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# Cell cycle and migration control in prostate and colon cancer cells by CLIC1 protein

Dottorando: **Dott.ssa Valentina Carlini**

Matricola R10590

Tutor e Coordinatore: **Prof. Michele Mazzanti**

Dip. Bioscienze, Università degli Studi di Milano

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# ABSTRACT

The intracellular chloride channel 1 (CLIC1) is a metamorphic protein, belonging to a recently discovered and still largely unexplored ion channel family. It displays the unique characteristic of being expressed both in a cytoplasmic and in a transmembrane form, the latter able to form a chloride selective ion channel. One of the main factors known to regulate this membrane insertion is the increase of oxidative level of the cells.

If on one hand, the transient CLIC1 functional expression in the membrane could mediate several physiological cell responses, on the other hand, its chronic membrane translocation can lead to severe pathological conditions, including cancer. In particular two tumors, prostate cancer (PCa) and colon cancer (CRC), are characterized by elevated oxidative level and growing scientific evidence have suggested the involvement of CLIC1 in the tumorigenesis of these diseases.

PCa and CRC are two of the most diffuse cancers and a leading cause of tumor fatality worldwide. Detecting the disease in a very early stage and finding a treatment able to prevent metastasis are critical clinical challenges to achieve a successful treatment for these malignancies.

This thesis was focused in the direction of understanding the possible role of CLIC1 in the development and progression of PCa and CRC.

Results obtained have shown that CLIC1 functional expression in plasma membrane occurs selectively in malignant cells, compared to benign or normal cells. Moreover, it has been demonstrated that CLIC1 membrane chloride current promotes proliferation, cell cycle progression from G1 to S phase and migration of cancer cells. All these findings suggest that CLIC1 may actively contribute to development of PCa and CRC and their progression towards a more aggressive form.

It can be reasonably hypothesize that elevated oxidative levels present in cancer cells compared to normal cells cause the chronic overexpression of CLIC1 in the plasma membrane only of malignant cells. Therefore, targeting this protein could make it possible to hit selectively the tumor cells without damaging their normal counterpart. In this scenario, CLIC1 appears not only as a promising pharmacological target but also as a suitable biomarker.

The only effective inhibitor of CLIC1 activity to date identified is highly toxic in vivo. For this reason, finding other compounds able to specifically block the channel but causing negligible side effects is necessary. In light with this purpose, our laboratory has recently proposed the anti-diabetic drug metformin as CLIC1 channel blocker.

Results obtained have demonstrated that metformin displays a significant antitumoral activity on PCa and CRC cells, inhibiting both cell proliferation and migration. This anti-neoplastic effect is dependent on the inhibition of CLIC1 channel together with other intracellular targets.

Overall these findings provide new support on the antineoplastic role of metformin and encourage the research of more specific and more effective compounds for CLIC1 channel, in order to minimize side effects.

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# INTRODUCTION

# 2.1 Chloride channels and cancer

In the last two decades, an emerging role for ion channels in driving tumor development and growth has been defined [1, 2]. It is now clear that the sequence of genetic alterations occurring during the switch from a physiological condition towards neoplastic transformation often involves ion channels [2, 3].

It has been proposed the involvement of ion channels activity in virtually all the defined hallmarks of cancer: 1) self-sufficiency in growth signals, 2) insensitivity to anti-growth signals, 3) evasion of apoptosis, 4) unlimited replicative potential, 5) sustained angiogenesis and 6) tissue invasion and metastasis (figure 1). The contribution of specific ion channels to these hallmarks varies for different types of cancer [4, 5].

Consistent with our evolving knowledge about the role of ion channels in tumorigenesis, cancer can be classified as a "channelopathy", or a disease brought about by altered ion channels expression or activity [6].

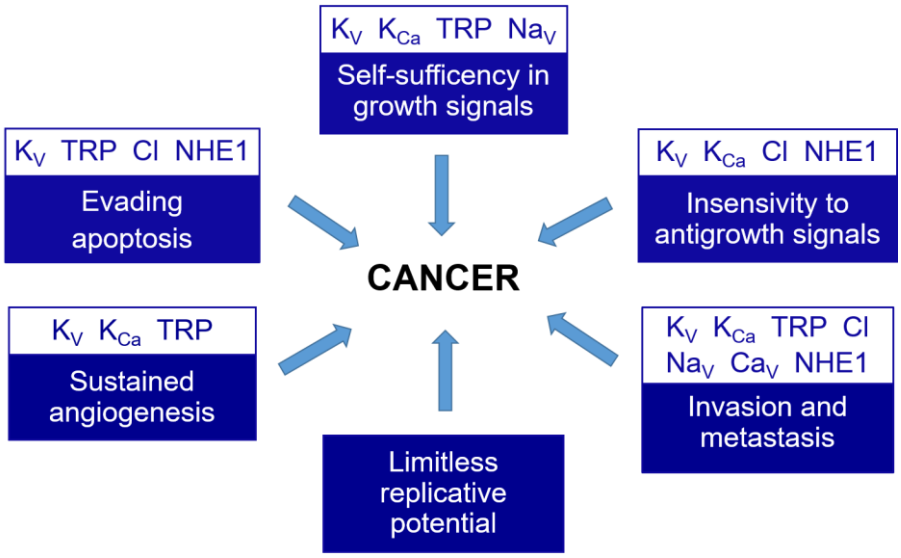


Figure 1. A schematic diagram illustrating the contribution of different ion channels in several aspects of the six cancer hallmarks. $K_V$, $K_{Ca}$: voltage-gated/$Ca^{2+}$ sensitive $K^+$ channels; Cl: $Cl^-$ channels; $Na_V$, $Ca_V$: voltage-gated $Na^+$, $Ca^{2+}$ channels; TRP: transient receptor potential channels: NHE1: $Na^+/H^+$ exchanger 1 (adapted from [5]).

Most of the studies have been focused on the role of potassium channels [7, 8], but with the flow of time, increasing scientific evidence has attributed to chloride channels a more prominent position in carcinogenesis [1, 9].

Chloride channels are transmembrane proteins that allow the flux of anions ($I^-$, $Br^-$, $Cl^-$, $HCO_3^-$) along their electrochemical gradient across the cell membranes [10, 11].
The different members of the chloride channel family display heterogeneous characteristics in the control of their activity. Specifically, they can be divided in four different classes: voltage-dependent chloride channels (ClC), calcium-activated chloride channels (CaCC), cystic fibrosis transmembrane conductance regulator (CFTR), and chloride intracellular channels (CLIC) [10].

