LC3-II to β-actin or other reference proteins, but not to that of LC3-I. However, this choice can be discussed in some cases [109]. For instance, when actin as well as other cytoplasmic proteins is degraded by autophagy, determination of the LC3-II/LC3-I ratio becomes more appropriate. Besides, by putting aside the turnover of the unconjugated form of LC3, we may miss part of the overall picture of the autophagic process.

Alternatively, chimeric constructs of LC3 fused with fluorescent proteins have been generated to follow by fluorescence microscopy the recruitment of LC3 into autophagosome membranes upon autophagy induction. Expression and degradation of p62 and NBR1 can also be measured to monitor autophagic activity [109].

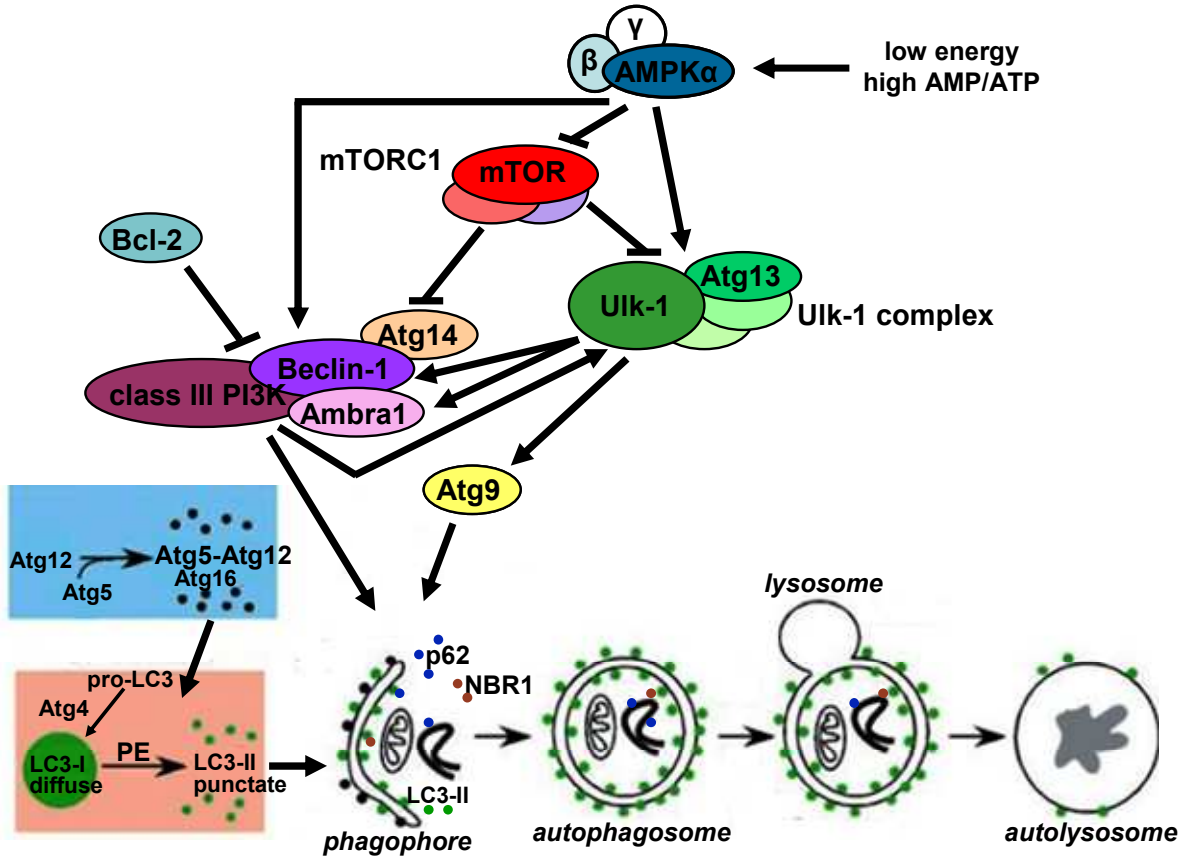

Fig. 9: The molecular mechanisms of the autophagosome formation. Adapted from [74]
Autophagy induction is dependent on the recruitment of different AuTophaGy-related (Atg) proteins. One of them is Ulk-1 (Unc-51-like kinase 1), the mammalian homologue of Atg1, which regulates the formation of the phagophore at the ER, in a complex with other Atg proteins including Atg13. The energy sensor AMPK (5' adenosine monophosphate-activated protein kinase) stimulates the Ulk-1 complex whereas mTOR complex 1 (mTORC1) represses it under nutrient-rich conditions. Activated Ulk-1 controls the activity of Atg9 protein and recruits the class III PI3K/Beclin-1 complex to the ER by phosphorylating Ambra1, one of its associated regulatory proteins. In turn, the class III PI3K/Beclin-1 complex recruits other proteins required for the phagophore formation by producing phosphatidylinositol-3-phosphate (PIP). Ulk-1 and AMPK stimulates the complex activity by phosphorylating Beclin-1 whereas mTORC1 and Bcl-2 represses it by respectively phosphorylating Atg14 and disrupting the interaction between Beclin-1 and class III PI3K.
Two ubiquitin-like conjugation systems are required for the phagophore expansion: the Atg5-Atg12 conjugation and the processing of LC3 (microtubule-associated protein, light chain 3). Atg12 covalently binds to Atg5 and associates with Atg16 on the autophagosomal membrane. Cytosolic pro-LC3 is cleaved by the protease Atg4 to produce LC3-I. The latter can be covalently linked to PE (phosphatidylethanolamine), so as to generate LC3-II which is integrated on both internal and external autophagosomal membranes. LC3-II can bind "adaptors" molecules including p62 and NBR1 (neighbour of BRCA1 gene 1) to target defined intracellular structures and to allow their selective degradation.
By its closure, the autophagosome sequesters various cytoplasmic components. Its fusion with a lysosome, thereby forming an autolysosome, results in their degradation by hydrolysis.

The maturation of autophagosomes seems to occur through several fusion events with vesicles originating from the endosomal compartment. Thus, autophagosomes have been reported to fuse with early endosomes and then with multivesicular endosomes, forming amphisomes. Finally, the outer membrane of amphisomes fuses with the lysosomal membrane, resulting in "fused organelles" called autolysosomes. Initially, autophagosome lumen has the same pH as the surrounding cytoplasm, but during this maturation process, autophagic vacuoles become acidic. This pH decrease activates lysosomal proteases such as cathepsins B, L and D, and results in the final degradation of the autophagic vacuoles content [110]. A main regulator of autophagosomes maturation is Rab7, a member of small GTPases, which is essential in the trafficking of vacuoles along microtubules and participates in the fusion with endo/lysosomal membranes [111]. The lysosomal membrane protein Lamp-2 is also critical for functional autophagy, due to its involvement in the fusion process. This is emphasized in Danon disease in which a mutation in the LAMP-2 gene causes hypertrophic cardiomyopathy, skeletal muscle myopathy and variable mental retardation [112].

7.3 - Autophagy functions

A strong increase of autophagy was observed to promote cell death. Thus, autophagy has long been perceived as a form of programmed cell death which has been called "type II" programmed cell death, in opposition to apoptosis, the classical "type I" programmed cell death. However, it is now well accepted that cell death is often associated with, but rarely due to, autophagy. Moreover, autophagy inhibition has more often been reported to enhance it rather than prevent cell death (see below). Thus, autophagy is instead regarded as a stress adaptation pathway that allows cell survival [113].

Besides this narrow and complex relationship with cell death, autophagy maintains a particular link with cellular growth control where mTORC1 still plays a central role. Autophagy is known to induce cell cycle arrest and to promote senescence while it is inhibited in mitosis [114]. Moreover, autophagic function is essential during embryogenesis and postnatal development [115]. Besides, its ability to remove damaged organelles such as stressed mitochondria provides its significance in aging. Furthermore, misregulation of autophagy can lead to the pathogenesis of a number of human diseases including neurodegenerative, muscle or heart diseases, and cancer [113].

7.4 - Dual-faced role of autophagy in cancer

A clear link is observed between autophagy defects and cancer. Autophagy can be perceived as a protective mechanism against cancerogenesis. However, autophagy can also promote survival of tumor cells in a stressful environment and thereby constitutes a significant contribution to tumor maintenance. A multitude of studies have investigated the role of autophagy in tumorigenesis, cancer progression and anticancer treatments. Although conflicting conclusions have emerged from this intense research effort, it now seems clear that autophagy involvement in cancer must be considered at two levels: (i) cancerogenesis and cancer development and (ii) treatment resistance (Fig. 10).

7.4.1 - Anti-tumor role of autophagy during cancerogenesis

Autophagy regulation closely overlaps with signaling pathways involved in tumorigenesis including mTOR pathway. As previously described, mTORC1 is the major negative regulator of autophagy. However, the mTOR protein regulates several other functions including cell survival, protein synthesis or cell growth. In tumor cells, constitutively active RTKs (receptor tyrosine kinases) and/or persistent production of autocrine growth factors are often observed and lead to activation of the mTOR pathway [116]. Besides, oncoproteins connecting RTKs to mTOR such as class I PI-3 kinase, Akt or the small GTPase Ras, are considered to inhibit autophagy. Conversely, several tumor suppressor genes involved in the upstream inhibition of mTOR such as TSC1/TSC2 and phosphatase PTEN activate autophagy [113]. The death-associated protein kinase 1 (DAPK1), a calcium-dependent serine/threonine kinase which promotes both apoptosis and autophagy, has also tumor suppressor properties and is frequently silenced in human cancers by epigenetic mechanisms [117].

Tumorigenesis seems to be favored by autophagy inhibition. Reduced expression of proteins required for autophagy has been noticed in cancer cells. For example, the essential autophagy gene, Beclin-1, is monoallelically deleted in many cases of human breast, ovarian and prostate cancer [118]. Likewise, numerous brain tumors have reduced levels of Beclin-1, which inversely correlate with clinical malignancy [119]. Thus, Beclin-1 can be considered as a tumor suppressor. Its gene transfer inhibits tumor cells growth while mutant mice with monoallelic deletion of Beclin-1 show increased frequency of spontaneous malignancies [118, 120]. Other components of the class III PI3K/Beclin-1 complex seem to share the same properties like UVRAG (UV radiation resistance-associated gene) which is often

monoallelically deleted in human colon cancers and the overexpression of which suppresses the proliferation and tumorigenicity of human colon cancer cells [121]. In mice, the loss of Bif-1, another regulatory protein of the complex, inhibits autophagosome formation and results in the development of spontaneous tumors [122]. Atg genes other than Beclin-1 also have tumor suppressor effects, such as Atg5 and Atg7 whose deletion in mice leads to the development of liver adenomas [123], or Atg4, whose absence in a mouse model favors the occurrence of chemically-induced fibrosarcomas [124]. Moreover, rearrangements in different types of leukemia are frequently met in a region of chromosome 3 where Rab7 gene is localized [125]. Besides, Beclin-1 interacts with Bcl-2 family proteins, which are well-known to be involved in tumorigenesis while regulating autophagic activity. Indeed, anti-apoptotic Bcl-2 family members (Bcl-2 proper, Bcl-xL, Bcl-w and Mcl-1) are prominent oncoproteins whereas pro-apoptotic members (Bax, Bak and BH3-only proteins) are considered as tumor suppressors [116]. By their regulation of the activity of class III PI3K/Beclin-1 complex, the anti-apoptotic factors inhibit autophagy and could therefore favor cancer development. Inversely, by freeing Beclin-1 from inhibitory interactions with Bcl-2 and its anti-apoptotic homologues, the BH3-only proteins such as Bad, Bik, Noxa or Puma promote autophagy [126].

In human cancers, the most commonly mutated tumor suppressor gene is p53. p53 is inactivated in more than 50% of human tumors [127]. p53 is crucial in eukaryotic cells where it controls numerous functions including cell cycle, senescence, DNA repair and apoptosis. Furthermore, p53 plays a role in the regulation of autophagy. It can induce the expression of damage-regulated autophagy modulator (DRAM), a lysosomal protein which activates autophagy and is also essential for p53-mediated apoptosis [128]. Moreover, DNA damage induces a p53-dependent autophagy via AMPK activation and inhibition of the mTOR pathway [129]. Otherwise, re-expression of p53 in p53-deficient cancer cells results in autophagosome formation [130]. The expression of $p14^{ARF}$, an alternate reading frame product of the CDKN2A locus which inhibits the ubiquitination-dependent degradation of p53, is frequently abolished in many cases of human cancers including PCa [131]. $p14^{ARF}$ is known to promote both p53-dependent and p53-independent autophagy [132]. Moreover, $p14^{ARF}$ mRNA produces a shorter isoform, short mitochondrial form of ARF (smARF), which also induces massive p53-independent autophagy [133]. However, it should be noted that while transcriptional activities of p53 promote autophagy, cytosolic p53 has been shown to inhibit it [134].

Except conflicting data about p53, numerous observations thus reinforce the idea that autophagy exerts a tumor suppressing role. Several explanations can be proposed. First, a cell can be eliminated by both apoptosis and autophagy that surpasses the safe threshold. And of course, the presence of an alternative pro-death mechanism is essential if one pathway is inactivated. Suppression of these two functions would further sustain tumor development. Moreover, autophagy defects are noted to increase necrotic cell death within tumors, associated with inflammation which facilitates angiogenesis and tumor growth [135]. It can also be highlighted that functional autophagy is necessary in the induction of an anticancer immune response by its role in the presentation of antigens [136, 137]. Furthermore, autophagy inactivation increases genomic instability that facilitates cancer progression. Indeed, autophagy limits metabolic stress and associated oxidative damage, thus protecting the genome [138]. The degradation of altered mitochondria as source of reactive oxygen species (ROS) is essential to prevent DNA damage [139]. Besides, autophagy inhibition has been shown to result in deficient repair of DNA damage although the role that autophagy plays in this function is still unclear [140, 141]. Finally, cell growth limitation by autophagy may also participate to preserve genome integrity [114]. The autophagic process therefore appears as an important mechanism by which cancer initiation and progression are prevented.

7.4.2 - Pro-tumor role of therapy-induced autophagy

In some cancers (especially when essential apoptotic modulator, like Bax and Bak or caspases, are defective), autophagy induction in response to chemotherapy has been reported to promote cell death [142, 143]. However, autophagy has most frequently been demonstrated to favor survival of tumor cells exposed to changes in their microenvironment or to various treatments. Indeed, when a solid tumor is growing, it quickly depletes its blood supply, leaving portions of the tumor undergoing hypoxia and imbalances in nutrient supply. Inhibition of autophagy induced by these conditions can promote cell death [144]. Moreover, in addition to conventional cytotoxic drugs, numerous pharmacological agents such as anti-angiogenic molecules, kinase inhibitors, proteasome inhibitors or HDAC inhibitors are known to induce autophagy [145]. Pharmacological inhibitors of autophagy (e.g., chloroquine and hydroxychloroquine, 3-methyladenine, bafilomycin A1, monensin, NH_4Cl) and siRNA targeting Atg genes (e.g., Atg5, Beclin-1, Atg7, Atg10) have been used to sensitize tumor cells to various stress inducers such as glucose and amino acid deprivation, growth factor withdrawal, radiation therapy or above mentioned pharmacological agents [116]. Several

ongoing phase I/II trials targeting autophagy in cancer have even been conducted with the use of chloroquine or its derivatives, safe antimalarial drugs which inhibit lysosome acidification [145].

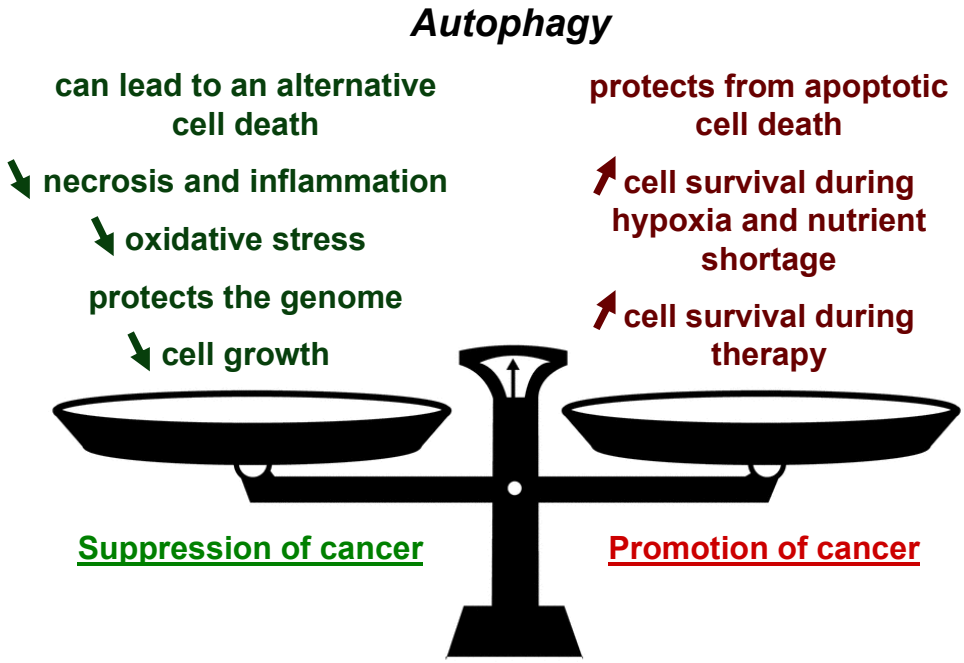

Fig. 10: Dual-faced role of autophagy in cancer.
Pathways involved in autophagy control are often deregulated during malignant transformation. Autophagic process is thus considered to have tumor suppressor properties. However, autophagy also represents a mechanism of cell survival providing resistance to various anti-tumoral treatments.

8 - Cell death and autophagy

Classically, two types of mechanisms leading to cell elimination are described: the programmed cell death, which is physiological and tolerogenic, and the necrosis, an accidental, pathological and immunogenic cell death. The programmed cell death is mainly related to apoptosis, a form of cell death which exhibits particular morphological features such as chromatin condensation, nuclear fragmentation or plasma membrane blebbing. Besides, for a long time, the terms apoptosis and programmed cell death had the same meaning, until autophagic cell death and programmed necrosis were described [146].

8.1 - Apoptosis

Two pathways regulating apoptosis have been described, the extrinsic pathway and the intrinsic or mitochondrial pathway [147, 148]. The extrinsic pathway is triggered by binding of lethal ligands, such as FAS ligand (FAS-L) but also TNFα or TRAIL to specific receptors (FAS, TNFα receptor 1 and TRAIL receptor 1-2 respectively). Combination of ligand with its death receptor results in the recruitment of different proteins including death domain-containing protein (FADD) and pro-caspase-8. This assembly forms the death-inducing signaling complex (DISC) where pro-caspase-8 is cleaved to give active caspase-8 (cysteinyl aspartate-specific protease-8). Activation of caspase-8 catalyses the proteolytic maturation of caspase-3 and triggers the execution phase of apoptosis.

The intrinsic pathway is dependent on various non-receptor-mediated stimuli that can consist in the absence of certain growth factors or cytokines, toxins, viral infections, hypoxia, oxidative stress, DNA damage, cytosolic calcium overload or radiation. These stimuli induce changes in mitochondrial membranes that result in mitochondrial outer membrane permeabilization (MOMP), loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) (due to permeabilization of the mitochondrial inner membrane) and arrest of ATP synthesis and $\Delta\Psi_m$-dependent transport activities. Besides, the respiratory chains get uncoupled, producing important amount of ROS which amplifies the apoptotic signal [149].

MOMP has been related to two mechanisms [147]. In the first, pores are generated through the formation of permeability transition pore complexes (PTPC) which raise the permeability of the inner mitochondrial membrane and induce, due to osmotic forces, a swelling of the matrix as water enters, which results in the outer membrane break. The molecular nature of the PTPCs has long been debated. However, convincing evidence in favor of formation of these complexes from the F0F1 ATP synthase was recently provided [150, 151]. The second mechanism involved in MOMP is mediated by proteins of the Bcl-2 family (Fig. 11). Indeed, in response to apoptotic stimuli, pro-apoptotic members Bax and Bak undergo conformational changes leading to their oligomerization on the mitochondrial outer membrane and the formation of pores. Conversely, anti-apoptotic members (e.g., Bcl-2, Bcl-xL, Bcl-w) can inhibit this process by interacting with Bax and Bak [152].

With MOMP, the release into the cytosol of proteins normally confined within the mitochondrial intermembrane space occurs. Among these, two groups can be described. The first group gathers cytochrome c, SMAC (second mitochondria-derived activator of caspases, also known as DIABLO) and HTRA2 (high temperature requirement protein A2). Released

cytochrome c binds and activates Apaf-1 (apoptotic peptidase activating factor 1) as well as pro-caspase-9, forming the "apoptosome". Activated caspase-9 can in turn cleave and activate pro-caspase-3, thus engaging the caspase-dependent proteolytic cascade. SMAC/DIABLO and HTRA2 inhibit the anti-apoptotic activities of members of the IAP family (inhibitors of apoptosis proteins).

A second group of mitochondrial proteins is released after the cell has committed to die. They include AIF (apoptosis-inducing factor), endonuclease G and CAD (caspase-activated DNase). They are translocated to the nucleus where they induce chromatin condensation and oligonucleosomal DNA fragmentation (Fig. 12).

The extrinsic and intrinsic pathways are connected by Bid (BH3-interacting domain death agonist), another pro-apoptotic Bcl-2 family protein. It is cleaved by active caspase-8, producing a mitochondrion-permeabilizing fragment known as truncated Bid (tBid). Accumulation of tBid at the mitochondria causes MOMP, $\Delta\Psi_m$ dissipation and cytochrome c release [149].

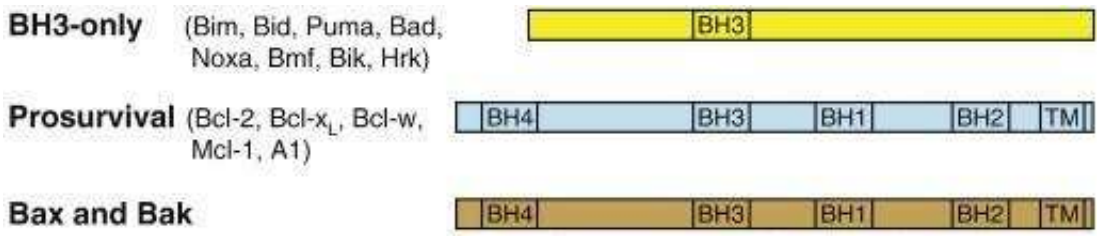

Fig. 11: The Bcl-2 protein family. Adapted from [153].

Bcl-2 (B-cell lymphoma-2) proto-oncogene was identified from the discovery of a chromosomal translocation involving chromosomes 14 and 18 in B-cell follicular lymphoma. This translocation places Bcl-2 under the control of an immunoglobulin gene enhancer thereby increasing its expression. Bcl-2 is the first of a family of genes that can be either anti-apoptotic or pro-apoptotic. Each Bcl-2 family protein contains at least one of the four characteristic Bcl-2 homology (BH) domains (named BH1, BH2, BH3 and BH4) which correspond to α-helical segments. Anti-apoptotic members (Bcl-2, Bcl-xL, Mcl-1) have the four BH domains and a hydrophobic transmembrane (TM) domain allowing their integration into lipid bilayer. For their part, pro-apoptotic members are distinguished into two groups, those that only have the BH3 domain (e.g. Bid, Bim and Bad) and those with several BH domains and a TM domain (e.g. Bax and Bak). Such pro-apoptotic proteins were long described as having three BH domains (BH1, BH2 and BH3), whereas anti-apoptotic proteins contain a supplementary BH4 domain conferring anti-apoptotic activity. However, the BH4 domain was recently redefined as a structural motif also present in Bax and Bak [152, 154].

Extrinsic and intrinsic pathways lead to the execution phase of apoptosis. Initiator caspases (caspase-8 and caspase-9) activate caspase-3 and effector caspases cascade. These caspases in turn activate cytoplasmic endonucleases and proteases, and cleave various cellular

components that cause the morphological changes seen in apoptotic cells. Indeed, apoptotic cells exhibit specific microscopic features. First, cell shrinkage and rounding are observed because of cytoskeleton disruption. The cytoplasm appears dense and the organelles more tightly packed. Chromatin undergoes condensation (pyknosis) until nuclear envelope becomes discontinuous and fragmented DNA escapes. Formation of irregular buds on cell membrane (blebbing) occurs followed by separation of cell fragments into several vesicles known as apoptotic bodies [155]. The latter are rapidly phagocytosed by macrophages due to the recognition of phosphatidylserine externalized during the apoptotic process and thereby displayed at their surface [156]. Therefore, apoptotic cells do not release their immunogenic constituents into the surrounding environment [155].

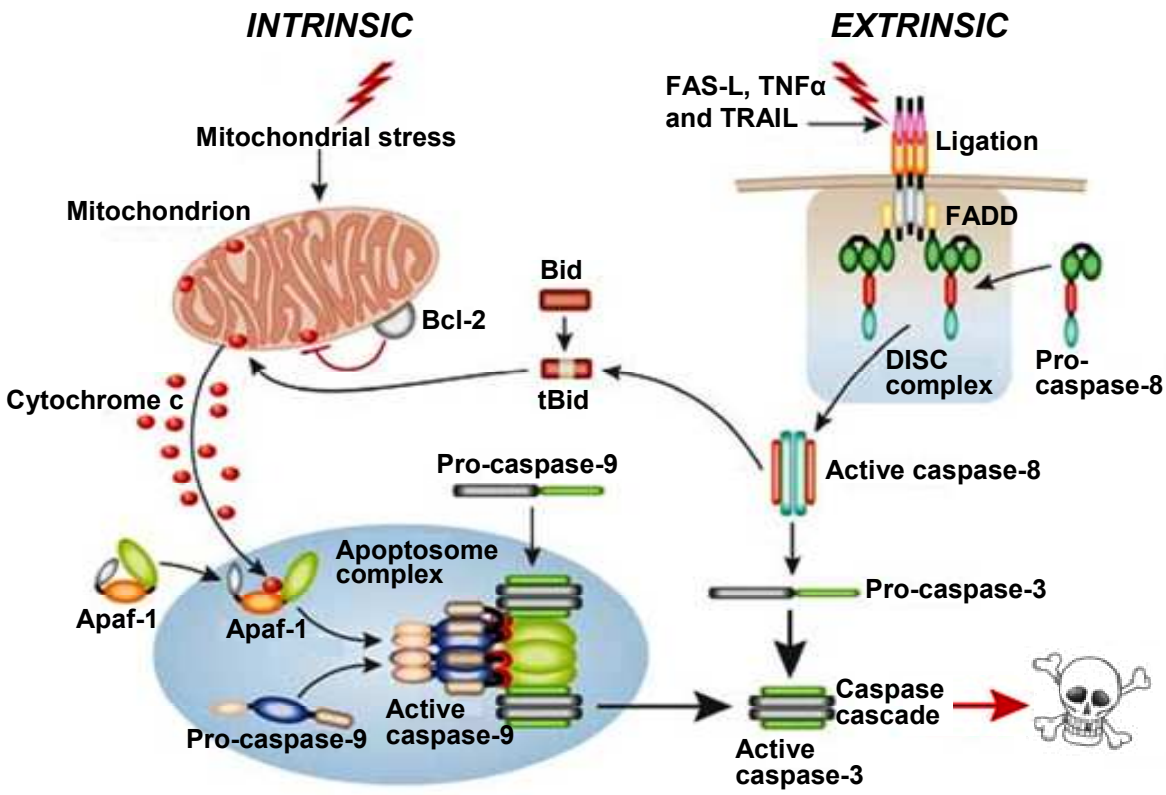

Fig. 12: Pathways regulating apoptosis. Adapted from [157]
Two distinct pathways have been reported to conduct to apoptosis: the extrinsic and intrinsic pathways. The extrinsic pathway is triggered via the stimulation of death receptors such as FAS, TNFα receptor and TRAIL receptor. Activated receptors recruit different proteins including death domain-containing protein (FADD) and pro-caspase-8 which form the death-inducing signaling complex (DISC) where pro-caspase-8 is cleaved to give active caspase-8. Caspase-8 in turn activates caspase-3 to trigger the execution phase of apoptosis.
The intrinsic pathway is induced in response to various intracellular stresses and involves the mitochondrial outer membrane permeabilization (MOMP) which results in the release of cytochrome c. The latter can bind and activate Apaf-1 (apoptotic peptidase activating factor 1) and pro-caspase-9, thereby forming the "apoptosome". Activated caspase-9 can in turn cleave pro-caspase-3, engaging the caspase-dependent proteolytic cascade which leads to the cell death. These two pathways are not independent, extrinsic pathway being able to activate intrinsic pathway through Bid (BH3-interacting domain death agonist) cleavage.

8.2 - A debatable autophagic cell death

An autophagic cell death has been described during the development of lower eukaryotes [158, 159] and *in vitro* in some cancer cells where apoptosis is disrupted (e.g. Bax/Bak double-knockout) [142, 143]. Besides, a nonphysiological over-activation of autophagy which exceeds a safe threshold has been reported to cause cell death [67]. Morphological features characteristic of autophagy and apoptosis have often been observed concomitantly. For instance, MG132 proteasome inhibitor has been noticed to induce apoptosis and autophagy-mediated cell death in PC3 prostate cancer cells [160]. In this model and in some others, inhibition of autophagy partially suppresses cell death. However, if it is well accepted that cells can undergo autophagy before or during their elimination, it seems rarely to be the process by which cells actually die [161]. Autophagy could rather play a role of partner of apoptosis [67]. By the interaction between p62 and caspase-8, the autophagy stimulated by the proteasome inhibition and the subsequent accumulation of p62 and LC3 have been noticed to favor caspase-8 aggregation and activation, thereby inducing apoptosis [162, 163]. Moreover, in Bax/Bak double-knockout or in Bcl-xL-overexpressing cells that are defective in apoptosis, prolonged ER stress and associated autophagy have been demonstrated to promote a necrosis-like cell death [164]. Some authors even speak of cell death impostor [165]. In fact, today, autophagy is further considered as a cytoprotective mechanism activated in response to various stress. It can serve to supply energy and nutrients when they are limited and to maintain genomic integrity by eliminating depolarized mitochondria that are a source of genotoxic ROS. Moreover, specific degradation of mitochondria by autophagic process (mitophagy) may circumscribe apoptosis by preventing MOMP and subsequent release of cytochrome c [166]. Besides, as explained above, the resistance to many anticancer drugs is related to the triggering of a prosurvival autophagy [145]. Several clinical research studies have been conducted to define optimal strategies to repress autophagy in tumor cells [167, 168].

8.3 - Cross-talk between autophagy and apoptosis

Although autophagy most frequently constitutes a prosurvival pathway, numerous conflicting data show that autophagy and apoptosis are closely related. It can be noted that several autophagy-related genes are involved in apoptosis regulation and their knockdown may not be without effect on apoptotic cell death. For instance, Atg3 and Atg12 have been reported to be

conjugated and localized to the mitochondrial outer membrane where they regulate mitochondrial fusion. Disruption of the formation of this complex inhibits mitochondrial pathway-mediated apoptosis [169]. Moreover, Atg12 knockdown is noticed to suppress intrinsic apoptosis. Indeed, free form of Atg12 promotes apoptosis by binding and inactivating prosurvival Bcl-2 family members, such as Bcl-2 and Mcl-1 [170]. For its part, Atg4 can be cleaved by caspase-3 during apoptosis generating fragments which enhance delipidation of the Atg8 homologue GABARAP and sensitizes cells to apoptosis [171]. Besides, calpain-mediated Atg5 cleavage is observed during apoptosis. Truncated Atg5 is translocated to the mitochondria and interacts with Bcl-xL to enhance cytochrome c release and caspases activation [172]. Beclin-1 can also be cleaved by caspase-3, losing its ability to promote autophagy. Moreover, one of its truncated fragments localizes to mitochondria and increases cell sensitivity to apoptosis [173]. Various proteins other than Atg family members but evenly participating in the regulation of autophagy also interfere with apoptosis mechanisms [174]. For instance, apoptosis induction leads to caspases and calpains-mediated cleavage of Ambra1, a member of class III PI3K/Beclin-1 complex, which inhibits pro-survival autophagy [175]. Moreover, UVRAG, another protein of the class III PI3K/Beclin-1 complex, is known to interact with Bax, thus inhibiting its translocation to mitochondria and the induction of apoptosis [176]. Otherwise, Bcl-2 family proteins are involved in the regulation of the two functions. As we have previously seen, anti-apoptotic members of Bcl-2 family (including Bcl-2 itself, but also Bcl-xL or Mcl-1) can interact with Beclin-1 to inhibit the formation of class III PI3K/Beclin-1 complex and the autophagy induction. Through their competitive interaction with Bcl-2, Bcl-xL or Mcl-1, BH3-only proteins suppress this inhibition and promote autophagy [126, 177]. Jnk1 (c-Jun N-terminal kinase) by phosphorylating Bcl-2 at multiple sites, thus decreasing its ability to bind Bax as Beclin-1, is reported to induce apoptosis and autophagy [178]. Besides, through its substrate c-Jun, Jnk1 increases the transcription of Beclin-1 gene [179]. Calpains, a family of Ca^{2+}-dependent cysteine proteases activated during execution phase of apoptosis, are also involved in autophagy process. Indeed, in calpain-deficient cells, an impaired autophagy has been observed [180]. Apoptosis and autophagy are also interconnected by several signaling pathways which share the control of these two functions. Indeed, as we have explained above, mTOR and p53 pathways regulate cell survival by playing on apoptosis and autophagy induction. Moreover, DAPK1, known to induce apoptosis in response to various stimuli, can also regulate autophagy in different ways. DAPK1 is noticed to interact with and to phosphorylate Beclin-1, which suppresses the inhibitory Beclin-1/Bcl-2 interaction. It also

interacts with MAP1B (microtubule-associated protein 1B), which facilitates autophagosomes trafficking along microtubules. Finally, it has been demonstrated to activate p53 and increase $p14^{ARF}$ mRNA splicing producing pro-autophagic smARF [181].

8.4 - Regulated necrosis

Although necrosis had been considered for a long time to be only an uncontrolled mechanism, a regulated necrosis was recently highlighted. This regulated necrosis (or necroptosis) is under the control of various proteins including RIPK (Receptor interacting protein kinases) or PARP1 (poly(ADP-ribose) polymerase-1) and responds to several stimuli such as DNA damage and activation of death receptors [182].

9 - Calcium signaling

In rat prostatic cells, the role of intracellular calcium in the programmed cell death triggered by androgen removal has been known for 25 years [183, 184]. The involvement of Ca^{2+} in the resistance to apoptosis in PCa cells is clearly established [185]. We have tried to understand more precisely how Ca^{2+} signaling participates to the mechanisms of hormone resistance.

Calcium is a versatile intracellular messenger, which regulates many different cellular functions such as gene transcription, muscular contraction, cell differentiation, cell cycle, migration, secretion and apoptosis. Ca^{2+} exerts its effects as a signal by binding to various proteins including calmodulin, a Ca^{2+}-binding messenger protein which plays the role of sensor of cytosolic [Ca^{2+}] and transduces calcium signals to target proteins. Ca^{2+} signaling is dependent on extensive cellular machinery allowing a precise spatiotemporal control of [Ca^{2+}]. While extracellular environment contains more than 1 mM Ca^{2+}, cytosolic [Ca^{2+}] remains very low (~100 nM), due to the active extrusion of Ca^{2+} through the plasma membrane by the plasma-membrane Ca^{2+}-ATPase (PMCA) and the Na+/Ca^{2+} exchanger (NCX) or through the membranes of intracellular organelles such as the ER by the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump. The presence of numerous Ca^{2+}-binding proteins in its lumen (calreticulin, calnexin, etc.) in addition to the SERCA activity makes ER the main intracellular Ca^{2+} store. A strong gradient is thus generated, ER accumulating Ca^{2+} in more than thousandfold excess in comparison to the cytosol [186] (Fig. 13).

Almost all Ca^{2+} signals correspond to brief pulses of Ca^{2+}, released in all the cytoplasmic compartments or limited to only sub-domains or specific organelles. [Ca^{2+}] perturbations can take a few milliseconds to several hours. These Ca^{2+} transients can have two origins : (i) the internal stores, due to the presence of two channels at their surface, the inositol trisphosphate receptor (IP3R) and its close relative, the ryanodine receptor (RyR), and (ii) the extracellular medium [187].

In the latter case, they are dependent on the opening of various plasma membrane channels in response to stimuli such as membrane depolarization, agonists, stretch or the depletion of internal stores. These channels are thus classified according to their mechanisms of activation: voltage-operated channels (VOC) present in excitable cells and essential for muscle contraction, for action potential generation and for exocytosis of neurotransmitters in synaptic cleft, receptor-operated channels (ROC), which bind external ligand that activates them, second-messenger-operated channels (SMOC) which respond to internal messengers, store-operated channels (SOC) opened by the depletion of intracellular Ca^{2+} store (particularly the ER), thermosensors and stretch-activated channels [187]. Some of them belong to the transient receptor potential (TRP) channel family, on which we focused our attention specifically on TRPM8 largely expressed in PCa cells [188].

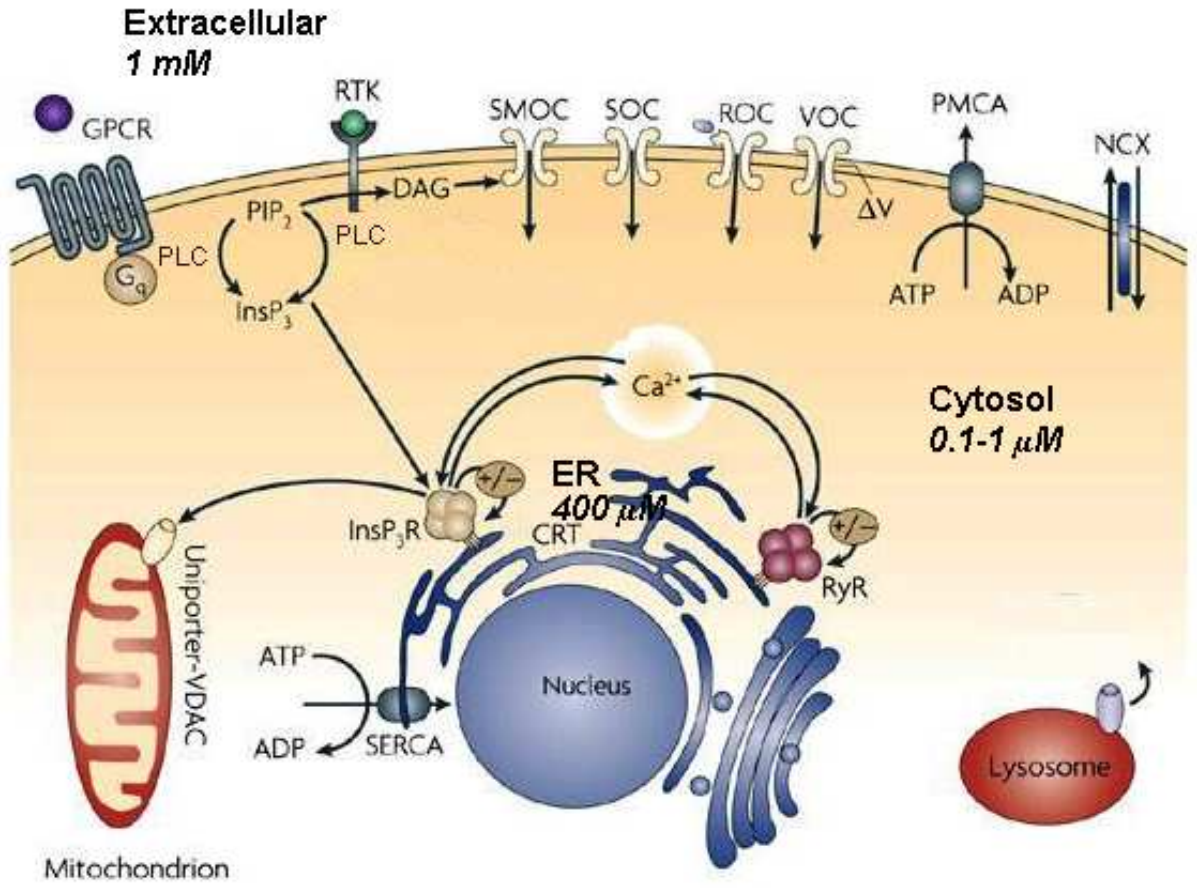

Fig. 13: The Ca^{2+} homeostasis. Adapted from [189]
Cellular Ca^{2+} homeostasis is finely regulated via a set of pumps, exchangers and channels. Ca^{2+} signals are the result of influx from the extracellular space mediated via diverse plasma membrane channels or originate from releases from internal stores through inositol trisphosphate receptors (IP3R) and ryanodine receptors (RyR). (Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CRT, calreticulin; DAG, diacylglycerol; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; $InsP_3$, inositol trisphosphate; $InsP_3R$, inositol trisphosphate receptor; NCX, Na^+/Ca^{2+} exchanger; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PMCA, plasma-membrane Ca^{2+}-ATPase; ROC, receptor-operated channel; RTK, receptor tyrosine kinase; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; SMOC, second-messenger-operated channel; SOC, store-operated channel; VDAC, voltage-dependent anion channel; VOC, voltage-operated channel.)