Chloride ($Cl^-$) channels are ubiquitously expressed and reside both in the plasma membrane and in the intracellular organelles [11, 12]. They are implicated in a wide range of biological processes [10, 11]. In the plasma membrane, they participate, for example, to ion homeostasis, cell volume regulation, pH modulation, epithelial fluid secretion and trans-epithelial salt transport, muscle contraction and neuroexcitation [10, 11, 13]. In the organelle membranes, they are implicated in endosomal, lysosomal and Golgi acidification [11, 13].
In addition, it has been shown that changing in intracellular chloride concentration could act like a second messenger to activate downstream pathways and modulate many physiological functions. As example, cells can use variation of $Cl^-$ concentration to regulate the ion channels mRNA expression [14, 15], the activation of apoptosis [15] or the release of prostaglandin [16]. Moreover, it has been recently confirmed that these channels play an active part in the regulation of cell proliferation and apoptotic cell death. Regulating ion transport across the cell membrane, indeed, $Cl^-$ channels provide the osmolyte flux required for cell volume changes associated with cell proliferation and apoptosis [17-19].
Chloride channels have also a role in the regulation of the cell cycle progression, probably as a key factor for the progression from G1 to S phase [20-22]. They display a cell cycle-dependent expression: for example, ClC-5 expression in myeloid cells ranged from high during S and G2/M to low in G0/G1 [23]. In lymphocytes, chloride permeability also changed with the cell cycle, being low in G0 and S phase and high in G1/S [24].

Chloride channels have recently gained a growing importance in tumor development and progression so that they are currently included among the novel targets for cancer therapy [25-27]. In literature, several examples of dysregulated chloride channels participating in the progression of a number of tumors have been reported [1].

Volume-activated chloride channels are involved in the regulation of cell shape and cell volume changes required for migration and invasion processes [28]. These channels may also have a role in the angiogenic process [29]. CLC-3, in particular, has an important role in prostate cancer [30], nasopharyngeal carcinoma [31] and glioma [32].

$Ca^{2+}$-activated chloride channels control fluid secretion [33] and have been found to be either up- or downregulated in many cancers [34]. CLCA2 inhibits cell proliferation [35], cell migration and invasion [36] and promotes apoptosis [35]. It has a tumor suppressor role and is down-regulated in breast cancer [37] and colorectal cancer [38]. ANO1 regulates cell proliferation by promoting the transition G1/S phase of cell cycle [39]. It is ubiquitously expressed in gastrointestinal stromal tumors [40] and promotes growth and invasion of androgen-independent prostate cancer [41].

CFTR modulates cell inflammation and apoptotic signalling and its expression has been found to be strongly correlated with cervical cancer progression [42].

Intracellular chloride channels, in particular CLIC1 and CLIC4, have recently attracted the attention of researchers in view of their important role in tumorigenesis and their peculiar functional expression. These two channels display different patterns of expression in different tissues and they have also different levels of expression in different tumors [1, 43-49]. CLIC channels have a peculiar behavior that differentiates them from the others membrane-resident chloride channels. They can undergo a modulated translocation and functional membrane insertion in response to external stimuli or internal perturbations in cell homeostasis. As the functional membrane insertion may be unique for each CLIC family member and cell type, these proteins represent a particularly interesting pharmacological target [1].

# 2.2 Chloride Intracellular Channels (CLICs)

The chloride intracellular channel (CLICs) protein family has been the last discovered and still largely underexplored among all types of chloride channels. To date, seven members of the family have been identified: CLIC1, CLIC2, CLIC3, CLIC4, CLIC5a, CLIC5b (or p64) and CLIC6 (or parchorin) [50, 51].

The CLIC family is defined by a conserved C-terminal module composed of approximately 240 amino acids that is common to all CLIC proteins. Several members containing also additional N-terminal domains, which are divergent both in sequence and in size [51, 56].

CLIC5b (or p64) was the first member of this family to be identified in 1989 by Landry and Al Awqati. It was isolated from microsomes of bovine kidney cortex and trachea apical epithelium and showed chloride selective channel function in lipid bilayers [52].

The cloning of p64 facilitated the identification of others CLICs: CLIC1 and CLIC4 were the first proteins to be cloned and functionally studied and they still remain the most characterized within the whole family [53, 54]. In particularly, CLIC1 (or NCC27) was first cloned in 1997 from a human monocytic cell line. It was initially detected on the nuclear membrane and then identified on the plasma membrane [53, 55].
The members of CLIC protein class are highly conserved in all vertebrates, suggesting their involvement in basic biological functions [57].

Differently from other ion channels families, CLIC proteins have the unique feature to exist either as cytoplasmic soluble proteins either as integral membrane proteins with ion channel activity [56-59]. Membrane insertion and channel conductance are regulated by a number of factors including cholesterol [60], increases of cytoplasmic oxidation [51, 61, 62], membrane phospholipid composition and pH changes [59, 63].

The CLIC proteins are localized in the plasma membranes and in a variety of intracellular membranes including nuclear membrane, endoplasmic reticular membrane, mitochondrial membrane, phagosomal and endosomal membranes, trans-Golgi vesicles and secretory vesicles [48, 57, 64].

They are expressed in a variety of tissues and regulate a wide range of important cellular events, including cell growth, cell division [65-67] and apoptosis [68, 69], cell motility [49, 70, 71], angiogenesis [47] and acidification of intracellular organelles [47, 72]. They are also involved in the processes of bone resorption [73], in tubulogenesis [74, 75], in formation of stereocilia [76], skeletal muscle and brain [77, 78] and in development of the organ of Corti [79, 80]. Furthermore, they participate to β-amyloid induced neurotoxicity [81], production of reactive oxygen species by activated microglia [82] and neurite elongation of retinal ganglion cell [83].

## 2.2.1 Structure and biophysical properties of CLIC1 protein

CLIC1 is a small protein composed by 241 amino acids, with a molecular weight of 27 kDa [53]. Being a metamorphic protein, it has different tertiary structures corresponding to the same primary sequence [84]. It is usually present in the cytoplasm and in the nucleoplasm in a soluble form, but following different stimuli it undergoes major structural changes and translocates into lipid membranes. Once inserted in the membranes it works as a chloride-selective ion channel [50, 57, 61].