9.1 - The TRPM8 channel

The family of TRP channels is so designated because they were originally described in *Drosophilia* whose photoreceptors with mutations in the *trp* locus responded to a continuous light with a transient receptor potential. The mammalian TRP channels mediate a variety of sensations like the sensations of pain, warmth or coldness, different kinds of tastes, olfaction, hearing, touch and vision [190, 191]. Furthermore, they are reported to respond to various agonists, modifications of pH or pressure, and oxidative stress. Nevertheless, the activation and regulation mechanisms of TRP channels are not well known. Structurally, TRP channels comprise four subunits, themselves composed of six transmembrane domains (Fig. 14). The mammalian TRPs are encoded by at least 28 genes. These are divided into two broad groups: group 1 including TRPC ("C" for canonical), TRPV ("V" for vanilloid), TRPM ("M" for melastatin) and TRPA ("A" for ankyrin) and group 2 including TRPP ("P" for polycystic) and TRPML ("ML" for mucolipin).

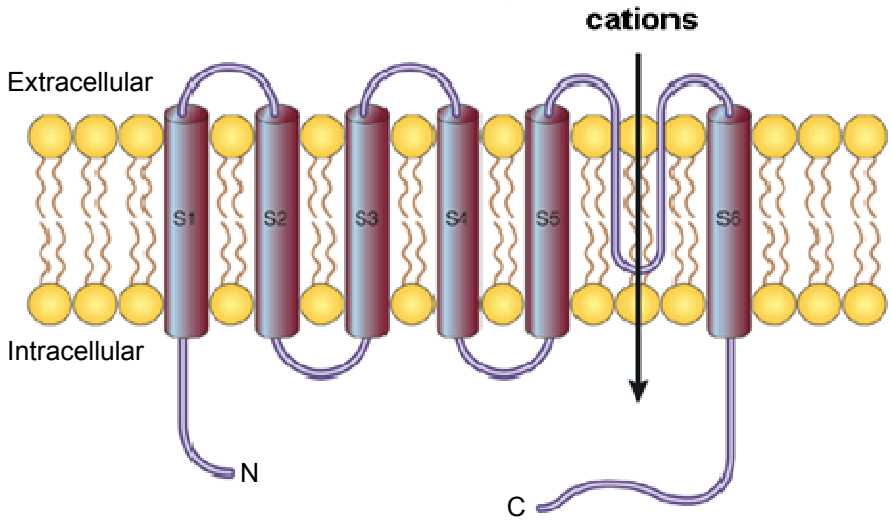

Fig. 14: The TRP architecture. Adapted from [192]
TRP channels present six membrane-spanning domains: S1 to S6 transmembrane domains. They assemble into homo- or heterotetramers to form non-selective cationic channels.

TRPM8 is a non-selective cation channel assembled in homotetramer and activated by temperatures below 26°C. While acid pH inhibits TRPM8, many chemicals compounds such as menthol, icilin or eucalyptol shift activation threshold to higher temperatures explaining the cold sensation produced by these molecules when they are in contact with nerve endings of the oral cavity. Temperature sensitivity of TRPM8 is also dependent on the transmembrane voltage. Moreover, cellular microenvironment finely regulates channel activity. Indeed,

presence of phosphatidylinositol 4,5-bisphosphate seems essential. Its depletion is involved in the desensitization of TRPM8 as well as PKC and calmodulin. Furthermore, GPCRs, in particular activated by inflammatory mediators and growth factors, have also been noticed to control TRPM8 opening [193]. Originally, TRPM8 is known to be expressed in thermosensitive neurons (trigeminal ganglia, dorsal root ganglia) but also in prostate and liver [190].

It is now well established that TRP channels are involved in PCa initiation and progression. TRPV6 expression is increased in advanced PCa and significantly correlated with the degree of aggressiveness of the cancer, measured by the Gleason score (which determines the histological grading of PCa and indicates the long-term prognosis of the disease [194]), making it a strong predictor of the clinical outcome [195]. For its part, TRPM8 is presented as a promising PCa biomarker and a putative therapeutic target in PCa treatment. Its expression in PCa increases more than any other prostate specific gene compared to non-malignant tissues. The channel is also overexpressed in other cancers such as breast, lung and colon cancers [193]. Dendritic cells loaded with a cocktail of peptides derived from different PCa-associated antigens including TRPM8 have been used for vaccination in a phase I clinical trial to treat patients reached by HRPCa [196].

In prostate, TRPM8 localization depends on the differentiation status of prostate epithelial cells. Whereas secretory mature prostate epithelial luminal cells expressed functional plasma membrane TRPM8, the channel activity is reported to be lost in the plasma membrane in dedifferentiated prostate epithelial cells but conserved on the ER membrane, due to a truncated splice variant of TRPM8 [188]. Cells obtained from PCa biopsies present a stronger TRPM8-mediated current density than normal prostate or benign prostate hyperplasia cells. But if TRPM8 expression has been noticed to be upregulated in PCa epithelia, it is mostly lost in dedifferentiated and androgen-independent cells [197, 198]. Indeed, TRPM8 expression in prostate cells is abolished after anti-androgen therapy [199]. Actually, one androgen response element (ARE) is present in the promoter of trpm8 gene and eleven AREs in its introns, making TRPM8 expression directly under control of AR activity [200].

Two studies have presented TRPM8 as an ER Ca^{2+} release channel in the PCa cell line LNCaP [200, 201]. Zhang and Barritt detected TRPM8 by immunofluorescence both in the plasma membrane and the ER of LNCaP cells and showed that, in the absence of extracellular Ca^{2+}, cooling and high concentrations of menthol were able to induce a small increase in intracellular Ca^{2+}. Nevertheless, the increase was much higher in presence of extracellular Ca^{2+}, suggesting an inward current from extracellular medium. Otherwise, they found that

both activation by menthol and inhibition by antagonist capsazepine or siRNA-mediated silencing of TRPM8 reduced LNCaP cells survival. Therefore, although TRPM8-mediated Ca^{2+} influx seems beneficial, a prolonged overstimulation and a subsequent Ca^{2+}-overload can become toxic [200]. In turn, Prevarskaya and her team also observed TRPM8 on intracellular structures in LNCaP cells. They also measured the menthol-induced Ca^{2+} release from the ER and an inward current carried not by TRPM8 but rather by SOCs activated by menthol-induced store depletion [201]. However, the localization of TRPM8 in LNCaP cells continues to be debated [202]. The specificity of antibodies directed against TRP channels is frequently questioned. Moreover, chemical compounds used at high concentrations to induce these currents could not be very specific of TRPM8. Thus, Voets and his collaborators showed that menthol could induce a Ca^{2+} release from the ER independently of TRPM8 channels. Besides, in LNCaP cells, they detected TRPM8 only in the plasma membrane [203].

In the prostate, cellular microenvironment is important in the regulation of TRPM8 opening. Indeed, TRPM8 activation in PCa cells has already been reported to depend on PSA-activated bradykinin 2 receptor pathway [204], phosphatidylinositol 4,5-bisphosphate [205], and lysophospholipid generated by the Ca^{2+}-independent phospholipase A2 [206].

In PCa, TRPM8 seems to be involved in the regulation of cell proliferation and migration. Thus, in androgen-independent PCa PC-3 cells, exogenous TRPM8 overexpression has been shown to inhibit cell cycle and migration while facilitating apoptosis induced by starvation [207]. Besides, PSA has been noted to activate and increase the number of functional TRPM8 channels thus reducing PC-3 cells motility [204]. Moreover, in the other androgen-independent DU145 cell line which endogenously expresses TRPM8, cell growth and migration have also been reported to be inhibited by menthol [208]. Migration control of PCa cells by TRPM8 seems to depend on their association with two recently discovered proteins named TAF1 and TAF2 (TRP channel-associated factors) [209].

9.2 - Inositol trisphosphate receptors

Among the elements of Ca^{2+} signaling machinery, IP3R channel also appears to be involved in the mechanisms of cancerogenesis [210].

The primary structure of IP3R presents different domains: an N-terminal IP3-binding domain, a regulatory/coupling domain in the middle of the molecule, a Ca^{2+} channel-forming region localized to the C-terminus, with six transmembrane domains, and a short cytoplasmic C-terminal tail, the gating domain. Three IP3R isoforms (IP3R1, 2 and 3), generated from ITPR1, ITPR2 and ITPR3 genes, form homo- and heterotetramers of molecular weight of

about 1.2 MDa (Fig. 15). Their opening is triggered by IP3, which is mainly produced at the cell membrane in response of GPCRs or RTKs activation by ligands such as hormones and growth factors. Activated membrane receptors stimulate phospholipase C (PLC) which carry out the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to release IP3 and diacylglycerol (DAG). Due to its hydrophilic composition, IP3 can diffuse away from its production site and binds IP3Rs on the ER surface. This binding increases the sensitivity of the receptor to cytosolic Ca^{2+}, which activates it at low concentrations and inhibits it at the higher concentrations that occur after Ca^{2+} release [211, 212]. For its part, luminal Ca^{2+} can also modulate IP3R activity, the depletion of ER Ca^{2+} store being known to decrease the channel sensitivity to IP3 [213, 214]. IP3R contains multiple phosphorylation sites and is associated with several regulatory proteins such as IRBIT (IP3R-binding protein released by IP3) and Bcl-2, themselves regulated by various kinases and phosphatases and making difficult the perfect understanding of IP3R regulation [212]. However, several mechanisms of control have been established. It has been demonstrated that PKA (cyclic AMP-dependent protein kinase) phosphorylates IP3R1 on two sites, S1589 and S1716, both located in the regulatory domain, leading to an increase of its sensitivity towards IP3 [215, 216]. Other kinases such as CaMKII (Ca^{2+}/Calmodulin dependent kinase II), PKG (cyclic GMP-dependent protein kinase), PKC and Akt modulate IP3R opening [212]. Besides, several Bcl-2 family members are involved in the control of IP3R activity. Indeed, beside their roles in the permeability of the mitochondrial outer membrane and in the regulation of class III PI3K/Beclin-1 complex, Bcl-2 family proteins have repeatedly been reported to modulate the ER Ca^{2+} content and its release. Although the involved mechanisms are still not fully understood (see the following chapter), a regulation of IP3R opening by the Bcl-2 family members has been highlighted [217].

IP3R channel seems involved in cancer progression. Indeed, IP3R expression profile is reported to be altered in several cancers such as glioblastoma [218] and gastric cancers [219]. The aggressiveness of colorectal carcinomas is also correlated to the expression of IP3R3 [220]. Moreover, modulation of IP3R expression seems involved in the growth of breast cancer cells [221]. We can also notice these conflicting data about IP3R involvement in the resistance to anti-tumor treatment : the overexpression of IP3R constitutes a mechanism of resistance to cisplatin in non-small-cell and small-cell lung cancer cells [222] while a reduced expression of IP3R1 decreases sensitivity to the same drug in bladder cancer cells [223]. Besides, numerous proto-oncogenes such as Akt [224], Bcl-2 or Bcl-xL [217] as explained

above, but also tumor suppressors such as the promyelocytic leukemia protein (PML) [225], BRCA1 [226] and Beclin-1 [227] regulate IP3R function and ER Ca^{2+} store content.

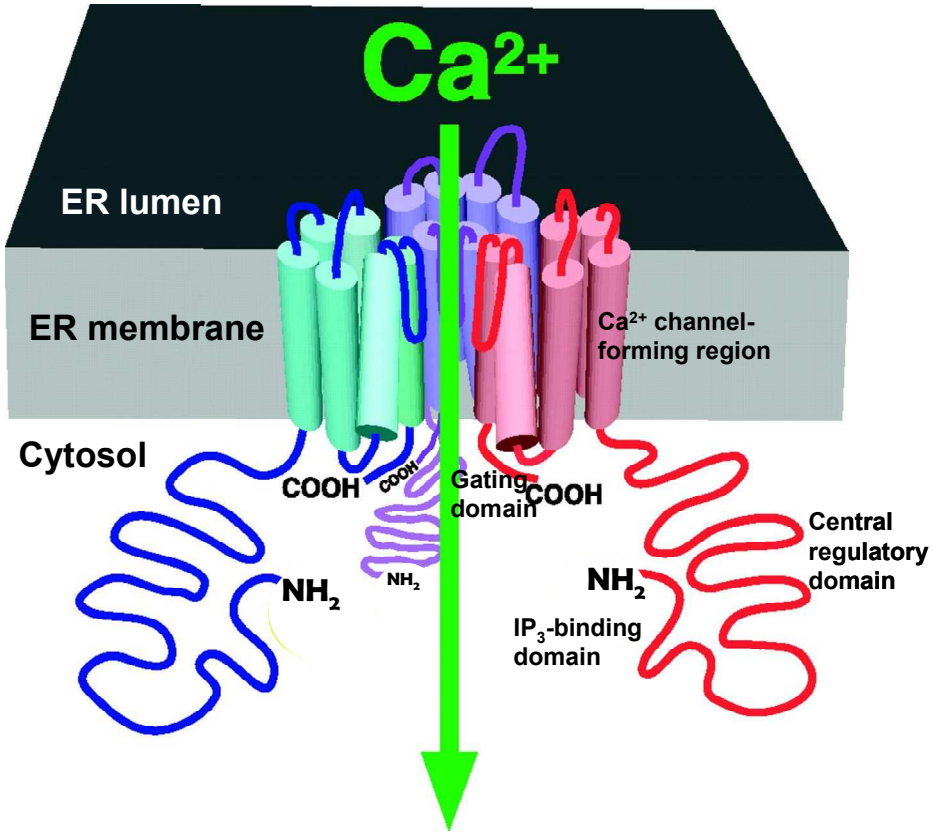

Fig. 15: The IP3R structure. Adapted from [228].
Three of the four IP3R subunits are represented on the figure. Each of them is composed of an N-terminal IP3-binding domain, a central regulatory domain, a Ca^{2+} channel-forming region with 6 transmembrane domains and a C-terminal gating domain.

As we have previously seen, several signaling pathways regulate both apoptosis and autophagy. Ca^{2+} signaling has also been demonstrated to be involved in the control of these two functions, which again supports the notion of a close link between them.

10 - Calcium and apoptosis

The high toxicity of a cellular Ca^{2+} overload has been known since the late 1960s. Indeed, one of the first effects described for Ca^{2+} ionophores was their toxicity [229]. Originally, the toxic role of Ca^{2+} was related to necrosis as in the case of excitotoxicity, in which necrotic death occurs in neurons after glutamate-dependent hyperstimulation and subsequent massive Ca^{2+} influx [230]. However, in recent years, numerous reports have indicated an involvement of

Ca^{2+} in apoptosis regulation. In particular, the ER Ca^{2+} store has been reported to modulate the sensitivity to apoptosis [231].

10.1 - Role of the ER Ca^{2+} store

The ER represents the main intracellular store of Ca^{2+} and serves many functions among which the folding, the post-translational modification and the trafficking of proteins. As ER chaperones require a high Ca^{2+} concentration for their activity, an $[Ca^{2+}]_{ER}$ reduction can promote the accumulation of unfolded proteins, a condition referred to as ER stress. To overcome this stress, a particular pathway called the unfolded protein response (UPR) is triggered (Fig. 16). It precludes the accumulation of unfolded proteins by the release of three proteins, PERK (PKR-like eIF2α kinase), IRE1 (inositol requiring enzyme 1) and ATF6 (activating transcription factor 6), normally bound to and inactivated by the luminal chaperone GRP78 (glucose regulation protein 78 also called BiP for Binding immunoglobulin protein). By their transcriptional and translational activities, these proteins inhibit general protein synthesis, while selected synthesis of chaperones and protein folding enzymes is favored. Moreover, they promote, through ERAD (ER-associated degradation) system, the retrograde translocation of ER proteins to the cytoplasm to allow their degradation by the proteasome [232, 233]. In the case of excess ER stress that UPR's mechanisms fail to reduce, the UPR activates apoptosis by upregulation of the proapoptotic factor CHOP (CCAAT/-enhancer-binding protein homologous protein) and Jnk-dependent inactivation of Bcl-2 [234, 235].

The concept wherein ER Ca^{2+} is closely related to apoptosis control also comes from the recognition that Bcl-2 proteins are localized on the membranes of organelles deeply involved in Ca^{2+} storage (the ER and the mitochondria). Indeed, in addition to their direct role in apoptosis and more precisely in the control of the permeability of the mitochondrial outer membrane, Bcl-2 family proteins have been reported to govern the ER Ca^{2+} content and its release (reviewed in [236]). The first observation suggesting a role of Bcl-2 on ER Ca^{2+} dates back to 1993, when Bcl-2 overexpression was shown to protect hematopoietic cells from apoptosis induced by interleukin-3 withdrawal by precluding Ca^{2+} transfer from the ER to the mitochondria [237]. Similar data were obtained in other studies. For example, Bcl-2 overexpression was demonstrated to abolish cytosolic Ca^{2+} increase provoked by serum withdrawal in murine fibroblasts [238].

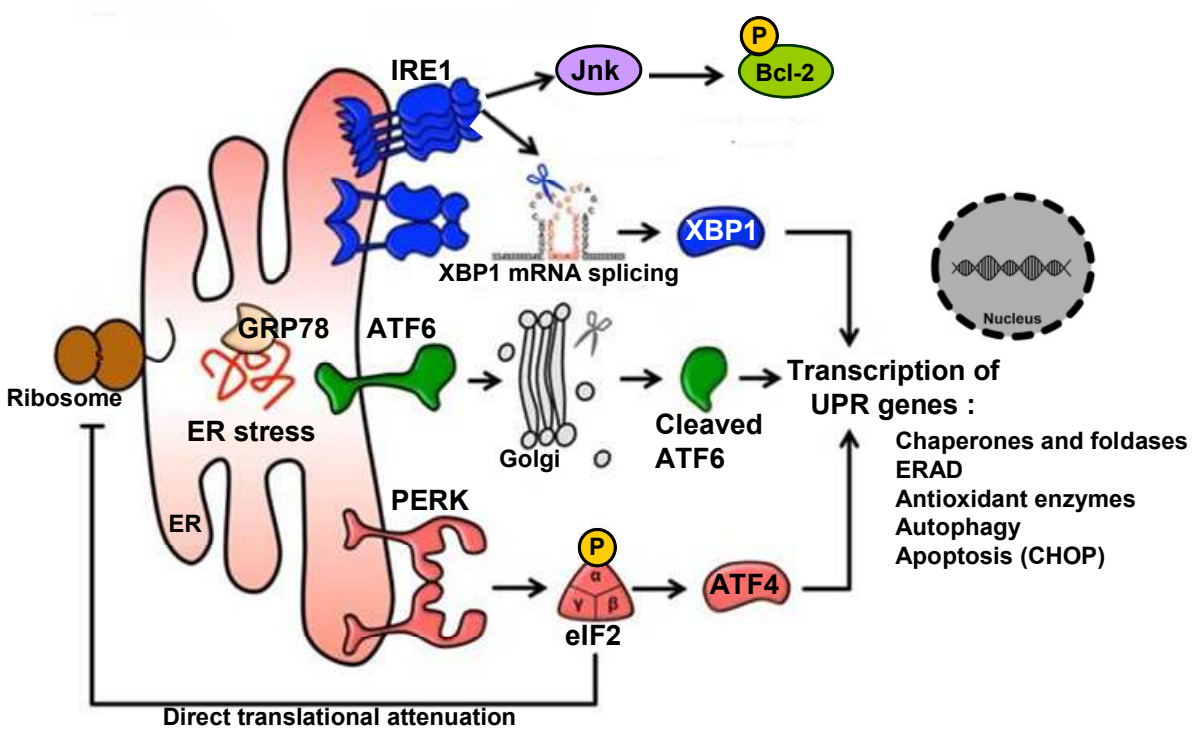

Fig. 16: The UPR signaling pathway. Adapted from [239]
An ER stress is known to trigger the unfolded protein response (UPR). Such a pathway depends on the release of PERK (PKR-like eIF2α kinase), IRE1 (inositol requiring enzyme 1) and ATF6 (activating transcription factor 6) from GRP78 (glucose regulation protein 78), which leads to their activation. Released and oligomerized IRE1 achieves the splicing of XBP1 mRNA, which generates the transcription factor XBP1. IRE1 also mediates the inactivation of Bcl-2 by stimulating Jnk (c-Jun N-terminal kinase). Released ATF6 translocates to the Golgi apparatus where it is cleaved to produce an active transcription factor. PERK undergoes oligomerization and phosphorylates the translation initiating factor eIF2α, thereby repressing the general protein synthesis while promoting the translation of the transcription factor ATF4 (activating transcription factor 4). ATF4, ATF6 and XBP1 promote the expression of various genes required for ERAD (ER-associated degradation) and for the restoration of ER homeostasis. However, upon severe ER stress, the UPR triggers apoptosis by increasing the expression of the pro-apoptotic transcription factor CHOP (CCAAT/-enhancer-binding protein homologous protein).

Moreover, Bcl-2 inhibits the induction of apoptosis by thapsigargin, a specific inhibitor of the SERCA pump, by reducing Ca^{2+} efflux through the ER membrane [240].

Actually, various stimuli among which oxidative stress [241], ozone [242], palmitate [243] or paclitaxel [244] induce Ca^{2+} release from the ER in order to trigger apoptosis. Bcl-2 overexpression counteracts this process in numerous models [245, 246]. However, one point remains controversial. Several reports emphasize if it decreases the Ca^{2+} release from the ER, Bcl-2 preserves rather than depletes ER Ca^{2+} store [236]. Nonetheless, more recent data, including direct measurements of luminal Ca^{2+} with ER-targeted Ca^{2+}-sensitive fluorescent probes, seem to demonstrate the opposite. Pinton et al. showed an increased ER Ca^{2+} leak, hence reducing the $[Ca^{2+}]_{ER}$ after Bcl-2 overexpression. Ca^{2+} release caused by IP3-generating agonists was subsequently decreased in both the cytosol and the mitochondria. Moreover, they observed a decrease of the amplitude of store-operated Ca^{2+} entry (SOCE), the Ca^{2+} influx through the plasma membrane activated in response to the depletion of intracellular Ca^{2+} store [247]. These reduced $[Ca^{2+}]_{ER}$ and increased permeability of the ER membrane have been confirmed in other models [248, 249]. Indeed, it has been demonstrated that Bcl-2 overexpression in the PCa-derived LNCaP cell line decreases $[Ca^{2+}]_{ER}$ through the reduction of calreticulin and SERCA2b expressions and restricts SOCE by decreasing the number of functional plasma membrane channels [250].

The mechanism by which Bcl-2 regulates Ca^{2+} store content is still a matter of debate. In addition to the possibility of a pore formation by Bcl-2 itself, it could reduce the activity of SERCA pumps. Interestingly, SERCA overexpression, by increasing ER Ca^{2+} store content, has been observed to improve the sensitivity to apoptosis [251]. Besides, an interaction between Bcl-2 and SERCA, which destabilizes SERCA pump, has been reported [252]. Furthermore, calreticulin overexpression has a similar effect on cell survival and Ca^{2+} homeostasis. Meanwhile, calreticulin depletion increases resistance to apoptosis [253]. Bcl-2 has also been reported to interact with IP3R, thus reducing its open probability and IP3-induced Ca^{2+} release (IICR) [254, 255]. This interaction involves the BH4 domain of Bcl-2 that binds to the regulatory domain in the middle of IP3R [256]. In addition, Bcl-2 but also Bcl-xL and Mcl-1 are demonstrated to bind another site in the C-terminal domain of IP3R, which in this case would enhance its gating. These last interactions lower $[Ca^{2+}]_{ER}$ and increase IICR at low [IP3] to likely stimulate bioenergetics but decrease it at high [IP3], thereby avoiding a lethal Ca^{2+} overload in the cytosol [217]. However, although Bcl-xL is also able to modulate Ca^{2+} release through IP3R, its anti-apoptotic activity seems less potent than Bcl-2 due to a single amino-acid difference [257]. Besides, the anti-apoptotic functions

of Bcl-2 through its regulation of ER Ca^{2+} store could depend on its phosphorylation status and interactions with pro-apoptotic family members [235]. Indeed, Bax and Bak also localize at the ER where they induce ER Ca^{2+} emptying leading to an accumulation of Ca^{2+} in the mitochondria and a subsequent release of cytochrome c [258]. Furthermore, Korsmeyer and his team observed that in Bax/Bak double-knockout cells, IP3R1 was hyperphosphorylated, in particular at Ser1756 (human Ser1716), a PKA and PKG phosphorylation site. This increases ER Ca^{2+} leak and thereby reducing [Ca^{2+}] in the ER. They also demonstrated that Bcl-2 knockdown is able to decrease IP3R1 phosphorylation and restore $[Ca^{2+}]_{ER}$ [259]. This last observation shows us how IP3R regulation by Bcl-2 can be complex and not just related to direct interactions.

The Ca^{2+} release from the ER is known to range from confined spikes produced by a single or a few channels through propagating repetitive waves to massive influx generated by the opening of the entirety of the IP3R and RyR channels throughout the ER. Whereas oscillatory and moderate Ca^{2+} pulses are recognized to be beneficial for the cell, a bulk release is deleterious. The effects of Ca^{2+} release from the ER on cell survival seem mainly dependent on the Ca^{2+} entry into the mitochondria [260].

10.2 - The mitochondrial Ca^{2+}

The ER is tightly associated to mitochondria, 20% of the mitochondrial surface being in direct contact with ER membranes [261]. The ER communicates with mitochondria through contiguous membranes, which have been reported to be expanded in some situations such as under ER stress and/or apoptotic conditions [262, 263]. These contact sites are called mitochondria-associated membranes (MAM) and support direct transfer of lipids, ATP and proteins between the two organelles [264]. Moreover, MAM are enriched in Ca^{2+} channels and Ca^{2+}-sensing ER chaperones allowing an easy exchange of cations [231]. Indeed, IP3R is incorporated in a protein complex with GRP75 and the outer mitochondrial membrane VDAC channel to promote a Ca^{2+} flux between the two organelles [265]. The close apposition of ER and mitochondria creates a confined space wherein Ca^{2+} released from the ER can reach concentrations in the millimolar range. Thus, despite the low affinity of mitochondrial transporters, large Ca^{2+} fluxes can cross the mitochondrial membranes (Fig. 17). The outer mitochondrial membrane is penetrated through VDAC while Ca^{2+} is taken up into the matrix through MCU, the mitochondrial Ca^{2+} uniporter whose molecular identity was discovered

only recently [266, 267]. This transport is primarily driven by the $\Delta\Psi_m$ generated by the electron transport in normally respiring mitochondria [260].

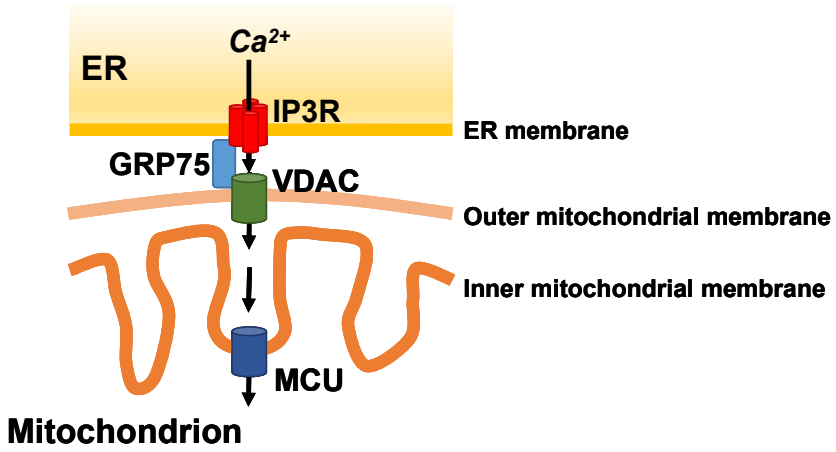

Fig. 17: The mitochondrial calcium uptake. Adapted from [268].
The close apposition of ER and mitochondrion allows a rapid Ca^{2+} transfer between these two organelles. Ca^{2+} is released in the intermembrane space through the complex formed by IP3R (inositol trisphosphate receptor), GRP75 (75 kDa glucose regulation protein) and VDAC channel (voltage-dependent anion channel), and passes through the inner mitochondrial membrane by the mitochondrial Ca^{2+} uniporter (MCU).

The capacity of mitochondria to accumulate Ca^{2+} seems, as for the ER, under the control of Bcl-2 family members. Thus, Bcl-2 has repeatedly been reported to enhance Ca^{2+} storage while protecting mitochondria from Ca^{2+} overload [269-271].

Mitochondrial Ca^{2+} uptake has a crucial physiological function. It contributes to the mitochondrial metabolism and ATP production. Actually, the activity of pyruvate dehydrogenase as those of two enzymes of the Krebs cycle (isocritrate and α-ketoglutarate dehydrogenases) is dependent on Ca^{2+}. Moreover, some metabolite transporters are regulated by Ca^{2+} [272]. Nevertheless, a direct effect of $[Ca^{2+}]_m$ increase on mitochondrial permeability transition has been known for almost 40 years [273]. Indeed, a bulk Ca^{2+} arrival is detrimental (Fig. 18). It impairs respiration chain, increasing $\Delta\Psi_m$. At high $\Delta\Psi_m$, harmful reactive oxygen species (ROS) production rises [274]. Moreover, high $\Delta\Psi_m$ promotes the mitochondrial sponging of Ca^{2+}, increasing $[Ca^{2+}]_m$ which may reach the activation threshold of the permeability transition pore, leading to $\Delta\Psi_m$ dissipation, mitochondria swelling and triggering of the intrinsic apoptosis pathway [271, 275].

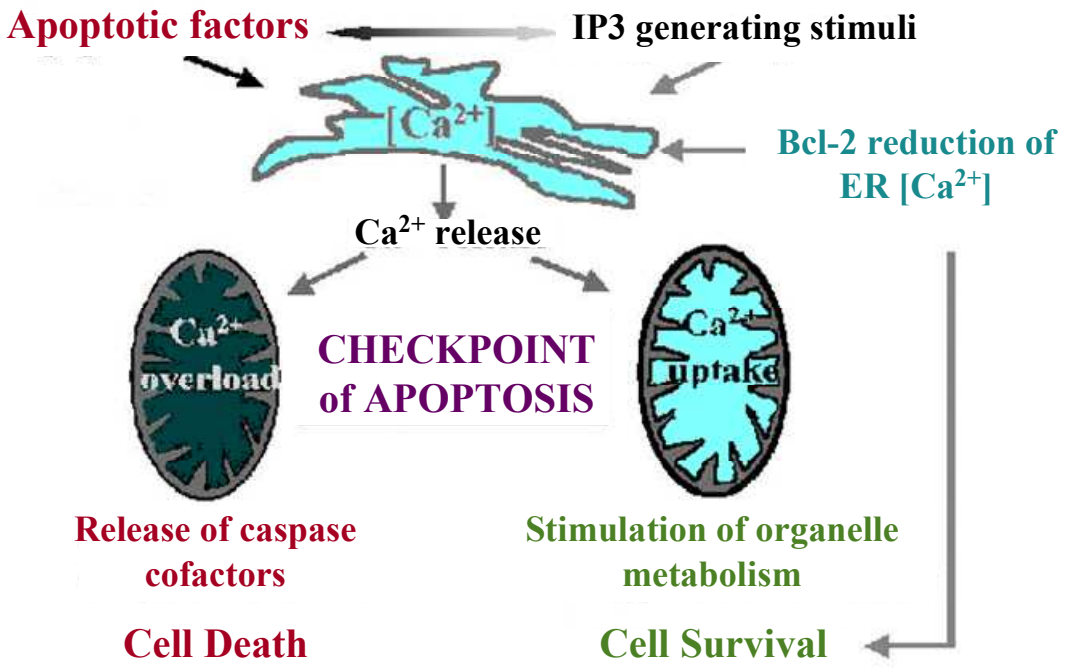

Fig. 18: The mitochondrial Ca^{2+} handling: checkpoint of apoptosis. Adapted from [276] Whereas moderate Ca^{2+} transfer from the ER to the mitochondria is beneficial, contributing to the mitochondrial metabolism, massive Ca^{2+} accumulation in the organelle triggers cell death. Bcl-2, by reducing $[Ca^{2+}]_{ER}$, restricts any mitochondrial Ca^{2+} overload and thereby protects from apoptosis.

10.3 - The cytosolic Ca^{2+}

Several agents such as TGFβ1, doxorubicin or radiation have been shown to induce apoptosis by elevating $[Ca^{2+}]_c$ in prostate and bladder cancer cells [277]. Indeed, the Ca^{2+} released from the ER into the cytosol paired with store-operated Ca^{2+} entry also contributes to the induction of apoptosis. Cytosolic Ca^{2+} increase allows activation of the cysteine proteases known as calpains by an autocatalytic cleavage. They are reported to cleave Bcl-2 and Bcl-xL thereby reducing their anti-apoptotic activities and leading to Bcl-2 conversion into a pro-apoptotic protein [278]. Moreover, calpains cleave pro-apoptotic Bid into its active form, inducing cytochrome c release [279]. These Ca^{2+}-dependent proteases can also cleave and activate caspase-12, localized on the cytoplasmic side of the ER, to trigger apoptosis in response to Ca^{2+} store depletion [280]. Besides, $[Ca^{2+}]_c$ rise directly or indirectly activates numerous kinases and phosphatases. Among them, the Ca^{2+}-dependent serine-threonine phosphatase calcineurin that is able, once activated, to dephosphorylate Bad, another BH3-only protein, thereby enhancing its heterodimerization with Bcl-xL and initiating the intrinsic mitochondrial pathway [281]. However, a modest increase of $[Ca^{2+}]_c$, on the contrary, can

lead to Bad phosphorylation and sequestration to protein 14-3-3 through activation of CaMKK, highlighting the versatility of Ca^{2+} signaling in cell survival [282]. Furthermore, caspases can cleave different components of the Ca^{2+} signaling machinery: IP3R1 has been reported to be cleaved by caspase-3 [283, 284] which would generate a constitutively open channel and would enhance Ca^{2+} release [285]; plasma membrane Na^{+}/Ca^{2+} exchanger and PMCA pumps, essential for the return of $[Ca^{2+}]_c$ to basal levels, can also be cleaved by caspases and calpains thereby increasing Ca^{2+} overload [286, 287].

11 - Calcium and autophagy

Ca^{2+} was first reported to be involved in autophagy control in 1993 when Gordon et al. discovered that chelation of either intra- and extracellular Ca^{2+} as well as overload of cytosolic Ca^{2+} inhibit autophagy [288]. Since then, several reports have confirmed the dual role that plays Ca^{2+} homeostasis in this catabolic process. Indeed, Ca^{2+} and some elements of Ca^{2+} signaling machinery have repeatedly been presented as negative regulators of autophagy whereas an increased Ca^{2+} influx has also been noticed to stimulate this function [289].

11.1 - Suppression of autophagy by Ca^{2+} signaling

The inhibitory function of Ca^{2+} signaling on autophagy is mainly related to IP3R. Indeed, the Ca^{2+} channel has been established to play an important role in autophagy. Its inhibition by xestospongin B (as the reduction of IP3 levels by lithium) and its knockdown lead to autophagy [290, 291]. Besides, basal autophagic flux is higher in IP3R triple knock out (TKO) chicken DT40 B lymphocytes than in their wild-type counterparts [292, 293]. Heterologous expression of IP3R1 or IP3R3, but not RyR, decreases autophagy levels in these TKO cells, thereby confirming an IP3R-mediated negative regulation of autophagy. Kroemer and his team, by demonstrating an interaction between Beclin-1 and the IP3-binding domain of the receptor, have proposed that the involvement of IP3R in autophagy would be explained by its role of scaffold protein facilitating Beclin-1 interaction with Bcl-2. Thus, xestospongin B would induce autophagy by dissociating Beclin-1 from IP3R/Bcl-2 complex. Besides, they have suggested that the Ca^{2+} channel function of IP3R would not be involved, Beclin-1 knockdown leaving unchanged the $[Ca^{2+}]_{ER}$ [294]. However, the Ca^{2+}-independent nature of the mechanism of autophagy control by IP3R is not unanimously accepted. Indeed, expression

of functionally inactive IP3R mutants in TKO cells has been observed as not suppressing autophagy in the same manner as wild-type IP3Rs, suggesting that the channel function of the molecule would be important in its properties of autophagy regulator [292, 293].

Foskett and his collaborators have demonstrated that a mitochondrial uptake of IP3R-released Ca^{2+} is required to provide optimal bioenergetics. In the absence of this Ca^{2+} transfer, as it is the case in TKO cells, metabolic enzymes such as pyruvate dehydrogenase are inactivated, ATP production decreased and AMPK activated, which induces a prosurvival autophagy [293]. In addition of these data, Bax inhibitor-1 (BI-1) overexpression has been noticed to promote autophagy by decreasing $[Ca^{2+}]_{ER}$, thereby reducing IP3R-mediated Ca^{2+} transfer to the mitochondria, which affects mitochondrial respiration and ATP production, and leads to AMPK activation and autophagy induction [295].

The induction of autophagy by inhibitors of high voltage-activated (L-type) Ca^{2+} channels also highlights the anti-autophagic properties of Ca^{2+} [296, 297]. Interestingly, these inhibitors, by influencing $[Ca^{2+}]_c$, impact the activity of calpains, which indirectly control the PLC activation and IP3 production [298, 299]. Therefore, blocking L-type Ca^{2+} channels may also lead to a reduced Ca^{2+} transfer to the mitochondria.

Besides, the late-phase of autophagy can also be inhibited by Ca^{2+} signaling. In obesity in particular, a chronic $[Ca^{2+}]_c$ increase in hepatocytes preventing autophagosome-lysosome fusion and deregulating autophagy has recently been described [300].

11.2 - Promotion of autophagy by Ca^{2+} signaling

If some modulations of Ca^{2+} homeostasis seem able to repress autophagy, a release of Ca^{2+} in the cytosol has repeatedly been reported to stimulate autophagy. Indeed, various drugs which elevate $[Ca^{2+}]_c$ such as SERCA inhibitor thapsigargin, Ca^{2+} ionophore ionomycin, ATP or vitamin D_3 compounds have been demonstrated to inhibit mTOR activity and induce autophagy. This process seems to be dependent on CaMKKβ and AMPK activation and can be inhibited by the localization of Bcl-2 to the ER membrane, where it lowers $[Ca^{2+}]_{ER}$ and reduces IP3-induced Ca^{2+} release into the cytosol [301]. The ability of Bcl-2 to decrease Ca^{2+} store content thereby appears as another anti-autophagic mechanism of the protein beyond its inhibitory interaction with Beclin-1. The signaling pathways involved in this autophagy triggering have been discussed, some studies showing that thapsigargin can induce autophagy without AMPK stimulation [302] and even apart from mTOR inhibition but through PKCθ

[303]. Besides, cadmium-induced $[Ca^{2+}]_c$ rise has been noted to lead to autophagy via the activation of Erk [304].

Although the buffering of cytosolic Ca^{2+} by BAPTA-AM prevents such autophagy, thereby proving the significance of Ca^{2+} in this function, prolonged treatment with these molecules could also result in ER Ca^{2+} store emptying and subsequent unfolded protein response, which itself can trigger autophagy through different pathways [232]. However, thapsigargin-induced autophagy is still present in UPR-deficient cells, therefore supporting the direct role of Ca^{2+} in autophagy induction [303].

The Ca^{2+} release from other compartments than ER can also promote autophagy. Indeed, the lysosomal two pore channels opening by nicotinic acid adenine dinucleotide phosphate (NAADP) has been reported to induce autophagy [305].

Actually, Ca^{2+} signaling modulation could even be a mechanism allowing the triggering of autophagy in response to numerous stimuli. Indeed, nutrient deprivation-induced autophagy is suppressed by the Ca^{2+} chelator BAPTA-AM [306]. A remodeling of the Ca^{2+} signalosome has even been observed during starvation-induced autophagy. At the beginning of the autophagic response, Bultynck and his collaborators have reported an overexpression of Ca^{2+}-binding chaperones calreticulin and GRP78 which increase the ER Ca^{2+} content. Furthermore, starvation leads to an increased Beclin-1 binding to the IP3R resulting in a higher sensitivity of the receptor towards IP3. The authors have suggested that by promoting the release of Ca^{2+} into the cytosol, Beclin-1/IP3R interaction would have a pro-autophagic function in starved cells, whereas it would inhibit autophagy in non-starved cells by facilitating Beclin-1 tethering to Bcl-2 and beneficial IP3R-mediated Ca^{2+} transfer to the mitochondria. They have shown that xestospongin B suppresses starvation-induced autophagy although it enhances basal levels of autophagy [227]. Moreover, they have recently highlighted that the mTOR inhibitor and autophagy inducer rapamycin also remodels Ca^{2+} homeostasis. They have found that rapamycin elevates the ER Ca^{2+} content by reducing Ca^{2+} leakage from the compartment and increases IP3-induced Ca^{2+} release, while buffering intracellular Ca^{2+} with BAPTA-AM precludes autophagy, indicating that cytosolic Ca^{2+} is essential for the induction of autophagy by rapamycin [307].

Besides, diverse autophagy regulators seem influenced by Ca^{2+}. Thus, the class III PI3K has been noticed to be under the control of Ca^{2+} and calmodulin [308], as well as DAPK [309], members of the S100 Ca^{2+}-binding protein family [310] and the kinase of the eukaryotic elongation factor (eEF-2 kinase) [311]. Although Ca^{2+}-dependent calpains are known to cleave Atg5, producing a pro-apoptotic residue [172], they seem also required for autophagy.

Indeed, as we have previously seen, in calpain-deficient cells, autophagy is impaired [180]. Furthermore, the Ca^{2+} channel TRPML3 is recruited to autophagosomes upon induction of autophagy [312]. Its overexpression promotes autophagy whereas its knockdown or the expression of a channel-dead dominant negative reduces it. Therefore, Ca^{2+} participates in the induction of autophagy but may also be required for the maturation of autophagosomes.

Aims and objectives

As explained in the introduction, a better understanding of the mechanisms of androgen independence is necessary to improve the management of HRPCa. More precisely, we have to determine why apoptosis is not significantly engaged after the pro-death AD in HRPCa. We made several assumptions. While autophagy protects many cancer cells from apoptosis, its possible involvement in HRPCa progression remained unexplored. Hence, we sought to clarify its activity in HRPCa cells and to understand its function. To this end, we used LNCaP cells, isolated in 1977 from a needle aspiration biopsy of a lymph node of a 50 year old man with confirmed diagnosis of metastatic prostate carcinoma [313]. We can reproduce AD therapy *in vitro* by culturing these cells in medium supplemented with charcoal stripped fetal calf serum, in which steroid hormones have been removed, or by treating them with AR antagonists such as bicalutamide. LNCaP cells can survive in culture after androgen withdrawal and therefore constitute a valid model of HRPCa. However, they are also considered as androgen-sensitive PCa cells since their growth is still dependent on the androgen concentration. Thus, they appear as a valid model for studying the transition to hormone independence.

In these cells, we have also observed various modifications of Ca^{2+} homeostasis after AD. These led us to explore the role of the Ca^{2+} signaling machinery in the apoptosis resistance of LNCaP cells to androgen deprivation. Indeed, as we have explained above, Ca^{2+} is known to participate in the regulation of both apoptosis and autophagy. We therefore wanted to study its involvement in these two processes in our model.

Thus, the aim of this work was to answer several issues allowing us to give new elements to the understanding of the cell survival mechanisms engaged in HRPCa in response to AD:

a) Does androgen control autophagy in HRPCa cells? And if so, by what mechanisms?
b) Is autophagy responsible in resistance to AD in HRPCa?
c) What are the mechanisms behind these Ca^{2+} homeostasis alterations?
d) Is Ca^{2+} signaling involved in androgen independence and apoptosis resistance?
e) Is there a crosstalk between these modifications in Ca^{2+} homeostasis and the autophagic process?

Two research papers and additional data answering these diverse items are presented in the following sections.

Results

Research paper 1 / Androgen deprivation and androgen receptor competition by bicalutamide induce autophagy of hormone-resistant prostate cancer cells and confer resistance to apoptosis.

Research paper 2 / Endoplasmic reticulum Ca^{2+} content decrease by PKA-dependent hyperphosphorylation of type 1 IP3 receptor contributes to prostate cancer cell resistance to androgen deprivation.

Supplementary data

Androgen Deprivation and Androgen Receptor Competition by Bicalutamide Induce Autophagy of Hormone-Resistant Prostate Cancer Cells and Confer Resistance to Apoptosis

Benoît Boutin,[1] Nicolas Tajeddine,[1] Patrick Vandersmissen,[2] Nadège Zanou,[1] Monique Van Schoor,[1] Ludivine Mondin,[1] Pierre J. Courtoy,[2] Bertrand Tombal,[1,3] and Philippe Gailly[1]*

[1]*Laboratory of Cell Physiology, Université catholique de Louvain, Brussels, Belgium*
[2]*Cell Unit, Université catholique de Louvain and de Duve Institute, Brussels, Belgium*
[3]*Urology Unit, Cliniques universitaires Saint-Luc, Brussels, Belgium*

BACKGROUND. Treatment of advanced prostate cancer (PCa) relies on pharmacological or surgical androgen deprivation. However, it is only temporarily efficient. After a few months or years, the tumor relapses despite the absence of androgenic stimulation: a state referred to as hormone-refractory prostate cancer (HRPCa). Although autophagy confers chemoresistance in some cancers, its role in the development of HRPCa remains unknown.

METHODS. Autophagic flux was assayed by GFP-LC3 clustering, by LC3-I to LC3-II conversion and transmission electron microscopy. Cell death was detected by sub-G1 quantification and concomitant measurement of transmembrane mitochondrial potential and plasma membrane permeabilization. Inhibition of autophagy was achieved by siRNAs and pharmacological inhibitors.

RESULTS. Androgen deprivation or treatment with the anti-androgen bicalutamide promoted autophagy in HRPCa-derived LNCaP cells. This effect was dramatically reduced after depletion of Atg5 and Beclin-1, two canonical autophagy genes, and was associated with an inhibition of the androgen-induced mTOR pathway. The depletion of Atg5 and Beclin-1 significantly increased the level of cell death induced by androgen deprivation or bicalutamide. Finally, the safe anti-malarial drug chloroquine, an inhibitor of autophagy, dramatically increased cell death after androgen deprivation or bicalutamide treatment.

CONCLUSION. Taken together, our data suggest that autophagy is a protective mechanism against androgen deprivation in HRPCa cells and that chloroquine could restore hormone dependence. This set of data could lead to the development of new therapeutic strategy against HRPCa. *Prostate* © 2013 Wiley Periodicals, Inc.

KEY WORDS: **prostate cancer; autophagy; apoptosis; androgen; chloroquine**

Additional supporting information may be found in the online version of this article.

Grant sponsor: General Direction of Scientific Research of the French Community of Belgium, Inter-University Attraction Pole (IUAP P7/13); Grant number: F.R.S.-FNRS—Télévie n°7. 4542 07; Grant sponsor: ''Fonds Joseph Maisin''; Grant number: n°7.4611.10, F.R.S.M. 3.4.515.09.F, ARC n°10-15/029.

Benoît Boutin and Nicolas Tajeddine contributed equally to this work.

Bertrand Tombal and Philippe Gailly share senior authorship.

The authors have declared that no conflict of interest exists.

*Correspondence to: Philippe Gailly, Laboratory of Cell Physiology, Université catholique de Louvain, Avenue Mounier, B1.55.12, Brussels 1200, Belgium. E-mail: philippe.gailly@uclouvain.be

Received 25 October 2012; Accepted 6 February 2013
DOI 10.1002/pros.22658
Published online in Wiley Online Library (wileyonlinelibrary.com).

INTRODUCTION

Prostate cancer (PCa) is the most frequent cancer in men above the age of 55 years. Only low-grade and locally advanced PCa are curable and, despite significant progresses in early detection and curative treatments, 30% of patients will develop an advanced or metastatic cancer requiring systemic therapies [1]. Reference treatment of advanced PCa relies on pharmacological or surgical androgen deprivation therapy. However, despite initial efficacy of androgen deprivation, the tumor inevitably relapses becoming hormone-refractory (HRPCa) and progresses to metastatic disease. Effective treatment at this stage is largely limited to docetaxel-based chemotherapies conferring a median survival of only 17–19 months [2,3]. Moreover, despite numerous clinical trials, consensus about the management of non-metastatic HRPCa has not emerged. All these observations lead the scientific community to decipher the mechanisms involved in androgen-independent phenotype development in PCa, and to develop novel strategies to circumvent hormone independence of advanced prostate cancers [4]. Deregulations of multiple pathways have been involved in the progression of PCa to HRPCa, including aberrant activation of the androgen receptor or abnormalities in the PI3K/AKT signaling and PTEN function [5–7]. However, the role of autophagy, which seems to be involved in apoptosis resistance in some cancers, remains unknown in the development of HRPCa.

The general concept of ''autophagy,'' introduced in 1963 by the Nobel Prize laureate Christian de Duve, has now been dismembered into a variety of intracellular processes, including chaperone-mediated autophagy, micro- and macro-autophagy, all of which eventually converge to a common degradation phase mediated by lysosomes [8,9]. Macroautophagy (hereafter referred to as autophagy) has recently been involved in the pathogenesis of diverse diseases including cancer [10]. During autophagy, bulk portions of the cytoplasm, including organelles and long-lived proteins, are sequestered in double- or multiple-membranes vacuoles (autophagosomes) that fuse with lysosomes that degrade their contents. Upon autophagy induction, the LC3-I protein is modified into the PE-conjugated form, LC3-II. This latter is associated with autophagosomes and its detection is routinely used to monitor autophagy [11]. Basal levels of autophagy contribute to cellular homeostasis by ensuring the turnover of supernumerary, aged, and damaged components. *Atg* genes encode proteins required in the various phases of autophagic pathways [12].

Dramatic increase of autophagy level has long been considered as a particular form of cell death (termed type II cell death), characterized by massive accumulation of autophagosomes and dependence on *Atg* genes [13–15]. In contrast, under stress conditions such as starvation or growth factor withdrawal, autophagic pathways operate to supply cells with metabolic substrates and hence represent an important pro-survival mechanism. Under carbon and nitrogen starvation, for instance, the activity of mammalian target of rapamycin (mTOR; the major negative regulator of autophagy) is rapidly shut down, and the ensuing up-regulation of autophagic pathway supplies cells with metabolic substrates to meet their energetic demands [12]. These observations prompted some investigators to consider that cell death is often associated with autophagy but is rarely due to autophagy [16].

Here, we investigated these processes in PCa cells derived from a lymph node metastasis of a hormone-refractory patient (LNCaP cells). In these cells, a mutation in the androgen receptor gene creates a promiscuous receptor able to bind to other steroids [17]. It turns out that LNCaP cells are able to grow in culture despite the absence of androgen and therefore constitute a valid cellular model of HRPCa. We show that either androgen deprivation or the specific antiandrogen agent bicalutamide induce mTOR-associated autophagy. Moreover, specific inhibition of autophagy by siRNAs targeting *Atg* genes restores sensitivity of LNCaP cells to androgen deprivation or bicalutamide treatment. Pharmacological inhibition of autophagy by the safe anti-malarial drug chloroquine was also able to increase susceptibility of LNCaP to androgen ablation and bicalutamide treatment. We hope that those finding will pave the way to clinical trials using chloroquine in combination with anti-androgen therapy in HRPCa.

MATERIALS AND METHODS

Cell Culture and Reagents

LNCaP and DU145 cells (American Type Culture Collection, CRL-1740, and HTB-81) were grown at 37°C in an humidified atmosphere of 5% CO2–95% air in RPMI 1640 (Gibco, 52400) supplemented with 10% fetal calf serum, 100 IU/ml penicillin (Gibco, 10270), and 100 μg/ml streptomycin (Gibco, 15140). Cells were cultured up to passage 30. Analysis of the effect of androgen deprivation was performed by culturing cells for 2 days in RPMI 1640 medium supplemented with charcoal-stripped FCS (Gibco, 12676) for serum steroid hormone removal (−R1881 condition). As control condition, the synthetic agonist of the androgen receptor (AR) methyltrienolone (R1881), was added to the medium at 0.2 nM (+R1881 condition).

The effects of the non-steroidal anti-androgen bicalutamide (Bic; Sigma–Aldrich, Saint Louis, MO, B9061) were tested by culturing LNCaP cells for 2 days in RPMI1640 supplemented with FCS, R1881 and at 50 μM Bic (+Bic condition) or without Bic (−Bic condition). Chloroquine (Sigma–Aldrich, C6628) was used at 100 μM, concanamycin A (Sigma–Aldrich, 27689) at 1 μM, rapamycin (Sigma–Aldrich, R8781) at 100 nM, E-64d (Sigma–Aldrich, E8640) at 10 μg/ml and pepstatin A (Sigma–Aldrich, P5318) at 10 μg/ml. Nuclei were stained with 10 μg/ml Hoechst 33342 (Molecular Probes-Invitrogen, H3570).

GFP-LC3 Transfection and Imaging

LNCaP cells were seeded in six-well culture plates until to reach ~80% confluence. Cells were then transfected with a plasmid coding for GFP-LC3 using Lipofectamine reagent (Invitrogen, 11668-019) according to manufacturer's instructions. After 24 hr, cells were plated on glass coverslips and 48 hr later, were subjected to various treatments during 2 days. GFP fluorescence was analyzed on an inverted Axiovert 200 microscope (Zeiss, Oberkochen, Germany) using FITC excitation and emission wavelengths (×100, NA 1.4, oil immersion). Images were acquired with a Zeiss Axiocam and analyzed by the Zeiss Axiovision software.

Electron Microscopy

After three washes with ice-cold PBS, samples were fixed in 1% glutaraldehyde, postfixed in osmium tetroxide and embedded in Epon. Ultrathin sections (80 nm) were contrasted with uranyl acetate and lead citrate, and were examined through a Philips CM12 (Philips) electron microscope operated at 80 kV.

Immunoblotting

Cells were harvested by scraping in PBS and re-suspended in lysis buffer containing (mM) 20 Tris–HCl (pH 7.5), 150 NaCl, 1 Na_2EDTA, 1 EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethanesulfonylfluoride. Extracts were diluted in a mix of LDS Sample Buffer and Sample Reducing Agent (NuPAGE®, Invitrogen, NP0007 and NP0009) and heated at 95°C for 3 min. Samples were electrophoresed on 10% or 12% SDS–polyacrylamide gels (Invitrogen, NP0301BOX and NP0341BOX) and transferred onto nitrocellulose membranes (Biorad, 162-0097). The blots were saturated in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.05% Tween 20, pH 7.6), containing 5% skimmed milk for 1 hr at room temperature, incubated overnight at 4°C with primary antibodies: anti-AR (Millipore Corporation 06-680) was used at a dilution of 1:250, anti-BECN1 (Santa Cruz Biotechnology, sc-1142) at a dilution of 1:500, anti-LC3 (Cell Signaling Technology, 3868), anti-p62 (Cell Signaling Technology, 8025), anti-Atg5 (Cell Signaling Technology, 2630), anti-Akt^{S473} (Cell Signaling Technology, 9271), anti-4EBP-1 (Cell Signaling Technology, 9452), anti-phospho-$p70S6k^{T389}$ (Santa Cruz Biotechnology, sc-11759) and anti-p70S6k (Santa Cruz Biotechnology, sc-230) at a dilution of 1:1,000, and anti-PI3 Kinase p85 (Millipore Corporation, 06-195) at a dilution of 1:3,000. Immunodetection of β-actin with monoclonal anti-actin antibody (Sigma–Aldrich, A5441) was used as loading control (dilution 1:10,000). After incubation with appropriate secondary antibodies coupled to peroxidase, peroxidase activity was detected with ECL+ (Amersham, RPN2132) on ECL hyperfilm. Immunoblots were quantified using ImageMaster densitometry program (GE Healthcare).

Co-Immunoprecipitation

Cells were harvested by scraping in PBS and re-suspended in lysis buffer. Cell lysates were centrifuged and 500 μg of total protein was subjected to immunoprecipitation using GammaBind G Sepharose (GE Healthcare, 17-0895-01) and anti-p85 or anti-Androgen Receptor antibodies overnight at 4°C. After four washes with PBS, immunocomplexes were eluted in a mix of LDS Sample Buffer and Sample Reducing Agent, heated at 95°C for 3 min and analyzed by immunoblotting as described above.

siRNA Transfection

The siRNAs targeting Atg5 and BECN1 as well as the non-silencing control siRNA (siUNR) were purchased from Thermo Fisher Scientific. LNCaP cells were transfected using DharmaFECT reagent (Thermo Fisher Scientific, T-2002-03) according to the manufacturer's instructions. Twenty-four hours later, cells were plated on six-well plates and after 48 additional hours, subjected to various treatments. Five days after transfection, efficiency of siRNA-mediated depletion of the genes of interest was assessed in Western blot.

Flow Cytometry

Cyto-ID™ Green autophagy assay (Enzo, ENZ-51031-K200) was performed as recommended by the manufacturers' instructions. Briefly, LNCaP cells were seeded into six-well plates and treated 48 hr in the different conditions in the presence of E64d/pepstatin A. Then, cells were trypsinized, washed, and

resuspended in Cyto-ID™ Green autophagy detection reagent and incubated at room temperature for 30 min. Cyto-ID Green autophagy reagent was measured in the FL-1 channel (530/30 nm bandpass filters with excitation at 488 nm).

Simultaneous detection of mitochondrial membrane potential ($\Delta\Psi_m$) dissipation and plasma membrane permeabilization was determined by staining with 40 nM $\Delta\Psi_m$-sensitive fluorochrome $DiOC_6(3)$ (Molecular Probes-Invitrogen, D273) and 1 μg/ml vital dye propidium iodide (PI; Sigma–Aldrich, P1304MP). All PI+ events have been considered as DiOC low. Cell cycle analysis was performed by permeabilizing cells with 0.01% triton X-100 and subsequent staining by 50 μg/ml PI. Cytofluorometric analyses were performed on a FACSCalibur equipped with CellQuest Pro software (Becton Dickinson).

Statistical Analysis

Data are presented as means ± SD. Student's *t*-test, two-way ANOVA, and two-sample Wilcoxon rank-sum (Mann–Whitney) analysis were used to determine statistical significance when appropriate.

RESULTS

Androgen Deprivation As Well As Bicalutamide Enhances Autophagic Flux in LNCaP Cells

Autophagy was first monitored by detection of GFP-LC3 accumulation into vacuoles. After 2 days of androgen deprivation or bicalutamide treatment, an increased number of LNCaP cells exhibited a relocalization of GFP-LC3 (Fig. 1A,B). The number of cells containing more that 10 cytoplasmic puncta increased approximately twofold after androgen removal and threefold after bicalutamide addition in comparison with control conditions. Transmission electron microscopy showed double- or multiple-membranes structures, considered as hallmarks of autophagy (Fig. 1C). Those observations were corroborated by LC3-I/LC3-II immunodetection. In response to androgen deprivation or bicalutamide, LC3-I significantly decreased suggesting its conversion into LC3-II. However, since LC3-II also tends to be degraded, the ratio LC3-II/LC3-I typically used to quantify autophagy did not increase significantly (Fig. 1D,E) [11]. To avoid this degradation, we used a combination of E64d and pepstatin A in order to inhibit lysosomal function [18]. In the presence of inhibitors, LC3-II/LC3-I ratio was significantly increased in control conditions showing the existence of a detectable basal autophagic flux in LNCaP cells. In those conditions, treatment with bicalutamide or androgen deprivation greatly enhanced LC3-II accumulation and therefore the LC3-II/LC3-I ratio (Fig. 1D,E). Similar results were observed after treatment with flutamide, another AR antagonist (Fig. S1).

Expression of the adaptor protein p62 mediating the interaction between ubiquitinated proteins and LC3 was also assessed in response to androgen deprivation and bicalutamide. In those conditions, the amount of p62 was significantly decreased ($P < 0.05$, three independent experiments). This effect was abolished by pretreating cells with E64d/pepstatin A inhibitors (Fig. 2A). Finally, a new flow cytometry-based strategy was used to monitor autophagy in cells stained with a fluorescent cationic amphiphilic tracer dye that rapidly partitions into the hydrophobic environment of multi-lamellar vesicles (Cyto-ID autophagy detection kit). As expected, fluorescent signal was significantly increased after androgen deprivation and bicalutamide treatment (Fig. 2B,C).

Androgen Deprivation and Bicalutamide-Induced Autophagy Are Mediated by the Androgen Receptor Signaling Pathway

To rule out that the increased level of autophagy after androgen deprivation or bicalutamide treatment could be an off-target effect, experiments were extended to the DU-145 cell line devoid of AR. Neither biochemical signs (LC3-I → LC3-II conversion) nor morphological manifestations (GFP-LC3 relocalization) of autophagy were observed in DU-145 after androgen deprivation or bicalutamide, confirming that autophagy induced by both treatments in LNCaP cells was mediated by the AR (Fig. 3A,B). The positive control rapamycin was able to increase LC3-I → LC3-II conversion demonstrating that autophagic pathways are functional in DU-145 cells (Fig. 3C).

Androgen Deprivation and Bicalutamide Induce mTOR-Associated Autophagy

To look for an implication of the mTOR signaling pathway in autophagy induced by disruption of the AR pathway, we checked by immunoblotting the phosphorylation of Akt^{S473}, the upstream kinase of mTOR, and of the substrates of this latter, $p70S6k^{T389}$ and 4EBP-1 (Fig. 4A). Androgen deprivation reduced phosphorylation of Akt^{S473} by 21 ± 18% ($P < 0.05$, N = 6) and phosphorylation of $p70S6k^{T389}$ by 44 ± 23% ($P < 0.01$, N = 4). Similarly, bicalutamide treatment reduced phosphorylation of Akt^{S473} by 27 ± 28% ($P < 0.05$, N = 6) and phosphorylation of $p70S6k^{T389}$ by 45 ± 21% ($P < 0.01$, N = 4). 4EBP-1 can be phosphorylated on various sites. Immunoblotting with the antibody against the total form of 4EBP-1 reveals a shift to a lower molecular weight when

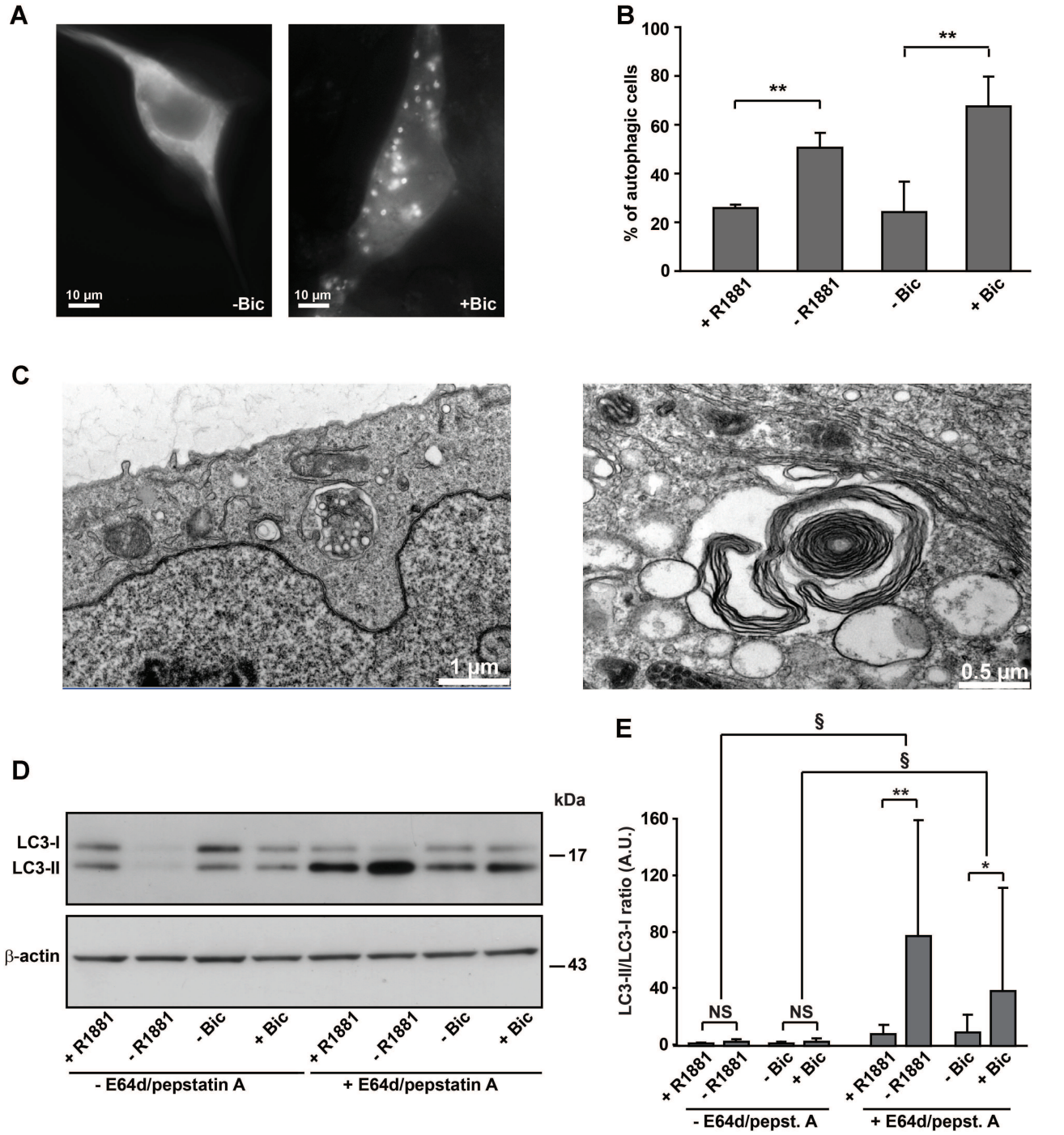

Fig. 1. Effects of androgen deprivation and bicalutamide treatment on the autophagic flux in LNCaP cells. **A**,**B**: Fluorescence monitoring of autophagy based on GFP-LC3 relocalization. A: Representative images of GFP-LC3-labeled punctae in LNCaP cells in control condition versus bicalutamide exposure. B: Histogram showing the number of cells exhibiting more than 10 fluorescent dots in the indicated conditions ($^{**}P < 0.01$, n = 3). **C**: Transmission electron microscopy of LNCaP cells cultured in the presence of Bic. Representative images of three independent experiments. **D**,**E**: Immunoblot analysis (D) and densitometry (E) of LC3-I → LC3-II conversion in LNCaP cells either deprived in androgen (−R1881), or treated with 50 μM bicalutamide (+Bic) for 48 hr, in the absence or in the presence of the lysosomal proteases inhibitors 10 μg/ml E64d and 10 μg/ml pepstatin A. β-actin immunodetection is used as a loading control. Bar histogram shows the LC3-II/LC3-I ratios in the indicated conditions. Data are presented as means ± SD of 7–19 independent experiments. *, **: Significantly different than control conditions in the presence of E64d/pepstatin A inhibitors ($^{*}P < 0.05$; $^{**}P < 0.01$). §: Significantly different than similar conditions in the absence of inhibitors ($^{§}P < 0.05$).

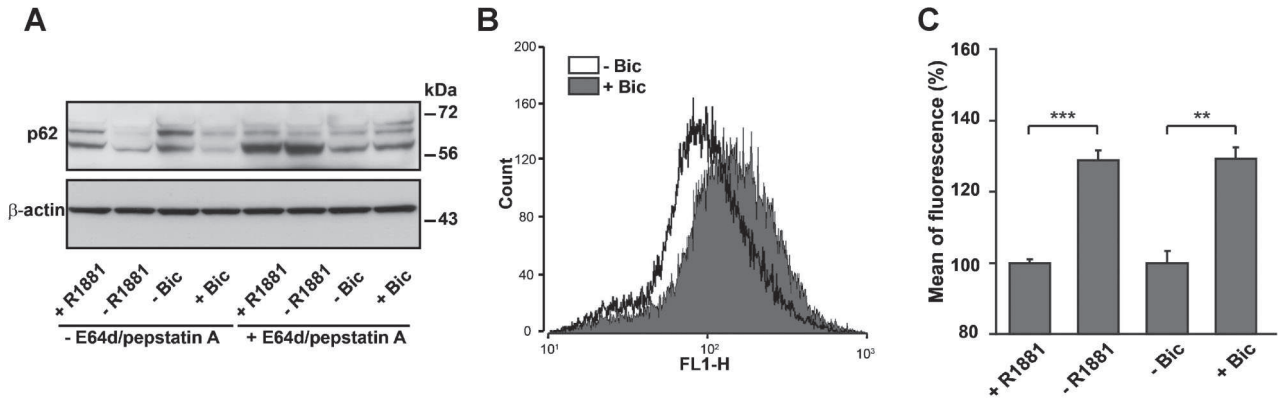

Fig. 2. Assessment of pro-autophagic effects of androgen deprivation and bicalutamide by detection of p62 degradation and accumulation of autophagic vacuoles. **A**: Immunoblot analysis of p62 expression in LNCaP cells either deprived in androgen (−R1881), or treated with 50 μM bicalutamide (+Bic) for 48 hr, in the absence or in the presence of the lysosomal proteases inhibitors 10 μg/ml E64d and 10 μg/ml pepstatin A (representative of three independent experiments). **B**: Flow cytometry-based profiling and (**C**) quantification of Cyto-ID™ probe accumulation in same conditions as in (A). Data are expressed mean of fluorescence ± SD of three independent experiments ($^{**}P < 0.05$; $^{***}P < 0.001$).

the protein is dephosphorylated. On this basis, we concluded that the non-phosphorylated form was predominant after androgen ablation. Previous studies showed that AR interacts with the regulatory subunit of class Ia PI3-K, p85α, and therefore stimulates the PI3K/Akt/mTOR pathway [19]. We thus monitored AR/p85 interaction in response to androgen deprivation and bicalutamide. Our results confirmed that p85 and AR cross-co-immunoprecipitate (Fig. 4B). However, AR expression was significantly decreased in LNCaP cells in which AR signaling was inhibited (Fig. 4C). Indeed, we observed a reduction of AR expression by 70 ± 21% after androgen depletion ($P < 0.05$, n = 6) and by 45 ± 22% after bicalutamide treatment ($P < 0.05$, n = 8).

siRNA-Mediated Inhibition of Autophagy Sensitizes LNCaP Cells to Bicalutamide-Induced Apoptosis

To test whether autophagy induced by androgen deprivation or bicalutamide was mediated by the canonical pathway, we transfected LNCaP cells with siRNAs targeted against Atg5 and Atg6/Beclin-1 (BECN1) genes, two autophagy-related genes. As shown in Figure 5A, pools of four siRNAs targeted against different regions of the Atg5 and BECN1 mRNAs dramatically reduced their protein levels and increased LC3-I content (Fig. 5A). In the presence of E64d/pepstatin A, Atg5 and BECN1 depletion significantly decreased basal LC3-I → LC3-II conversion (Fig. 5B,C) and GFP-LC3 recruitment to punctae

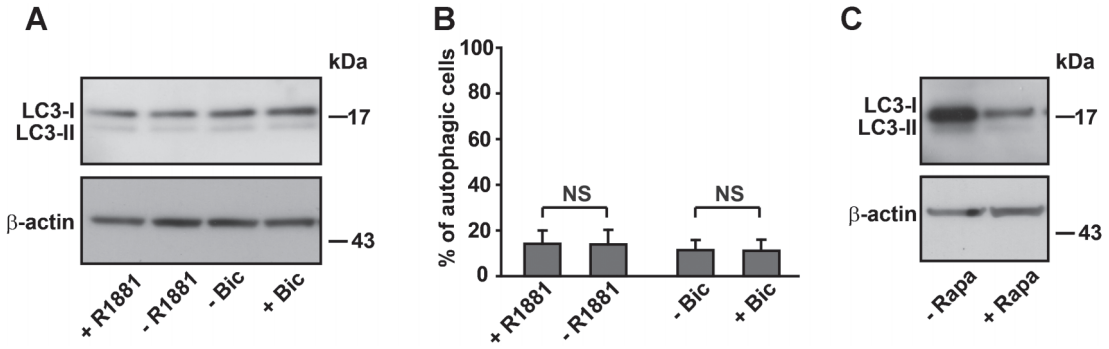

Fig. 3. Lack of effect of androgen deprivation or bicalutamide on the autophagic flux of AR-deficient DU-145 cells. **A**: Immunoblot analysis of LC3-I → LC3-II conversion in DU-145 either deprived in androgen (−R1881), or treated with 50 μM bicalutamide (+Bic) for 48 hr. **B**: Autophagy monitoring by GFP-LC3 relocalization in the same conditions. Bar histogram shows the number of cells with more of 10 fluorescent dots after culture in the indicated conditions. Data are presented as means ± SD of three independent experiments. **C**: Immunoblot analysis of LC3-I → LC3-II conversion in DU-145 treated with 100 nM rapamycin for 24 hr.

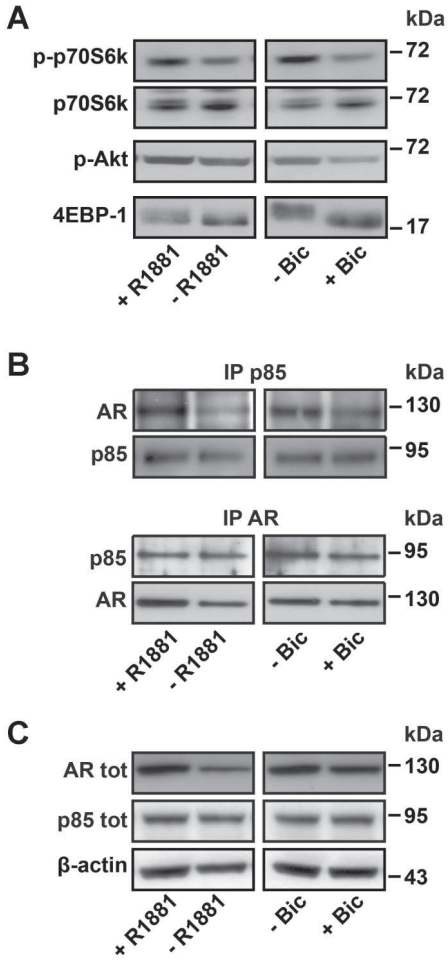

Fig. 4. Both androgen deprivation and bicalutamide induce mTOR-associated autophagy by regulating AR/PI3-K interaction in LNCaP cells. **A**: Immunoblot analysis of phosphorylation of p70S6k^{T389}, Akt^{S473} and 4EBP-1 in LNCaP cells either deprived in androgen (−R1881), or treated with 50 μM bicalutamide (+Bic) for 48 hr. **B**: Cross co-immunoprecipitation of AR and p85 of LNCaP cells treated as in (A). Total protein extracts were immunoprecipitated (IP) for p85 (upper panel) or AR (lower panel), followed by SDS–PAGE separation and immunoblotting for AR and p85. Representative of three independent experiments (**C**) Abundance of total AR and p85 in the total proteins extracted from LNCaP treated as in (A). Representative of six to eight independent experiments.

(Fig. 5D). Depletion of Atg5 completely inhibited bicalutamide-induced autophagy whereas the effect of BECN1 depletion was only partial.

To distinguish whether autophagy induced by bicalutamide represents a mechanism of cell survival or cell death, we measured the consequences of Atg5 and BECN1 depletion on bicalutamide-induced apoptosis. Measurements of DNA content by PI staining on permeabilized cells showed that neither treatment induced significant cytotoxicity. However, Atg5 or BECN1 repression markedly increased sub-G1 cell population after bicalutamide treatment (Fig. 5E). Mitochondrial membrane potential ($\Delta\Psi_m$) dissipation and plasma membrane permeabilization, determined by simultaneous assessment of $\Delta\Psi_m$ (with the $\Delta\Psi_m$-sensitive fluorochrome $DiOC_6(3)$) and cell viability (with the vital dye PI) similarly showed an increase in apoptosis after Atg5 repression after bicalutamide treatment (Fig. 5F). This assay failed to reveal a statistically significant effect of BECN1 depletion in restoring sensitivity to apoptosis. Interestingly, Atg5 depletion was more efficient than BECN1 to both block autophagy (Fig. 5C,D) and restore cytotoxicity (Fig. 5F).

Chloroquine Strongly Increases Apoptosis Induced by Androgen Disruption

Considering that autophagy induced by androgen disruption could be a therapeutic target in the management of HRPCa, we tested the effect of the safe anti-malarial drug chloroquine on androgen deprivation and bicalutamide-induced autophagy. Chloroquine dramatically reduced LC3-II degradation, in line with the fact that chloroquine is a well known inhibitor of lysosomal activity (Fig. 6A). This reinforces the fact that autophagic basal flux is important in LNCaP cells (see also Fig. 1D). To validate the inhibitory effect of chloroquine on autophagy, we therefore measured the level of GFP released from autophagic cleavage of the GFP-LC3 fusion protein in LNCaP cells. A recent study has indeed demonstrated that accumulation of GFP in the presence of lysosomal inhibitors is a marker of autophagy [20]. We confirmed that GFP accumulated upon chloroquine treatment and found that this was exacerbated by bicalutamide treatment. Similar results were obtained in the presence of cathepsin inhibitors E64d and pepstatin A (Fig. 6B). Chloroquine also dramatically increased the level of apoptosis induced by androgen deprivation and bicalutamide, as assessed by sub-G1 detection (Fig. 6C) and simultaneous measurement of $\Delta\Psi_m$ dissipation and plasma membrane permeabilization (Fig. 6D). Indeed, the proportion of cells exhibiting DNA degradation was increased about tenfold upon combined bicalutamide and chloroquine treatment compared to the situation where bicalutamide is added alone. Similar observations were obtained by associating androgen deprivation and chloroquine. Importantly, the inhibitor of the vacuolar H^+-ATPase, concanamycin A, a well-known inhibitor of autophagy also enhanced toxicity of androgen and bicalutamide treatment (Fig. 6E).

DISCUSSION

Previous studies have investigated the inhibitory role of androgen on autophagy in PCa cells. In

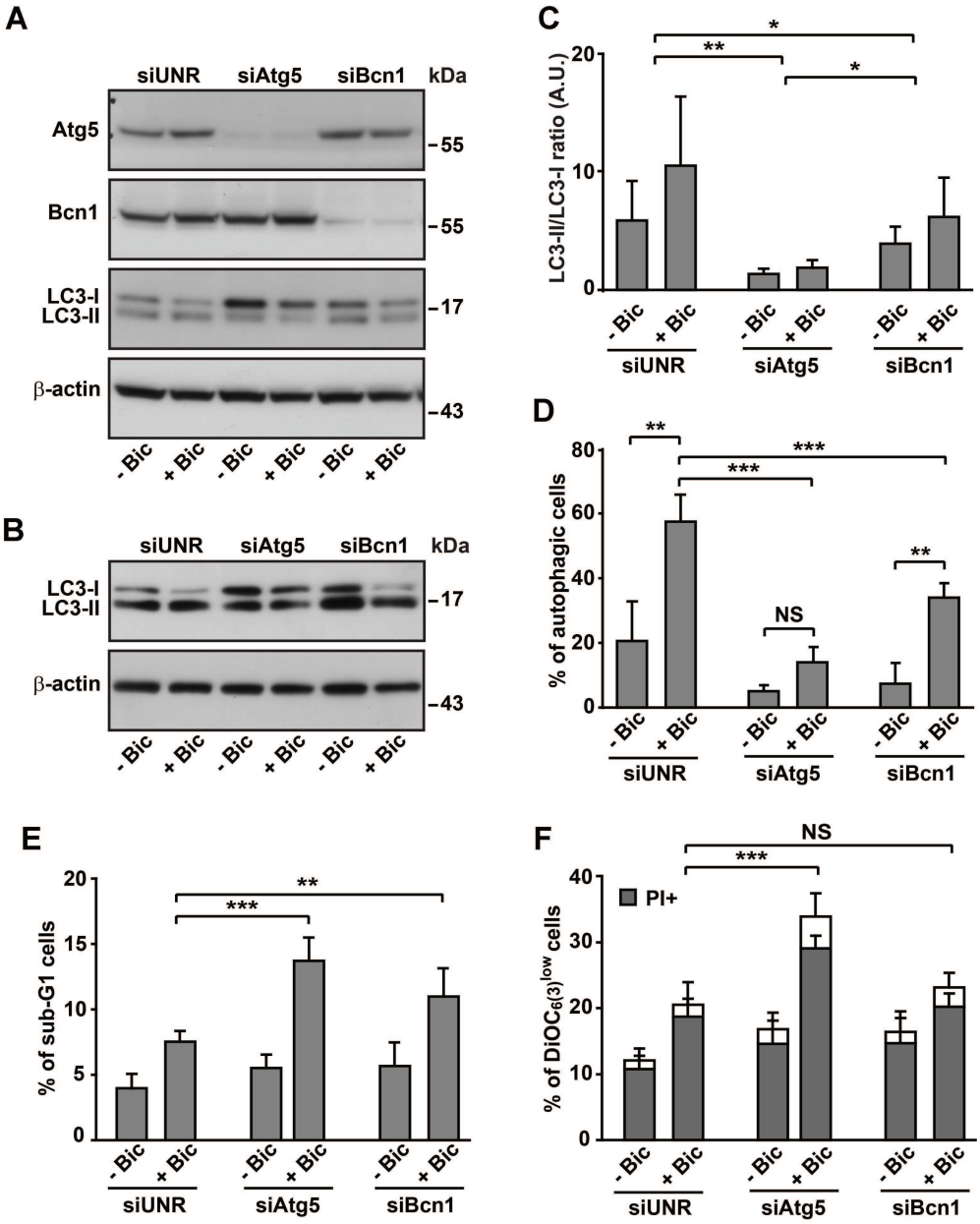

Fig. 5. Effects of siRNA-mediated inhibition of autophagy on cell death induced by bicalutamide. **A**: Immunoblot analysis showing the depletion of Atg5 and BECNI 5 days after transfection of LNCaP cells with the indicated siRNAs pools and the LC3-I → LC3-II conversion after 48 hr treatment with or without 50 μM Bic for 48 hr after transfection with the indicated siRNAs pool. Representative of three independent experiments. **B**: LC3-I → LC3-II conversion in LNCaP treated as in (A) in the presence of E64d/pepstatin A. Representative of six independent experiments. **C**: Quantification of experiments showed in B. Bars present means ± SD of LC3-II/LC3-I ratios from six independent experiments. Two-way ANOVA revealed a significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$) between the transfections with the indicated siRNAs. **D**: Bar histogram showing the number of cells exhibiting more than 10 fluorescent dots in LNCaP co-transfected by the indicated siRNAs and the GFP-LC3 construct, and cultured with or without 50 μM Bic for 48 hr (means ± SD of three independent experiments, $^{**}P < 0.01$, $^{***}P < 0.001$). **E**: Bar histogram showing the proportion of sub-G1 cells detected by DNA content analysis after PI staining in permeabilized LNCaP cells previously transfected with the indicated siRNAs and cultured in the absence or in the presence of 50 μM Bic for 48 hr. Data are presented as means ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$. **F**: Bar histogram showing cytofluorimetric analysis of the effects of siRNA-mediated autophagy inhibition on apoptosis-associated mitochondrial transmembrane potential ($\Delta\Psi_m$) dissipation and plasma membrane permeabilization. Transfected cells were labeled with the $\Delta\Psi_m$-sensitive probe $DiOC_6(3)$ and with PI. White and gray columns represent percentages of cells exhibiting $\Delta\Psi_m$ loss alone ($DiOC_6(3)^{low}$) or in association with plasma membrane breakdown (PI+), respectively. Data are presented as means ± SD of three independent experiments, $^{***}P < 0.001$.