The crystal structure of the soluble form of CLIC1 has been resolved few years ago by Harrop and colleagues in two crystal forms at 1.4-Å and 1.75-Å resolution (figure 2). It shares structural homology with the members of omega Glutathione S-transferases (Ω-GST) superfamily [56].

The N-terminus and the C- terminus domains are connected by a proline-rich region, in which proline 91 has been suggested to play a main role in the changes from the soluble to the membrane-inserted form of the protein, due to its change from cis to trans configuration [56]. The C-domain is all α-helical, closely resembling the Ω-GST. The N-domain (residues 1–90) has indeed a thioredoxin fold that consists of a four-stranded mixed β-sheet plus three α-helices. This domain has also a well conserved glutaredoxin-like site for interaction with glutathione (GSH), which renders this protein sensitive to the oxidative state of the cell. GSH appears to be covalently attached to Cys-24 through disulfide bond.

In oxidizing conditions, glutathione detaches from its binding site and the N-terminal domain of the protein undergoes conformational rearrangements that expose hydrophobic regions that are able to interact with cellular membranes [56].

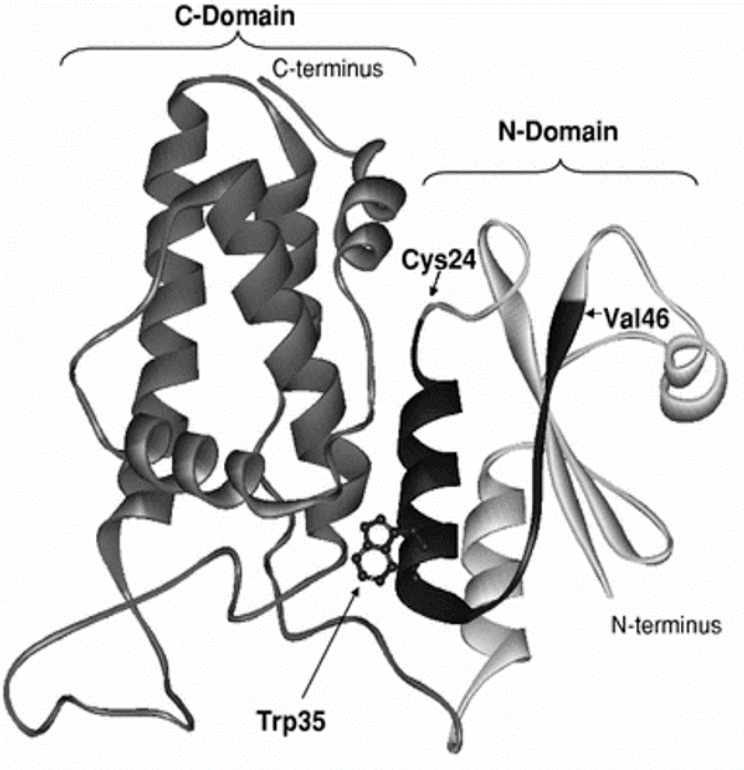


Figure 2. Crystal structure of CLIC1 monomer. The all α-helical C-terminal domain is shown on the left in grey. The N-terminal domain containing the thioredoxin fold is represented on the right in white. The putative transmembrane region (residues Cys25-Val46) is shown in black [61].

However, the crystal structure of the transmembrane form has not yet been resolved. It has been speculated that the region between Cys-24 and Val-46 located in the N-terminal domain may constitute a transmembrane helix to form CLIC1 transmembrane region. In the structure of the monomeric soluble form of CLIC1, this putative segment forms α-helix and a β strand within the glutaredoxin-like-N-domain. In the transition from the hydrophilic to the membrane binding form many structural rearrangement occur that may involve the N-domain and disrupt the glutathione-binding site [51, 59].

Changes in the cellular redox state determine a reversible transition of CLIC1 protein between a reduced, soluble, monomeric form and an oxidized, soluble, dimeric form, resolved by Littler and

colleagues in 2004 (figure 3) [51]. In this form, the glutaredoxin-like-N-domain has undergone a radical rearrangement to expose an extended hydrophobic region, which forms the dimer interface. The reversible transition is stabilized through the formation of an intramolecular disulfide bond between two cysteine residues (Cys-24 and Cys-59) [51, 85]. This dimer is able to form, in artificial lipid bilayers, chloride ion channels similar to the native channel [51].

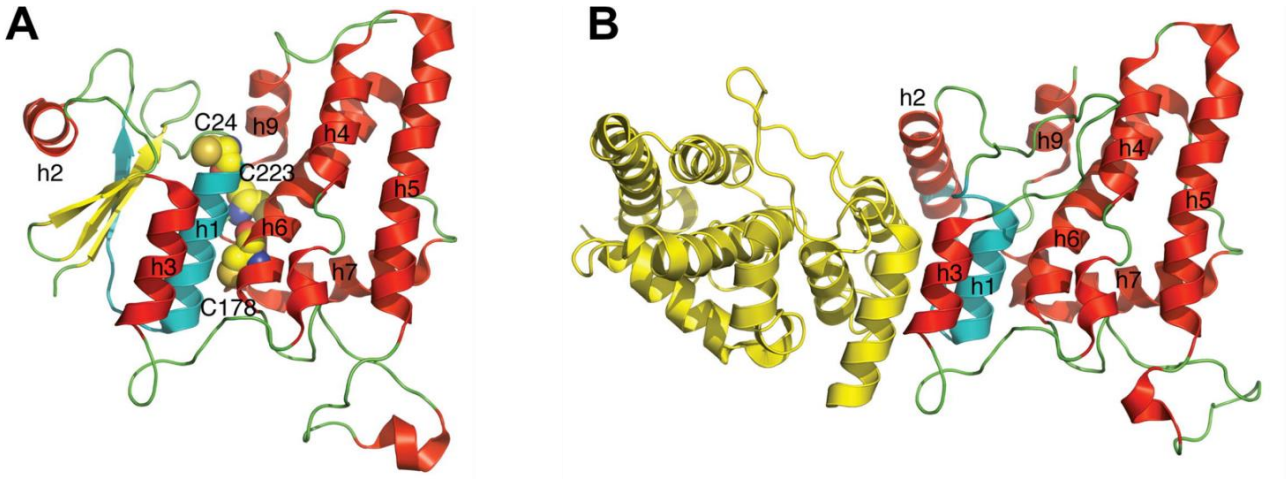


Figure 3. CLIC1 structural transition. (A) Structure of CLIC1 in the reduced monomeric form coloured by secondary structure (helices in red, strands in yellow, loops in green). The putative TM region is shown in cyan. (B) Crystal structure of the oxidized CLIC1 dimer. The left hand side subunit is coloured yellow, while the right hand side subunit is coloured and oriented as the CLIC1 monomer in panel (A) [51].