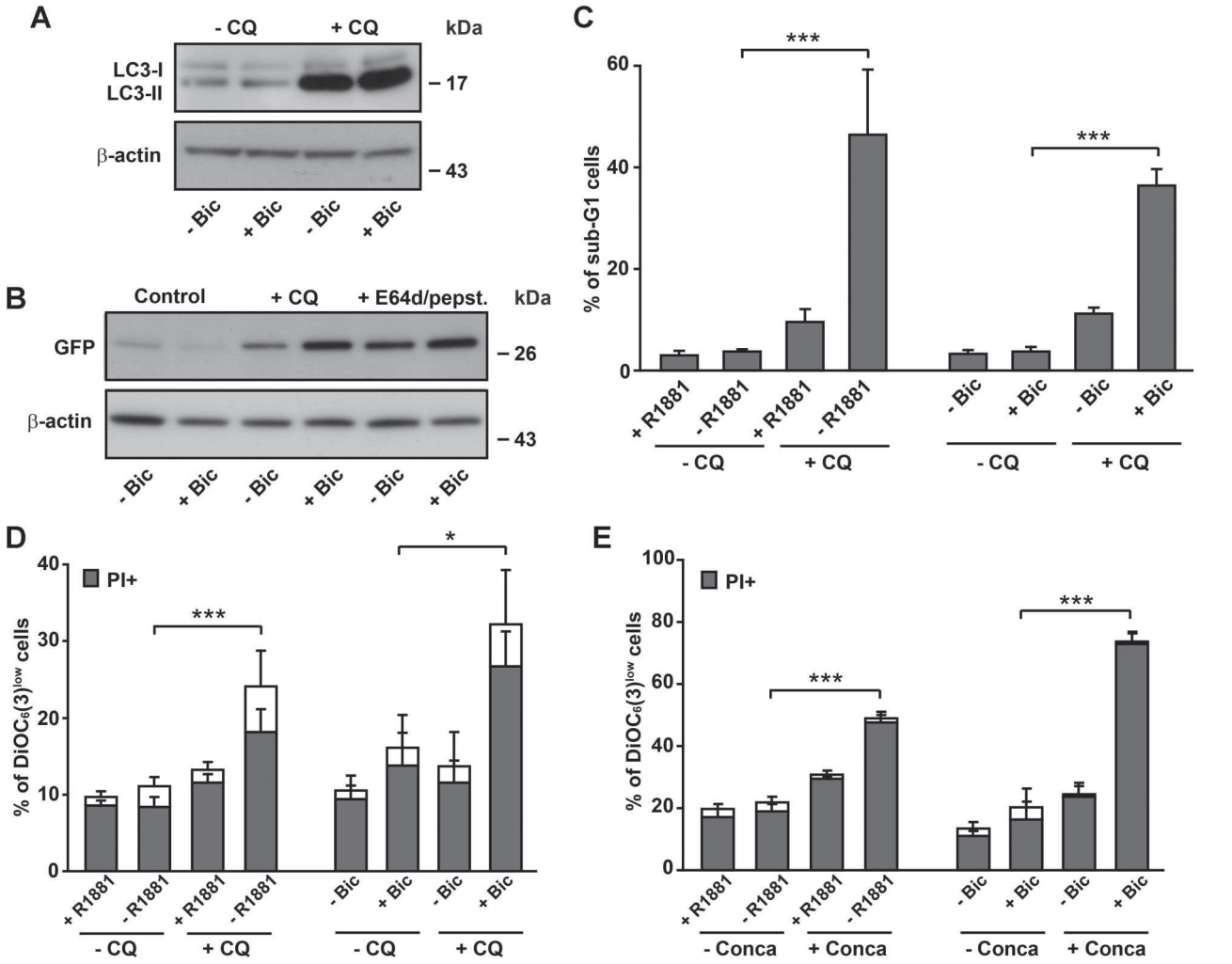

Fig. 6. Effects of the association of bicalutamide or androgen deprivation and chloroquine on cell death induced by androgen deprivation or bicalutamide treatment. **A**: Immunoblot showing LC3-II level in the absence (−CQ) or in the presence (+CQ) of 100 μM chloroquine and with or without 50 μM bicalutamide for 72 hr. **B**: Immunoblot showing GFP level in LNCaP cells treated in the presence of 100 μM CQ or E64d/pepstatin A with or without 50 μM bicalutamide for 48 hr. Representative of two independent experiments. **C**: Bar histogram showing the proportion of sub-G1 cells detected by DNA content analysis after PI staining in permeabilized LNCaP cells previously cultured in the indicated conditions. Data are presented as means ± SD of three independent experiments. $^{***}P < 0.001$. **D**: Bar histogram showing cytofluorimetric analysis of apoptosis-associated mitochondrial transmembrane potential ($\Delta\Psi_m$) dissipation and plasma membrane permeabilization on LNCaP cells cultured in an androgen-depleted medium (−R1881) or with 50 μM Bic in the absence or in the presence of 100 μM CQ for 72 hr. White and gray columns represent percentages of cells exhibiting $\Delta\Psi_m$ loss alone ($DiOC_6(3)^{low}$) or in association with plasma membrane breakdown (PI+), respectively. Data are presented as means ± SD of three independent experiments, $^{*}P < 0.05$, $^{***}P < 0.001$. **E**: Same experiments as in (D) except that chloroquine treatment was replaced by 1 μM concanamycin A (Conca) treatment and that the duration of treatment was 48 hr. Data are presented as means ± SD of four independent experiments, $^{***}P < 0.001$.

particular, androgen supplementation was shown to inhibit autophagy induced by serum starvation in LNCaP cells [21,22]. Moreover, association of hypoxia and androgen deprivation induced an AMPK-mediated autophagy in PCa cells but, in this study, androgen deprivation alone was not sufficient to promote the same effect [23]. In the present study, we clearly show that androgen deprivation by itself and, importantly, AR blockade by the clinically used bicalutamide increase the autophagic flux in LNCaP cells. In this cellular model, we observed an important amount of LC3-II. This could be due to a high basal flux of autophagy in LNCaP cells. However, this has to be interpreted in light of the fact that antibodies against LC3 better recognized LC3-II form. This rendered detection of LC3-I → LC3-II conversion by immunoblotting not optimal. Indeed, androgen deprivation or bicalutamide induced only a minor decrease of LC3-I

but no obvious increase of LC3-II. However, addition of E64d/pepstatin A clearly revealed the increase of LC3-II suggesting that androgen deprivation or bicalutamide do stimulate autophagy. In contrast to assessment of LC3-I → LC3-II conversion by immunoblotting, detection of GFP-LC3 clustering showed a dramatic increase in autophagy after both treatments. Transmission electron microscopy showed typical multiple-membrane structures compatible with macro-autophagy. All these results were confirmed by p62 degradation and by a new flow cytometry assay based on the fluorimetric detection of autophagic vacuoles. This was associated with a reduction of phosphorylated forms of Akt and p70S6k, suggesting that autophagy induced by AR signaling disruption is associated to the inhibition of the PI3-K/Akt/mTOR pathway, a master regulator of multiple forms of autophagy. Several reports indicated that androgens trigger the PI3-K/Akt/mTOR pathway by increasing the interaction between the activated AR and p85, the regulatory subunit of class Ia PI3-K [19,21,24,25]. Consistently, we observed that AR interacted with p85. However, our data also showed that AR expression was decreased after androgen deprivation or bicalutamide treatment. We therefore suggest that androgen ablation, by decreasing AR expression, increased the cytosolic amount of free p85 that in turn inhibits the PI3-K activity. This observation gives a novel insight to explain the inhibition of mTOR after androgen signaling pathway disruption.

The impact of autophagy in neoplastic transformation and cancer therapy remains speculative and cross-talk between apoptosis and autophagy is a crucial question to address to develop optimal treatments for cancer patients [26]. On the one hand, several tumor suppressors involved in the inhibition of mTOR, including PTEN or TSC1 and TSC2, stimulate autophagy and, conversely, mTOR-activating oncogene products such as class I PI3-K and Akt inhibit autophagy. The p53 tumor suppressor gene promotes autophagy in DNA-damaged cells whereas apoptosis inhibitors Bcl-2 and Bcl-X_L overexpressed in a series of cancers also inhibit autophagy. Moreover, deficiencies in the autophagic machinery are associated with an increase in tumor susceptibility and several anticancer therapies induce accumulation of autophagosomes in cancer cell lines in vitro (for a review see ref. 10). On the other hand, the prosurvival properties of autophagy can be exploited by cancer cells to resist to metabolic stress or cytotoxic therapies. Indeed, autophagy has recently been pointed out as an important mechanism of resistance to chemotherapy, radiation therapy or immunotherapy since pharmacological or siRNAs-mediated inhibition of autophagy usually accelerates, rather than prevents, cell death in these settings [27,28].

Our study provides novel insights in the role of autophagy in PCa by demonstrating the possibility to restore sensitivity of HRPCa cells to hormone therapy by inactivating autophagic degradation, suggesting that autophagy is a prosurvival mechanism in LNCaP cells. Chloroquine and concanamycin A, two well-known inhibitors of autophagy, dramatically increased cell death after androgen depletion or bicalutamide treatment. Inhibition of autophagy by Atg5 or BECN1 depletion had significant but smaller effects on bicalutamide-induced cell death than pharmacological inhibitors. This might be due to the fact that siRNA-mediated depletion of Atg5 and BECN1 was not complete and that therefore autophagy was not entirely blocked. Chloroquine is already being tested to restore sensitivity to cytostatic drugs in lung cancer, breast cancer and metastatic HRPCa. Because of the lack of effective therapy in the management of non-metastatic HRPCa, the combination of chloroquine and hormone therapy could constitute a useful strategy at this stage of the disease.

In conclusion, our study clearly suggests that clinical trials investigating the association between hormone therapy and chloroquine should be considered to improve the management of HRPCa patients and delay the apparition of metastasis and the recourse to chemotherapy.

ACKNOWLEDGMENTS

We thank Mrs. Thanh Vollemaere-Lac for technical expertise. N.T. and L.M. are post-doctoral fellows of the F.N.R.S.

REFERENCES

1. Han M, Partin AW, Pound CR, Epstein JI, Walsh PC. Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience. Urol Clin North Am 2001;28(3):555–565.
2. Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004;351(15):1513–1520.
3. Berthold DR, Pond GR, Soban F, de Wit R, Eisenberger M, Tannock IF. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: Updated survival in the TAX 327 study. J Clin Oncol 2008;26(2):242–245.
4. Knudsen KE, Scher HI. Starving the addiction: New opportunities for durable suppression of AR signaling in prostate cancer. Clin Cancer Res 2009;15(15):4792–4798.

5. Devlin HL, Mudryj M. Progression of prostate cancer: Multiple pathways to androgen independence. Cancer Lett 2009;274(2): 177–186.
6. Shen MM, Abate-Shen C. Pten inactivation and the emergence of androgen-independent prostate cancer. Cancer Res 2007; 67(14):6535–6538.
7. Sarker D, Reid AH, Yap TA, de Bono JS. Targeting the PI3K/AKT pathway for the treatment of prostate cancer. Clin Cancer Res 2009;15(15):4799–4805.
8. Galluzzi L, Vicencio JM, Kepp O, Tasdemir E, Maiuri MC, Kroemer G. To die or not to die: That is the autophagic question. Curr Mol Med 2008;8(2):78–91.
9. Cuervo AM. Autophagy many paths to the same end. Mol Cell Biochem 2004;263(1–2):55–72.
10. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008;132(1):27–42.
11. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, Ahn HJ, Ait-Mohamed O, Ait-Si-Ali S, Akematsu T, Akira S, Al-Younes HM, Al-Zeer MA, Albert ML, Albin RL, Alegre-Abarrategui J, Aleo MF, Alirezaei M, Almasan A, Almonte-Becerril M, Amano A, Amaravadi R, Amarnath S, Amer AO, Andrieu-Abadie N, Anantharam V, Ann DK, Anoopkumar-Dukie S, Aoki H, Apostolova N, Arancia G, Aris JP, Asanuma K, Asare NY, Ashida H, Askanas V, Askew DS, Auberger P, Baba M, Backues SK, Baehrecke EH, Bahr BA, Bai XY, Bailly Y, Baiocchi R, Baldini G, Balduini W, Ballabio A, Bamber BA, Bampton ET, Banhegyi G, Bartholomew CR, Bassham DC, Bast RC Jr, Batoko H, Bay BH, Beau I, Bechet DM, Begley TJ, Behl C, Behrends C, Bekri S, Bellaire B, Bendall LJ, Benetti L, Berliocchi L, Bernardi H, Bernassola F, Besteiro S, Bhatia-Kissova I, Bi X, Biard-Piechaczyk M, Blum JS, Boise LH, Bonaldo P, Boone DL, Bornhauser BC, Bortoluci KR, Bossis I, Bost F, Bourquin JP, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady NR, Brancolini C, Brech A, Brenman JE, Brennand A, Bresnick EH, Brest P, Bridges D, Bristol ML, Brookes PS, Brown EJ, Brumell JH, Brunetti-Pierri N, Brunk UT, Bulman DE, Bultman SJ, Bultynck G, Burbulla LF, Bursch W, Butchar JP, Buzgariu W, Bydlowski SP, Cadwell K, Cahova M, Cai D, Cai J, Cai Q, Calabretta B, Calvo-Garrido J, Camougrand N, Campanella M, Campos-Salinas J, Candi E, Cao L, Caplan AB, Carding SR, Cardoso SM, Carew JS, Carlin CR, Carmignac V, Carneiro LA, Carra S, Caruso RA, Casari G, Casas C, Castino R, Cebollero E, Cecconi F, Celli J, Chaachouay H, Chae HJ, Chai CY, Chan DC, Chan EY, Chang RC, Che CM, Chen CC, Chen GC, Chen GQ, Chen M, Chen Q, Chen SS, Chen W, Chen X, Chen X, Chen X, Chen YG, Chen Y, Chen Y, Chen YJ, Chen Z, Cheng A, Cheng CH, Cheng Y, Cheong H, Cheong JH, Cherry S, Chess-Williams R, Cheung ZH, Chevet E, Chiang HL, Chiarelli R, Chiba T, Chin LS, Chiou SH, Chisari FV, Cho CH, Cho DH, Choi AM, Choi D, Choi KS, Choi ME, Chouaib S, Choubey D, Choubey V, Chu CT, Chuang TH, Chueh SH, Chun T, Chwae YJ, Chye ML, Ciarcia R, Ciriolo MR, Clague MJ, Clark RS, Clarke PG, Clarke R, Codogno P, Coller HA, Colombo MI, Comincini S, Condello M, Condorelli F, Cookson MR, Coombs GH, Coppens I, Corbalan R, Cossart P, Costelli P, Costes S, Coto-Montes A, Couve E, Coxon FP, Cregg JM, Crespo JL, Cronje MJ, Cuervo AM, Cullen JJ, Czaja MJ, D'Amelio M, Darfeuille-Michaud A, Davids LM, Davies FE, De Felici M, de Groot JF, de Haan CA, De Martino L, De Milito A, De Tata V, Debnath J, Degterev A, Dehay B, Delbridge LM, Demarchi F, Deng YZ, Dengjel J, Dent P, Denton D, Deretic V, Desai SD, Devenish RJ, Di Gioacchino M, Di Paolo G, Di Pietro C, Diaz-Araya G, Diaz-Laviada I, Diaz-Meco MT, Diaz-Nido J, Dikic I, Dinesh-Kumar SP, Ding WX, Distelhorst CW, Diwan A, Djavaheri-Mergny M, Dokudovskaya S, Dong Z, Dorsey FC, Dosenko V, Dowling JJ, Doxsey S, Dreux M, Drew ME, Duan Q, Duchosal MA, Duff K, Dugail I, Durbeej M, Duszenko M, Edelstein CL, Edinger AL, Egea G, Eichinger L, Eissa NT, Ekmekcioglu S, El-Deiry WS, Elazar Z, Elgendy M, Ellerby LM, Eng KE, Engelbrecht AM, Engelender S, Erenpreisa J, Escalante R, Esclatine A, Eskelinen EL, Espert L, Espina V, Fan H, Fan J, Fan QW, Fan Z, Fang S, Fang Y, Fanto M, Fanzani A, Farkas T, Farre JC, Faure M, Fechheimer M, Feng CG, Feng J, Feng Q, Feng Y, Fesus L, Feuer R, Figueiredo-Pereira ME, Fimia GM, Fingar DC, Finkbeiner S, Finkel T, Finley KD, Fiorito F, Fisher EA, Fisher PB, Flajolet M, Florez-McClure ML, Florio S, Fon EA, Fornai F, Fortunato F, Fotedar R, Fowler DH, Fox HS, Franco R, Frankel LB, Fransen M, Fuentes JM, Fueyo J, Fujii J, Fujisaki K, Fujita E, Fukuda M, Furukawa RH, Gaestel M, Gailly P, Gajewska M, Galliot B, Galy V, Ganesh S, Ganetzky B, Ganley IG, Gao FB, Gao GF, Gao J, Garcia L, Garcia-Manero G, Garcia-Marcos M, Garmyn M, Gartel AL, Gatti E, Gautel M, Gawriluk TR, Gegg ME, Geng J, Germain M, Gestwicki JE, Gewirtz DA, Ghavami S, Ghosh P, Giammarioli AM, Giatromanolaki AN, Gibson SB, Gilkerson RW, Ginger ML, Ginsberg HN, Golab J, Goligorsky MS, Golstein P, Gomez-Manzano C, Goncu E, Gongora C, Gonzalez CD, Gonzalez R, Gonzalez-Estevez C, Gonzalez-Polo RA, Gonzalez-Rey E, Gorbunov NV, Gorski S, Goruppi S, Gottlieb RA, Gozuacik D, Granato GE, Grant GD, Green KN, Gregorc A, Gros F, Grose C, Grunt TW, Gual P, Guan JL, Guan KL, Guichard SM, Gukovskaya AS, Gukovsky I, Gunst J, Gustafsson AB, Halayko AJ, Hale AN, Halonen SK, Hamasaki M, Han F, Han T, Hancock MK, Hansen M, Harada H, Harada M, Hardt SE, Harper JW, Harris AL, Harris J, Harris SD, Hashimoto M, Haspel JA, Hayashi S, Hazelhurst LA, He C, He YW, Hebert MJ, Heidenreich KA, Helfrich MH, Helgason GV, Henske EP, Herman B, Herman PK, Hetz C, Hilfiker S, Hill JA, Hocking LJ, Hofman P, Hofmann TG, Hohfeld J, Holyoake TL, Hong MH, Hood DA, Hotamisligil GS, Houwerzijl EJ, Hoyer-Hansen M, Hu B, Hu CA, Hu HM, Hua Y, Huang C, Huang J, Huang S, Huang WP, Huber TB, Huh WK, Hung TH, Hupp TR, Hur GM, Hurley JB, Hussain SN, Hussey PJ, Hwang JJ, Hwang S, Ichihara A, Ilkhanizadeh S, Inoki K, Into T, Iovane V, Iovanna JL, Ip NY, Isaka Y, Ishida H, Isidoro C, Isobe K, Iwasaki A, Izquierdo M, Izumi Y, Jaakkola PM, Jaattela M, Jackson GR, Jackson WT, Janji B, Jendrach M, Jeon JH, Jeung EB, Jiang H, Jiang H, Jiang JX, Jiang M, Jiang Q, Jiang X, Jiang X, Jimenez A, Jin M, Jin S, Joe CO, Johansen T, Johnson DE, Johnson GV, Jones NL, Joseph B, Joseph SK, Joubert AM, Juhasz G, Juillerat-Jeanneret L, Jung CH, Jung YK, Kaarniranta K, Kaasik A, Kabuta T, Kadowaki M, Kagedal K, Kamada Y, Kaminskyy VO, Kampinga HH, Kanamori H, Kang C, Kang KB, Kang KI, Kang R, Kang YA, Kanki T, Kanneganti TD, Kanno H, Kanthasamy AG, Kanthasamy A, Karantza V, Kaushal GP, Kaushik S, Kawazoe Y, Ke PY, Kehrl JH, Kelekar A, Kerkhoff C, Kessel DH, Khalil H, Kiel JA, Kiger AA, Kihara A, Kim DR, Kim DH, Kim DH, Kim EK, Kim HR, Kim JS, Kim JH, Kim JC, Kim JK, Kim PK, Kim SW, Kim YS, Kim Y, Kimchi A, Kimmelman AC, King JS, Kinsella TJ, Kirkin V, Kirshenbaum LA, Kitamoto K, Kitazato K, Klein L, Klimecki WT, Klucken J, Knecht E, Ko BC, Koch JC, Koga H, Koh JY, Koh YH, Koike M, Komatsu M, Kominami E, Kong HJ, Kong WJ, Korolchuk VI, Kotake Y, Koukourakis MI, Kouri Flores, Kovacs JB, Kraft AL, Krainc C, Kramer D, Kretz-Remy H, Krichevsky C, Kroemer AM, Kruger G, Krut R, Ktistakis O, Kuan NT, Kucharczyk CY, Kumar R, Kumar A, Kumar R, Kundu S, Kung M, Kurz HJ, Kwon T, La Spada HJ, Lafont AR, Lamark F, Landry T, Lane J, Lapaquette JD, Laporte P, Laszlo

JF, Lavandero L, Lavoie S, Layfield JN, Lazo R, Le W PA, Le Cam L, Ledbetter DJ, Lee AJ, Lee BW, Lee GM, Lee J, Lee JH, Lee M, Lee MS, Lee SH, Leeuwenburgh C, Legembre P, Legouis R, Lehmann M, Lei HY, Lei QY, Leib DA, Leiro J, Lemasters JJ, Lemoine A, Lesniak MS, Lev D, Levenson VV, Levine B, Levy E, Li F, Li JL, Li L, Li S, Li W, Li XJ, Li YB, Li YP, Liang C, Liang Q, Liao YF, Liberski PP, Lieberman A, Lim HJ, Lim KL, Lim K, Lin CF, Lin FC, Lin J, Lin JD, Lin K, Lin WW, Lin WC, Lin YL, Linden R, Lingor P, Lippincott-Schwartz J, Lisanti MP, Liton PB, Liu B, Liu CF, Liu K, Liu L, Liu QA, Liu W, Liu YC, Liu Y, Lockshin RA, Lok CN, Lonial S, Loos B, Lopez-Berestein G, Lopez-Otin C, Lossi L, Lotze MT, Low P, Lu B, Lu B, Lu B, Lu Z, Luciano F, Lukacs NW, Lund AH, Lynch-Day MA, Ma Y, Macian F, MacKeigan JP, Macleod KF, Madeo F, Maiuri L, Maiuri MC, Malagoli D, Malicdan MC, Malorni W, Man N, Mandelkow EM, Manon S, Manov I, Mao K, Mao X, Mao Z, Marambaud P, Marazziti D, Marcel YL, Marchbank K, Marchetti P, Marciniak SJ, Marcondes M, Mardi M, Marfe G, Marino G, Markaki M, Marten MR, Martin SJ, Martinand-Mari C, Martinet W, Martinez-Vicente M, Masini M, Matarrese P, Matsuo S, Matteoni R, Mayer A, Mazure NM, McConkey DJ, McConnell MJ, McDermott C, McDonald C, McInerney GM, McKenna SL, McLaughlin B, McLean PJ, McMaster CR, McQuibban GA, Meijer AJ, Meisler MH, Melendez A, Melia TJ, Melino G, Mena MA, Menendez JA, Menna-Barreto RF, Menon MB, Menzies FM, Mercer CA, Merighi A, Merry DE, Meschini S, Meyer CG, Meyer TF, Miao CY, Miao JY, Michels PA, Michiels C, Mijaljica D, Milojkovic A, Minucci S, Miracco C, Miranti CK, Mitroulis I, Miyazawa K, Mizushima N, Mograbi B, Mohseni S, Molero X, Mollereau B, Mollinedo F, Momoi T, Monastyrska I, Monick MM, Monteiro MJ, Moore MN, Mora R, Moreau K, Moreira PI, Moriyasu Y, Moscat J, Mostowy S, Mottram JC, Motyl T, Moussa CE, Muller S, Muller S, Munger K, Munz C, Murphy LO, Murphy ME, Musaro A, Mysorekar I, Nagata E, Nagata K, Nahimana A, Nair U, Nakagawa T, Nakahira K, Nakano H, Nakatogawa H, Nanjundan M, Naqvi NI, Narendra DP, Narita M, Navarro M, Nawrocki ST, Nazarko TY, Nemchenko A, Netea MG, Neufeld TP, Ney PA, Nezis IP, Nguyen HP, Nie D, Nishino I, Nislow C, Nixon RA, Noda T, Noegel AA, Nogalska A, Noguchi S, Notterpek L, Novak I, Nozaki T, Nukina N, Nurnberger T, Nyfeler B, Obara K, Oberley TD, Oddo S, Ogawa M, Ohashi T, Okamoto K, Oleinick NL, Oliver FJ, Olsen LJ, Olsson S, Opota O, Osborne TF, Ostrander GK, Otsu K, Ou JH, Ouimet M, Overholtzer M, Ozpolat B, Paganetti P, Pagnini U, Pallet N, Palmer GE, Palumbo C, Pan T, Panaretakis T, Pandey UB, Papackova Z, Papassideri I, Paris I, Park J, Park OK, Parys JB, Parzych KR, Patschan S, Patterson C, Pattingre S, Pawelek JM, Peng J, Perlmutter DH, Perrotta I, Perry G, Pervaiz S, Peter M, Peters GJ, Petersen M, Petrovski G, Phang JM, Piacentini M, Pierre P, Pierrefite-Carle V, Pierron G, Pinkas-Kramarski R, Piras A, Piri N, Platanias LC, Poggeler S, Poirot M, Poletti A, Pous C, Pozuelo-Rubio M, Praetorius-Ibba M, Prasad A, Prescott M, Priault M, Produit-Zengaffinen N, Progulske-Fox A, Proikas-Cezanne T, Przedborski S, Przyklenk K, Puertollano R, Puyal J, Qian SB, Qin L, Qin ZH, Quaggin SE, Raben N, Rabinowich H, Rabkin SW, Rahman I, Rami A, Ramm G, Randall G, Randow F, Rao VA, Rathmell JC, Ravikumar B, Ray SK, Reed BH, Reed JC, Reggiori F, Regnier-Vigouroux A, Reichert AS, Reiners JJ Jr, Reiter RJ, Ren J, Revuelta JL, Rhodes CJ, Ritis K, Rizzo E, Robbins J, Roberge M, Roca H, Roccheri MC, Rocchi S, Rodemann HP, Rodriguez deCordoba S, Rohrer B, Roninson IB, Rosen K, Rost-Roszkowska MM, Rouis M, Rouschop KM, Rovetta F, Rubin BP, Rubinsztein DC, Ruckdeschel K, Rucker EB III, Rudich A, Rudolf E, Ruiz-Opazo N, Russo R, Rusten TE, Ryan KM, Ryter SW, Sabatini DM, Sadoshima J, Saha T, Saitoh T, Sakagami H, Sakai Y, Salekdeh GH, Salomoni P, Salvaterra PM, Salvesen G, Salvioli R, Sanchez AM, Sanchez-Alcazar JA, Sanchez-Prieto R, Sandri M, Sankar U, Sansanwal P, Santambrogio L, Saran S, Sarkar S, Sarwal M, Sasakawa C, Sasnauskiene A, Sass M, Sato K, Sato M, Schapira AH, Scharl M, Schatzl HM, Scheper W, Schiaffino S, Schneider C, Schneider ME, Schneider-Stock R, Schoenlein PV, Schorderet DF, Schuller C, Schwartz GK, Scorrano L, Sealy L, Seglen PO, Segura-Aguilar J, Seiliez I, Seleverstov O, Sell C, Seo JB, Separovic D, Setaluri V, Setoguchi T, Settembre C, Shacka JJ, Shanmugam M, Shapiro IM, Shaulian E, Shaw RJ, Shelhamer JH, Shen HM, Shen WC, Sheng ZH, Shi Y, Shibuya K, Shidoji Y, Shieh JJ, Shih CM, Shimada Y, Shimizu S, Shintani T, Shirihai OS, Shore GC, Sibirny AA, Sidhu SB, Sikorska B, Silva-Zacarin EC, Simmons A, Simon AK, Simon HU, Simone C, Simonsen A, Sinclair DA, Singh R, Sinha D, Sinicrope FA, Sirko A, Siu PM, Sivridis E, Skop V, Skulachev VP, Slack RS, Smaili SS, Smith DR, Soengas MS, Soldati T, Song X, Sood AK, Soong TW, Sotgia F, Spector SA, Spies CD, Springer W, Srinivasula SM, Stefanis L, Steffan JS, Stendel R, Stenmark H, Stephanou A, Stern ST, Sternberg C, Stork B, Stralfors P, Subauste CS, Sui X, Sulzer D, Sun J, Sun SY, Sun ZJ, Sung JJ, Suzuki K, Suzuki T, Swanson MS, Swanton C, Sweeney ST, Sy LK, Szabadkai G, Tabas I, Taegtmeyer H, Tafani M, Takacs-Vellai K, Takano Y, Takegawa K, Takemura G, Takeshita F, Talbot NJ, Tan KS, Tanaka K, Tanaka K, Tang D, Tang D, Tanida I, Tannous BA, Tavernarakis N, Taylor GS, Taylor GA, Taylor JP, Terada LS, Terman A, Tettamanti G, Thevissen K, Thompson CB, Thorburn A, Thumm M, Tian F, Tian Y, Tocchini-Valentini G, Tolkovsky AM, Tomino Y, Tonges L, Tooze SA, Tournier C, Tower J, Towns R, Trajkovic V, Travassos LH, Tsai TF, Tschan MP, Tsubata T, Tsung A, Turk B, Turner LS, Tyagi SC, Uchiyama Y, Ueno T, Umekawa M, Umemiya-Shirafuji R, Unni VK, Vaccaro MI, Valente EM, Van den Berghe G, van der Klei IJ, van Doorn W, van Dyk LF, van Egmond M, van Grunsven LA, Vandenabeele P, Vandenberghe WP, Vanhorebeek I, Vaquero EC, Velasco G, Vellai T, Vicencio JM, Vierstra RD, Vila M, Vindis C, Viola G, Viscomi MT, Voitsekhovskaja OV, von Haefen C, Votruba M, Wada K, Wade-Martins R, Walker CL, Walsh CM, Walter J, Wan XB, Wang A, Wang C, Wang D, Wang F, Wang F, Wang G, Wang H, Wang HG, Wang HD, Wang J, Wang K, Wang M, Wang RC, Wang X, Wang X, Wang YJ, Wang Y, Wang Z, Wang ZC, Wang Z, Wansink DG, Ward DM, Watada H, Waters SL, Webster P, Wei L, Weihl CC, Weiss WA, Welford SM, Wen LP, Whitehouse CA, Whitton JL, Whitworth AJ, Wileman T, Wiley JW, Wilkinson S, Willbold D, Williams RL, Williamson PR, Wouters BG, Wu C, Wu DC, Wu WK, Wyttenbach A, Xavier RJ, Xi Z, Xia P, Xiao G, Xie Z, Xie Z, Xu DZ, Xu J, Xu L, Xu X, Yamamoto A, Yamamoto A, Yamashina S, Yamashita M, Yan X, Yanagida M, Yang DS, Yang E, Yang JM, Yang SY, Yang W, Yang WY, Yang Z, Yao MC, Yao TP, Yeganeh B, Yen WL, Yin JJ, Yin XM, Yoo OJ, Yoon G, Yoon SY, Yorimitsu T, Yoshikawa Y, Yoshimori T, Yoshimoto K, You HJ, Youle RJ, Younes A, Yu L, Yu L, Yu SW, Yu WH, Yuan ZM, Yue Z, Yun CH, Yuzaki M, Zabirnyk O, Silva-Zacarin E, Zacks D, Zacksenhaus E, Zaffaroni N, Zakeri Z, Zeh HJ III, Zeitlin SO, Zhang H, Zhang HL, Zhang J, Zhang JP, Zhang L, Zhang L, Zhang MY, Zhang XD, Zhao M, Zhao YF, Zhao Y, Zhao ZJ, Zheng X, Zhivotovsky B, Zhong Q, Zhou CZ, Zhu C, Zhu WG, Zhu XF, Zhu X, Zhu Y, Zoladek T, Zong WX, Zorzano A, Zschocke J, Zuckerbraun B. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012;8(4):445–544.

12. Galluzzi L, Morselli E, Vicencio JM, Kepp O, Joza N, Tajeddine N, Kroemer G. Life, death and burial: Multifaceted impact of autophagy. Biochem Soc Trans 2008;36(Pt 5):786–790.
13. Tsujimoto Y, Shimizu S. Another way to die: Autophagic programmed cell death. Cell Death Differ 2005;12(Suppl 2):1528–1534.
14. Bursch W, Ellinger A, Gerner C, Frohwein U, Schulte-Hermann R. Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? Ann N Y Acad Sci 2000;926:1–12.
15. Glick D, Barth S, Macleod KF. Autophagy cellular and molecular mechanisms. J Pathol 2010;221(1):3–12.
16. Kroemer G, Levine B. Autophagic cell death: The story of a misnomer. Nat Rev Mol Cell Biol 2008;9(12):1004–1010.
17. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 1990;173(2):534–540.
18. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy 2007;3(6):542–545.
19. Baron S, Manin M, Beaudoin C, Leotoing L, Communal Y, Veyssiere G, Morel L. Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells. J Biol Chem 2004;279(15): 14579–14586.
20. Ni HM, Bockus A, Wozniak AL, Jones K, Weinman S, Yin XM, Ding WX. Dissecting the dynamic turnover of GFP-LC3 in the autolysosome. Autophagy 2011;7(2):188–204.
21. Li M, Jiang X, Liu D, Na Y, Gao GF, Xi Z. Autophagy protects LNCaP cells under androgen deprivation conditions. Autophagy 2008;4(1):54–60.
22. Bennett HL, Fleming JT, O'Prey J, Ryan KM, Leung HY. Androgens modulate autophagy and cell death via regulation of the endoplasmic reticulum chaperone glucose-regulated protein 78/BiP in prostate cancer cells. Cell Death Dis 2010; 1:e72.
23. Chhipa RR, Wu Y, Ip C. AMPK-mediated autophagy is a survival mechanism in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia. Cell Signal 2011;23(9):1466–1472.
24. Xu Y, Chen SY, Ross KN, Balk SP. Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins. Cancer Res 2006;66(15):7783–7792.
25. Sun M, Yang L, Feldman RI, Sun XM, Bhalla KN, Jove R, Nicosia SV, Cheng JQ. Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src. J Biol Chem 2003;278(44):42992–43000.
26. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: Apoptosis, autophagy and the cross-talk between them. Cell Death Differ 2009;16(7):966–975.
27. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: Crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 2007;8(9):741–752.
28. Livesey KM, Tang D, Zeh HJ, Lotze MT. Autophagy inhibition in combination cancer treatment. Curr Opin Investig Drugs 2009;10(12):1269–1279.

Cell Calcium 57 (2015) 312–320

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Endoplasmic reticulum Ca^{2+} content decrease by PKA-dependent hyperphosphorylation of type 1 IP3 receptor contributes to prostate cancer cell resistance to androgen deprivation

Benoît Boutin[a,1], Nicolas Tajeddine[a,1], Giovanni Monaco[b], Jordi Molgo[c], Didier Vertommen[d], Mark Rider[d], Jan B. Parys[b], Geert Bultynck[b], Philippe Gailly[a,*]

[a] Laboratory of Cell Physiology, Institute of Neuroscience, Université catholique de Louvain, Brussels, Belgium
[b] Laboratory of Molecular and Cellular Signaling, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium
[c] CNRS, Institut de Neurobiologie Alfred Fessard, Laboratoire de Neurobiologie et Développement, UPR 3294, 1 Avenue de la Terrasse, bâtiments 32-33, 91198 Gif sur Yvette Cedex, France
[d] de Duve Institute, Université catholique de Louvain, Brussels, Belgium

ARTICLE INFO

Article history:
Received 3 November 2014
Received in revised form 27 January 2015
Accepted 10 February 2015
Available online 18 February 2015

Keywords:
IP3R1
Apoptosis
Endoplasmic reticulum
Prostate cancer
PKA
Hormone therapy

ABSTRACT

Reference treatment of advanced prostate cancer (PCa) relies on pharmacological or surgical androgen deprivation therapy. However, it is only temporarily efficient as tumor cells inevitably adapt to the low testosterone environment and become hormone-refractory (HRPCa). We observed that androgen removal in HRPCa-derived LNCaP cells causes different alterations in their Ca^{2+} homeostasis among which a reduction of ER Ca^{2+} content. We show that the decrease in $[Ca^{2+}]_{ER}$ is due to a modest overexpression of type 1 IP3R and a threefold increased phosphorylation of IP3R1 on Ser-1716, a protein kinase A (PKA) consensus site, both implicated in ER Ca^{2+} leak. Accordingly, ER Ca^{2+} content was restored by siRNA-mediated down-regulation of IP3R1 or by inhibition of its phosphorylation by competition with a permeant TAT-peptide containing the Ser-1716 consensus phosphorylation sequence or by treatment with the PKA inhibitor H89. Moreover, inhibition of the IP3R1 phosphorylation by both methods sensitized the LNCaP cells to androgen deprivation-induced apoptosis. In addition, SERCA2b overexpression precluded the effect of androgen deprivation on ER Ca^{2+} store content and reduced resistance to androgen deprivation. Taken together, these results indicate that lowering the ER Ca^{2+}-store content by increasing IP3R1 levels and IP3R1 phosphorylation by PKA is a protective mechanism by which HRPCa-derived cells escape cell death in the absence of androgenic stimulation.

1. Introduction

Metastatic prostate cancer (PCa) is associated with a poor prognosis with a 5-year relative survival of 27% [1]. Reference treatment of metastatic PCa consists in pharmacological or surgical androgen deprivation therapy. However, androgen deprivation is only temporarily efficient. After a few months or years, the tumor inevitably relapses despite the absence of androgenic stimulation and becomes hormone-refractory (HRPCa). Effective treatment at this stage is largely limited to docetaxel-based chemotherapies conferring a median survival of only 17–19 months [2,3]. Among commercially available HRPCa cell lines, only LNCaP cells harbor the androgen receptor (AR) [4].