To further support the ion channel nature of CLIC1, it has been reported that a single point mutation (C24A, R29A, K37A) in CLIC1 putative transmembrane region results in alterations of the electrophysiological characteristics of the channel [85, 86].

The mechanisms by which CLIC protein undergoes membrane insertion and forms an active channel remains not fully elucidated. According to the model of Littler and colleagues [51], the hydrophobic region exposed after the oxidation-dependent transition may represent the membrane docking interface. Probably, an additional structural change is required to integrate the transmembrane domain into the membrane. This is also likely to be follow by oligomerization to form the active ion channel [51].

In a different study, Goodchild and colleagues suggest that the dimerization process during oxidation is not necessary for the insertion of the protein into membranes [61]. In this model, at first CLIC1 monomer interacts by itself with the membrane and only successively cytoplasmic oxidation promotes the structural changes that allow the protein to cross the membrane and form a functional ion channel (figure 4). They showed that both monomeric and dimeric forms are able to form functional ion channels in artificial membranes [61].

It has been demonstrated that, once inserted in the membrane, the protein exposes its N-terminus to the extracellular side, whereas the C-terminus remains on the intracellular side of the membrane [55]. Although it is still uncertain how many CLIC1 monomers participate to form the functionally active structure, once inserted in the membrane, CLIC1 is able to act as a selective chloride channel. Some hypothesis propose the association of two or four subunits, until the formation of small oligomers of six or eight subunits, to constitute one single ion channel [50, 55, 57, 61, 62, 87].

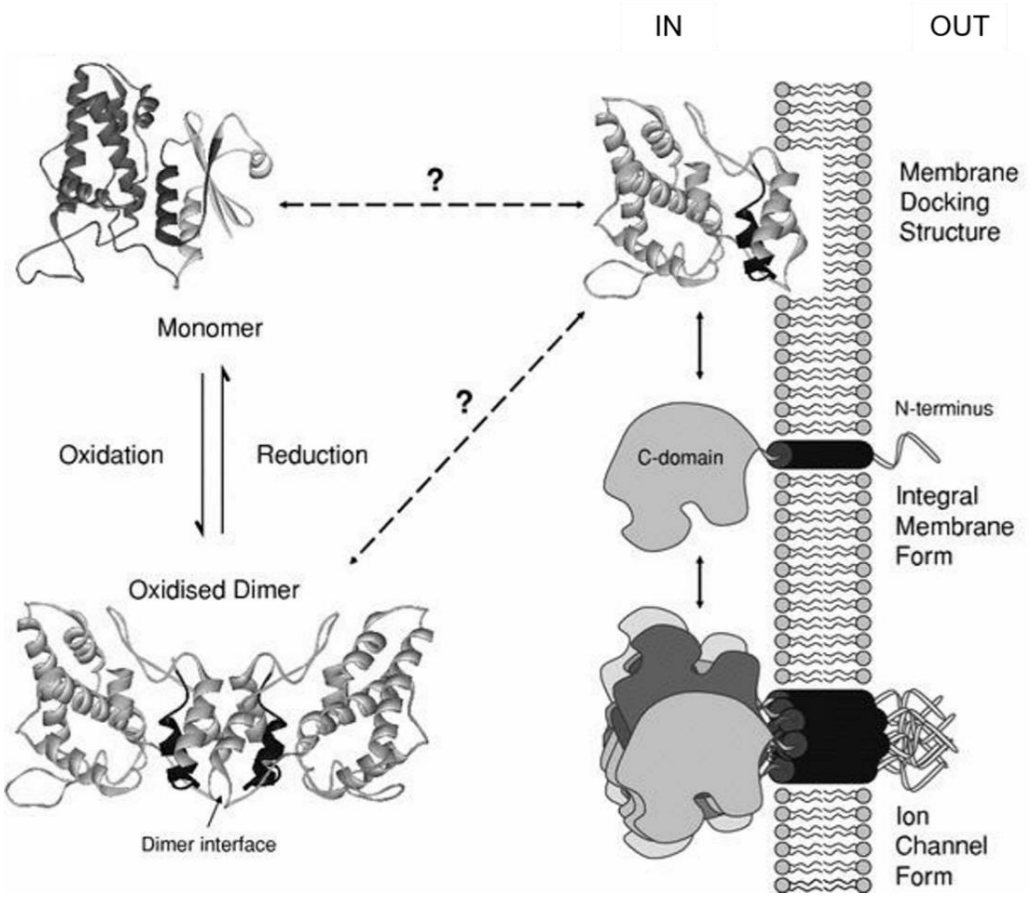


Figure 4. Proposed model of the CLIC1 membrane insertion to form an active ion channel. Following oxidation, the soluble CLIC1 monomer undergoes a reversible structural transition to an all α-helical, non-covalent dimer. As initial step in the process of membrane insertion, the hydrophobic surface in the dimer-interface may dock to the bilayer. After membrane docking, the second stage involves insertion of the N-terminus and putative TM domain (Cys24– Val46, black in all structures) across the lipid bilayer, while the C-domain remains in the cytosol. This results in the CLIC1 integral membrane form, while a number of integral membrane CLIC1 subunits then converge to form the active CLIC1 ion channel form [61].

In addition to oxidation, one of the contributing factor to CLIC1 membrane translocation is the variation of intracellular pH between the cytosol and the membrane [88]. In artificial lipid bilayers, CLIC1 channel activity has been demonstrated to be dependent from hydrogen ion concentration. Activity is minimal at pH 7 and it significantly increases at lower and higher pH values [59]. It has been hypothesized that soluble CLIC1 responds to the low pH found at membrane surface by

partially unfolding and restructuring into a membrane-competent conformation. This transition is proposed to be controlled by strategically located residues (Glu-85 and Glu-228) that become protonated at acidic pH and act as "pH-sensor" [89]. Recently, have been also identified two histidine residues (His-74 and His-185) which are involved in triggering the pH changes to CLIC1 conformational stability via their protonation [88].

Electrophysiology experiments performed in CHO (Chinese hamster ovary) cells transfected with a plasmid for CLIC1 overexpression allowed understanding the biophysical characteristics of the channel [65]. In physiological conditions, when the membrane voltage of the cells is more positive than the chloride reversal potential, CLIC1 mediates an outward current that rectified at +40/+50 mV. Conversely, a small inward current is recorded at potential more negative than the chloride reversal potential [55].

It has also been shown that CLIC1 activity is slightly voltage-dependent. Channel closures were rarely observed at voltages between ±50 mV, but occurred more frequently as membranes were largely hyperpolarized or depolarized [59].

The only CLIC1 specific inhibitor so far identified is IAA94 (Indanyloxyacetic acid 94), that completely and reversely blocked the channel mediated current [59, 90]. On the contrary, the most common chloride channels blocker DIDS (4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid) does not have any effect on the conductance of this channel [67]. Recently, the antidiabetic drug Metformin has been proposed as a blocker of CLIC1 channel activity [91].

## 2.2.2 CLIC1 role in cancer

CLIC1 is a dimorphic protein able to modulate its membrane insertion in response to oxidative conditions [61, 62]. It is possible to hypothesize that CLIC1 could serve as master regulator linking oxidoreductive state of the cell and extracellular environment with organization of the cytoskeleton, membrane trafficking, ion permeability, and gene expression [92].

Cell homeostatic mechanisms maintain redox balance with a fine regulation between production of reactive oxygen species (ROS) and their removal through the antioxidant systems [93]. A shift in the balance between oxidants and antioxidant defenses in favor of oxidants results in a condition of oxidative stress [94].

ROS are highly reactive molecules and can damage cell structures leading to loss of function and even cell death [94]. However, they play also important roles as secondary messengers in many intracellular signalling pathways that involve changes in the cellular redox state such as migration, differentiation and cell replication [95].

Altered oxidative state is a major feature of several pathological conditions, including many cancers [96-98] and neurodegenerative diseases [99-101]. In these pathological states, that share an overproduction of ROS as common characteristic, CLIC1 transmembrane form appears to have a crucial role [1, 81, 82, 102].

It has been previously demonstrated by our group that CLIC1 could act as both a "sensor" and an effector of oxidative stress [62]. In highly proliferating activated microglia cells, CLIC1 translocates to the plasma membrane in response to increase ROS generation by NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidase. Once inserted in the membrane, CLIC1 promotes an anionic current that, balancing the excess charge extruded by the active NADPH oxidase, supports ROS production by the enzyme [82]. It has been shown by our laboratory that in stimulated microglia cells CLIC1 is over-expressed [81] and its blockade limits the detrimental effects due to the over-activation of microglia [82].

It can be hypothesized that, like activated microglia, cancer cells could obtain selective advantages by the feed-forward mechanisms between CLIC1 channel activity and ROS production. Indeed, it is known that alterations in the oxidative basal level in the intracellular compartment are fundamental for the progression of cell cycle through the different phases [103]. Consistently with this idea, increased ROS levels in hyper-proliferating tumor cells could promote CLIC1 membrane insertion. CLIC1 chloride current in turn could sustain ROS production necessary for the progression through the cell cycle. Thus, increased CLIC1 expression and activity could lead to an increase of proliferation, migration and invasiveness of tumor cells.

Several studies have recently indicated that CLIC1 protein is significantly overexpressed in different tumors, compared to normal tissue, including: human breast ductal carcinoma [104], gastric cancer [105], gallbladder carcinoma [106], ovarian cancer [107], nasopharyngeal carcinoma [108]. Other malignancies in which CLIC1 is found to be up-regulated are: pancreatic ductal adenocarcinoma [109], hepatocellular carcinoma [110], high-grade gliomas [111], colorectal cancer [112] and prostate cancer [113]. Moreover, it has been reported that CLIC1 overexpression is generally correlated with a poor prognosis [105,106, 109,111]. Furthermore, this protein is sometimes detectable even in the plasma of patients [107,108, 112]. All these findings propose CLIC1 as possible tumor biomarker and so very useful in clinic [1].

Our idea is that the upregulation of CLIC1 protein in the plasma membrane is a precise sign of a cell in unbalanced redox state. The membrane translocation is a transient event in physiological conditions, but it can become chronic under conditions of prolonged ROS over-production, as occurs in tumors. This differential membrane insertion could allow discriminating malignant cells form non-cancerous cells, making CLIC1 an attracting pharmacological target. It is likely that in this way is possible selectively hit only the tumor cells and therefore limit the toxicity often associated with conventional antitumor therapies [1].

# 2.3 Colorectal cancer

Colorectal cancer is a major cause of morbidity and mortality throughout the world [114]. Accounting for over 9% of all cancer incidence, it is the third most common diagnosed cancer worldwide and represents the second leading cause of cancer-related death [115, 116].

The incidence rates are higher in more developed countries than in less developed ones, with the developed world that accounts for over 63% of all cases [116, 117].

Several risk factors are associated with the development of colorectal cancer, including advanced age [118-120], personal history of adenomatous polyps [117, 120] or inflammatory bowel disease [117, 119] and hereditary factors [121]. In addition, different environmental and lifestyle risk factors may play an important role in the incidence of colorectal cancer such as diets high in fats [116, 117], physical inactivity and excess body weight [115, 116], smoking and regular alcohol consumption [122].

About 70% of all cases of colorectal cancers (CRC) are sporadic, whereas about 10–30% are familiar and about 5–7% are hereditary [123].

In the biological behaviour and at the molecular level, CRCs are a very heterogeneous group of diseases. CRC originates from epithelial cells of gastrointestinal tract, which undergo sequential genetic and epigenetic mutations that alter normal mechanisms of proliferation and self-renewal [124]. These patterns of different mutations not only contribute to the onset and multistep progression of the CRC, but are also responsible for specific aggressiveness and response to the therapy [124].

According to the model proposed by Fearon and collegues, CRC development occurs through a series of steps morphologically identifiable. Initially normal epithelium becomes hyperproliferative mucosa and originates small benign adenomas, which progressively grow with dysplasia and ultimately evolve in invasive carcinoma and metastasis in about 10 years (figure 5) [125, 126].