The role of Ca^{2+} in prostate involution induced by androgen deprivation (AD) has been recognized for more than two decades [5]. Mitochondrial membrane permeabilization (MMP) constitutes a key event in apoptotic cell death [6]. At least, two mechanisms can enhance the Ca^{2+} transfer between ER and mitochondria. On the one hand, this could be related to an increase in Ca^{2+} conductance between ER and mitochondria after apoptosis induction. Indeed, it has been proposed that excessive transfer of Ca^{2+} from the ER to the mitochondria possibly through a protein complex comprising the inositol trisphosphate receptor, the glucose-regulated protein 75 and the voltage-dependent anion channel (IP3R/grp75/VDAC) [7] via MMP induction, release of intermembrane proapoptotic factors, dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$), formation of apoptosomes and subsequent cell death [8,9]. Thus, overactivation/overexpression of one member of the IP3R/grp75/VDAC complex could enhance

* Corresponding author at: Laboratory of Cell Physiology, Institute of Neuroscience, Université catholique de Louvain, Avenue Mounier, B1.53.17, 1200 Brussels, Belgium. Tel.: +32 2 764 55 42; fax: +32 2 764 55 80.
E-mail address: philippe.gailly@uclouvain.be (P. Gailly).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ceca.2015.02.004
0143-4160/

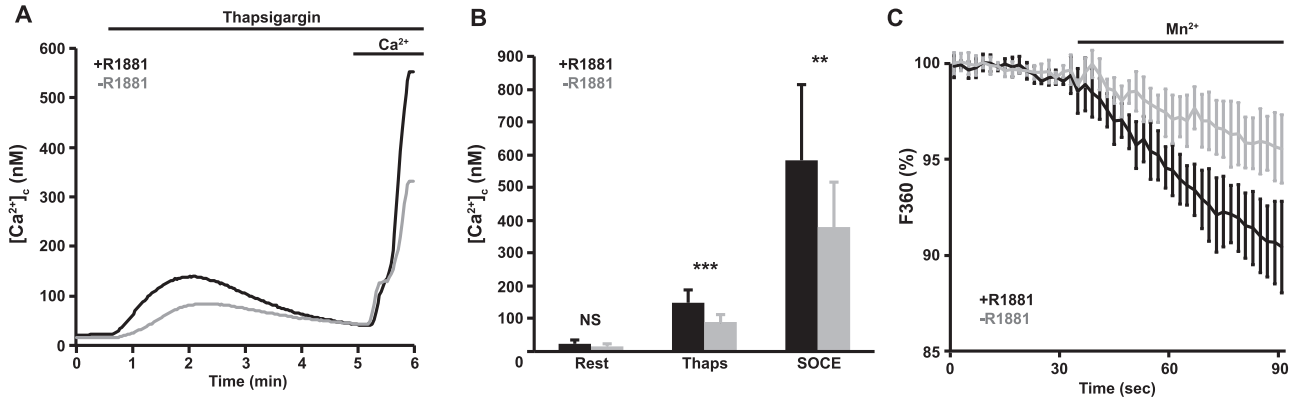

Fig. 1. Intracellular Ca^{2+} homeostasis alterations after androgen deprivation. (A) LNCaP cells cultured for 48 h in the presence of 0.2 nM R1881 (+R1881 condition) or in its absence (−R1881 condition) were loaded with Fura-2/AM and stimulated with 1 μM thapsigargin in the absence of extracellular Ca^{2+}. After 5 min, Ca^{2+} was reintroduced in the external medium to evaluate SOCE amplitude. Representative thapsigargin-evoked changes in $[Ca^{2+}]_c$ are presented for both conditions. (B) Bar histogram showing in both conditions the $[Ca^{2+}]_c$ measured in Fura-2/AM stained LNCaP cells first in the absence of extracellular Ca^{2+}, then after addition of 1 μM thapsigargin (Thaps) and finally after addition of 1.8 mM extracellular Ca^{2+} (SOCE) (means ± S.D. of five independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$). (C) By measurement of Fura-2/AM emission intensity (excitation at 360 nm), the progressive fluorescence quenching due to the influx of Mn^{2+}, taken as a surrogate of Ca^{2+}, was monitored in LNCaP cells in both conditions. The slope of the Mn^{2+}-induced fluorescence quenching was decreased more than twice in the −R1881 condition, revealing that the calcium influx through the cell membrane was strongly decreased after AD (means ± S.D. of three independent experiments).

the sensitivity to proapoptotic agents. On the other hand, the ER-mitochondria Ca^{2+} flux might be enhanced by an increase in the electrochemical gradient, e.g. subsequently to an increase in $[Ca^{2+}]_{ER}$. Indeed, antiapoptotic proteins such as Bcl-2 or Bcl-X_L decrease $[Ca^{2+}]_{ER}$ whereas proapoptotic proteins such as Bax or Bak have the opposite effect, at least in some cell systems [10]. This suggests that $[Ca^{2+}]_{ER}$ is closely related to the sensitivity to cytotoxic stimuli [11]. Increase in $[Ca^{2+}]_{ER}$ can be secondary to an increase in the ER refilling, e.g. due to an increase in SERCA activity or expression, or to a decrease in Ca^{2+} leak from ER. In mouse embryonic fibroblasts, the group of Korsmeyer showed several years ago that IP3R1 constituted an ER Ca^{2+} leak channel, the permeability of which was modulated by a protein kinase A (PKA)-mediated phosphorylation, itself increased by Bcl-2 and decreased by Bax or Bak expression [12]. In T-lymphocytes however Bcl-2 can dock both DARPP-32 and calcineurin to the IP3R, whereby the PKA-mediated phosphorylation of the latter is antagonized, leading to decreased IP3R-mediated Ca^{2+} release [13]. Very recently, in an siRNA screen for the identification of ER Ca^{2+}-leak channels, IP3R1 channels emerged as one of the most prominent Ca^{2+}-transport systems implicated in ER Ca^{2+} leak [14].

In the present study, we show that AD decreases $[Ca^{2+}]_{ER}$ in LNCaP cells and suggest that this is mediated by a phosphorylation-dependent increase in IP3R1 permeability. Inhibition of IP3R1 phosphorylation restores LNCaP cells sensitivity to AD and could be a novel therapeutic target for the treatment of HRPCa.

2. Results

2.1. Androgen deprivation alters intracellular Ca^{2+} homeostasis

In order to study the effects of AD on $[Ca^{2+}]_c$ and on $[Ca^{2+}]_{ER}$, we measured $[Ca^{2+}]_c$ in LNCaP cells cultured in the absence or in the presence of synthetic androgen R1881 for 48 h. Fura-2 loaded cells were treated with the SERCA inhibitor thapsigargin in the absence of external Ca^{2+} and then, Ca^{2+} was reintroduced in the external medium to evaluate the amplitude of store-operated Ca^{2+} entry (SOCE). We observed that AD had no effect on $[Ca^{2+}]_c$ but reduced by about 40% the amount of thapsigargin-induced Ca^{2+} release, suggesting a decrease of the ER Ca^{2+} store content. SOCE was also decreased after AD (Fig. 1A and B). Interestingly, treatment of the cells with the AR specific inhibitor bicalutamide (50 μM) for 48 h also decreased releasable Ca^{2+} from the ER (data not shown).

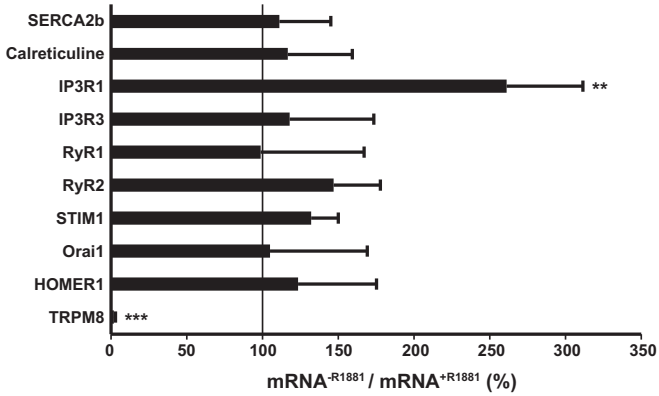

Fig. 2. Bar histogram showing the relative mRNA expression of genes involved in calcium homeostasis after AD in LNCaP cells. Two days after AD treatment, total RNA was extracted and reverse-transcribed. qPCR was performed with specific primers. The mRNA expression of each gene was normalized to β2-microglobulin expression (means ± S.D. of four independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$).

We also observed that basal influx of Ca^{2+} through the plasma membrane as measured by the Mn^{2+}-induced Fura-2 quenching technique was diminished after androgen removal (Fig. 1C).

We therefore tried to decipher the mechanisms underlying these modifications and measured the expression of a series of proteins possibly involved in Ca^{2+} homeostasis (Ca^{2+} pumps, channels and buffers). AD induced a strong decrease in TRPM8 expression and a more than twofold increase in IP3R1 expression at the mRNA level (Fig. 2). Previous studies have shown that the expression of the Ca^{2+} channel TRPM8 is highly dependent on the presence of androgens and is overexpressed in prostate cancer cells [15,16]. Its repression after androgen removal might therefore explain alterations in Ca^{2+} homeostasis. IP3R1 beside its well-known role in agonist-induced Ca^{2+} signaling, might also function as an ER Ca^{2+} leak channel [12,17], thereby controlling resting $[Ca^{2+}]_{ER}$. Importantly, its activity has been reported to be phosphorylation-dependent [18].

2.2. Involvement of TRPM8 in Ca^{2+} homeostasis alterations induced by AD

Given the importance of AD on TRPM8 expression, we tried to mimic this effect by depleting TRPM8 in LNCaP cells using specific

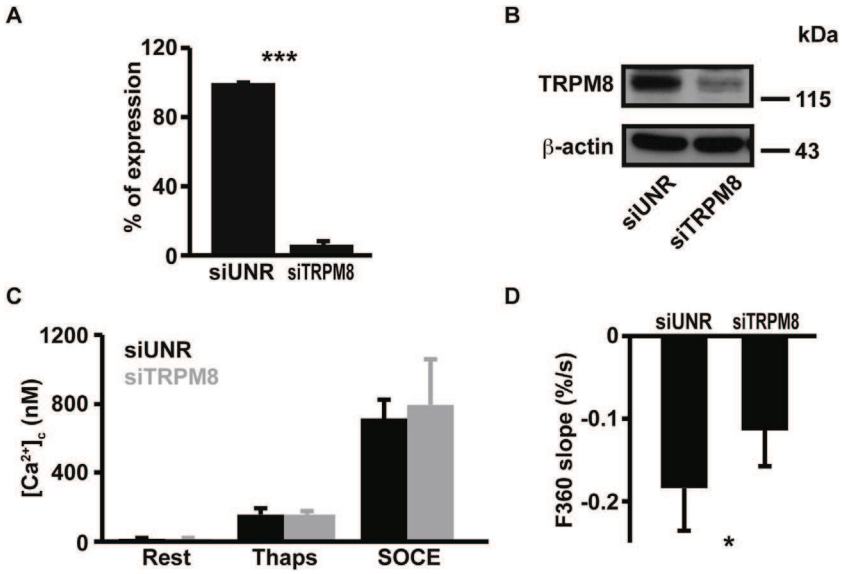

Fig. 3. Role of TRPM8 in LNCaP cells. (A, B) LNCaP cells were transfected with a siRNA targeting TRPM8 mRNA (siTRPM8) or with a non-silencing control siRNAs (siUNR) pool and then cultured in the presence of 0.2 nM R1881. The silencing of TRPM8 was proved by RT-qPCR (A) and immunoblot (B). β-Actin was used as loading control. Four days after transfection, total RNA was extracted and reverse-transcribed. qPCR was performed with specific primers. The results are expressed as the level of TRPM8 expression normalized to β2-microglobulin expression (means ± S.D. of three independent experiments, $^{***}p < 0.001$). (B) Bar histogram showing in siUNR and siTRPM8 conditions the intracellular Ca^{2+} concentrations measured in Fura-2/AM stained LNCaP cells first in the absence of extracellular Ca^{2+} (Rest), then after addition of 1 μM thapsigargin (Thaps) and finally after addition of 1.8 mM extracellular Ca^{2+} (SOCE) (means ± S.D. of three independent experiments). (C) Mn^{2+}-induced fluorescence quenching of Fura-2/AM in LNCaP cells four days after transfection with siUNR or siTRPM8 (means ± S.D. of three independent experiments, $^{*}p < 0.05$).

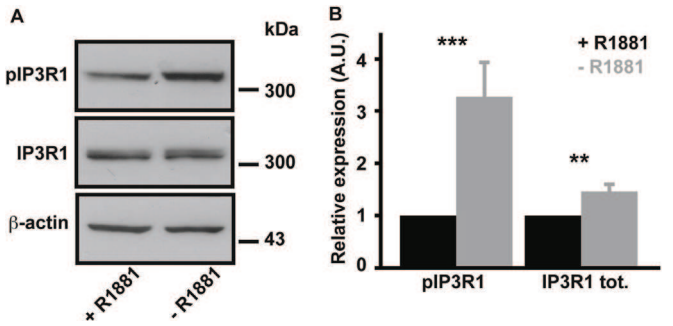

Fig. 4. Phosphorylation of IP3R1 on Ser-1716 after AD. (A) Immunoblot analysis of the expression of IP3R1 and of its phosphorylation on Ser-1716 in LNCaP cells cultured for 48 h in the presence of 0.2 nM R1881 (+R1881 condition) or in its absence (−R1881 condition). β-Actin was used as a loading control. Data presented are representative of seventeen independent experiments. (B) Bar histogram showing phosphorylation and total expression level of IP3R1 in both conditions (means ± S.D. of seventeen independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$).

siRNA. The silencing of TRPM8 was proved by RT-qPCR (Fig. 3A) and was correlated with a significant decrease of basal influx of Ca^{2+} (Fig. 3C). This indicates that the channel is expressed, at least partially, at the plasma membrane and that the decrease of its expression due to AD could be responsible for basal Ca^{2+} influx reduction. However, we did not observe any subsequent decrease in $[Ca^{2+}]_c$, in thapsigargin-evoked Ca^{2+} release or in SOCE (Fig. 3B).

2.3. Involvement of IP3R1 in Ca^{2+} homeostasis alterations induced by AD

It has been suggested that IP3R1 could control resting $[Ca^{2+}]_{ER}$ [12,14]. After AD treatment in LNCaP cells, we observed a modest increase in the amount of IP3R1 protein (Fig. 4A and B) and a dramatic increase in IP3R1 phosphorylation on the PKA consensus site Ser-1716 (equivalent to Ser-1756 in the rat IP3R1 amino acid sequence). Incidentally, IP3R2 was not expressed in LNCaP cells [19] and expression of IP3R3 mRNA was not modified by AD (Fig. 2).

In order to evaluate the effects of IP3R1 phosphorylation and expression on $[Ca^{2+}]_{ER}$, we first measured the Ca^{2+} content in the ER after inhibition of PKA-mediated IP3R1 phosphorylation. As also performed in Fig. 1, we tried to evaluate $[Ca^{2+}]_{ER}$ after thapsigargin treatment. However, we observed that acute inhibition (5 min pretreatement) of IP3R by xestospongin B decreased by itself the amount of releasable Ca^{2+} (Fig. 5A). This suggested that IP3R is the Ca^{2+} leak channel in thapsigargin-induced ER depletion. Since we were interested in the permeability of IP3R, a procedure using thapsigargin to evaluate $[Ca^{2+}]_{ER}$ was obviously inadequate. We then decided to use ionomycin to permeabilize the ER membrane and measure releasable Ca^{2+} independently of the IP3R-mediated leak. We observed that the ionomycin-induced Ca^{2+} release was decreased by AD (Fig. 5E and F), confirming the results obtained with thapsigargin (Fig. 1B). We then interfered with IP3R1 phosphorylation by using a cell-permeable TAT-peptide (TAT-IP3R1^{S1716}) containing the Ser-1716 consensus phosphorylation sequence. AD-induced phosphorylation of IP3R1 was abolished after treatment with 80 μM TAT-IP3R1^{S1716} (Fig. 5B and C). As Ser-1716 is known to be phosphorylated by PKA [20,21], we treated LNCaP cells with H89, a selective inhibitor of this kinase. We observed that 10 μM H89 treatment strongly reduced IP3R1 phosphorylation (Fig. 5B and 5D). This effect was stronger than that observed with TAT-IP3R1^{S1716} treatment and occurred even in the presence of androgen. In the presence of 80 μM TAT-IP3R1^{S1716}, the effect of AD on ionomycin-induced Ca^{2+} release was abolished (Fig. 5E). Inhibition of PKA by H89 strongly increased $[Ca^{2+}]_{ER}$ and also inhibited AD-induced decrease in $[Ca^{2+}]_{ER}$ (Fig. 5F).

2.4. Involvement of IP3R1 phosphorylation in the resistance to AD

The results presented above suggest that the decrease in $[Ca^{2+}]_{ER}$ is due to an increase of the amount of IP3R1 phosphorylated on its serine 1716 residue consecutively to AD. We therefore investigated whether this process was involved in the resistance of LNCaP cells to AD. LNCaP cells were treated for 72 h with 80 μM TAT-IP3R1^{S1716} in the presence or in the absence of androgen. Simultaneous

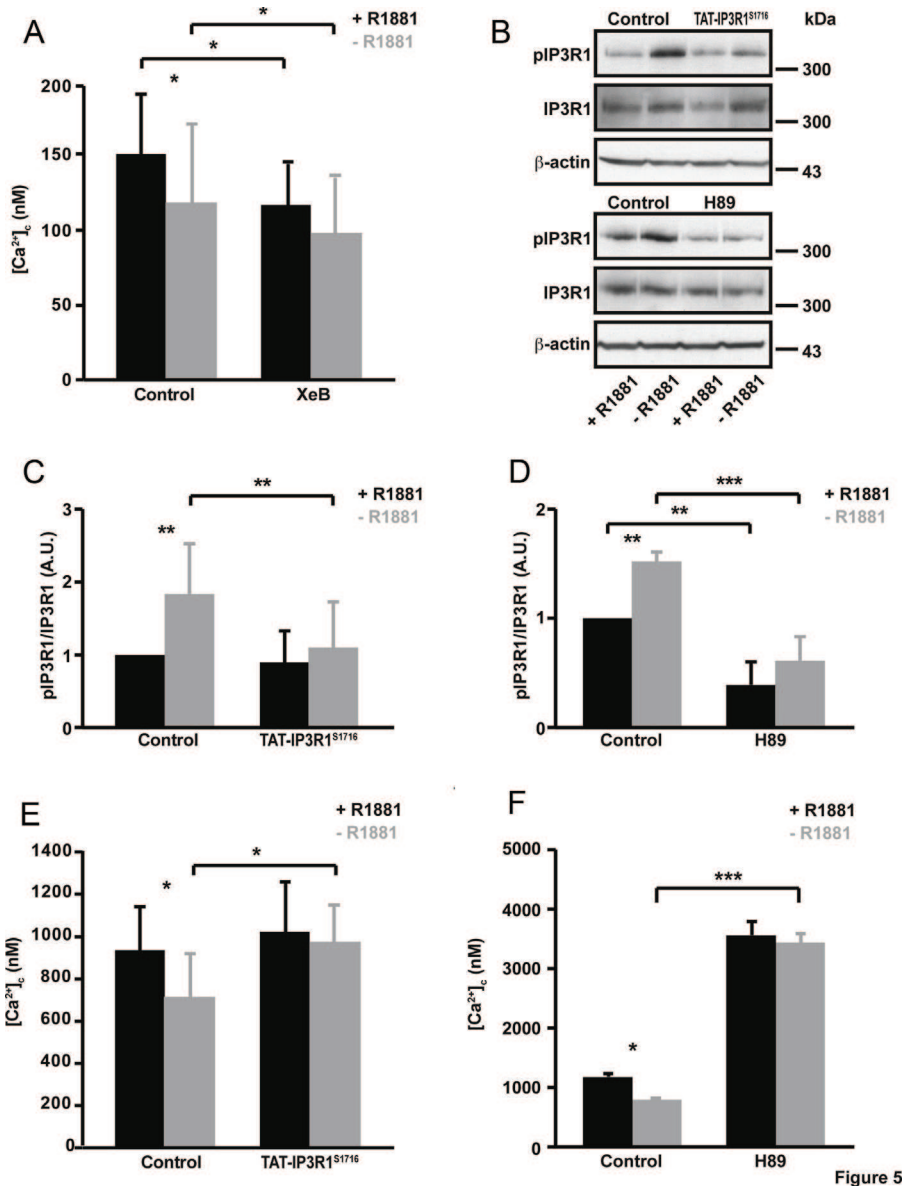

Fig. 5. Involvement of IP3R1 phosphorylation in Ca^{2+} homeostasis alterations induced by AD. (A) Bar histogram showing the thapsigargin-induced Ca^{2+} release in the absence of extracellular Ca^{2+} measured in Fura-2/AM-loaded LNCaP cells cultured in the presence or in the absence of 0.2 nM R1881 and pretreated or not with 2 μM Xestospongin B (means ± S.D. of three independent experiments, $^{*}p < 0.05$). (B) Immunoblot analysis of the expression of IP3R1 and of its phosphorylation on Ser-1716 in LNCaP cells cultured in the presence or in the absence of 0.2 nM R1881 and treated for 48 h with 80 μM cell-permeant TAT-peptide (TAT-IP3R1^{S1716}) containing the Ser-1716 consensus phosphorylation sequence or with 10 μM PKA inhibitor H89. β-Actin was used as a loading control. Data presented are representative of three independent experiments. (C, D) Quantification of data presented in A and B (means ± S.D. of three to seven independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$). (E) Bar histograms showing the effects of TAT-IP3R1^{S1716} on the $[Ca^{2+}]_c$ measured in Fura-2/AM-loaded LNCaP cells cultured in the presence or in the absence of 0.2 nM R1881 after addition of 10 μM ionomycin in the absence of extracellular Ca^{2+} (means ± S.D. from 6 to 7 independent experiments, $^{*}p < 0.05$). (F) Bar histograms showing the effects of H89 on the $[Ca^{2+}]_c$ measured in Fura-2/AM-loaded LNCaP cells cultured in the presence or in the absence of 0.2 nM R1881 after addition of 10 μM ionomycin in the absence of extracellular Ca^{2+} (means ± S.D. from 5 to 6 independent experiments, $^{*}p < 0.05$, $^{***}p < 0.001$).

fluorimetric detection of mitochondrial membrane potential ($\Delta\Psi_m$) dissipation and plasma membrane permeabilization, two markers associated with cell death, showed that TAT-IP3R1^{S1716} strongly sensitized LNCaP cells to AD (Fig. 6A and B). TAT-IP3R1^{S1716} did however not exert any effect on cell death in the presence of androgen.

Additionally, we observed that 10 μM H89 also restored AD-dependent cell death but did not affect cell viability in the presence of androgen (Fig. 6C and D). Its effect on AD-dependent cell death was strongly diminished by adding 0.5 μM thapsigargin during the last 24 h of AD treatment. This suggests that the inhibition of PKA by H89 exerts its effect on cell death by modulating $[Ca^{2+}]_{ER}$.

To confirm the role of Ca^{2+} store in the resistance to AD, we partially prevented AD-induced Ca^{2+} store depletion by overexpressing SERCA2b, the isoform the most ubiquitously expressed in non-muscle tissues (Fig. 7A) and measured the effects of such $[Ca^{2+}]_{ER}$ stabilization on cell survival. We observed that SERCA2b overexpression increased the proportion of apoptotic cells after AD treatment proving ER Ca^{2+} store control on LNCaP sensitivity to AD (Fig. 7B).

2.5. Effects of IP3R1 phosphorylation on autophagy

We previously showed that AD induces autophagy in LNCaP cells and that genetic or pharmacological inhibition of

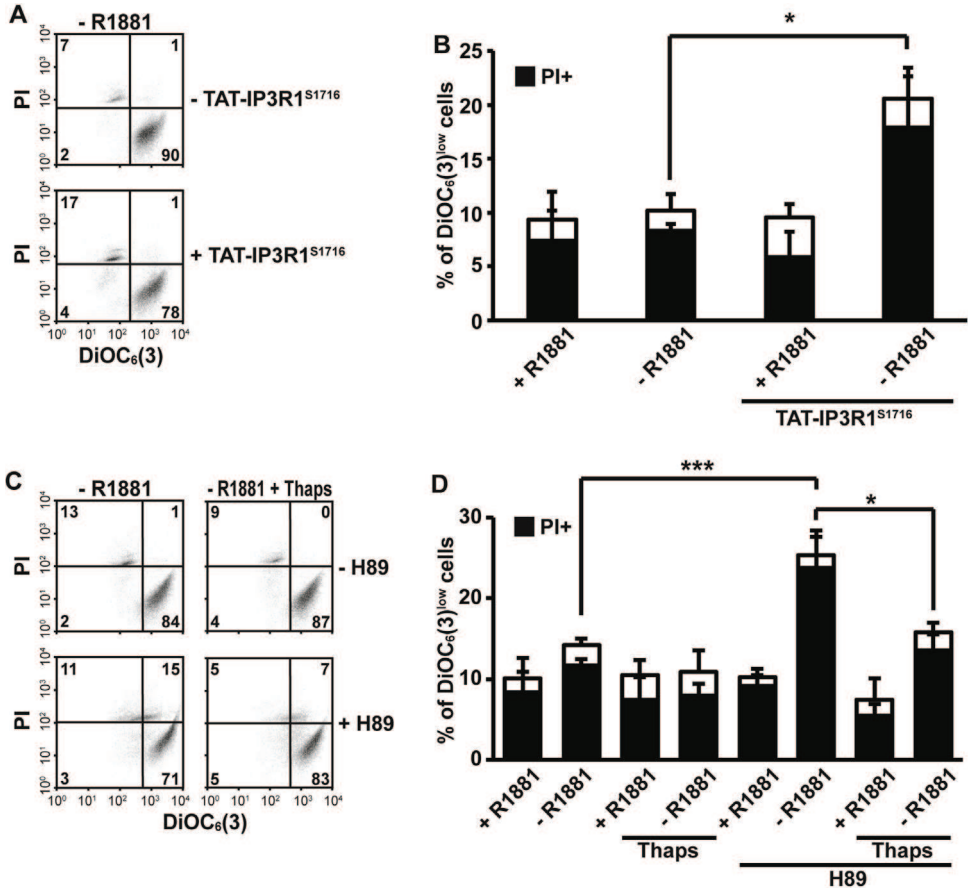

Fig. 6. Inhibition of IP3R1 phosphorylation sensitizes LNCaP cells to AD. (A) Cytofluorimetric assessment of the effects of AD in the absence or in the presence of 80 μM TAT-IP3R1^{S1716} on apoptosis-associated mitochondrial transmembrane potential ($\Delta\Psi_m$) dissipation and plasma membrane permeabilization. Cells were treated for 72 h and then labeled with the $\Delta\Psi_m$-sensitive probe $DiOC_6(3)$ and with propidium iodide (PI). Representative dot plots recorded upon AD treatment in the absence and in the presence of TAT-IP3R1^{S1716} are shown. Numbers in each quadrant indicate the percentage of cells. (B) Quantification of data illustrated in A as well as of cells cultured in the presence of R1881 and similarly treated. White and black columns represent percentages of cells exhibiting $\Delta\Psi_m$ loss alone ($DiOC_6(3)^{low}$) or in association with plasma membrane breakdown (PI+), respectively. Data are presented as means ± S.D. of three independent experiments ($^*p < 0.05$). (C, D) Similar experiments were performed in the absence or in the presence of 10 μM H89 for 72 h. Effects of thapsigargin (Thaps) were also monitored. Data are presented as means ± S.D. of three independent experiments ($^*p < 0.05$, $^{***}p < 0.001$).

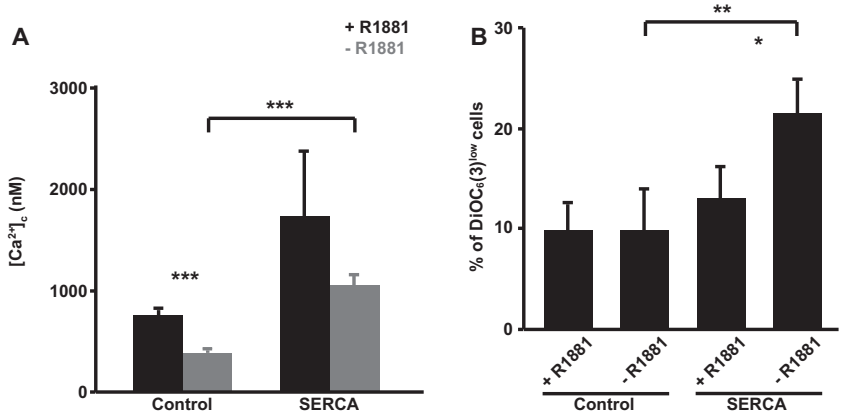

Fig. 7. SERCA2b overexpression prevents Ca^{2+} store depletion and promotes cell death after AD. (A) LNCaP cells cultured in the presence or in the absence of 0.2 nM R1881 were transfected with a control plasmid or a plasmid encoding SERCA2b and with a dsRed expressing plasmid allowing detection of transfected cells. Cells were loaded with Fura-2/AM and stimulated with 10 μM ionomycin in the absence of extracellular Ca^{2+}. Bar histogram showing the effects of SERCA2b overexpression on the $[Ca^{2+}]_c$ in dsRed-positive cells after addition of ionomycin (means ± S.D. from 4 to 6 independent experiments, $^{***}p < 0.001$). (B) Cytofluorimetric assessment of the effects of AD in dsRed-positive cells on apoptosis-associated mitochondrial transmembrane potential ($\Delta\Psi_m$) dissipation. Transfected LNCaP cells were cultured for 72 h in the presence or in the absence of 0.2 nM R1881 and then labeled with the $\Delta\Psi_m$-sensitive probe $DiOC_6(3)$. Bar histogram shows percentages of dsRed-positive cells exhibiting $\Delta\Psi_m$ loss for each condition. Data are presented as means ± S.D. of three independent experiments ($^*p < 0.05$, $^{**}p < 0.01$).

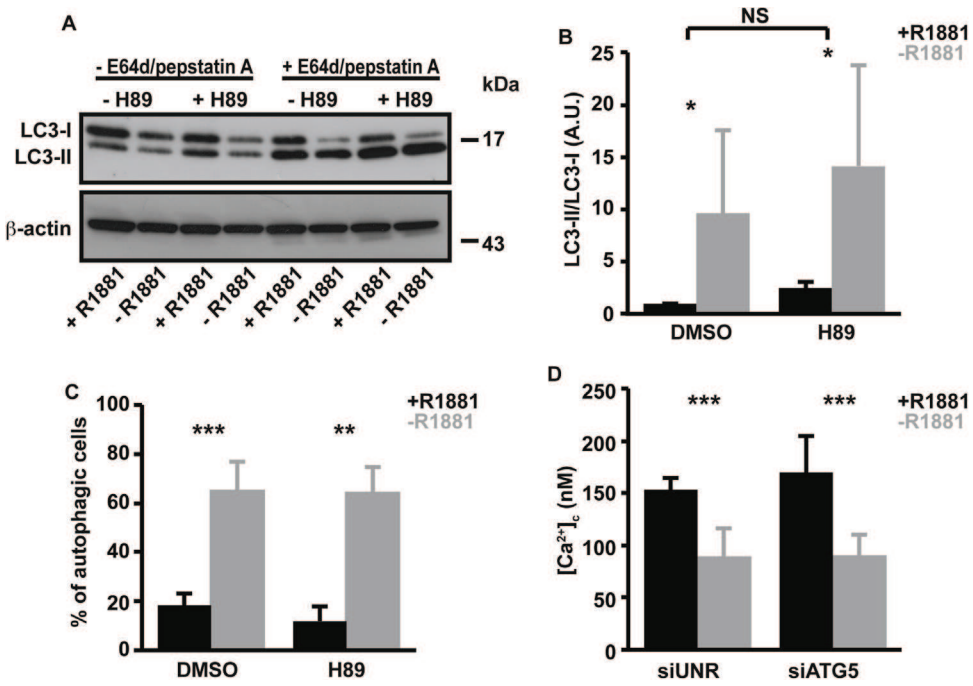

Fig. 8. $[Ca^{2+}]_{ER}$ and autophagy. (A) Immunoblot analysis assessing LC3-II protein levels after 48 h AD treatment in with the absence or presence of 10 μM H89. E64d (10 μg/ml) and pepstatin A (10 μg/ml) were added during the treatment. β-Actin was used as a loading control. Immunoblots are representative of three independent experiments. (B) Quantification of data illustrated in A. Data are presented as means ± S.D. of at least three independent experiments ($^{*}p < 0.05$). (C) LNCaP cells were transfected with a plasmid encoding a GFP-LC3 fusion protein. Bar histogram showing the number of cells exhibiting more than 10 fluorescent dots after treatment by 10 μM H89 for 48 h in the presence or in the absence of 0.2 nM R1881 (means ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$). (D) LNCaP cells were transfected with a pool of 4 siRNAs targeting Atg5 mRNA (siATG5) or with a non-silencing control siRNAs (siUNR) pool and then cultured in the presence or in the absence of 0.2 nM R1881 for 48 h. LNCaP cells were loaded with Fura-2/AM and stimulated with 1 μM thapsigargin in the absence of extracellular Ca^{2+}. Bar histogram showing thapsigargin-evoked changes in $[Ca^{2+}]_c$ for each condition (means ± S.D. of three independent experiments, *** $p < 0.001$).

autophagy also restores AD-dependent cell death [22]. Furthermore, IP3Rs and downstream Ca^{2+} signaling have been implicated in mTOR-controlled autophagy [23,24]. We therefore studied here whether AD-induced $[Ca^{2+}]_{ER}$ modulation was involved in autophagy.

Upon autophagy induction, the LC3-I protein is modified into the lipidated form, LC3-II. This latter is associated with autophagosomes and its detection is routinely used to monitor autophagy [25]. We observed that LC3-II levels declined after AD (Fig. 8A and B). To distinguish whether this observation was due to a decrease in the on-rate of autophagic LC3-II formation or an increase in the off-rate of, the lysosomal LC3-II degradation, we used a combination of E64d and pepstatin A to inhibit lysosomal function and block LC3-II degradation. The use of inhibitors revealed that LC3-II was increased, demonstrating that AD promotes autophagy at the level of the autophagosomal/lysosomal fusion and/or degradation events. Together, these observations confirm our previous study showing that AD increases autophagic rate in LNCaP cells [22].

H89 treatment did not preclude AD-induced LC3-II degradation, suggesting that phosphorylation of IP3R1 is not responsible for the AD-induced autophagy flux at the level of autophagosomes/lysosomes (Fig. 8A and B). These results were corroborated by detection of another biochemical sign of autophagy, namely the redistribution of GFP-LC3 fusion protein from a ubiquitous, diffuse pattern toward autophagosomes, which became visible as cytoplasmic puncta, independently of the presence of H89 (Fig. 8C).

Consistent with these findings, genetic inhibition of autophagy by siRNA-mediated depletion of the essential autophagy gene product Atg5 did not modify AD-induced decrease of the thapsigargin-releasable pool of Ca^{2+} (Fig. 8D). This suggests that the effects of AD on Ca^{2+} stores were not due to an autophagic degradation of ER membranes or ER proteins involved in intracellular Ca^{2+} handling in addition to the control of IP3R1 permeability.

3. Discussion

Several reports have demonstrated the role of $[Ca^{2+}]_{ER}$ in the resistance to proapoptotic stimuli and increased proliferation, two major hallmarks of cancer cells [26,27]. In LNCaP cells, AD for 96 h has been shown to induce neuroendocrine differentiation associated with a reduction in $[Ca^{2+}]_{ER}$. This observation was attributed to a decreased expression of SERCA2b and of the luminal Ca^{2+}-binding/storage chaperone protein calreticulin and was correlated with a decrease in sensitivity to thapsigargin and TNF-α [28]. In our experiments, we confirmed the decrease in $[Ca^{2+}]_{ER}$ after AD but expression levels of SERCA2b and calreticulin were not modified. This apparent discrepancy could be attributed to the fact that in our study, AD was maintained for only 48 h. The TRPM8 expression was however strongly decreased and we also observed that basal Ca^{2+} influx through the plasma membrane was diminished after AD. This observation could explain the decrease in $[Ca^{2+}]_{ER}$. Since expression of TRPM8 is known to be dependent of androgen receptor activation [16], we measured basal Ca^{2+} influx after siRNA-mediated TRPM8 depletion. It was decreased in TRPM8-depleted cells suggesting that it was, at least partially, localized in the plasma membrane. However, neither thapsigargin-induced Ca^{2+} release nor SOCE were modified by repression of TRPM8. We therefore conclude that TRPM8 repression and decrease in basal Ca^{2+} influx after AD were not related to the decrease in $[Ca^{2+}]_{ER}$. As expected, AD induced-TRPM8 decrease or siRNA-mediated TRPM8 depletion decreased Ca^{2+} entry. However, they did not modify resting cytosolic $[Ca^{2+}]$, suggesting that their effects on Ca^{2+} entry is

compensated by the activity of transporters allowing fluxes of Ca^{2+} out of the cells or into the ER.

As mentioned above, phosphorylated IP3R1 was reported as an ER leak channel in mouse embryonic fibroblasts cells [12]. We therefore measured the expression and the phosphorylation of IP3R1 in AD conditions. Expression was increased by only 50% but phosphorylation on Ser-1716 increased threefold after AD for 48 h. Inhibiting PKA-dependent phosphorylation of $IP3R1^{S1716}$ by competition with TAT-$IP3R1^{S1716}$ abolished the effect of AD on $[Ca^{2+}]_{ER}$. This underpins the concept that beside its role in response to agonists, IP3R1 also constitutes in LNCaP cells an ER Ca^{2+} leak channel that is modulated by AD-dependent phosphorylation. In those cells, IP3R1 could therefore regulate $[Ca^{2+}]_{ER}$ at rest. Inhibition of PKA by H89 also abolished AD-dependent decrease in $[Ca^{2+}]_{ER}$ but also dramatically increased $[Ca^{2+}]_{ER}$ in the presence of androgen. This could be due to the higher efficiency of H89 to reduce IP3R1 phosphorylation in comparison with TAT-$IP3R1^{S1716}$ (Fig. 5B and D). However, we cannot exclude an alternative pathway connecting PKA and $[Ca^{2+}]_{ER}$.

Our results also clearly show that AD-dependent phosphorylation of IP3R1 is an important mediator of androgen resistance in PCa cells. Indeed, inhibition of IP3R1 phosphorylation with H89 or TAT-$IP3R1^{S1716}$ dramatically increased sensitivity to AD in LNCaP cells. Interestingly, this effect was seriously decreased after pretreatment with thapsigargin, indicating that the effect of PKA on AD-induced cell death is dependent on the Ca^{2+} content in the ER. The role of $[Ca^{2+}]_{ER}$ in androgen resistance was also confirmed by the fact that SERCA2b overexpression limited the effect of AD on Ca^{2+} store content and increased AD-induced apoptosis. Therefore, we show for the first time that survival of PCa cells in response to androgen removal is dependent on the (partial) depletion of their Ca^{2+} stores.

It has been reported that PKA is overexpressed in PCa and constitutes a marker of poor prognosis [7]. Moreover, PKA hyperactivity could contribute to resistance of PCa cells to AD [29]. Our results suggest that IP3R1 phosphorylation by PKA could explain the decreased AD-induced cell death in HRPCa. Targeting the PKA-IP3R1 pathway could therefore constitute an interesting strategy to restore sensitivity to AD in HRPCa.

Since AD itself induced phosphorylation on the PKA consensus site $IP3R1^{S1716}$, we suggest that, beside its proapoptotic effect, AD concomitantly triggers a prosurvival pathway. Bagchi et al. showed that androgen stimulation induced PKA activation by increasing cAMP production [30]. This result is apparently contradictory to our data but it is noteworthy that in contrast to our experimental design (48 h AD), Bagchi et al. investigated short-term (10 min) effect of androgen on PKA-related pathways. Furthermore, prolonged AD treatment has been noticed to increase the expression of different catalytic subunits of PKA in LNCaP cells [31].

In a previous study, we showed that AD induced autophagy in LNCaP cells and that this process in turn inhibited AD-triggered cell death [22]. Therefore, we have investigated in the present study whether $IP3R1^{S1716}$ phosphorylation and Ca^{2+} store depletion could be intermediates between AD and autophagy. Our data showed that inhibition of $IP3R1^{S1716}$ phosphorylation does not interfere with AD-induced autophagy. Moreover, siRNA-mediated inhibition of autophagy has no effect on $[Ca^{2+}]_{ER}$. This suggests that AD-induced autophagy and AD-induced $IP3R1^{S1716}$ phosphorylation constitute two independent mechanisms of resistance to AD.

In conclusion, we show that phosphorylation of IP3R1 on a PKA consensus site modulates $[Ca^{2+}]_{ER}$ and renders LNCaP cells more resistant to AD-induced apoptosis. This demonstrates that the common anti-prostate cancer strategy consisting in androgen removal could interfere with ER Ca^{2+} homeostasis, highlighting the role of ER Ca^{2+} in the mechanisms of cancer aggressiveness.

4. Materials and methods

4.1. Cell culture and reagents

LNCaP cells (American Type Culture Collection, Manassas, VA, USA, CRL-1740) were grown at 37 °C in an humidified atmosphere of 5% CO2–95% air in RPMI 1640 (Gibco Invitrogen, Carlsbad, CA, USA, 52400) supplemented with 10% fetal calf serum, 100 IU/ml penicillin (Gibco, 10270) and 100 μg/ml streptomycin (Gibco, 15140). Cells were cultured up to passage 30. Analysis of the effect of androgen deprivation was performed by culturing cells for 2 days in RPMI 1640 medium supplemented with charcoal-treated FCS (Gibco, 12676) for serum steroid hormone removal (−R1881 condition). As control condition, the synthetic agonist of the androgen receptor (AR), methyltrienolone (R1881), was added to the medium at 0.2 nM (+R1881 condition). H89 (Sigma–Aldrich, Saint Louis, MO, B1427) was used at 10 μM, thapsigargin (Sigma–Aldrich, T9033) at 0.5 and 1 μM, ionomycin (Invitrogen, Carlsbad, CA, USA, I24222) at 10 μM. Xestospongin B was purified from Xestospongia exigua as previously described [32]. The synthetic peptide TAT-$IP3R1^{S1716}$ (NH2-RKKRRQRRRGGRPSGRRESLTSFGNG-COOH) was obtained from ThermoFisher Scientific (Waltham, MA, USA) and used at 80 μM.

4.2. siRNA transfection

The siRNA targeting TRPM8 mRNA (siTRPM8; 5′-GCAAACUGGUUGCGAACUU-3′) as well as the non-silencing control siRNAs (siUNR) pool were purchased from Thermo Fisher Scientific (Lafayette, CO, USA). LNCaP cells were transfected using DharmaFECT reagent (Thermo Fisher Scientific, T-2002-03) according to the manufacturer's instructions. Twenty-four hours later, cells were plated on six-well plates and after 48 additional hours subjected to AD treatment. Four days after transfection, efficiency of siRNA-mediated depletion of the TRPM8 mRNA was assessed by RT-qPCR.

4.3. SERCA2b transfection

LNCaP cells, seeded in six-well culture plates until ∼80% confluence, were co-transfected with a plasmid coding a DsRed fluorescent protein and an empty plasmid or a plasmid coding for SERCA2b using Lipofectamine reagent (Invitrogen, 11668-019) according to manufacturer's instructions. After 24 h, cells were plated on six-well plates and 48 h later, were subjected to AD treatment during 2 at 3 days.

4.4. Quantitative RT-PCR

LNCaP mRNAs were extracted with Trizol reagent (Invitrogen). Gene-specific PCR primers were designed using Primer3 and purchased from Eurogentec (Seraing, Belgium). Primer sequences are given Table 1. The β2-microglobulin was used as housekeeping gene. Quantitative RT-PCR was performed using 5 μL of cDNA, 12.5 μL of SYBRGreen Mix (Bio-Rad, Hercules, CA, USA) and 300 nM of each primer in a total reaction volume of 25 μL. The reaction was initiated at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min, and extension at 72 °C for 10 s. Data were recorded on a MyiQ qPCR detection system (Bio-Rad), and cycle threshold (*Ct*) values for each reaction were determined using analytical software from the same manufacturer. Each cDNA was amplified in duplicate, and *Ct* values were averaged for each duplicate. The average *Ct* value for β2-microglobulin was subtracted from the average *Ct* value for the gene of interest. This Δ*Ct* value obtained in–R1881 condition or in siRNA-TRPM8 silenced LNCaP was then subtracted from the Δ*Ct* value obtained

Table 1
Primers used in Real-Time PCR analyses.

Gene	Forward primer	Reverse primer	PCR Product (bp)
Serca2b	ACCCACATTCGAGTTGGAAG	CCAACGAAGGTCAGATTGGT	209
Calreticuline	TCTCAGTTCCGGCAAGTTCT	TCTGAGTCTCCGTGCATGTC	231
IP3R1	CTGATTCACCCACGAAGGTT	TGCAAATCAGGTGCTTTCTG	200
IP3R3	AACTACCTGGCTGCTGAGGA	CGAAAGAGTCGGTTTTCTGC	205
RyR1	TGTCGCTCTGCAGGTACATC	CCCGTCGGTAGGCAGTAGTA	157
RyR2	CAACCGGACTCGTCGTATTT	TTGGCTTTCTCTTTGGCTGT	249
Stim1	CCAGAGCCTCAGCCATAGTC	CTTCCACATCCACATCACCA	200
Orai1	GACTGGATCGGCCAGAGTTA	CACTGAAGGCGATGAGCAG	214
Homer1	AGCTGCTCGACTAGCAAAGG	CCTCCCAATGTTTGCTGATT	230
Trpm8	ACTTAGCCAATGATGAGATTT	CTTTATGAGAGCCGTAAACAT	86

in control conditions (+R1881 or siUNR in +R1881 condition) giving a $\Delta\Delta Ct$ value. As amplification efficiencies of the genes of interest and β2-microglobulin were comparable, the amount of mRNA, normalized to β2-microglobulin, was given by the relation $2^{-\Delta\Delta Ct}$.

4.5. Immunoblotting

Cells were harvested by scraping in PBS and re-suspended in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonylfluoride and 1 μg/mL leupeptin. Extracts were diluted in a mix of LDS Sample Buffer and Sample Reducing Agent (NuPAGE®, Invitrogen, NP0007 and NP0009) and heated at 95 °C for 3 min. Samples were electrophoresed on 4–12% or 12% SDS-polyacrylamide gels (Invitrogen, NP0321BOX and NP0341BOX) and transferred onto nitrocellulose membranes (Biorad, 162-0097). The blots were saturated in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.05% Tween 20, pH 7.6) containing 5% skimmed milk for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies: anti-TRPM8 (Abcam, Cambridge, UK, ab3243, dilution 1:10,000), and anti-phospho-IP3R^{S1756} (Cell Signaling Technology, Beverly, MA, USA, 3760; recognizing human IP3R1 phosphorylated on Ser-1716), anti-IP3R1 (Cell Signaling Technology, 8568), anti-LC3 (Cell Signaling Technology, 3868) all used at a dilution of 1:1000. Immunodetection of β-actin with monoclonal anti-actin antibody (Sigma–Aldrich, A5441) was used as loading control (dilution 1:10,000). After incubation with appropriate secondary antibodies coupled to peroxidase, peroxidase activity was detected with ECL Prime (GE Healthcare, Uppsala, Sweden, RPN2236) on ECL hyperfilm. Immunoblots were quantified using the *ImageMaster densitometry* program.

4.6. Calcium measurements

Cells were plated on glass coverslips, subjected to various treatments during 2 days and then incubated for 1 h at room temperature with 1 μM Fura-2/AM (Calbiochem, Camarillo, CA, USA) in Krebs-HEPES buffer containing 11.5 mM HEPES, 135.5 mM NaCl, 5.9 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM D-glucose, pH 7.4. After rinsing, measurements were then realized in Krebs-HEPES buffer without $CaCl_2$ and supplemented with 0.2 mM EGTA. Coverslips were mounted in a heated (37 °C) microscope chamber. Cells were alternately excited (0.5 Hz) at 340 and 380 nm using a Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instrument, Novato, CA, USA) coupled to a Zeiss Axiovert 200 M inverted microscope (Zeiss Belgium, Zaventem, Belgium). Images were acquired with a Zeiss Axiocam camera coupled to a 510-nm emission filter and analyzed with Axiovision software. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) was evaluated from the ratio of fluorescence emission intensities excited at the two wavelengths using the Grynkiewicz equation [33].

The progressive fluorescence quenching due to the influx of 100 μM Mn^{2+} (Sigma–Aldrich) was monitored by measurement of Fura-2/AM emission intensity (excitation at 360 nm). The slope of the Mn^{2+}-induced fluorescence quenching was measured on the first 30 s after adding Mn^{2+}.

4.7. Flow cytometry

Simultaneous detection of mitochondrial membrane potential ($\Delta\Psi_m$) dissipation and plasma membrane permeabilization was determined by staining with 40 nM $\Delta\Psi_m$-sensitive fluorochrome $DiOC_6(3)$ (Molecular Probes-Invitrogen, Carlsbad, CA, USA, D273) and 1 μg/ml vital dye propidium iodide, PI (Sigma–Aldrich, P1304MP). All PI+ events have been considered at DiOC low. Cytofluorometric analyses were performed on a FACSCalibur equipped with CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA).

4.8. GFP-LC3 transfection and imaging

LNCaP cells, seeded in six-well culture plates until ~80% confluence, were transfected with a plasmid coding for GFP-LC3 using Lipofectamine reagent. After 24 h, cells were plated on glass coverslips and 48 h later, were subjected to various treatments during 2 days. GFP fluorescence was analyzed on an inverted Axiovert 200 microscope (Zeiss) using FITC excitation and emission wavelengths (100×, NA 1.4, oil immersion). Images were acquired with a Zeiss Axiocam and analyzed by the Zeiss Axiovision software.

4.9. Statistical analysis

Data are presented as means ± S.D. Student's *t*-test and two-way ANOVA were used to determine statistical significance when appropriate.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

The work was funded by the Belgian agencies "Fonds National de la Recherche Scientifique" and "Fonds de la Recherche Scientifique Médicale", "Fonds Joseph Maisin", "Télévie", Concerted Research Action (10-15/029) and the Interuniversity Poles of Attraction Belgian Science Policy (P7/13). D.V. was "Collaborateur Logistique" of the Fund for Medical Scientific Research (FNRS, Belgium) and Chercheur Qualifé (Université catholique de Louvain). GM is a post-doctoral researcher of the FWO. We thank Dr. Nadège Zanou and Mrs. Valentina Butoescu for helpful comments and technical assistance.

References

[1] N.A. Howlader, A.M. Noone, M. Krapcho, et al., SEER Cancer Statistics Review, 1975–2010, 2013 http://seer.cancer.gov/csr/1975_2010/

[2] D.R. Berthold, G.R. Pond, F. Soban, R. de Wit, M. Eisenberger, I.F. Tannock, Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study, J. Clin. Oncol. 26 (2008) 242–245.

[3] D.P. Petrylak, C.M. Tangen, M.H. Hussain, et al., Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer, N. Engl. J. Med. 351 (2004) 1513–1520.

[4] A. van Bokhoven, M. Varella-Garcia, C. Korch, et al., Molecular characterization of human prostate carcinoma cell lines, Prostate 57 (2003) 205–225.

[5] J. Connor, I.S. Sawczuk, M.C. Benson, et al., Calcium channel antagonists delay regression of androgen-dependent tissues and suppress gene activity associated with cell death, Prostate 13 (1988) 119–130.

[6] D.R. Green, G. Kroemer, The pathophysiology of mitochondrial cell death, Science 305 (2004) 626–629.

[7] C. Giorgi, D. De Stefani, A. Bononi, R. Rizzuto, P. Pinton, Structural and functional link between the mitochondrial network and the endoplasmic reticulum, Int. J. Biochem. Cell Biol. 41 (2009) 1817–1827.

[8] J.P. Decuypere, G. Monaco, G. Bultynck, L. Missiaen, H. De Smedt, J.B. Parys, The IP(3) receptor–mitochondria connection in apoptosis and autophagy, Biochim. Biophys. Acta 1813 (2011) 1003–1013.

[9] G. Szabadkai, R. Rizzuto, Participation of endoplasmic reticulum and mitochondrial calcium handling in apoptosis: more than just neighborhood? FEBS Lett. 567 (2004) 111–115.

[10] C.W. Distelhorst, M.D. Bootman, Bcl-2 interaction with the inositol 1,4,5-trisphosphate receptor: role in Ca(2+) signaling and disease, Cell Calcium 50 (2011) 234–241.

[11] P. Pinton, C. Giorgi, R. Siviero, E. Zecchini, R. Rizzuto, Calcium and apoptosis. ER-mitochondria Ca^{2+} transfer in the control of apoptosis, Oncogene 27 (2008) 6407–6418.

[12] S.A. Oakes, L. Scorrano, J.T. Opferman, et al., Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 105–110.

[13] M.J. Chang, F. Zhong, A.R. Lavik, J.B. Parys, M.J. Berridge, C.W. Distelhorst, Feedback regulation mediated by Bcl-2 and DARPP-32 regulates inositol 1,4,5-trisphosphate receptor phosphorylation and promotes cell survival, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 1186–1191.

[14] S. Bandara, S. Malmersjo, T. Meyer, Regulators of calcium homeostasis identified by inference of kinetic model parameters from live single cells perturbed by siRNA, Sci. Signal. 6 (2013) ra56.

[15] S.M. Henshall, D.E. Afar, J. Hiller, et al., Survival analysis of genome-wide gene expression profiles of prostate cancers identifies new prognostic targets of disease relapse, Cancer Res. 63 (2003) 4196–4203.

[16] L. Zhang, G.J. Barritt, Evidence that TRPM8 is an androgen-dependent Ca^{2+} channel required for the survival of prostate cancer cells, Cancer Res. 64 (2004) 8365–8373.

[17] C. Camello, R. Lomax, O.H. Petersen, A.V. Tepikin, Calcium leak from intracellular stores – the enigma of calcium signalling, Cell Calcium 32 (2002) 355–361.

[18] V. Vanderheyden, B. Devogelaere, L. Missiaen, H. De Smedt, G. Bultynck, J.B. Parys, Regulation of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by reversible phosphorylation and dephosphorylation, Biochim. Biophys. Acta 1793 (2009) 959–970.

[19] F. Vanden Abeele, L. Lemonnier, S. Thebault, et al., Two types of store-operated Ca^{2+} channels with different activation modes and molecular origin in LNCaP human prostate cancer epithelial cells, J. Biol. Chem. 279 (2004) 30326–30337.

[20] S. Nakade, S.K. Rhee, H. Hamanaka, K. Mikoshiba, Cyclic AMP-dependent phosphorylation of an immunoaffinity-purified homotetrameric inositol 1,4,5-trisphosphate receptor (type I) increases Ca^{2+} flux in reconstituted lipid vesicles, J. Biol. Chem. 269 (1994) 6735–6742.

[21] T.S. Tang, H. Tu, Z. Wang, I. Bezprozvanny, Modulation of type 1 inositol (1,4,5)-trisphosphate receptor function by protein kinase a and protein phosphatase 1alpha, J. Neurosci. 23 (2003) 403–415.

[22] B. Boutin, N. Tajeddine, P. Vandersmissen, et al., Androgen deprivation and androgen receptor competition by bicalutamide induce autophagy of hormone-resistant prostate cancer cells and confer resistance to apoptosis, Prostate 73 (2013) 1090–1102.

[23] J.P. Decuypere, D. Kindt, T. Luyten, et al., mTOR-controlled autophagy requires intracellular Ca(2+) Signaling, PLOS ONE 8 (2013) e61020.

[24] J.P. Decuypere, K. Welkenhuyzen, T. Luyten, et al., Ins(1,4,5)P3 receptor-mediated Ca^{2+} signaling and autophagy induction are interrelated, Autophagy 7 (2011) 1472–1489.

[25] D.J. Klionsky, F.C. Abdalla, H. Abeliovich, et al., Guidelines for the use and interpretation of assays for monitoring autophagy, Autophagy 8 (2012) 445–544.

[26] A. Bergner, R.M. Huber, Regulation of the endoplasmic reticulum Ca(2+)-store in cancer, Anticancer Agents Med. Chem. 8 (2008) 705–709.

[27] H. Akl, G. Bultynck, Altered Ca(2+) signaling in cancer cells: proto-oncogenes and tumor suppressors targeting IP3 receptors, Biochim. Biophys. Acta 1835 (2013) 180–193.

[28] K. Vanoverberghe, F. Vanden Abeele, P. Mariot, et al., Ca^{2+} homeostasis and apoptotic resistance of neuroendocrine-differentiated prostate cancer cells, Cell Death Differ. 11 (2004) 321–330.

[29] D. Merkle, R. Hoffmann, Roles of cAMP and cAMP-dependent protein kinase in the progression of prostate cancer: cross-talk with the androgen receptor, Cell. Signal. 23 (2011) 507–515.

[30] G. Bagchi, J. Wu, J. French, J. Kim, N.H. Moniri, Y. Daaka, Androgens transduce the G alphas-mediated activation of protein kinase A in prostate cells, Cancer Res. 68 (2008) 3225–3231.

[31] A.K. Kvissel, H. Ramberg, T. Eide, A. Svindland, B.S. Skalhegg, K.A. Tasken, Androgen dependent regulation of protein kinase A subunits in prostate cancer cells, Cell. Signal. 19 (2007) 401–409.

[32] E. Jaimovich, C. Mattei, J.L. Liberona, et al., Xestospongin B, a competitive inhibitor of IP3-mediated Ca^{2+} signalling in cultured rat myotubes, isolated myonuclei, and neuroblastoma (NG108-15) cells, FEBS Lett. 579 (2005) 2051–2057.

[33] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.

Supplementary data

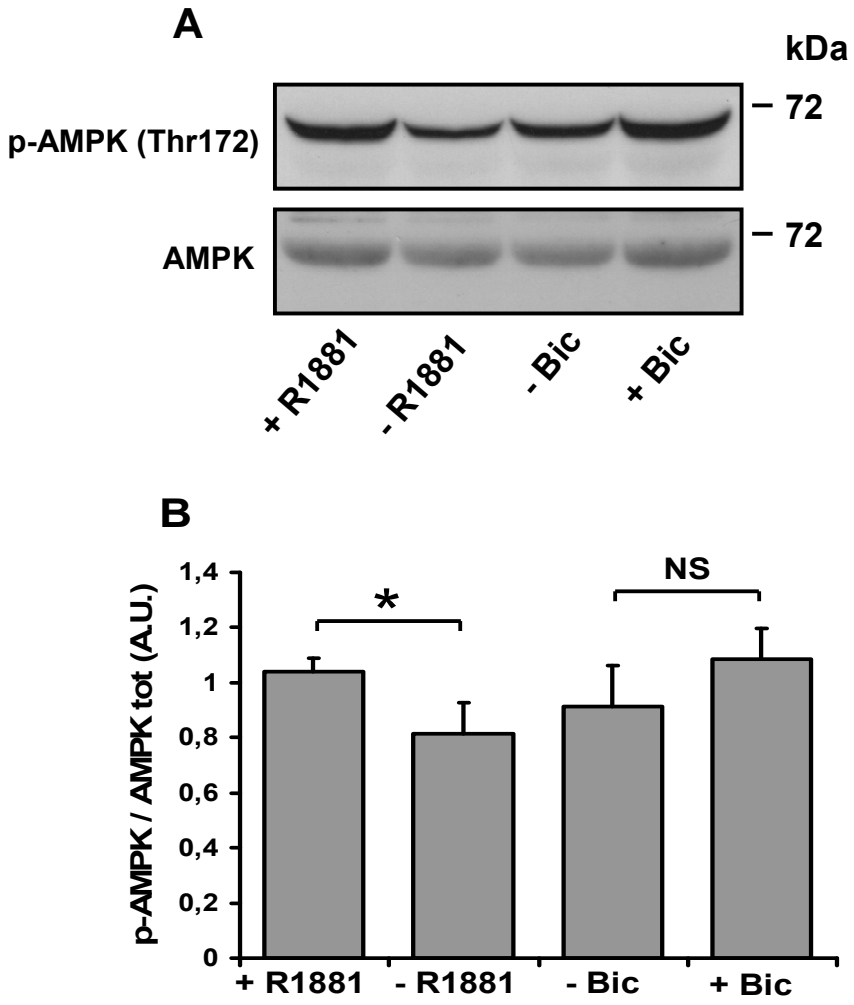

Fig. S1: Effects of AD and bicalutamide treatment on AMPK activity.
(A) Immunoblot analysis of phosphorylation of $AMPK^{Thr172}$ in LNCaP cells either deprived in androgen (- R1881) or treated with 50 µM bicalutamide (+ Bic) for 48 hr. Data presented are representative of three independent experiments. (B) Quantification of data illustrated in A. Data are presented as means ± S.D. of at least three independent experiments (* $p < 0.05$).

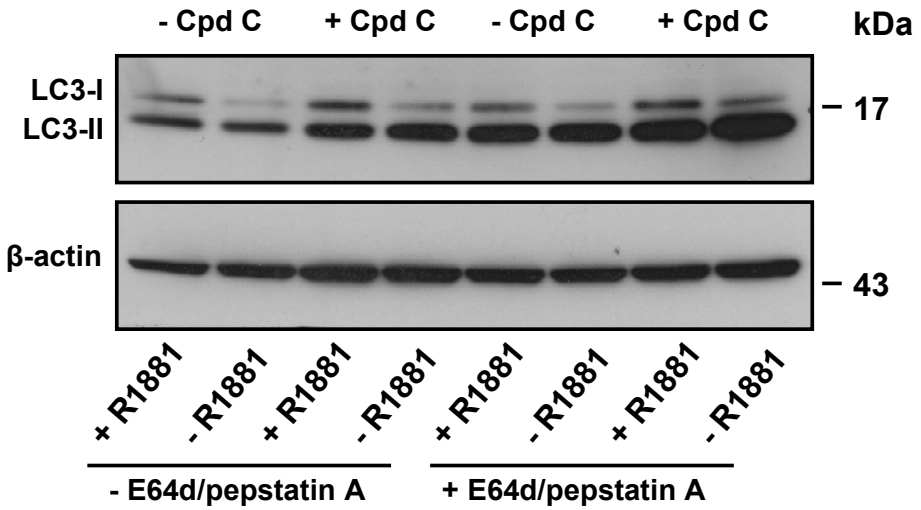

Fig. S2: Effects of AMPK inhibition on AD-induced autophagy.
Immunoblot analysis of LC3-I → LC3-II conversion in LNCaP cells either deprived in androgen (- R1881), or/and treated with the AMPK inhibitor 10 µM compound C (+ Cpd C) for 48 hr, in the absence or in the presence of 10 µg/ml E64d and 10 µg/ml pepstatin A. β-actin immunodetection is used as a loading control. Data presented are representative of two independent experiments.

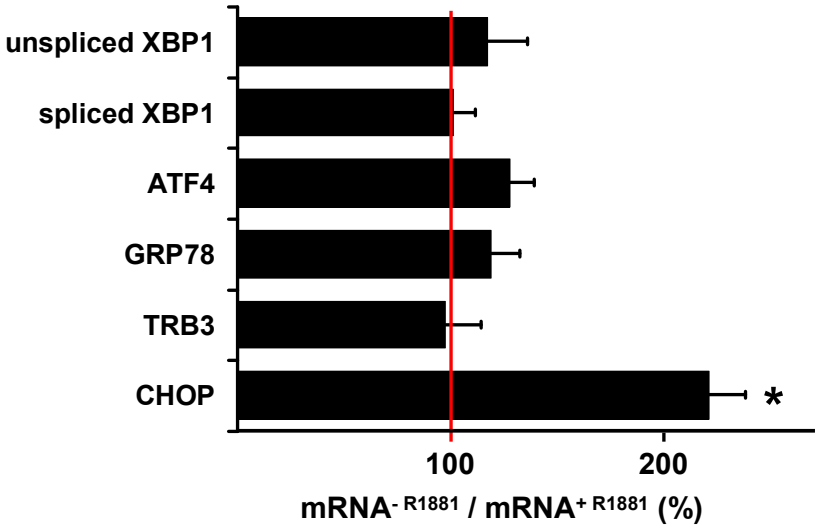

Fig. S3: Effects of AD on the UPR signaling pathway.
Bar histogram showing the relative mRNA expression of genes involved in UPR pathway after AD in LNCaP cells. Two days after AD treatment, total RNA was extracted and reverse-transcribed. A real-time PCR was performed with specific primers. The mRNA expression of each genes was reported to β2-microglobulin expression (means ± S.D. of three independent experiments, * $p < 0.05$).

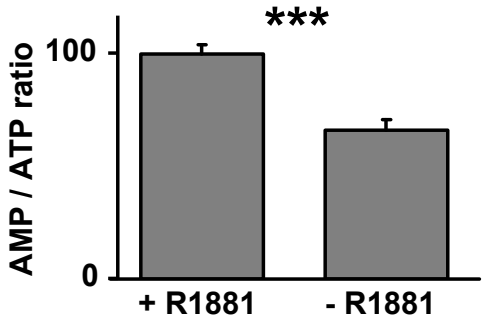

Fig. S4: Effects of AD on the cellular energy homeostasis.
Bar histogram showing the AMP/ATP ratios in LNCaP cells cultured in the presence or in the absence of androgen (- R1881) for 48 hr. These ratio were calculated from ATP and AMP concentrations determined by high-performance liquid chromatography (means ± S.D. of three independent experiments, *** $p < 0.001$).

General conclusions and future perspectives

The results presented above allow us to clarify cell survival mechanisms of PCa subjected to AD. Indeed, we have highlighted the substantial roles of autophagy and Ca^{2+} signaling in apoptosis resistance of HRPCa. Five demonstrations are provided:

a) Androgen deprivation or treatment with the anti-androgen bicalutamide promotes autophagy in the LNCaP model of HRPCa.

By using several techniques, we observed that androgen withdrawal induced increased autophagy in LNCaP cells. The AR is a ligand-dependent transcriptional factor which keeps playing an important function in PCa [9]. 1,5% to 4,3% of the LNCaP transcriptome is directly or indirectly regulated by androgens [314]. Androgens have been observed to promote the expression of genes favoring nutrient availability which activates the energy sensor mTOR and stimulates the growth of LNCaP cells [45]. The downexpression of these genes after AD could explain the activation of autophagy. Besides, as explained in the introduction, AR also has non-genomic activities [9]. For instance, AR bound to its ligand, by interacting with p85, the regulatory subunit of class Ia PI3K, has been shown to directly stimulate the PI3-K/Akt/mTOR pathway in LNCaP cells [16]. We confirmed this interaction and observed an inhibition of PI3-K/Akt/mTOR pathway after AD and bicalutamide treatment. We suggest that the lower AR expression measured after AD for 48 hr could limit the formation of the AR/p85 complex, thereby inhibiting the PI3K activity. However, it would be better to evaluate more accurately the direct effects of AD on the complex using a time-course experiment. Moreover, the nature of the AR involved in this interaction remains unknown. Non-genomic activities of AR have repeatedly been associated to AR tethered to the plasma membrane [12]. It would be interesting to assess the effects of non-internalizable testosterone-BSA conjugates on the p85/AR interaction and the PI3-K/Akt/mTOR pathway. Nevertheless, the modulation of Akt activity by direct binding of AR highlights that other regulation mechanisms of this signaling pathway should not be ignored [18].

It can be noticed that while we suggest a direct control of AD on autophagy through an inhibition of the PI3-K/Akt/mTOR pathway, another team has recently reported that the autophagic process observed after AD could be dependent on AMPK stimulation [315]. Furthermore, AD therapy promotes hypoxia in the tumor microenvironment, likely due to a concomitant rarefaction of tumor vessels [316]. Hypoxia, which has clearly been established

as being able to induce autophagy [317], could participate to the triggering of AD-induced autophagy. Chhipa et al. have shown that AD and hypoxia together stimulate an AMPK-dependent autophagy in LNCaP cells although they did not find that androgen withdrawal alone was able to sufficiently activate AMPK so as to induce autophagy [318]. In our hands, the monitoring of the phosphorylation status of AMPK did not suggest involvement of this pathway. Although bicalutamide treatment for 48 hr seems to result in a small increase of AMPK phosphorylation, it was not the case after AD. In line with other studies [45, 46], AMPK is even stimulated by androgens rather than their withdrawal (Fig. S1). Furthermore, the AMPK inhibitor compound C was not able to suppress the enhancement of LC3-I → LC3-II conversion after AD (Fig. S2).

Interestingly, autophagy induced by AD has also been related to lipolysis. Lipid droplet degradation mediated by autophagy in PCa cells could contribute to their survival by providing energy [319]. It can also be noticed that, despite the fact that the unfolded protein response does not seem involved, the expression of GRP78 has been related to AD-induced autophagy, suggesting a role of androgens in the ER stability [320].

Arguably, culture of LNCaP cells in phenol red-free medium would have been preferable. Indeed, the pH indicator phenol red is a weak estrogen that can impact the growth of estrogen-sensitive cells [321, 322]. It is likely to modulate AR activity, oestradiol receptor β interacting with AR in LNCaP cells [15]. The only use of charcoal stripped fetal calf serum would not be enough to remove any traces of hormone in the medium. Such a serum always contains itself very low concentrations of steroid hormones [56]. The presence of estrogens could reduce the impact of androgen withdrawal, oestradiol stimulating the growth of LNCaP cells [15]. However, such cell culture conditions are already far different from the physiological ones. Estrogens are always present in men undergoing an AD therapy. The use of a specific AR antagonist such as bicalutamide on cells cultured in regular medium thereby seems preferable to reproduce AD therapy *in vitro*.

Although LNCaP cells are by far the most popular model of HRPCa expressing the AR, it may be objected that our results have been obtained in a single PCa cell line. 22Rv1 prostate carcinoma cells also seemed to be a good model for our purpose but they were no longer commercially available due to a viral contamination. Other PCa cell lines such as VCaP and MDA-PCa-2b were tested but they were very difficult to culture and presented very high basal level of apoptosis making them an unsuitable model for our study. However, it can be noticed that AR knockdown has been shown to promote autophagy in PCa cell lines [323]. Furthermore, Beclin-1 and LC3 expressions are increased in PCa and hyperplasia prostate

tissues from patients treated by 5-α reductase inhibitor finasteride which lowers the level of DHT, the form of testosterone with the greatest affinity for the AR [324, 325].

b) AD-induced autophagy seems to constitute a mechanism of resistance against anticancer therapy.

Silencing of the two canonical autophagic products, Atg5 and Beclin-1, significantly increases the level of cell death induced by androgen depletion or bicalutamide. Moreover, concanamycin A and chloroquine, two autophagy inhibitors, reproduce these effects by dramatically increasing cell death after AD or bicalutamide treatment. These results are in line with other studies [319, 326-329]. Besides, the inhibition of autophagy has recently been shown to overcome anti-androgen resistance in a prostate xenograft mouse model, thereby confirming *in vivo* our observations [315]. These data lead us to support the idea that clinical trials using chloroquine or its derivates in combination with anti-androgen therapy should be considered in the management of HRPCa patients. Although some anti-tumor agents have been reported to promote an autophagic cell death in PCa cells [330-332], the interest of autophagy blockade combined with chemo- or radiotherapies to treat patients reached by advanced PCa has already been noticed [333, 334]. A phase II clinical trial has even been conducted to evaluate docetaxel and hydroxychloroquine association in treating patients with metastatic HRPCa. However, the fact that androgen withdrawal results by itself in autophagy leads us to believe that pharmacological inhibition of autophagy should be envisaged earlier, in parallel with AD therapy, in order to delay onset of metastasis and recourse to chemotherapy. The recent discovery of new anticancer properties of chloroquine supports this idea: independently of autophagy blockade, it tightens tumor vessels, impairs cancer cells invasion and metastasis and enhances chemotherapy delivery [335].

It can be noted that AD-induced autophagy in PCa cells parallels autophagy stimulation in breast cancer in response to anti-hormone therapies. As demonstrated in PCa, such autophagy also allows the survival of breast cancer cells and facilitates the progression to anti-estrogen resistance [336].

It does not seem so surprising to observe that the removal of an anabolic hormone promotes a catabolic pathway. However, a recent report indicated that, if low concentrations of androgen repress autophagy in LNCaP cells, higher androgen levels would also induce autophagy as well as result in senescence [66]. Interestingly, autophagy therefore seems associated with this form of cell cycle arrest, AD and likely high concentrations of androgen both promoting

autophagy and senescence. If autophagy protects from cell death triggered after AD, its relationship with the cell cycle remains to explore. Several pathways including mTOR and p53 regulate both cell growth and autophagy. The latter is known to be inhibited during the mitosis [337] whereas it is more active during the G1 and S phases of the cell cycle [338]. The disruption of Atg genes such as Beclin-1 seems to lead to an increased cell growth [120]. Autophagy could allow a rapid protein turnover required for the establishment of cellular senescence [339]. Nevertheless, autophagy impairment has also been reported to promote it [340]. Interestingly, overexpression of $p27^{Kip1}$ could stimulate autophagy [341, 342]. Let us remember that the CDK inhibitor is upregulated after AD [40]. Its possible involvement in AD-induced autophagy thereby remains to be determined, as well as the consequences of an autophagy inhibition on the senescence arrest that AD causes.

c) Overphosphorylation of IP3R1 and altered expression profile of TRP channels modify Ca^{2+} homeostasis after androgen deprivation.

Prevarskaya and her team discovered that neuroendocrine differentiation associated to prolonged AD treatment (96 h) resulted in decreases in the $[Ca^{2+}]_{ER}$ and store-operated Ca^{2+} current in LNCaP cells [343]. They explained the reduction of Ca^{2+} store content by their observation of both SERCA2b pump and luminal Ca^{2+} binding chaperone calreticulin underexpression. After only 48 hr of AD treatment, we measured no change in the SERCA2b and calreticulin expression although we observed that ER Ca^{2+} store content was already reduced. Besides, we noted that SOCE amplitude and Ca^{2+} influx through cell membrane were also decreased after 2 days without androgen.

In seeking to explain these changes, we found that the mRNA expression of IP3R1 was increased. The protein was only weakly overexpressed, but we observed that it was phosphorylated on Ser-1716, a PKA phosphorylation site known to regulate the sensitivity of the channel to IP3 [215, 216]. Actually, Ca^{2+} concentration in the ER is known to be controlled by the SERCA pumps activity and the Ca^{2+}-binding proteins availability. However, steady state calcium level within the ER is also reported to be dependent on a Ca^{2+} leak through ER membrane which balances the influx created by the pumps [344]. The molecular nature of the leak mechanism is still debated and different proteins have been proposed such as Bcl-2 [247], BI-1 [345, 346], CALHM1 (calcium homeostasis modulator 1) [347], pannexin [348], presenillins [349], translocon [350] and SERCA splice variants [351]. Caspase-3 is able to cleave IP3R and the resulting truncated IP3R mediates an important Ca^{2+}

flux through ER membrane [285]. However, native form of IP3R has long been considered uninvolved in the leak [344], despite the fact that basal IP3 levels in non-stimulated conditions have been noted to increase the leakiness of the stores [352]. Ten years ago, Korsmeyer and his collaborators were the first to establish that unaltered IP3R1 could also have a function of leak channel. As explained in the introduction, they observed that the channel became leakier after its phosphorylation on Ser-1756, the PKA phosphorylation site in mouse embryonic fibroblasts [259]. In human cells, the site primarily phosphorylated by PKA is Ser1716. Thus, we assume that phosphorylation on this site after AD leads to a Ca^{2+} leak responsible for the $[Ca^{2+}]_{ER}$ reduction. The fact that inhibition of IP3R1 phosphorylation by a permeant TAT-peptide containing the Ser-1716 consensus phosphorylation sequence prevented the AD-induced decrease in $[Ca^{2+}]_{ER}$ is in line with our hypothesis. Moreover, IP3R inhibition by xestospongin B reduced Ca^{2+} release after thapsigargin-mediated SERCA inhibition, therefore reinforcing the idea that IP3R contributes to the leak of Ca^{2+} through the ER membrane.

Furthermore, a screening of genes involved in the control of Ca^{2+} homeostasis revealed that TRPM8 expression was suppressed after AD for 48 hr. This observation was expected since expression of the TRPM8 gene is controlled by several AREs [200]. The downregulation of TRPM8 could explain the Ca^{2+} influx reduction observed after androgen withdrawal. Indeed, we showed that siRNA-mediated TRPM8 silencing reproduced at least in part the decrease in the Ca^{2+} influx resulting from AD. However, AD-induced TRPM8 depletion is not involved in the reduction of $[Ca^{2+}]_{ER}$ since siRNA against TRPM8 did not modify releasable Ca^{2+} from the ER. Incidentally, our data also showed that TRPM8 is not involved in the SOCE.

d) $[Ca^{2+}]_{ER}$ reduction allows the resistance of HRPCa cells to androgen deprivation.

We observed that inhibition of the $[Ca^{2+}]_{ER}$ reduction by TAT-peptide containing the Ser-1716 consensus phosphorylation sequence of IP3R1 or by PKA inhibitor H89 restored the sensitivity of LNCaP cells to AD. Upon this result, we cannot exclude that dephosphorylation of IP3R1 by itself promotes cell death in response to AD without any involvement of ER Ca^{2+}. Phosphorylation profile of IP3R1 may affect numerous interactions of the protein including that with the anti-apoptotic members of the Bcl-2 protein family. However, SERCA2b overexpression, which increased $[Ca^{2+}]_{ER}$, made LNCaP cells sensitive to AD. This

observation prompted us to consider that it is rather the reconstitution of ER Ca^{2+} store which leads to the induction of cell death after AD treatment.

A role for Ca^{2+} in the prostate involution in rats after castration has already been highlighted, Ca^{2+} channel antagonists delaying regression of the androgen-dependent tissues [184]. Furthermore, testosterone and its analogues do not only exert their effects through nuclear AR regulating transcription of various genes but also via membrane AR activating different signaling pathways. Stimulation of such AR has been reported to induce intracellular Ca^{2+} increases in diverse cellular models [353]. Prevarskaya and her collaborators also showed that Ca^{2+} pool content could regulate apoptosis resistance and cell growth in PCa cells [185, 343, 354, 355]. Fluctuation of $[Ca^{2+}]_{ER}$ in a narrow range (≈30%) could cause massive changes in cell survival [356]. In this study, we suggest that AD, which triggers apoptosis in normal prostate cells, induces by itself a mechanism of resistance in HRPCa cells that suppresses AD-induced cell death. In other words, in HRPCa cells, AD triggers both apoptotic pathways and resistance mechanisms against apoptosis. Reduction of $[Ca^{2+}]_{ER}$ induced by AD allows the survival of PCa cells in response to AD. Moreover, we explained the main involved mechanism: the leak channel properties of IP3R1 and their regulation through phosphorylation of a PKA consensus site (Fig. 19).

Thereby, inositol trisphosphate receptor could constitute another interesting therapeutic target in PCa treatment. As explained in the introduction, the channel seems to be involved in the mechanisms of cancerogenesis [210]. Furthermore, IP3R is involved in apoptosis regulation. Some pro-apoptotic stimuli such as Fas lead to PLC activation and IP3 synthesis [357, 358], although this has not been demonstrated for every pro-apoptotic stimuli causing Ca^{2+} release from the ER [356]. The importance of IP3R in cell death is also supported by the fact that antisense knockdown or genetic deletion of IP3R gene modify apoptosis sensitivity in numerous models [231]. However, the various IP3R isoforms could play distinct roles. IP3R3 has been reported to be more significantly localized in the MAM where it would preferentially transmit apoptotic Ca^{2+} signals into mitochondria, whereas IP3R1 would preferentially allows Ca^{2+} release into cytosol [359]. This could explain why apoptosis induction is most effectively reduced by silencing IP3R3 than other isoforms [359-361]. Nevertheless, although the repression of IP3R1 expression can sensitize cells to apoptosis in some models [362], it has also been shown to inhibit cell death in others [284, 357, 363].

In addition to its role in the apoptosis triggering via the ER-mitochondria Ca^{2+} flux, IP3R can amplify apoptotic pathways by different ways [356]. Its cleavage by caspases (and calpains) which accelerates ER Ca^{2+} emptying through a constitutively open IP3R fragment could act as

a feed-forward mechanism [285, 364]. Similarly, its potential binding with released cytochrome c promotes IP3R opening, also facilitating Ca^{2+} release from the stores [365].
Phosphorylation of different sites on the IP3R protein also controls the induction of cell death. Indeed, Akt phosphorylates IP3Rs, in particular in LNCaP cells, thereby reducing cellular sensitivity to pro-apoptotic stimuli but without modifying the permeability of the channel, this last point being debated [224, 366]. As mentioned before, IP3R can play a role of scaffold protein and the multiplicity of its interactions, in particular with Bcl-2 family members, could explain its Ca^{2+}-independent contribution in the regulation of cell death. It would be interesting to determine the effects of the inhibition of PI3K/Akt/mTOR pathway that we observed after AD on the IP3 receptors and the resistance to apoptosis of LNCaP cells. However, we can already assert that they probably would not be mediated by IP3R1, Akt primarily acting on IP3R3 [210].
Cross-talk between PKA and IP3R has already been explored. Korsmeyer's team previously showed the importance of IP3R1 phosphorylation on its PKA consensus site in the control of the ER Ca^{2+} leak and apoptosis [259]. They observed that phosphorylation was dependent on the presence of Bcl-2 at the ER membrane. Although Bcl-2 protein was hardly detected in LNCaP cells, we measured a small increase in the Bcl-2 mRNA level after AD (only Bcl-2 mRNA itself increased while the expression of other members of Bcl-2 family remained stable). It will be interesting to study its possible control on the phosphorylation and the activity of IP3R1. Indeed, in addition of the regulation of IP3R phosphorylation and its properties of leak channel, Bcl-2, as explained in the introduction, also regulates IP3-induced Ca^{2+} release through direct interaction with IP3R [217, 254-256]. Moreover, Bcl-2 has been noticed to compete with IP3R1 for the binding of protein phosphatase 1 (PP1), which preserves IP3R1 phosphorylation by PKA and protect cells from apoptosis [367]. Nonetheless, a negative feedback has recently been highlighted, allowing Bcl-2, through its interactions with DARPP-32 (a PP1 inhibitor) and calcineurin, to accurately control IP3R1 phosphorylation on PKA consensus site and limit Ca^{2+} release [368]. It will also be interesting to monitor the RyRs permeability in our model, PKA being reported to phosphorylate these channels too, which results in an important Ca^{2+} leak from the stores [369]. Furthermore, it is also conceivable that PKA regulates other components of Ca^{2+} machinery. Thus, interestingly, TRPM8 channel opening has been noticed to be inhibited by the PKA pathway [370].
In this work, we did not investigate the mechanism of PKA-activation by AD. However, previous studies give us some insights about this phenomenon. As mentioned above, PKA and its activator cAMP play an important role in the mechanisms of carcinogenesis. PKA-1

isoform is often overexpressed in cancer cells including PCa cells and constitutes a marker of poor prognosis [371]. This pathway is known to regulate tumor cells proliferation. Otherwise, it controls the activity of androgen receptor [372]. It can also be noted that treatment of LNCaP cells with testosterone for 10 min increases cAMP level and PKA activity [373]. This was mediated via a $G_s\alpha$-dependent pathway that was independent of nuclear AR. Interestingly, activation of PKA pathway in PCa cells (via cAMP analogs, adenylate cyclase stimulators or phosphodiesterase inhibitors) results in their NE differentiation [372]. In addition of PKA activation, other stimuli lead to NE differentiation including long-term AD and treatment with interleukin-1β and -6 or VIP (vasoactive intestinal peptide). For the latter, the induced NE differentiation is partially dependent on PKA [374]. Besides, NE differentiation associated to prolonged AD in LNCaP cells increases the expression of different catalytic subunits of PKA [375].