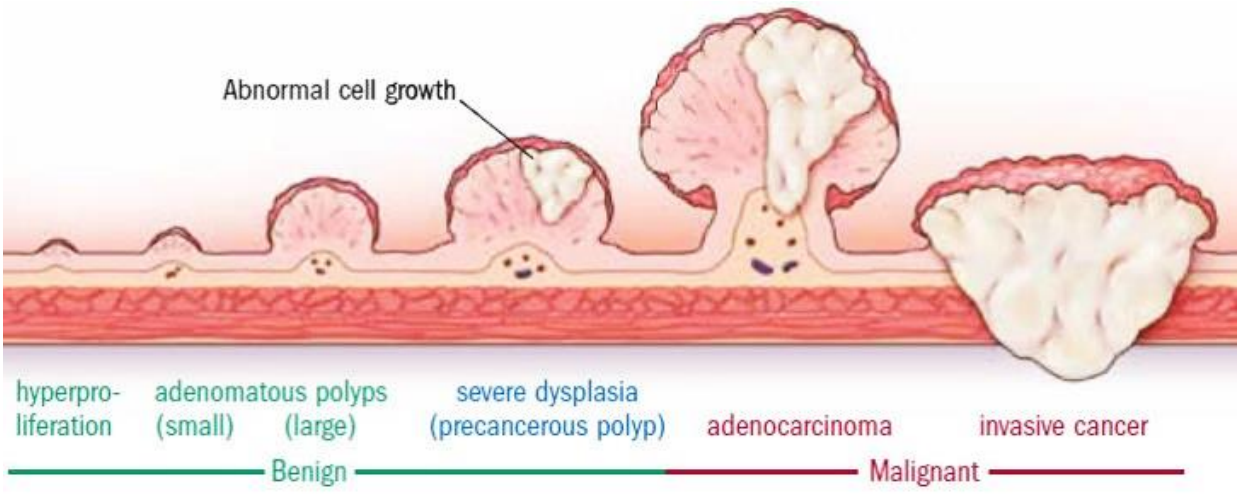


Figure 5. Stages of progression of human colon cancer (adapted from Johns Hopkins colon Cancer Center).

CRCs can arise from one or a combination of four different mechanisms: chromosomal instability, microsatellite instability, aberrant DNA methylation and DNA repair defects [127-130].

Several molecular signalling pathways are often dysfunctional in CRC. Among these, Wnt/APC/β-catenin pathway is the most frequently altered in sporadic CRC. It promotes tumor cell proliferation, regulates endothelial function and inhibits differentiation [131, 132]. PI3K/AKT/PTEN pathway is often found deregulated in both sporadic and hereditary CRC. It controls metabolism, activates cell growth and inhibits apoptosis in response to several extracellular stimuli [133, 134]. RAS/RAF pathway is dysregulated in at least 50% of CRCs and leads to increased proliferation and reduced apoptosis [135]. TGF-β/Smad pathway, usually highly expressed during the late stages of CRC, stimulates tumor cell proliferation and angiogenesis and promotes invasion and immuno-suppression [136]. NF-κB pathway is found constitutively activated in almost 40% of CRC and promotes proliferation and resistance to chemotherapy [137]. GSK-3β is another pathway frequently overexpressed in CRC and acts as a tumor promoter [138], regulating the cross-talk between Wnt and NF-κB [139].

Although the symptoms of CRC may vary depending on the location of the neoplastic lesions, the tumor generally causes only occult bleeding [124]. For this reason, 20–25% of patients at the moment of the first diagnosis have already developed metastasis [140, 141], most frequently in the liver [124].

Appropriate diagnosis and staging are crucial to predict clinical outcomes and ensure a correct treatment strategy [140]. Currently, optical colonoscopy is considered the gold-standard investigation in the early detection of CRC thanks to its high diagnostic performance [142]. Instead, the most used imaging modalities for staging of CRC are chest/abdomen/pelvis computed tomography (CT) [141] and magnetic resonance imaging (MRI) [144].
CRCs are commonly classified on the basis of the tumor, node, metastases (TNM) staging system, according to local tumor penetration (T stage), lymph node involvement (N stage) and presence of distant metastases (M stage) [143].
Surgical resection represents the mainstay treatment for early CRC [145], but in patients with advanced or metastatic disease the administration of systemic chemotherapy cocktails (5-fluorouracil, irinotecan, and oxaliplatin) is required [146].

The prognosis of CRC is closely related to the stage of disease at time of the first diagnosis [147]. The 5-year survival rate typically ranges from a 90% for cancers detected at the localized stage, 70% for regional diffused cancers, to 10% for distant metastatic cancers [148, 149]. Thus, the possibility to detect the disease in an earlier stage is crucial to ensure an effective treatment strategy of CRCs.

In the last decade mortality rate of CRC has significantly decreased, due to advances in diagnostic techniques, improvement in surgical techniques and more effective chemotherapy approaches [150]. Despite these clinical progresses, metastatic CRC remains incurable [124, 140]. One of the primary reasons for treatment failure and tumor relapse is believed to be the presence of intrinsic and acquired resistance to conventional therapies [151, 152]. Drug resistance occurs in nearly all patients with CRC [153] and is mainly due to overexpression of ATP-binding cassette (ABC) transporters [154, 155] and defects in the apoptotic signalling pathways [156]. In this scenario, finding novel pharmacological targets that allow to overcoming drug resistance is fundamental to achieve successful therapy for colon cancer [154].

CLIC1 expression has been found to be up-regulated in colorectal cancer, compared to normal colon tissue [112]. Moreover, Wang and colleagues have reported that CLIC1 modulates migration and invasion of LOVO colon cancer cells through its regulatory volume decrease (RVD)-mediating chloride channel function [157]. In another paper, they have also shown that CLIC1 promotes the metastasis of LOVO cells via regulating the ROS/ERK pathway under hypoxia-reoxygenation (H-R) conditions [158].

All these findings suggest an involvement of CLIC1 in development and progression of CRC; however, the role performed by this protein in the tumorigenic process remains unknown.

# 2.4 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is the most common proliferative abnormality of the human prostate throughout the world [159]. It is a non-malignant enlargement of the prostate gland caused by progressive hyperplasia of stromal and glandular prostatic cells [160].

BPH is an age-associated disease: half of all men aged 51–60 have histologically identifiable BPH and by age 85, the prevalence increases to approximately 90% [159].

Large-scale epidemiological studies have suggested that men with BPH have an increased risk of prostate cancer, but there is still no confirmation whether this association reflects a causal relation [61, 62]. Conversely, BPH has been directly associated with diabetes mellitus [163].