We still have to understand how the decrease in $[Ca^{2+}]_{ER}$ favors cell survival. As explained above in the introduction, this is probably dependent on the Ca^{2+} transfer into the mitochondria. The increased leak of Ca^{2+} from the ER after AD could enhance spontaneous IP3R-dependent oscillations which promote cell survival and limit any possible massive arrival of Ca^{2+} to the mitochondria, thereby protecting cells from apoptosis. A measure of $[Ca^{2+}]_m$ after androgen removal but also following a pro-apoptotic stimulus in the presence and the absence of androgen (with a cameleon probe targeted to mitochondria) would allow us to ensure the involvement of mitochondrial Ca^{2+} in the survival of LNCaP cells.

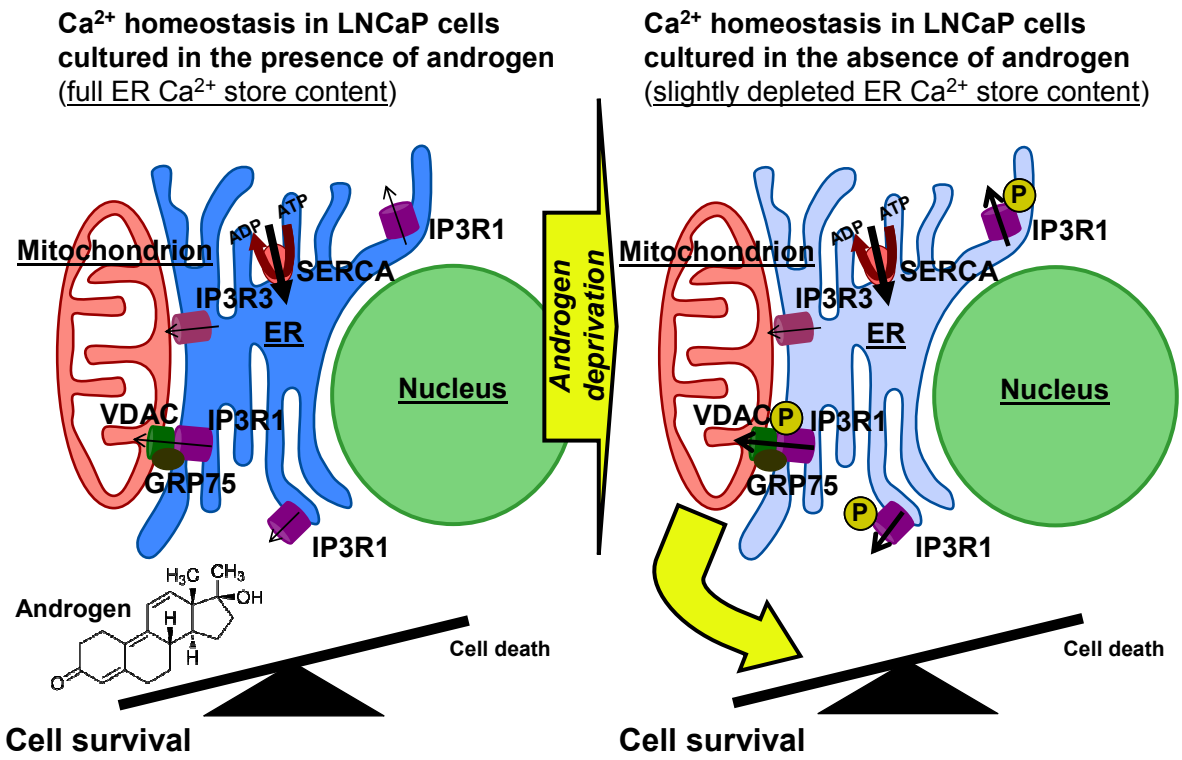

Fig. 19: The ER calcium store content and its effects on cell survival.
AD treatment modifies the permeability of ER membrane through the PKA-mediated phosphorylation of IP3R1 in LNCaP cells. The generated Ca^{2+} leakage and the resulting $[Ca^{2+}]_{ER}$ decrease would likely improve pro-survival moderated Ca^{2+} pulses but also avoid any deleterious Ca^{2+} overload into the mitochondria that could cause androgen withdrawal. (Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; ER, endoplasmic reticulum; GRP75, 75 kDa glucose regulation protein; IP3R1, inositol trisphosphate receptor isoform 1; IP3R3, inositol trisphosphate receptor isoform 3; MCU, mitochondrial Ca^{2+} uniporter; P, phosphorylated residues; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; VDAC, voltage-dependent anion channel.)

The decrease in the store-operated Ca^{2+} current after AD requires further clarification. Several store-operated currents have been described and can restore the Ca^{2+} content of the ER after its depletion. The best characterized is the Ca^{2+} release-activated Ca^{2+} current, I(CRAC) where ORAI1, the pore-forming subunit of the CRAC channel, is activated by STIM1 (stromal interaction molecule 1) which is able to sense ER Ca^{2+} store emptying [376]. We can imagine that the SOCE decrease observed after AD is subsequent to the $[Ca^{2+}]_{ER}$ reduction. Indeed, Ca^{2+} store being reduced after AD, a SOCE of large amplitude could not be necessary to fill them after their eventual emptying. Nevertheless, it must remembered that the overexpression of calreticulin allowing a Ca^{2+} storage increase has also been demonstrated to decrease SOCE amplitude [377]. Prevarskaya and her collaborators have already observed this decrease in the SOCE after AD. They have explained it by a downregulation of ORAI1 and a cytoskeleton reorganization limiting the integration of store-operated channels in the

plasma membrane [378, 379]. However, in our study, we did not note any variation in mRNA expression of ORAI1 and STIM1 after AD treatment for 48 hr.

Various studies have shown a crucial role of SOCE in cell death. Only a Ca^{2+} store release paired with sustained store-operated Ca^{2+} entry seems to allow a massive triggering of apoptosis. Indeed, it has been shown that cell death induced by Ca^{2+} store depletion is improved by high concentrations of extracellular Ca^{2+} [250]. A SOCE decrease could reduce any detrimental Ca^{2+} overload into the mitochondria. Furthermore, Prevarskaya's team showed that the downregulation of ORAI1, and consequently SOCE, participates to the apoptosis resistance acquisition of LNCaP cells [378]. Besides, they have recently highlighted an overexpression of ORAI3 isoform in human PCa which leads to an ORAI1 protein redistribution, a subsequent SOCE limitation and the cell survival promotion [380].

e) Autophagy and $[Ca^{2+}]_{ER}$ reduction are independently induced in response to androgen deprivation.

As explained before, Ca^{2+} signaling is known to participate in the regulation of autophagy. Although, in contrast with the effects of AD on PI3-K/Akt/mTOR pathway and $[Ca^{2+}]_{ER}$, rapamycin, the selective mTOR inhibitor and autophagy inducer, has been related to an increased $[Ca^{2+}]_{ER}$ consecutive to a reduced Ca^{2+} leak from the ER [307], we then hypothesized that the decrease of $[Ca^{2+}]_{ER}$ observed after AD treatment could lead to the induction of autophagy.

Firstly, the unfolded protein response (UPR) could connect the two processes. Indeed, ER Ca^{2+} emptying can stimulate UPR while this one can lead to ER /Ca^{2+} store expansion but also cause autophagy [232, 381]. But a report from Bennett et al. [320] and a preliminary screening of UPR genes did not suggest a stimulation of the UPR pathway after AD (Fig. S3). Apart for a two-fold overexpression of the pro-apoptotic transcription factor CHOP, no variation in the splicing of XBP1 mRNA or in the expression of various genes related to ER stress was detected. However, a complete monitoring of the UPR pathway remains to be performed.

The determination of ATP and AMP concentrations in LNCaP cells by high-performance liquid chromatography associated to the monitoring of the phosphorylation status of AMPK revealed no decrease in the cellular metabolism after AD. AMP/ATP ratio is even lowered after two days of treatment (Fig. S4), while AMPK is dephosphorylated (Fig. S1). This suggests that the $[Ca^{2+}]_{ER}$ decrease does not result in the suppression of the ER-mitochondria

Ca^{2+} exchange which has been proved to inhibit ATP synthesis and promote autophagy [293]. Besides, we observed that inhibition of IP3R1 phosphorylation which restores the ER Ca^{2+} store content could not inhibit AD-induced autophagy, demonstrating that the alterations of Ca^{2+} homeostasis are not responsible for the autophagy triggering in our model. However, a long-term control of autophagy by the Ca^{2+} leak can not be excluded. Since IP3R1 is known to regulate autophagy by allowing Bcl-2 interaction with Beclin-1, it would be interesting to evaluate the effects of the PKA-mediated phosphorylation of IP3R1 and its subsequent opening on its function of scaffold protein [294].

Several reports show that autophagosomes are partially formed from ER membranes [80] and a remodeling of Ca^{2+} signaling has already been observed during a starvation-induced autophagy [227]. We therefore sought to verify that autophagy did not participate to the ER Ca^{2+} store reduction. We found that autophagy inhibition by siRNA-mediated depletion of Atg5 did not modify the AD-induced decrease of releasable Ca^{2+} pool, suggesting that Ca^{2+} store alterations are not subsequent to autophagic process.

Therefore, the activation of autophagy and the decrease of $[Ca^{2+}]_{ER}$ appears as two independent mechanisms allowing the survival of PCa cells in response to AD (Fig. 20).

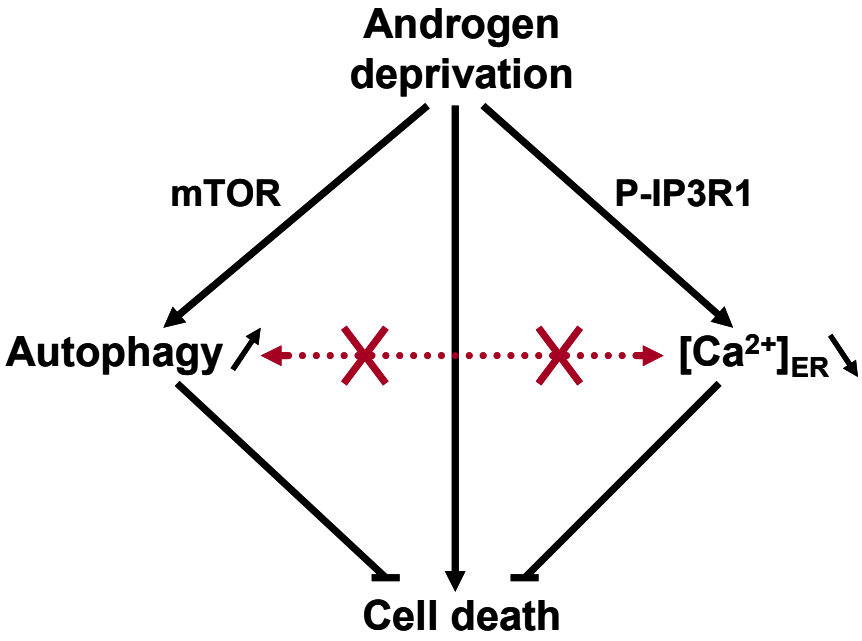

Fig. 20: Two mechanisms of adaptation for HRPCa-derived LNCaP cells in response to androgen deprivation. AD treatment leads to autophagy enhancement and a decrease of the ER Ca^{2+} store content. These two mechanisms that no link seems to connect allow the LNCaP cells to survive in the absence of androgenic stimulation.

Summary

Prostate cancer (PCa) is the most frequent cancer in men above the age of 50 years. Despite radical prostatectomy or radiation therapy as primary treatment, 15-30% of patients will develop an advanced or metastatic cancer requiring systemic therapies. Reference treatment of advanced PCa relies on pharmacological or surgical androgen deprivation therapy. However, despite initial efficacy of androgen deprivation (AD), the tumor inevitably adapts to low testosterone environment and becomes hormone-refractory (HRPCa). A better understanding of the mechanisms of androgen independence is necessary to improve the management of HRPCa.

Although autophagy confers chemoresistance in some cancers, its role in the development of HRPCa remained unknown. We found that AD or treatment with the anti-androgen bicalutamide promoted autophagy in HRPCa-derived LNCaP cells. This effect was associated with an inhibition of the PI3-K/Akt/mTOR pathway and with a disruption of the complex formed by androgen receptor and the regulatory subunit of PI3-K p85. Moreover, genetic or pharmacological inhibition of autophagy restored AD-dependent cell death indicating that autophagy is a protective mechanism against AD in HRPCa cells.

Besides, in these same cells, we also observed different modifications of Ca^{2+} homeostasis after androgen withdrawal among which a reduction of the ER Ca^{2+} content. We discovered that the $[Ca^{2+}]_{ER}$ decrease was due to the presence of IP3R1 made leakier by their phosphorylation on the PKA consensus site Ser-1716. Inhibition of the IP3R1 phosphorylation by PKA inhibitor H89 or permeant TAT-peptide containing the Ser-1716 consensus phosphorylation sequence sensitized LNCaP cells to AD, suggesting that $[Ca^{2+}]_{ER}$ can control HRPCa cells resistance to AD.

Therefore, we identified two distinct mechanisms by which HRPCa-derived cells manage to overcome cell death that they face in the absence of androgenic stimulation. We hope that these findings will help to device new therapeutic strategies to circumvent hormone independence of advanced PCa.

Résumé

Le cancer de la prostate (CaP) est le cancer le plus fréquent chez l'homme de plus de 50 ans. Malgré l'efficacité de la prostatectomie radicale et de la radiothérapie comme traitement initial à un stade précoce, 15 à 30% des patients développeront un cancer métastatique nécessitant un traitement systémique. Le traitement de référence des CaP à un stade avancé est l'hormonothérapie, consistant à inhiber l'effet stimulant des androgènes sur les cellules cancéreuses. Cependant, malgré son efficacité initiale, le CaP finit inévitablement par s'adapter à l'absence de stimulation androgénique et devient hormono-résistant (HRCaP). Une meilleure compréhension des mécanismes d'échappement à l'hormonothérapie est nécessaire dans le but d'améliorer la prise en charge des patients malades.

Bien que l'autophagie soit impliquée dans la chimiorésistance de certains cancers, son rôle dans le développement des HRCaP n'avait pas été exploré. Nous avons observé que le retrait des androgènes ainsi que l'utilisation de l'anti-androgène bicalutamide activaient l'autophagie dans la lignée cellulaire LNCaP dérivée d'un HRCaP. Cet effet était associé à l'inhibition de la voie de signalisation PI3-K/Akt/mTOR et à la diminution de l'interaction entre le récepteur aux androgènes et la sous-unité régulatrice p85 de la PI3-kinase. Par ailleurs, l'inhibition génétique ou pharmacologique de l'autophagie rétablissait la mort cellulaire induite par l'absence de stimulation androgénique, indiquant que l'autophagie est un mécanisme de résistance à l'hormonothérapie pour ces cellules prostatiques.

En outre, dans ce même modèle cellulaire, nous avons observé différentes modifications de l'homéostasie calcique suite au retrait des androgènes, parmi lesquelles une réduction du contenu en calcium du réticulum endoplasmique (RE). Nous avons découvert que cette diminution de la $[Ca^{2+}]_{RE}$ était due à une augmentation de la perméabilité du récepteur à l'IP3 de type 1 du fait de sa phosphorylation sur le résidu sérine 1716 par la PKA. L'inhibition de cette phosphorylation par le H89, un inhibiteur de la PKA, ou par un peptide TAT contenant le même site de phosphorylation entraînait la mort des cellules LNCaP en l'absence d'androgènes, suggérant que la réduction de la $[Ca^{2+}]_{RE}$ participe à l'hormono-résistance des cellules cancéreuses prostatiques.

Ainsi, nous avons identifié deux mécanismes distincts par lesquels les cellules d'un HRCaP parviennent à survivre dans un milieu hormonal qui leur est défavorable. Nous espérons que ces découvertes aideront à la mise au point de nouvelles stratégies thérapeutiques pour répondre à l'hormono-résistance des CaP à un stade avancé.

Bibliography

1. Zerbib, M. and S. Conquy, *La prostate.* Editors Doin, 2001.
2. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, 2015. **136**(5): p. E359-86.
3. Han, M., et al., *Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer.* J Urol, 2003. **169**(2): p. 517-23.
4. Huggins, C. and P.J. Clark, *Quantitative Studies of Prostatic Secretion : Ii. The Effect of Castration and of Estrogen Injection on the Normal and on the Hyperplastic Prostate Glands of Dogs.* J Exp Med, 1940. **72**(6): p. 747-62.
5. Edwards, J. and J.M. Bartlett, *The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 1: Modifications to the androgen receptor.* BJU Int, 2005. **95**(9): p. 1320-6.
6. *The prostate gland.* http://ourhealth.actwritepoetry.com/?paged=2.
7. Venyo, A. and K. Baiden-Amissah, *Gleason 4+4 = 8 Advanced Adenocarcinoma Of Prostate In A 44 Years-old Patient: A Case Report And A Review Of The Literature.* Webmedcentral, Case Report, http://www.webmedcentral.com/article_view/1391, 2010.
8. Wilson, J.D., *Role of dihydrotestosterone in androgen action.* Prostate Suppl, 1996. **6**: p. 88-92.
9. Gao, W., C.E. Bohl, and J.T. Dalton, *Chemistry and structural biology of androgen receptor.* Chem Rev, 2005. **105**(9): p. 3352-70.
10. Kaarbo, M., T.I. Klokk, and F. Saatcioglu, *Androgen signaling and its interactions with other signaling pathways in prostate cancer.* Bioessays, 2007. **29**(12): p. 1227-38.
11. Pascal, L.E. and Z. Wang, *Unzipping androgen action through ZIP9: a novel membrane androgen receptor.* Endocrinology, 2014. **155**(11): p. 4120-3.
12. Papadopoulou, N., et al., *Membrane androgen receptor activation in prostate and breast tumor cells: molecular signaling and clinical impact.* IUBMB Life, 2009. **61**(1): p. 56-61.
13. Yang, X., et al., *Novel membrane-associated androgen receptor splice variant potentiates proliferative and survival responses in prostate cancer cells.* J Biol Chem, 2011. **286**(41): p. 36152-60.
14. Peterziel, H., et al., *Rapid signalling by androgen receptor in prostate cancer cells.* Oncogene, 1999. **18**(46): p. 6322-9.
15. Migliaccio, A., et al., *Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation.* EMBO J, 2000. **19**(20): p. 5406-17.
16. Sun, M., et al., *Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src.* J Biol Chem, 2003. **278**(44): p. 42992-3000.
17. Baron, S., et al., *Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells.* J Biol Chem, 2004. **279**(15): p. 14579-86.
18. Cinar, B., et al., *Phosphoinositide 3-kinase-independent non-genomic signals transit from the androgen receptor to Akt1 in membrane raft microdomains.* J Biol Chem, 2007. **282**(40): p. 29584-93.

19. Sun, Y.H., et al., *Androgens induce increases in intracellular calcium via a G protein-coupled receptor in LNCaP prostate cancer cells.* J Androl, 2006. **27**(5): p. 671-8.
20. Hatzoglou, A., et al., *Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo.* J Clin Endocrinol Metab, 2005. **90**(2): p. 893-903.
21. Papadopoulou, N., et al., *Rho/ROCK/actin signaling regulates membrane androgen receptor induced apoptosis in prostate cancer cells.* Exp Cell Res, 2008. **314**(17): p. 3162-74.
22. Thomas, P., et al., *Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis.* Endocrinology, 2014. **155**(11): p. 4250-65.
23. Berges, R. and B. Tombal, *Androgens and prostate cancer.* Ismar Healthcare, 2009.
24. Petrylak, D., *Therapeutic options in androgen-independent prostate cancer: building on docetaxel.* BJU Int, 2005. **96 Suppl 2**: p. 41-6.
25. Chi, K.N., et al., *Castration-resistant prostate cancer: from new pathophysiology to new treatment targets.* Eur Urol, 2009. **56**(4): p. 594-605.
26. Karantanos, T., P.G. Corn, and T.C. Thompson, *Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches.* Oncogene, 2013. **32**(49): p. 5501-11.
27. Devlin, H.L. and M. Mudryj, *Progression of prostate cancer: multiple pathways to androgen independence.* Cancer Lett, 2009. **274**(2): p. 177-86.
28. Kraus, S., et al., *Receptor for activated C kinase 1 (RACK1) and Src regulate the tyrosine phosphorylation and function of the androgen receptor.* Cancer Res, 2006. **66**(22): p. 11047-54.
29. Dillard, P.R., M.F. Lin, and S.A. Khan, *Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol.* Mol Cell Endocrinol, 2008. **295**(1-2): p. 115-20.
30. Miyake, H., et al., *Overexpression of insulin-like growth factor binding protein-5 helps accelerate progression to androgen-independence in the human prostate LNCaP tumor model through activation of phosphatidylinositol 3'-kinase pathway.* Endocrinology, 2000. **141**(6): p. 2257-65.
31. Rocchi, P., et al., *Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer.* Cancer Res, 2004. **64**(18): p. 6595-602.
32. Deocampo, N.D., H. Huang, and D.J. Tindall, *The role of PTEN in the progression and survival of prostate cancer.* Minerva Endocrinol, 2003. **28**(2): p. 145-53.
33. Lin, Y., et al., *Up-regulation of Bcl-2 is required for the progression of prostate cancer cells from an androgen-dependent to an androgen-independent growth stage.* Cell Res, 2007. **17**(6): p. 531-6.
34. Castilla, C., et al., *Bcl-xL is overexpressed in hormone-resistant prostate cancer and promotes survival of LNCaP cells via interaction with proapoptotic Bak.* Endocrinology, 2006. **147**(10): p. 4960-7.
35. Nesslinger, N.J., X.B. Shi, and R.W. deVere White, *Androgen-independent growth of LNCaP prostate cancer cells is mediated by gain-of-function mutant p53.* Cancer Res, 2003. **63**(9): p. 2228-33.
36. Grossmann, M.E., H. Huang, and D.J. Tindall, *Androgen receptor signaling in androgen-refractory prostate cancer.* J Natl Cancer Inst, 2001. **93**(22): p. 1687-97.

37. Harris, W.P., et al., *Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion.* Nat Clin Pract Urol, 2009. **6**(2): p. 76-85.
38. Westin, P., et al., *Castration therapy rapidly induces apoptosis in a minority and decreases cell proliferation in a majority of human prostatic tumors.* Am J Pathol, 1995. **146**(6): p. 1368-75.
39. Lee, Y.M. and P. Sicinski, *Targeting cyclins and cyclin-dependent kinases in cancer: lessons from mice, hopes for therapeutic applications in human.* Cell Cycle, 2006. **5**(18): p. 2110-4.
40. Knudsen, K.E., K.C. Arden, and W.K. Cavenee, *Multiple G1 regulatory elements control the androgen-dependent proliferation of prostatic carcinoma cells.* J Biol Chem, 1998. **273**(32): p. 20213-22.
41. Balk, S.P. and K.E. Knudsen, *AR, the cell cycle, and prostate cancer.* Nucl Recept Signal, 2008. **6**: p. e001.
42. Lu, S., et al., *Androgen regulation of the cyclin-dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter.* Mol Endocrinol, 1999. **13**(3): p. 376-84.
43. LaBaer, J., et al., *New functional activities for the p21 family of CDK inhibitors.* Genes Dev, 1997. **11**(7): p. 847-62.
44. Sherr, C.J. and J.M. Roberts, *CDK inhibitors: positive and negative regulators of G1-phase progression.* Genes Dev, 1999. **13**(12): p. 1501-12.
45. Xu, Y., et al., *Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins.* Cancer Res, 2006. **66**(15): p. 7783-92.
46. Tennakoon, J.B., et al., *Androgens regulate prostate cancer cell growth via an AMPK-PGC-1alpha-mediated metabolic switch.* Oncogene, 2014. **33**(45): p. 5251-61.
47. Giacinti, C. and A. Giordano, *RB and cell cycle progression.* Oncogene, 2006. **25**(38): p. 5220-7.
48. Tricoli, J.V., et al., *Alterations of the retinoblastoma gene in human prostate adenocarcinoma.* Genes Chromosomes Cancer, 1996. **15**(2): p. 108-14.
49. Sharma, A., et al., *Retinoblastoma tumor suppressor status is a critical determinant of therapeutic response in prostate cancer cells.* Cancer Res, 2007. **67**(13): p. 6192-203.
50. Tso, C.L., et al., *Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells.* Cancer J, 2000. **6**(4): p. 220-33.
51. Chuu, C.P., et al., *Androgen suppresses proliferation of castration-resistant LNCaP 104-R2 prostate cancer cells through androgen receptor, Skp2, and c-Myc.* Cancer Sci, 2011. **102**(11): p. 2022-8.
52. Kokontis, J.M., et al., *Androgen suppresses the proliferation of androgen receptor-positive castration-resistant prostate cancer cells via inhibition of Cdk2, CyclinA, and Skp2.* PLoS One, 2014. **9**(10): p. e109170.
53. Sato, N., et al., *Intermittent androgen suppression delays progression to androgen-independent regulation of prostate-specific antigen gene in the LNCaP prostate tumour model.* J Steroid Biochem Mol Biol, 1996. **58**(2): p. 139-46.
54. Kratiras, Z., C. Konstantinidis, and K. Skriapas, *A review of continuous vs intermittent androgen deprivation therapy: redefining the gold standard in the treatment of advanced prostate cancer. Myths, facts and new data on a "perpetual dispute".* Int Braz J Urol, 2014. **40**(1): p. 3-15; discussion 15.

55. de Launoit, Y., et al., *Characteristics of the biphasic action of androgens and of the potent antiproliferative effects of the new pure antiestrogen EM-139 on cell cycle kinetic parameters in LNCaP human prostatic cancer cells.* Cancer Res, 1991. **51**(19): p. 5165-70.
56. Song, W. and M. Khera, *Physiological normal levels of androgen inhibit proliferation of prostate cancer cells in vitro.* Asian J Androl, 2014. **16**(6): p. 864-8.
57. Khera, M., et al., *A new era of testosterone and prostate cancer: from physiology to clinical implications.* Eur Urol, 2014. **65**(1): p. 115-23.
58. Szmulewitz, R., et al., *A randomized phase 1 study of testosterone replacement for patients with low-risk castration-resistant prostate cancer.* Eur Urol, 2009. **56**(1): p. 97-103.
59. Morgentaler, A., et al., *Testosterone therapy in men with untreated prostate cancer.* J Urol, 2011. **185**(4): p. 1256-60.
60. Pastuszak, A.W., et al., *Testosterone replacement therapy in patients with prostate cancer after radical prostatectomy.* J Urol, 2013. **190**(2): p. 639-44.
61. Ewald, J.A., et al., *Androgen deprivation induces senescence characteristics in prostate cancer cells in vitro and in vivo.* Prostate, 2013. **73**(4): p. 337-45.
62. Pernicova, Z., et al., *Androgen depletion induces senescence in prostate cancer cells through down-regulation of Skp2.* Neoplasia, 2011. **13**(6): p. 526-36.
63. Barakat, D.J., et al., *CCAAT/Enhancer binding protein beta controls androgen-deprivation-induced senescence in prostate cancer cells.* Oncogene, 2015.
64. Campisi, J., *Aging, cellular senescence, and cancer.* Annu Rev Physiol, 2013. **75**: p. 685-705.
65. Burton, D.G., et al., *Androgen deprivation-induced senescence promotes outgrowth of androgen-refractory prostate cancer cells.* PLoS One, 2013. **8**(6): p. e68003.
66. Roediger, J., et al., *Supraphysiological androgen levels induce cellular senescence in human prostate cancer cells through the Src-Akt pathway.* Mol Cancer, 2014. **13**: p. 214.
67. Eisenberg-Lerner, A., et al., *Life and death partners: apoptosis, autophagy and the cross-talk between them.* Cell Death Differ, 2009. **16**(7): p. 966-75.
68. Deter, R.L. and C. De Duve, *Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes.* J Cell Biol, 1967. **33**(2): p. 437-49.
69. Cuervo, A.M., *Autophagy: many paths to the same end.* Mol Cell Biochem, 2004. **263**(1-2): p. 55-72.
70. Kaushik, S. and A.M. Cuervo, *Chaperone-mediated autophagy.* Methods Mol Biol, 2008. **445**: p. 227-44.
71. Martinez-Vicente, M. and A.M. Cuervo, *Autophagy and neurodegeneration: when the cleaning crew goes on strike.* Lancet Neurol, 2007. **6**(4): p. 352-61.
72. Feng, Y., et al., *The machinery of macroautophagy.* Cell Res, 2014. **24**(1): p. 24-41.
73. Russell, R.C., H.X. Yuan, and K.L. Guan, *Autophagy regulation by nutrient signaling.* Cell Res, 2014. **24**(1): p. 42-57.
74. Glick, D., S. Barth, and K.F. Macleod, *Autophagy: cellular and molecular mechanisms.* J Pathol, 2010. **221**(1): p. 3-12.
75. Diaz-Troya, S., et al., *The role of TOR in autophagy regulation from yeast to plants and mammals.* Autophagy, 2008. **4**(7): p. 851-65.
76. Sancak, Y., et al., *Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids.* Cell, 2010. **141**(2): p. 290-303.
77. Dunlop, E.A. and A.R. Tee, *mTOR and autophagy: a dynamic relationship governed by nutrients and energy.* Semin Cell Dev Biol, 2014. **36**: p. 121-9.

78. Nakatogawa, H., et al., *Dynamics and diversity in autophagy mechanisms: lessons from yeast.* Nat Rev Mol Cell Biol, 2009. **10**(7): p. 458-67.
79. Hamasaki, M., et al., *Autophagosomes form at ER-mitochondria contact sites.* Nature, 2013. **495**(7441): p. 389-93.
80. Axe, E.L., et al., *Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum.* J Cell Biol, 2008. **182**(4): p. 685-701.
81. Hayashi-Nishino, M., et al., *A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation.* Nat Cell Biol, 2009. **11**(12): p. 1433-7.
82. Yen, W.L., et al., *The conserved oligomeric Golgi complex is involved in double-membrane vesicle formation during autophagy.* J Cell Biol, 2010. **188**(1): p. 101-14.
83. Hailey, D.W., et al., *Mitochondria supply membranes for autophagosome biogenesis during starvation.* Cell, 2010. **141**(4): p. 656-67.
84. Ravikumar, B., et al., *Plasma membrane contributes to the formation of pre-autophagosomal structures.* Nat Cell Biol, 2010. **12**(8): p. 747-57.
85. English, L., M. Chemali, and M. Desjardins, *Nuclear membrane-derived autophagy, a novel process that participates in the presentation of endogenous viral antigens during HSV-1 infection.* Autophagy, 2009. **5**(7): p. 1026-9.
86. Zoncu, R., A. Efeyan, and D.M. Sabatini, *mTOR: from growth signal integration to cancer, diabetes and ageing.* Nat Rev Mol Cell Biol, 2011. **12**(1): p. 21-35.
87. Mizushima, N., *The role of the Atg1/ULK1 complex in autophagy regulation.* Curr Opin Cell Biol, 2010. **22**(2): p. 132-9.
88. Egan, D.F., et al., *Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy.* Science, 2011. **331**(6016): p. 456-61.
89. Mack, H.I., et al., *AMPK-dependent phosphorylation of ULK1 regulates ATG9 localization.* Autophagy, 2012. **8**(8): p. 1197-214.
90. Dunlop, E.A., et al., *ULK1 inhibits mTORC1 signaling, promotes multisite Raptor phosphorylation and hinders substrate binding.* Autophagy, 2011. **7**(7): p. 737-47.
91. Jung, C.H., et al., *ULK1 inhibits the kinase activity of mTORC1 and cell proliferation.* Autophagy, 2011. **7**(10): p. 1212-21.
92. Loffler, A.S., et al., *Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop.* Autophagy, 2011. **7**(7): p. 696-706.
93. Funderburk, S.F., Q.J. Wang, and Z. Yue, *The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond.* Trends Cell Biol, 2010. **20**(6): p. 355-62.
94. Polson, H.E., et al., *Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation.* Autophagy, 2010. **6**(4): p. 506-22.
95. Karanasios, E., et al., *Dynamic association of the ULK1 complex with omegasomes during autophagy induction.* J Cell Sci, 2013. **126**(Pt 22): p. 5224-38.
96. Fimia, G.M., et al., *Unleashing the Ambra1-Beclin 1 complex from dynein chains: Ulk1 sets Ambra1 free to induce autophagy.* Autophagy, 2011. **7**(1): p. 115-7.
97. Russell, R.C., et al., *ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase.* Nat Cell Biol, 2013. **15**(7): p. 741-50.
98. Kim, J., et al., *Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy.* Cell, 2013. **152**(1-2): p. 290-303.
99. Yuan, H.X., R.C. Russell, and K.L. Guan, *Regulation of PIK3C3/VPS34 complexes by MTOR in nutrient stress-induced autophagy.* Autophagy, 2013. **9**(12): p. 1983-95.
100. Pattingre, S., et al., *Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy.* Cell, 2005. **122**(6): p. 927-39.

101. Maiuri, M.C., et al., *Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1.* EMBO J, 2007. **26**(10): p. 2527-39.
102. Noda, N.N., et al., *Structure of the Atg12-Atg5 conjugate reveals a platform for stimulating Atg8-PE conjugation.* EMBO Rep, 2013. **14**(2): p. 206-11.
103. Mihaylova, M.M. and R.J. Shaw, *The AMPK signalling pathway coordinates cell growth, autophagy and metabolism.* Nat Cell Biol, 2011. **13**(9): p. 1016-23.
104. Slobodkin, M.R. and Z. Elazar, *The Atg8 family: multifunctional ubiquitin-like key regulators of autophagy.* Essays Biochem, 2013. **55**: p. 51-64.
105. Fujita, N., et al., *An Atg4B mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure.* Mol Biol Cell, 2008. **19**(11): p. 4651-9.
106. Kirkin, V., et al., *A role for ubiquitin in selective autophagy.* Mol Cell, 2009. **34**(3): p. 259-69.
107. Lim, J., et al., *Proteotoxic stress induces phosphorylation of p62/SQSTM1 by ULK1 to regulate selective autophagic clearance of protein aggregates.* PLoS Genet, 2015. **11**(2): p. e1004987.
108. Novak, I., et al., *Nix is a selective autophagy receptor for mitochondrial clearance.* EMBO Rep, 2010. **11**(1): p. 45-51.
109. Klionsky, D.J., et al., *Guidelines for the use and interpretation of assays for monitoring autophagy.* Autophagy, 2012. **8**(4): p. 445-544.
110. Eskelinen, E.L., *Maturation of autophagic vacuoles in Mammalian cells.* Autophagy, 2005. **1**(1): p. 1-10.
111. Hyttinen, J.M., et al., *Maturation of autophagosomes and endosomes: a key role for Rab7.* Biochim Biophys Acta, 2013. **1833**(3): p. 503-10.
112. Saftig, P., W. Beertsen, and E.L. Eskelinen, *LAMP-2: a control step for phagosome and autophagosome maturation.* Autophagy, 2008. **4**(4): p. 510-2.
113. Levine, B. and G. Kroemer, *Autophagy in the pathogenesis of disease.* Cell, 2008. **132**(1): p. 27-42.
114. Wang, R.C. and B. Levine, *Autophagy in cellular growth control.* FEBS Lett, 2010. **584**(7): p. 1417-26.
115. Cecconi, F. and B. Levine, *The role of autophagy in mammalian development: cell makeover rather than cell death.* Dev Cell, 2008. **15**(3): p. 344-57.
116. Morselli, E., et al., *Anti- and pro-tumor functions of autophagy.* Biochim Biophys Acta, 2009. **1793**(9): p. 1524-32.
117. Gozuacik, D. and A. Kimchi, *DAPk protein family and cancer.* Autophagy, 2006. **2**(2): p. 74-9.
118. Liang, X.H., et al., *Induction of autophagy and inhibition of tumorigenesis by beclin 1.* Nature, 1999. **402**(6762): p. 672-6.
119. Miracco, C., et al., *Protein and mRNA expression of autophagy gene Beclin 1 in human brain tumours.* Int J Oncol, 2007. **30**(2): p. 429-36.
120. Qu, X., et al., *Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene.* J Clin Invest, 2003. **112**(12): p. 1809-20.
121. Liang, C., et al., *Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG.* Nat Cell Biol, 2006. **8**(7): p. 688-99.
122. Takahashi, Y., et al., *Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis.* Nat Cell Biol, 2007. **9**(10): p. 1142-51.
123. Takamura, A., et al., *Autophagy-deficient mice develop multiple liver tumors.* Genes Dev, 2011. **25**(8): p. 795-800.
124. Marino, G., et al., *Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3.* J Biol Chem, 2007. **282**(25): p. 18573-83.

125. Kashuba, V.I., et al., *NotI linking/jumping clones of human chromosome 3: mapping of the TFRC, RAB7 and HAUSP genes to regions rearranged in leukemia and deleted in solid tumors.* FEBS Lett, 1997. **419**(2-3): p. 181-5.
126. Erlich, S., et al., *Differential interactions between Beclin 1 and Bcl-2 family members.* Autophagy, 2007. **3**(6): p. 561-8.
127. Freed-Pastor, W.A. and C. Prives, *Mutant p53: one name, many proteins.* Genes Dev, 2012. **26**(12): p. 1268-86.
128. Crighton, D., et al., *DRAM, a p53-induced modulator of autophagy, is critical for apoptosis.* Cell, 2006. **126**(1): p. 121-34.
129. Feng, Z., et al., *The coordinate regulation of the p53 and mTOR pathways in cells.* Proc Natl Acad Sci U S A, 2005. **102**(23): p. 8204-9.
130. Amaravadi, R.K., et al., *Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma.* J Clin Invest, 2007. **117**(2): p. 326-36.
131. Gonzalez, S. and M. Serrano, *A new mechanism of inactivation of the INK4/ARF locus.* Cell Cycle, 2006. **5**(13): p. 1382-4.
132. Abida, W.M. and W. Gu, *p53-Dependent and p53-independent activation of autophagy by ARF.* Cancer Res, 2008. **68**(2): p. 352-7.
133. Reef, S., et al., *A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death.* Mol Cell, 2006. **22**(4): p. 463-75.
134. Tasdemir, E., et al., *Regulation of autophagy by cytoplasmic p53.* Nat Cell Biol, 2008. **10**(6): p. 676-87.
135. Degenhardt, K., et al., *Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis.* Cancer Cell, 2006. **10**(1): p. 51-64.
136. Michaud, M., et al., *An autophagy-dependent anticancer immune response determines the efficacy of melanoma chemotherapy.* Oncoimmunology, 2014. **3**(7): p. e944047.
137. Li, Y., et al., *Efficient cross-presentation depends on autophagy in tumor cells.* Cancer Res, 2008. **68**(17): p. 6889-95.
138. Karantza-Wadsworth, V., et al., *Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis.* Genes Dev, 2007. **21**(13): p. 1621-35.
139. Lemasters, J.J., *Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging.* Rejuvenation Res, 2005. **8**(1): p. 3-5.
140. Bae, H. and J.L. Guan, *Suppression of autophagy by FIP200 deletion impairs DNA damage repair and increases cell death upon treatments with anticancer agents.* Mol Cancer Res, 2011. **9**(9): p. 1232-41.
141. Rodriguez-Rocha, H., et al., *DNA damage and autophagy.* Mutat Res, 2011. **711**(1-2): p. 158-66.
142. Fazi, B., et al., *Fenretinide induces autophagic cell death in caspase-defective breast cancer cells.* Autophagy, 2008. **4**(4): p. 435-41.
143. Shimizu, S., et al., *Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes.* Nat Cell Biol, 2004. **6**(12): p. 1221-8.
144. Song, J., et al., *Autophagy in hypoxia protects cancer cells against apoptosis induced by nutrient deprivation through a Beclin1-dependent way in hepatocellular carcinoma.* J Cell Biochem, 2011. **112**(11): p. 3406-20.
145. Yang, Z.J., et al., *Autophagy modulation for cancer therapy.* Cancer Biol Ther, 2011. **11**(2): p. 169-76.
146. Ouyang, L., et al., *Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis.* Cell Prolif, 2012. **45**(6): p. 487-98.
147. Green, D.R. and G. Kroemer, *The pathophysiology of mitochondrial cell death.* Science, 2004. **305**(5684): p. 626-9.