Androgenic signalling is the primary stimuli for prostatic proliferation and BPH development. However, estrogen action via estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) seems to be also implicated [164]. ER-β regulates cellular growth and promotes apoptotic signalling pathways, whereas ER-α promotes cellular proliferation and enhances survival and mitogenic pathway [164, 165]. Several growth factors contribute to BPH progression. In particular, insulin growth factor-1 (IGF-1) action via its receptor IGF-1R has been shown to promote prostatic growth and development via activation of phosphoinositol-3-kinase (PI3K)/protein kinase B (PKB)/Akt/cyclin D pathway [166, 167].

BPH is a serious urinary system disorder and may cause physical compression of the prostatic urethra leading to impaired voiding [168]. It can clinically present as lower urinary tract symptoms, urinary tract infections, acute urinary retention, renal failure haematuria, and bladder calculi [169].

Medical therapy with 5-alpha-reductase inhibitors and alpha-blockers is the most frequently used treatment for BPH [170]. In the setting, when medical therapy becomes ineffective, prostatectomy by open surgery or transurethral resection of the prostate is considered the primary method of treatment [171].

A recent study has demonstrated that anti-diabetic drug metformin inhibits the proliferation of BPH cells by suppressing the expression of IGF-1R and IGF-1 secretion in stromal cells [172].

# 2.5 Prostate cancer

Prostate cancer (PCa) is the most common non-cutaneous malignancy in American men, currently accounting for 29% of all diagnosed cancers [173]. It ranks second as leading cause of male cancer mortality in both the US and Europe [174, 175], representing 13% of all cancer fatalities [173].

Well-known risk factors for PCa include old age, black ethnicity, and a family history of the disease [176]. Moreover, some lifestyle risk factors such as endogenous hormones, obesity, cancer-related infections or inflammations and sexually transmitted diseases have also been suggested to predispose to PCa [176, 177].

PCa is generally a slowly progressing disease [178] and in most cases it has a long preclinical phase from the onset to the appearance of clinical symptoms [179]. To date it is not possible to diagnostically distinguish between an indolent localized tumor with low metastatic potential and an aggressive localized tumor with high metastatic potential [180].

PCa is a heterogeneous disease with multifocal nature [181]. Around 70% of PCa cases originate in the peripheral zone, 15%–20% in the central zone, and only 10%–15% in the transition zone of the prostate gland [182]. Most PCa cases are acinar adenocarcinomas that arise from prostatic epithelial cells expressing androgen receptor (AR) [183].

According to model of prostate cancer progression proposed by Pienta and colleagues, normal prostate epithelium gives rise to localized prostate cancer, that evolves in adenocarcinoma, and finally progresses to metastatic androgen-independent prostate cancer (figure 6) [184].

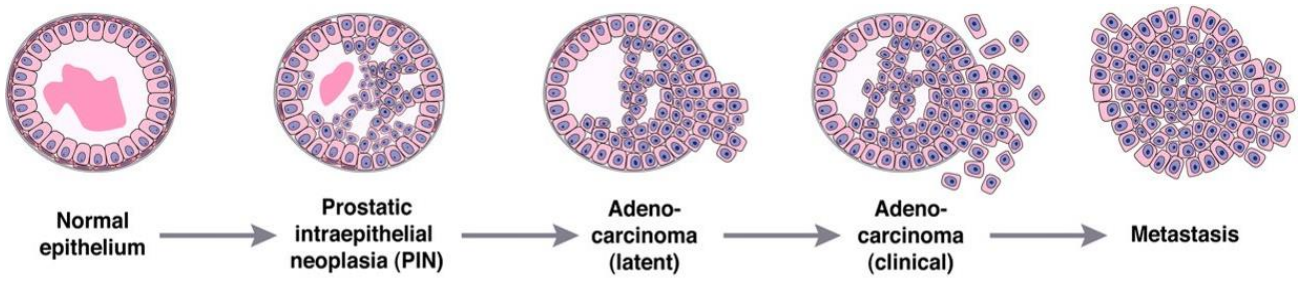


Figure 6. Stages of progression of human prostate cancer (adapted from Shen MM., Abate-Shen C. [180]).

The pattern of differentiation and proliferation abnormalities that trigger the PCa initiation and development involves multiple genetic and epigenetic modifications, such as loss of heterozygosity, activation of oncogenes, and loss of tumor suppressor genes [185].

Early steps of cancer progression include a down-regulation of epithelial cadherin (E-cadherin) and abnormal β-catenin signalling, that compromise cell-cell binding. Next, Ras and other GTP-binding proteins promote malignant cell motility and migration. Malignant cells become motile and acquire the ability to degradate the extracellular matrix. They enter in the bloodstream and, binding to endothelial surfaces at secondary sites, undergo trans-endothelial migration. Once in a secondary site, tumor cells perturb the normal microenvironment and establish metastasis [186].

Several signalling pathways appear to play a critical role in PCa tumorigenesis. Among these, the androgen receptor signalling pathway promotes cell proliferation, differentiation, apoptosis, and secretion [187, 188]. Estrogens signalling pathway induces DNA damage and production of high levels of ROS [189], promotes metastasis formation [190], stimulates proliferation and differentiation of PCa cells [191]. TGF-β signalling exhibits growth inhibitory effects in the early stages of PCa and promotes malignancy in later stages. It enhances prostate tumor growth, angiogenesis, immunosuppression, remodelling of the extracellular matrix, and bone metastasis formation [192]. IGF-1 signalling pathway is responsible for sustained proliferation of PCa cells [193] and tumor progression [194]. PI3K pathway promotes cell proliferation, survival [195] and cell invasion [196]. EGF, FGF and VEGF pathways are frequently up-regulated in advanced PCa and are involved in cell growth, proliferation, migration, and angiogenesis [197].

Prostatic cancer is rarely symptomatic early in its course and it is most commonly detected through screening [178]. Currently, conventional screening for PCa involves digital rectal examination (DRE), PSA blood test, and transrectal ultrasonography (TRUS), whereas definitive diagnosis is done with prostate biopsy [197].
PCa are commonly classified according with the Gleason score from 1 to 5, with 5 being the most aggressive and undifferentiated, based on their most prevalent architecture [200].