148. Hengartner, M.O., *The biochemistry of apoptosis.* Nature, 2000. **407**(6805): p. 770-6.
149. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.
150. Bonora, M., et al., *Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition.* Cell Cycle, 2013. **12**(4): p. 674-83.
151. Giorgio, V., et al., *Dimers of mitochondrial ATP synthase form the permeability transition pore.* Proc Natl Acad Sci U S A, 2013. **110**(15): p. 5887-92.
152. Westphal, D., R.M. Kluck, and G. Dewson, *Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis.* Cell Death Differ, 2014. **21**(2): p. 196-205.
153. Westphal, D., et al., *Molecular biology of Bax and Bak activation and action.* Biochim Biophys Acta, 2011. **1813**(4): p. 521-31.
154. Kvansakul, M., et al., *Vaccinia virus anti-apoptotic F1L is a novel Bcl-2-like domain-swapped dimer that binds a highly selective subset of BH3-containing death ligands.* Cell Death Differ, 2008. **15**(10): p. 1564-71.
155. Galluzzi, L., et al., *Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012.* Cell Death Differ, 2012. **19**(1): p. 107-20.
156. Greenberg, M.E., et al., *Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells.* J Exp Med, 2006. **203**(12): p. 2613-25.
157. MacFarlane, M. and A.C. Williams, *Apoptosis and disease: a life or death decision.* EMBO Rep, 2004. **5**(7): p. 674-8.
158. Berry, D.L. and E.H. Baehrecke, *Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila.* Cell, 2007. **131**(6): p. 1137-48.
159. Denton, D., et al., *Autophagy, not apoptosis, is essential for midgut cell death in Drosophila.* Curr Biol, 2009. **19**(20): p. 1741-6.
160. Yang, W., et al., *Proteasome inhibition induces both pro- and anti-cell death pathways in prostate cancer cells.* Cancer Lett, 2006. **243**(2): p. 217-27.
161. Kroemer, G. and B. Levine, *Autophagic cell death: the story of a misnomer.* Nat Rev Mol Cell Biol, 2008. **9**(12): p. 1004-10.
162. Pan, J.A., et al., *Inhibition of protein degradation induces apoptosis through a microtubule-associated protein 1 light chain 3-mediated activation of caspase-8 at intracellular membranes.* Mol Cell Biol, 2011. **31**(15): p. 3158-70.
163. Young, M.M., et al., *Autophagosomal membrane serves as platform for intracellular death-inducing signaling complex (iDISC)-mediated caspase-8 activation and apoptosis.* J Biol Chem, 2012. **287**(15): p. 12455-68.
164. Ullman, E., et al., *Autophagy promotes necrosis in apoptosis-deficient cells in response to ER stress.* Cell Death Differ, 2008. **15**(2): p. 422-5.
165. Levine, B. and G. Kroemer, *Autophagy in aging, disease and death: the true identity of a cell death impostor.* Cell Death Differ, 2009. **16**(1): p. 1-2.
166. Kim, I., S. Rodriguez-Enriquez, and J.J. Lemasters, *Selective degradation of mitochondria by mitophagy.* Arch Biochem Biophys, 2007. **462**(2): p. 245-53.
167. Rangwala, R., et al., *Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma.* Autophagy, 2014. **10**(8): p. 1391-402.
168. Sehgal, A.R., et al., *You eat what you are: autophagy inhibition as a therapeutic strategy in leukemia.* Leukemia, 2015. **29**(3): p. 517-525.
169. Radoshevich, L., et al., *ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death.* Cell, 2010. **142**(4): p. 590-600.

170. Rubinstein, A.D., et al., *The autophagy protein Atg12 associates with antiapoptotic Bcl-2 family members to promote mitochondrial apoptosis.* Mol Cell, 2011. **44**(5): p. 698-709.
171. Betin, V.M. and J.D. Lane, *Caspase cleavage of Atg4D stimulates GABARAP-L1 processing and triggers mitochondrial targeting and apoptosis.* J Cell Sci, 2009. **122**(Pt 14): p. 2554-66.
172. Yousefi, S., et al., *Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis.* Nat Cell Biol, 2006. **8**(10): p. 1124-32.
173. Wirawan, E., et al., *Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria.* Cell Death Dis, 2010. **1**: p. e18.
174. Fan, Y.J. and W.X. Zong, *The cellular decision between apoptosis and autophagy.* Chin J Cancer, 2013. **32**(3): p. 121-9.
175. Pagliarini, V., et al., *Proteolysis of Ambra1 during apoptosis has a role in the inhibition of the autophagic pro-survival response.* Cell Death Differ, 2012. **19**(9): p. 1495-504.
176. Yin, X., et al., *UV irradiation resistance-associated gene suppresses apoptosis by interfering with BAX activation.* EMBO Rep, 2011. **12**(7): p. 727-34.
177. Maiuri, M.C., et al., *BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2/Bcl-X(L).* Autophagy, 2007. **3**(4): p. 374-6.
178. Wei, Y., et al., *JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy.* Mol Cell, 2008. **30**(6): p. 678-88.
179. Park, K.J., et al., *Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death.* Biochem Biophys Res Commun, 2009. **382**(4): p. 726-9.
180. Demarchi, F., et al., *Calpain is required for macroautophagy in mammalian cells.* J Cell Biol, 2006. **175**(4): p. 595-605.
181. Bialik, S. and A. Kimchi, *Lethal weapons: DAP-kinase, autophagy and cell death: DAP-kinase regulates autophagy.* Curr Opin Cell Biol, 2010. **22**(2): p. 199-205.
182. Pasparakis, M. and P. Vandenabeele, *Necroptosis and its role in inflammation.* Nature, 2015. **517**(7534): p. 311-20.
183. Martikainen, P. and J. Isaacs, *Role of calcium in the programmed death of rat prostatic glandular cells.* Prostate, 1990. **17**(3): p. 175-87.
184. Connor, J., et al., *Calcium channel antagonists delay regression of androgen-dependent tissues and suppress gene activity associated with cell death.* Prostate, 1988. **13**(2): p. 119-30.
185. Prevarskaya, N., R. Skryma, and Y. Shuba, *Ca2+ homeostasis in apoptotic resistance of prostate cancer cells.* Biochem Biophys Res Commun, 2004. **322**(4): p. 1326-35.
186. Decuypere, J.P., et al., *IP(3) Receptors, Mitochondria, and Ca Signaling: Implications for Aging.* J Aging Res, 2011. **2011**: p. 920178.
187. Berridge, M.J., M.D. Bootman, and H.L. Roderick, *Calcium signalling: dynamics, homeostasis and remodelling.* Nat Rev Mol Cell Biol, 2003. **4**(7): p. 517-29.
188. Bidaux, G., et al., *Prostate cell differentiation status determines transient receptor potential melastatin member 8 channel subcellular localization and function.* J Clin Invest, 2007. **117**(6): p. 1647-57.
189. Roderick, H.L. and S.J. Cook, *Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival.* Nat Rev Cancer, 2008. **8**(5): p. 361-75.

190. Venkatachalam, K. and C. Montell, *TRP channels.* Annu Rev Biochem, 2007. **76**: p. 387-417.
191. Gees, M., et al., *TRP channels.* Compr Physiol, 2012. **2**(1): p. 563-608.
192. Clapham, D.E., L.W. Runnels, and C. Strubing, *The TRP ion channel family.* Nat Rev Neurosci, 2001. **2**(6): p. 387-96.
193. Yudin, Y. and T. Rohacs, *Regulation of TRPM8 channel activity.* Mol Cell Endocrinol, 2012. **353**(1-2): p. 68-74.
194. Egevad, L., et al., *Prognostic value of the Gleason score in prostate cancer.* BJU Int, 2002. **89**(6): p. 538-42.
195. Fixemer, T., et al., *Expression of the Ca2+-selective cation channel TRPV6 in human prostate cancer: a novel prognostic marker for tumor progression.* Oncogene, 2003. **22**(49): p. 7858-61.
196. Fuessel, S., et al., *Vaccination of hormone-refractory prostate cancer patients with peptide cocktail-loaded dendritic cells: results of a phase I clinical trial.* Prostate, 2006. **66**(8): p. 811-21.
197. Tsavaler, L., et al., *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, 2001. **61**(9): p. 3760-9.
198. Bidaux, G., et al., *Evidence for specific TRPM8 expression in human prostate secretory epithelial cells: functional androgen receptor requirement.* Endocr Relat Cancer, 2005. **12**(2): p. 367-82.
199. Henshall, S.M., et al., *Survival analysis of genome-wide gene expression profiles of prostate cancers identifies new prognostic targets of disease relapse.* Cancer Res, 2003. **63**(14): p. 4196-203.
200. Zhang, L. and G.J. Barritt, *Evidence that TRPM8 is an androgen-dependent Ca2+ channel required for the survival of prostate cancer cells.* Cancer Res, 2004. **64**(22): p. 8365-73.
201. Thebault, S., et al., *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, 2005. **280**(47): p. 39423-35.
202. Voets, T., G. Owsianik, and B. Nilius, *Trpm8.* Handb Exp Pharmacol, 2007(179): p. 329-44.
203. Mahieu, F., et al., *TRPM8-independent menthol-induced Ca2+ release from endoplasmic reticulum and Golgi.* J Biol Chem, 2007. **282**(5): p. 3325-36.
204. Gkika, D., et al., *PSA reduces prostate cancer cell motility by stimulating TRPM8 activity and plasma membrane expression.* Oncogene, 2010. **29**(32): p. 4611-6.
205. Rohacs, T., et al., *PI(4,5)P2 regulates the activation and desensitization of TRPM8 channels through the TRP domain.* Nat Neurosci, 2005. **8**(5): p. 626-34.
206. Vanden Abeele, F., et al., *Ca2+-independent phospholipase A2-dependent gating of TRPM8 by lysophospholipids.* J Biol Chem, 2006. **281**(52): p. 40174-82.
207. Yang, Z.H., et al., *Effects of TRPM8 on the proliferation and motility of prostate cancer PC-3 cells.* Asian J Androl, 2009. **11**(2): p. 157-65.
208. Wang, Y., et al., *Menthol inhibits the proliferation and motility of prostate cancer DU145 cells.* Pathol Oncol Res, 2012. **18**(4): p. 903-10.
209. Gkika, D., et al., *TRP channel-associated factors are a novel protein family that regulates TRPM8 trafficking and activity.* J Cell Biol, 2015. **208**(1): p. 89-107.
210. Akl, H. and G. Bultynck, *Altered Ca(2+) signaling in cancer cells: proto-oncogenes and tumor suppressors targeting IP3 receptors.* Biochim Biophys Acta, 2013. **1835**(2): p. 180-93.

211. Taylor, C.W. and S.C. Tovey, *IP(3) receptors: toward understanding their activation.* Cold Spring Harb Perspect Biol, 2010. **2**(12): p. a004010.
212. Vanderheyden, V., et al., *Regulation of inositol 1,4,5-trisphosphate-induced Ca2+ release by reversible phosphorylation and dephosphorylation.* Biochim Biophys Acta, 2009. **1793**(6): p. 959-70.
213. Missiaen, L., et al., *Ca2+ release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca2+ in permeabilized cells.* Nature, 1992. **357**(6379): p. 599-602.
214. Nunn, D.L. and C.W. Taylor, *Luminal Ca2+ increases the sensitivity of Ca2+ stores to inositol 1,4,5-trisphosphate.* Mol Pharmacol, 1992. **41**(1): p. 115-9.
215. Tang, T.S., et al., *Modulation of type 1 inositol (1,4,5)-trisphosphate receptor function by protein kinase a and protein phosphatase 1alpha.* J Neurosci, 2003. **23**(2): p. 403-15.
216. Nakade, S., et al., *Cyclic AMP-dependent phosphorylation of an immunoaffinity-purified homotetrameric inositol 1,4,5-trisphosphate receptor (type I) increases Ca2+ flux in reconstituted lipid vesicles.* J Biol Chem, 1994. **269**(9): p. 6735-42.
217. Eckenrode, E.F., et al., *Apoptosis protection by Mcl-1 and Bcl-2 modulation of inositol 1,4,5-trisphosphate receptor-dependent Ca2+ signaling.* J Biol Chem, 2010. **285**(18): p. 13678-84.
218. Kang, S.S., et al., *Caffeine-mediated inhibition of calcium release channel inositol 1,4,5-trisphosphate receptor subtype 3 blocks glioblastoma invasion and extends survival.* Cancer Res, 2010. **70**(3): p. 1173-83.
219. Sakakura, C., et al., *Possible involvement of inositol 1,4,5-trisphosphate receptor type 3 (IP3R3) in the peritoneal dissemination of gastric cancers.* Anticancer Res, 2003. **23**(5A): p. 3691-7.
220. Shibao, K., et al., *The type III inositol 1,4,5-trisphosphate receptor is associated with aggressiveness of colorectal carcinoma.* Cell Calcium, 2010. **48**(6): p. 315-23.
221. Szatkowski, C., et al., *Inositol 1,4,5-trisphosphate-induced Ca2+ signalling is involved in estradiol-induced breast cancer epithelial cell growth.* Mol Cancer, 2010. **9**: p. 156.
222. Schrodl, K., et al., *Altered Ca2+-homeostasis of cisplatin-treated and low level resistant non-small-cell and small-cell lung cancer cells.* Cell Oncol, 2009. **31**(4): p. 301-15.
223. Tsunoda, T., et al., *Inositol 1,4,5-trisphosphate (IP3) receptor type1 (IP3R1) modulates the acquisition of cisplatin resistance in bladder cancer cell lines.* Oncogene, 2005. **24**(8): p. 1396-402.
224. Szado, T., et al., *Phosphorylation of inositol 1,4,5-trisphosphate receptors by protein kinase B/Akt inhibits Ca2+ release and apoptosis.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2427-32.
225. Giorgi, C., et al., *PML regulates apoptosis at endoplasmic reticulum by modulating calcium release.* Science, 2010. **330**(6008): p. 1247-51.
226. Hedgepeth, S.C., et al., *The BRCA1 Tumor Suppressor Binds to Inositol 1,4,5-Trisphosphate Receptors to Stimulate Apoptotic Calcium Release.* J Biol Chem, 2015. **290**(11): p. 7304-13.
227. Decuypere, J.P., et al., *Ins(1,4,5)P3 receptor-mediated Ca2+ signaling and autophagy induction are interrelated.* Autophagy, 2011. **7**(12): p. 1472-89.
228. Foskett, J.K., et al., *Inositol trisphosphate receptor Ca2+ release channels.* Physiol Rev, 2007. **87**(2): p. 593-658.

229. Fariss, M.W., G.A. Pascoe, and D.J. Reed, *Vitamin E reversal of the effect of extracellular calcium on chemically induced toxicity in hepatocytes.* Science, 1985. **227**(4688): p. 751-4.
230. Sattler, R. and M. Tymianski, *Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death.* Mol Neurobiol, 2001. **24**(1-3): p. 107-29.
231. Pinton, P., et al., *Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis.* Oncogene, 2008. **27**(50): p. 6407-18.
232. Benbrook, D.M. and A. Long, *Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis.* Exp Oncol, 2012. **34**(3): p. 286-97.
233. Hetz, C., E. Chevet, and H.P. Harding, *Targeting the unfolded protein response in disease.* Nat Rev Drug Discov, 2013. **12**(9): p. 703-19.
234. Li, Y., et al., *New insights into the roles of CHOP-induced apoptosis in ER stress.* Acta Biochim Biophys Sin (Shanghai), 2015. **47**(2): p. 146-7.
235. Bassik, M.C., et al., *Phosphorylation of BCL-2 regulates ER Ca2+ homeostasis and apoptosis.* EMBO J, 2004. **23**(5): p. 1207-16.
236. Distelhorst, C.W. and G.C. Shore, *Bcl-2 and calcium: controversy beneath the surface.* Oncogene, 2004. **23**(16): p. 2875-80.
237. Baffy, G., et al., *Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production.* J Biol Chem, 1993. **268**(9): p. 6511-9.
238. Magnelli, L., et al., *Bcl-2 overexpression abolishes early calcium waving preceding apoptosis in NIH-3T3 murine fibroblasts.* Biochem Biophys Res Commun, 1994. **204**(1): p. 84-90.
239. Kato, H. and H. Nishitoh, *Stress responses from the endoplasmic reticulum in cancer.* Front Oncol, 2015. **5**: p. 93.
240. Lam, M., et al., *Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes.* Proc Natl Acad Sci U S A, 1994. **91**(14): p. 6569-73.
241. Farrukh, M.R., et al., *Oxidative stress mediated Ca(2+) release manifests endoplasmic reticulum stress leading to unfolded protein response in UV-B irradiated human skin cells.* J Dermatol Sci, 2014. **75**(1): p. 24-35.
242. Li, Y., et al., *Ozone (O3) elicits neurotoxicity in spinal cord neurons (SCNs) by inducing ER Ca(2+) release and activating the CaMKII/MAPK signaling pathway.* Toxicol Appl Pharmacol, 2014. **280**(3): p. 493-501.
243. Egnatchik, R.A., et al., *ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload.* Mol Metab, 2014. **3**(5): p. 544-53.
244. Liao, P.C., et al., *Involvement of endoplasmic reticulum in paclitaxel-induced apoptosis.* J Cell Biochem, 2008. **104**(4): p. 1509-23.
245. Distelhorst, C.W., M. Lam, and T.S. McCormick, *Bcl-2 inhibits hydrogen peroxide-induced ER Ca2+ pool depletion.* Oncogene, 1996. **12**(10): p. 2051-5.
246. Ichimiya, M., et al., *Effect of Bcl-2 on oxidant-induced cell death and intracellular Ca2+ mobilization.* Am J Physiol, 1998. **275**(3 Pt 1): p. C832-9.
247. Pinton, P., et al., *Reduced loading of intracellular Ca(2+) stores and downregulation of capacitative Ca(2+) influx in Bcl-2-overexpressing cells.* J Cell Biol, 2000. **148**(5): p. 857-62.
248. Foyouzi-Youssefi, R., et al., *Bcl-2 decreases the free Ca2+ concentration within the endoplasmic reticulum.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 5723-8.

249. Palmer, A.E., et al., *Bcl-2-mediated alterations in endoplasmic reticulum Ca2+ analyzed with an improved genetically encoded fluorescent sensor.* Proc Natl Acad Sci U S A, 2004. **101**(50): p. 17404-9.
250. Vanden Abeele, F., et al., *Bcl-2-dependent modulation of Ca(2+) homeostasis and store-operated channels in prostate cancer cells.* Cancer Cell, 2002. **1**(2): p. 169-79.
251. Ma, T.S., et al., *SR compartment calcium and cell apoptosis in SERCA overexpression.* Cell Calcium, 1999. **26**(1-2): p. 25-36.
252. Dremina, E.S., et al., *Anti-apoptotic protein Bcl-2 interacts with and destabilizes the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA).* Biochem J, 2004. **383**(Pt 2): p. 361-70.
253. Nakamura, K., et al., *Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis.* J Cell Biol, 2000. **150**(4): p. 731-40.
254. Chen, R., et al., *Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate.* J Cell Biol, 2004. **166**(2): p. 193-203.
255. Hanson, C.J., et al., *Bcl-2 suppresses Ca2+ release through inositol 1,4,5-trisphosphate receptors and inhibits Ca2+ uptake by mitochondria without affecting ER calcium store content.* Cell Calcium, 2008. **44**(3): p. 324-38.
256. Rong, Y.P., et al., *The BH4 domain of Bcl-2 inhibits ER calcium release and apoptosis by binding the regulatory and coupling domain of the IP3 receptor.* Proc Natl Acad Sci U S A, 2009. **106**(34): p. 14397-402.
257. Monaco, G., et al., *Selective regulation of IP3-receptor-mediated Ca2+ signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-Xl.* Cell Death Differ, 2012. **19**(2): p. 295-309.
258. Nutt, L.K., et al., *Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores.* J Biol Chem, 2002. **277**(11): p. 9219-25.
259. Oakes, S.A., et al., *Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum.* Proc Natl Acad Sci U S A, 2005. **102**(1): p. 105-10.
260. Spat, A., et al., *High- and low-calcium-dependent mechanisms of mitochondrial calcium signalling.* Cell Calcium, 2008. **44**(1): p. 51-63.
261. Rizzuto, R., et al., *Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses.* Science, 1998. **280**(5370): p. 1763-6.
262. Csordas, G., et al., *Structural and functional features and significance of the physical linkage between ER and mitochondria.* J Cell Biol, 2006. **174**(7): p. 915-21.
263. Tiwari, M., et al., *Mechanism of 4-HPR-induced apoptosis in glioma cells: evidences suggesting role of mitochondrial-mediated pathway and endoplasmic reticulum stress.* Carcinogenesis, 2006. **27**(10): p. 2047-58.
264. Fujimoto, M. and T. Hayashi, *New insights into the role of mitochondria-associated endoplasmic reticulum membrane.* Int Rev Cell Mol Biol, 2011. **292**: p. 73-117.
265. Szabadkai, G., et al., *Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels.* J Cell Biol, 2006. **175**(6): p. 901-11.
266. De Stefani, D., et al., *A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter.* Nature, 2011. **476**(7360): p. 336-40.
267. Baughman, J.M., et al., *Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter.* Nature, 2011. **476**(7360): p. 341-5.
268. Rizzuto, R., et al., *Mitochondria as sensors and regulators of calcium signalling.* Nat Rev Mol Cell Biol, 2012. **13**(9): p. 566-78.
269. Murphy, A.N., et al., *Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9893-8.

270. Hirata, H., et al., *Bcl-2 modulates endoplasmic reticulum and mitochondrial calcium stores in PC12 cells.* Neurochem Res, 2012. **37**(2): p. 238-43.
271. Bonneau, B., et al., *Non-apoptotic roles of Bcl-2 family: The calcium connection.* Biochim Biophys Acta, 2013. **1833**(7): p. 1755-65.
272. Giacomello, M., et al., *Mitochondrial Ca2+ as a key regulator of cell life and death.* Cell Death Differ, 2007. **14**(7): p. 1267-74.
273. Hunter, D.R., R.A. Haworth, and J.H. Southard, *Relationship between configuration, function, and permeability in calcium-treated mitochondria.* J Biol Chem, 1976. **251**(16): p. 5069-77.
274. Kadenbach, B., *Intrinsic and extrinsic uncoupling of oxidative phosphorylation.* Biochim Biophys Acta, 2003. **1604**(2): p. 77-94.
275. Deniaud, A., et al., *Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis.* Oncogene, 2008. **27**(3): p. 285-99.
276. Campanella, M., P. Pinton, and R. Rizzuto, *Mitochondrial Ca2+ homeostasis in health and disease.* Biol Res, 2004. **37**(4): p. 653-60.
277. Tombal, B., et al., *A supramicromolar elevation of intracellular free calcium ([Ca(2+)](i)) is consistently required to induce the execution phase of apoptosis.* Cell Death Differ, 2002. **9**(5): p. 561-73.
278. Gil-Parrado, S., et al., *Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members.* J Biol Chem, 2002. **277**(30): p. 27217-26.
279. Chen, M., et al., *Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion.* J Biol Chem, 2001. **276**(33): p. 30724-8.
280. Nakagawa, T. and J. Yuan, *Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis.* J Cell Biol, 2000. **150**(4): p. 887-94.
281. Wang, H.G., et al., *Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD.* Science, 1999. **284**(5412): p. 339-43.
282. Yano, S., H. Tokumitsu, and T.R. Soderling, *Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway.* Nature, 1998. **396**(6711): p. 584-7.
283. Hirota, J., T. Furuichi, and K. Mikoshiba, *Inositol 1,4,5-trisphosphate receptor type 1 is a substrate for caspase-3 and is cleaved during apoptosis in a caspase-3-dependent manner.* J Biol Chem, 1999. **274**(48): p. 34433-7.
284. Assefa, Z., et al., *Caspase-3-induced truncation of type 1 inositol trisphosphate receptor accelerates apoptotic cell death and induces inositol trisphosphate-independent calcium release during apoptosis.* J Biol Chem, 2004. **279**(41): p. 43227-36.
285. Verbert, L., et al., *Caspase-3-truncated type 1 inositol 1,4,5-trisphosphate receptor enhances intracellular Ca2+ leak and disturbs Ca2+ signalling.* Biol Cell, 2008. **100**(1): p. 39-49.
286. Schwab, B.L., et al., *Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis.* Cell Death Differ, 2002. **9**(8): p. 818-31.
287. Bano, D., et al., *Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity.* Cell, 2005. **120**(2): p. 275-85.
288. Gordon, P.B., et al., *Dependence of hepatocytic autophagy on intracellularly sequestered calcium.* J Biol Chem, 1993. **268**(35): p. 26107-12.
289. Decuypere, J.P., G. Bultynck, and J.B. Parys, *A dual role for Ca(2+) in autophagy regulation.* Cell Calcium, 2011. **50**(3): p. 242-50.
290. Sarkar, S., et al., *Lithium induces autophagy by inhibiting inositol monophosphatase.* J Cell Biol, 2005. **170**(7): p. 1101-11.

291. Criollo, A., et al., *Regulation of autophagy by the inositol trisphosphate receptor.* Cell Death Differ, 2007. **14**(5): p. 1029-39.
292. Khan, M.T. and S.K. Joseph, *Role of inositol trisphosphate receptors in autophagy in DT40 cells.* J Biol Chem, 2010. **285**(22): p. 16912-20.
293. Cardenas, C., et al., *Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria.* Cell, 2010. **142**(2): p. 270-83.
294. Vicencio, J.M., et al., *The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1.* Cell Death Differ, 2009. **16**(7): p. 1006-17.
295. Sano, R., et al., *Endoplasmic reticulum protein BI-1 regulates Ca(2)(+)-mediated bioenergetics to promote autophagy.* Genes Dev, 2012. **26**(10): p. 1041-54.
296. Bao, X.X., et al., *Nifedipine induced autophagy through Beclin1 and mTOR pathway in endometrial carcinoma cells.* Chin Med J (Engl), 2012. **125**(17): p. 3120-6.
297. Pajak, B., et al., *Verapamil-induced autophagy-like process in colon adenocarcinoma COLO 205 cells; the ultrastructural studies.* Pharmacol Rep, 2012. **64**(4): p. 991-6.
298. Williams, A., et al., *Novel targets for Huntington's disease in an mTOR-independent autophagy pathway.* Nat Chem Biol, 2008. **4**(5): p. 295-305.
299. Sarkar, S., et al., *Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies.* Cell Death Differ, 2009. **16**(1): p. 46-56.
300. Park, H.W., et al., *Pharmacological correction of obesity-induced autophagy arrest using calcium channel blockers.* Nat Commun, 2014. **5**: p. 4834.
301. Hoyer-Hansen, M., et al., *Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2.* Mol Cell, 2007. **25**(2): p. 193-205.
302. Grotemeier, A., et al., *AMPK-independent induction of autophagy by cytosolic Ca2+ increase.* Cell Signal, 2010. **22**(6): p. 914-25.
303. Sakaki, K., J. Wu, and R.J. Kaufman, *Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum.* J Biol Chem, 2008. **283**(22): p. 15370-80.
304. Wang, S.H., et al., *Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway.* Cell Mol Life Sci, 2008. **65**(22): p. 3640-52.
305. Pereira, G.J., et al., *Nicotinic acid adenine dinucleotide phosphate (NAADP) regulates autophagy in cultured astrocytes.* J Biol Chem, 2011. **286**(32): p. 27875-81.
306. Brady, N.R., et al., *The autophagic response to nutrient deprivation in the hl-1 cardiac myocyte is modulated by Bcl-2 and sarco/endoplasmic reticulum calcium stores.* FEBS J, 2007. **274**(12): p. 3184-97.
307. Decuypere, J.P., et al., *mTOR-Controlled Autophagy Requires Intracellular Ca(2+) Signaling.* PLoS One, 2013. **8**(4): p. e61020.
308. Vergne, I., J. Chua, and V. Deretic, *Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade.* J Exp Med, 2003. **198**(4): p. 653-9.
309. Kuczera, K. and P. Kursula, *Interactions of calmodulin with death-associated protein kinase peptides: experimental and modeling studies.* J Biomol Struct Dyn, 2012. **30**(1): p. 45-61.
310. Vig, P.J., et al., *Bergmann glial S100B activates myo-inositol monophosphatase 1 and Co-localizes to purkinje cell vacuoles in SCA1 transgenic mice.* Cerebellum, 2009. **8**(3): p. 231-44.
311. Hait, W.N., et al., *Elongation factor-2 kinase: its role in protein synthesis and autophagy.* Autophagy, 2006. **2**(4): p. 294-6.
312. Kim, H.J., et al., *The Ca(2+) channel TRPML3 regulates membrane trafficking and autophagy.* Traffic, 2009. **10**(8): p. 1157-67.

313. Horoszewicz, J.S., et al., *LNCaP model of human prostatic carcinoma.* Cancer Res, 1983. **43**(4): p. 1809-18.
314. Dehm, S.M. and D.J. Tindall, *Molecular regulation of androgen action in prostate cancer.* J Cell Biochem, 2006. **99**(2): p. 333-44.
315. Nguyen, H.G., et al., *Targeting autophagy overcomes Enzalutamide resistance in castration-resistant prostate cancer cells and improves therapeutic response in a xenograft model.* Oncogene, 2014. **33**(36): p. 4521-30.
316. Jain, R.K., et al., *Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor.* Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10820-5.
317. Kroemer, G., G. Marino, and B. Levine, *Autophagy and the integrated stress response.* Mol Cell, 2010. **40**(2): p. 280-93.
318. Chhipa, R.R., Y. Wu, and C. Ip, *AMPK-mediated autophagy is a survival mechanism in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia.* Cell Signal, 2011. **23**(9): p. 1466-72.
319. Kaini, R.R., et al., *Autophagy regulates lipolysis and cell survival through lipid droplet degradation in androgen-sensitive prostate cancer cells.* Prostate, 2012. **72**(13): p. 1412-22.
320. Bennett, H.L., et al., *Androgens modulate autophagy and cell death via regulation of the endoplasmic reticulum chaperone glucose-regulated protein 78/BiP in prostate cancer cells.* Cell Death Dis, 2010. **1**: p. e72.
321. Berthois, Y., J.A. Katzenellenbogen, and B.S. Katzenellenbogen, *Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture.* Proc Natl Acad Sci U S A, 1986. **83**(8): p. 2496-500.
322. Welshons, W.V., et al., *Estrogenic activity of phenol red.* Mol Cell Endocrinol, 1988. **57**(3): p. 169-78.
323. Jiang, Q., et al., *Targeting androgen receptor leads to suppression of prostate cancer via induction of autophagy.* J Urol, 2012. **188**(4): p. 1361-8.
324. Li, M., et al., *Inhibition of androgen induces autophagy in benign prostate epithelial cells.* Int J Urol, 2013.
325. Liu, C., et al., *Roles of autophagy-related genes Beclin-1 and LC3 in the development and progression of prostate cancer and benign prostatic hyperplasia.* Biomed Rep, 2013. **1**(6): p. 855-860.
326. Bennett, H.L., et al., *Does androgen-ablation therapy (AAT) associated autophagy have a pro-survival effect in LNCaP human prostate cancer cells?* BJU Int, 2013. **111**(4): p. 672-82.
327. Kaini, R.R. and C.A. Hu, *Synergistic killing effect of chloroquine and androgen deprivation in LNCaP cells.* Biochem Biophys Res Commun, 2012. **425**(2): p. 150-6.
328. Ziparo, E., et al., *Autophagy in prostate cancer and androgen suppression therapy.* Int J Mol Sci, 2013. **14**(6): p. 12090-106.
329. Li, M., et al., *Autophagy protects LNCaP cells under androgen deprivation conditions.* Autophagy, 2008. **4**(1): p. 54-60.
330. Tai, S., et al., *Combination of Rad001 (everolimus) and propachlor synergistically induces apoptosis through enhanced autophagy in prostate cancer cells.* Mol Cancer Ther, 2012. **11**(6): p. 1320-31.
331. Chen, R.J., et al., *Monascuspiloin induces apoptosis and autophagic cell death in human prostate cancer cells via the Akt and AMPK signaling pathways.* J Agric Food Chem, 2012. **60**(29): p. 7185-93.

332. Bommareddy, A., et al., *Atg5 regulates phenethyl isothiocyanate-induced autophagic and apoptotic cell death in human prostate cancer cells.* Cancer Res, 2009. **69**(8): p. 3704-12.
333. Lamoureux, F., et al., *Blocked autophagy using lysosomotropic agents sensitizes resistant prostate tumor cells to the novel Akt inhibitor AZD5363.* Clin Cancer Res, 2013. **19**(4): p. 833-44.
334. Bristol, M.L., et al., *Autophagy inhibition for chemosensitization and radiosensitization in cancer: do the preclinical data support this therapeutic strategy?* J Pharmacol Exp Ther, 2013. **344**(3): p. 544-52.
335. Maes, H., et al., *Tumor vessel normalization by chloroquine independent of autophagy.* Cancer Cell, 2014. **26**(2): p. 190-206.
336. Schoenlein, P.V., et al., *Autophagy facilitates the progression of ERalpha-positive breast cancer cells to antiestrogen resistance.* Autophagy, 2009. **5**(3): p. 400-3.
337. Eskelinen, E.L., et al., *Inhibition of autophagy in mitotic animal cells.* Traffic, 2002. **3**(12): p. 878-93.
338. Tasdemir, E., et al., *Cell cycle-dependent induction of autophagy, mitophagy and reticulophagy.* Cell Cycle, 2007. **6**(18): p. 2263-7.
339. Young, A.R., et al., *Autophagy mediates the mitotic senescence transition.* Genes Dev, 2009. **23**(7): p. 798-803.
340. Kang, H.T., et al., *Autophagy impairment induces premature senescence in primary human fibroblasts.* PLoS One, 2011. **6**(8): p. e23367.
341. Liang, J., et al., *The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis.* Nat Cell Biol, 2007. **9**(2): p. 218-24.
342. Sun, X., et al., *p27 protein protects metabolically stressed cardiomyocytes from apoptosis by promoting autophagy.* J Biol Chem, 2014. **289**(24): p. 16924-35.
343. Vanoverberghe, K., et al., *Ca2+ homeostasis and apoptotic resistance of neuroendocrine-differentiated prostate cancer cells.* Cell Death Differ, 2004. **11**(3): p. 321-30.
344. Camello, C., et al., *Calcium leak from intracellular stores--the enigma of calcium signalling.* Cell Calcium, 2002. **32**(5-6): p. 355-61.
345. Kim, H.R., et al., *Bax Inhibitor-1 Is a pH-dependent regulator of Ca2+ channel activity in the endoplasmic reticulum.* J Biol Chem, 2008. **283**(23): p. 15946-55.
346. Bultynck, G., et al., *The C terminus of Bax inhibitor-1 forms a Ca2+-permeable channel pore.* J Biol Chem, 2012. **287**(4): p. 2544-57.
347. Gallego-Sandin, S., M.T. Alonso, and J. Garcia-Sancho, *Calcium homoeostasis modulator 1 (CALHM1) reduces the calcium content of the endoplasmic reticulum (ER) and triggers ER stress.* Biochem J, 2011. **437**(3): p. 469-75.
348. Vanden Abeele, F., et al., *Functional implications of calcium permeability of the channel formed by pannexin 1.* J Cell Biol, 2006. **174**(4): p. 535-46.
349. Tu, H., et al., *Presenilins form ER Ca2+ leak channels, a function disrupted by familial Alzheimer's disease-linked mutations.* Cell, 2006. **126**(5): p. 981-93.
350. Van Coppenolle, F., et al., *Ribosome-translocon complex mediates calcium leakage from endoplasmic reticulum stores.* J Cell Sci, 2004. **117**(Pt 18): p. 4135-42.
351. Chami, M., et al., *SERCA1 truncated proteins unable to pump calcium reduce the endoplasmic reticulum calcium concentration and induce apoptosis.* J Cell Biol, 2001. **153**(6): p. 1301-14.
352. Missiaen, L., et al., *Ins(1,4,5)P3 and glutathione increase the passive Ca2+ leak in permeabilized A7r5 cells.* Biochem Biophys Res Commun, 1993. **193**(1): p. 6-12.

353. Vicencio, J.M., et al., *Anabolic androgenic steroids and intracellular calcium signaling: a mini review on mechanisms and physiological implications.* Mini Rev Med Chem, 2011. **11**(5): p. 390-8.
354. Legrand, G., et al., *Ca2+ pools and cell growth. Evidence for sarcoendoplasmic Ca2+-ATPases 2B involvement in human prostate cancer cell growth control.* J Biol Chem, 2001. **276**(50): p. 47608-14.
355. Humez, S., et al., *Role of endoplasmic reticulum calcium content in prostate cancer cell growth regulation by IGF and TNFalpha.* J Cell Physiol, 2004. **201**(2): p. 201-13.
356. Joseph, S.K. and G. Hajnoczky, *IP3 receptors in cell survival and apoptosis: Ca2+ release and beyond.* Apoptosis, 2007. **12**(5): p. 951-68.
357. Wozniak, A.L., et al., *Requirement of biphasic calcium release from the endoplasmic reticulum for Fas-mediated apoptosis.* J Cell Biol, 2006. **175**(5): p. 709-14.
358. Binah, O., et al., *The Fas receptor-1,4,5-IP3 cascade: a potential target for treating heart failure and arrhythmias.* Ann N Y Acad Sci, 2004. **1015**: p. 338-50.
359. Mendes, C.C., et al., *The type III inositol 1,4,5-trisphosphate receptor preferentially transmits apoptotic Ca2+ signals into mitochondria.* J Biol Chem, 2005. **280**(49): p. 40892-900.
360. Blackshaw, S., et al., *Type 3 inositol 1,4,5-trisphosphate receptor modulates cell death.* FASEB J, 2000. **14**(10): p. 1375-9.
361. Khan, A.A., et al., *Lymphocyte apoptosis: mediation by increased type 3 inositol 1,4,5-trisphosphate receptor.* Science, 1996. **273**(5274): p. 503-7.
362. Higo, T., et al., *Mechanism of ER stress-induced brain damage by IP(3) receptor.* Neuron, 2010. **68**(5): p. 865-78.
363. Jayaraman, T. and A.R. Marks, *T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis.* Mol Cell Biol, 1997. **17**(6): p. 3005-12.
364. Kopil, C.M., et al., *Calpain-cleaved type 1 inositol 1,4,5-trisphosphate receptor (InsP(3)R1) has InsP(3)-independent gating and disrupts intracellular Ca(2+) homeostasis.* J Biol Chem, 2011. **286**(41): p. 35998-6010.
365. Boehning, D., et al., *Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis.* Nat Cell Biol, 2003. **5**(12): p. 1051-61.
366. Khan, M.T., et al., *Akt kinase phosphorylation of inositol 1,4,5-trisphosphate receptors.* J Biol Chem, 2006. **281**(6): p. 3731-7.
367. Xu, L., et al., *Suppression of IP3-mediated calcium release and apoptosis by Bcl-2 involves the participation of protein phosphatase 1.* Mol Cell Biochem, 2007. **295**(1-2): p. 153-65.
368. Chang, M.J., et al., *Feedback regulation mediated by Bcl-2 and DARPP-32 regulates inositol 1,4,5-trisphosphate receptor phosphorylation and promotes cell survival.* Proc Natl Acad Sci U S A, 2014. **111**(3): p. 1186-91.
369. Wehrens, X.H., et al., *Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression.* Proc Natl Acad Sci U S A, 2006. **103**(3): p. 511-8.
370. Bavencoffe, A., et al., *The transient receptor potential channel TRPM8 is inhibited via the alpha 2A adrenoreceptor signaling pathway.* J Biol Chem, 2010. **285**(13): p. 9410-9.
371. Cho, Y.S., Y.N. Lee, and Y.S. Cho-Chung, *Biochemical characterization of extracellular cAMP-dependent protein kinase as a tumor marker.* Biochem Biophys Res Commun, 2000. **278**(3): p. 679-84.
372. Merkle, D. and R. Hoffmann, *Roles of cAMP and cAMP-dependent protein kinase in the progression of prostate cancer: cross-talk with the androgen receptor.* Cell Signal, 2011. **23**(3): p. 507-15.

373. Bagchi, G., et al., *Androgens transduce the G alphas-mediated activation of protein kinase A in prostate cells.* Cancer Res, 2008. **68**(9): p. 3225-31.
374. Gutierrez-Canas, I., et al., *Vasoactive intestinal peptide induces neuroendocrine differentiation in the LNCaP prostate cancer cell line through PKA, ERK, and PI3K.* Prostate, 2005. **63**(1): p. 44-55.
375. Kvissel, A.K., et al., *Androgen dependent regulation of protein kinase A subunits in prostate cancer cells.* Cell Signal, 2007. **19**(2): p. 401-9.
376. Hogan, P.G. and A. Rao, *Dissecting ICRAC, a store-operated calcium current.* Trends Biochem Sci, 2007. **32**(5): p. 235-45.
377. Mery, L., et al., *Overexpression of calreticulin increases intracellular Ca2+ storage and decreases store-operated Ca2+ influx.* J Biol Chem, 1996. **271**(16): p. 9332-9.
378. Flourakis, M., et al., *Orai1 contributes to the establishment of an apoptosis-resistant phenotype in prostate cancer cells.* Cell Death Dis, 2010. **1**: p. e75.
379. Vanoverberghe, K., et al., *Cytoskeleton reorganization as an alternative mechanism of store-operated calcium entry control in neuroendocrine-differentiated cells.* PLoS One, 2012. **7**(9): p. e45615.
380. Dubois, C., et al., *Remodeling of channel-forming ORAI proteins determines an oncogenic switch in prostate cancer.* Cancer Cell, 2014. **26**(1): p. 19-32.
381. Ribeiro, C.M., et al., *Chronic airway infection/inflammation induces a Ca2+i-dependent hyperinflammatory response in human cystic fibrosis airway epithelia.* J Biol Chem, 2005. **280**(18): p. 17798-806.