PCa has a high propensity to metastasize, mainly in the bone [198]. The potential presence of bone metastasis is detected using bone scintigraphy (BS) and X-ray radiography [199].
Localized tumors are treated by radical prostatectomy or radiotherapy, while metastatic prostate cancers are treated by androgen deprivation therapy [180, 197]. Initially the majority of metastatic PCa, being androgen-dependent for their growth, are very responsive to androgen deprivation [188]. However, they eventually become androgen-independent and the treatment lately results in the progression to a hormone-refractory cancer [180, 187, 197, 201]. For the advanced PCa the treatment of choice is systemic chemotherapy with taxanes, but the overall benefit was modest [201]. Indeed, although localized prostate cancer is potentially curable, when cancer becomes metastatic no curative therapy is nowadays available [202], with only 10% of patients living 10 years beyond diagnosis [203].

Intrinsic and acquired drug resistance represents the main obstacle for an effective cure of PCa [204]. Different mechanisms contribute to the insurgence of PCa chemoresistance, including activation of aberrant AR and ABC transporters, inactivation of tumor suppressor genes, evasion of apoptosis and cross-talk among key signalling pathways [180, 201]. Therefore, the discover of new therapeutic approaches able to overcoming drug resistance will provide a significant improvement in the treatment of advanced PCa [180].

Concerning the research of new pharmacological target for PCa, CLIC1 protein appears as potential candidate. Indeed, it has been found overexpressed in prostate cancer tissue, compared to normal prostate [113]. In addition, Tian and colleagues have proposed that CLIC1 could regulate prostate cancer cell proliferation and migration [205]. However, the effective involvement of CLIC1 in PCa has not yet been elucidated.

# 2.6 Metformin and cancer

## 2.6.1 Pharmacological properties

Metformin (1,1-dimethylbiguanide) is an anti-hyperglycaemic drug belonging to the biguanides family. It is a small-molecule weight (129.2 KDa), water-soluble, which at physiological pH values exists as organic cation [206, 207].

Firstly discovered in the 1920s in extracts of the plant *Galega officinalis* (French lilac), metformin has been used as glucose-lowering compound since 1957 in Europe and since 1995 in USA [207, 208]. Nowadays it represents one of the most commonly prescribed drugs, with nearly 120 million prescriptions filled yearly worldwide [209].

The physicochemical properties of metformin do not promote its efficient membrane permeation by passive diffusion [206]. The drug is widely distributed into body tissue by organic cation transporters [210]. The main active transporters, which mediate the uptake of metformin across the liver, intestine and kidney tissues, are solute carrier family 22 members (SLC22A) 1, 2, 3 and 4 (also known as OCT1-4), multidrug and toxin extrusion protein (MATE) 1 and 2, and the plasma membrane monoamine transporter hENT4 (also known as PMAT) [208, 210, 211]. It is also able to cross the blood-brain barrier [212, 213].

Metformin has an absolute oral bioavailability of 40 to 60% [214] and gastrointestinal absorption is apparently complete within 6 hours of ingestion [215]. An inverse relationship was observed between the dose ingested and the relative absorption with therapeutic doses ranging from 0.5 to 2.5g, suggesting the involvement of an active, saturable absorption process. It is rapidly distributed following absorption and does not bind to plasma proteins [215]. Metformin is not metabolized [216] and 90% is excreted unchanged in the urine with a plasma elimination half-life after oral administration of ~5 h [210].

Although metformin has been representing for many years the most widely used drug against type 2 diabetes, the exact molecular mechanisms underlying its anti-diabetic action are not completely understood [208]. Metformin lowers blood glucose levels and improves peripheral tissue sensitivity to insulin [208]. Its antihyperglycemic action is mainly due to the inhibition of hepatic glucose production and, in lesser extent, to an increased insulin-mediated glucose uptake in the skeletal muscle and a decreased intestinal glucose absorption [208, 217, 218]. The liver is considered to be the main site of action of metformin. In this organ, biguanide increases the activity of the insulin receptor and of its substrate (IRS-2) and enhances glucose uptake via increased membrane translocation of glucose transporters. The net effect is that metformin inhibits the gluconeogenesis, abrogates the glucagon's action and stimulates the glycolysis [208]. This drug also appears to have additional beneficial effects, including body weight reduction, lowering plasma and hepatic lipid levels, decreasing of fibrinolytic activity, modulation of inflammatory markers and prevention of some cardiovascular complications [208, 219, 220].

The most common side effects associated with metformin are mild, transient, gastrointestinal symptoms, which are usually self-limiting [217]. In addition, it does not stimulate insulin secretion thus it has the clinical advantage of not inducing hypoglycaemia [218]. The low occurrence of side effects and the good tolerability of metformin have made this drug the first-line therapy for treatment of type 2 diabetes mellitus [218, 219].

## 2.6.2 Role as anticancer agent

Recently, the possible antitumor effect of metformin has become the subject of a number of investigations. The potential for application of metformin in oncology was first recognized in retrospective epidemiological studies of diabetic patients with cancer. Numerous observational studies have indeed reported a reduced cancer incidence [121, 122] and mortality rate [123, 124] and an improved cancer prognosis [125] in patients treated with standard doses of metformin.

Accumulating evidence suggest an antineoplastic activity of metformin in different human solid tumors, including colorectal and prostate cancer [209]. It has been reported that metformin inhibits proliferation, migration and invasion of both PCa [225-229] and CRC cells [230, 231] *in vitro*. It also inhibits the growth of colon cancer [232, 233] and prostate cancer [227, 228, 234] *in vivo*.

Several studies have further supported the idea that metformin plays an antineoplastic role in prostate and colorectal cancer. In diabetic patients the use of metformin seems to decrease the risk of incidence [235, 236, 317] and of recurrence of PCa [231, 237] and improve the overall survival [238, 239]. Metformin treatment was also associated with reduced incidence of CRC [240, 318] and improved survival outcome [241- 243] among diabetic patients. In addition, a recent clinical trial has shown that short-term use of metformin suppressed both colonic epithelial proliferation and colorectal aberrant crypt foci formation in non-diabetic patients [244-246].

There are also a number of findings showing that metformin has synergistic or enhanced effects when used in combination with a range of other therapies [247, 248] and it increases the sensitivity of prostate cancer cells to radiotherapy treatment [249, 250].

Furthermore, recent studies have demonstrated that metformin may also target cancer stem cells (CSCs) in different cancers, including prostate and colon cancer [251, 252]. These findings are particularly relevant considering that CSCs are resistant to most conventional cancer therapies and are the main responsible for metastasis and tumor relapse [253].

### 2.6.3 Mechanisms of action in cancer

The proposed anticancer activity of metformin is a combination of both indirect and direct effects (figure 7) [254, 255].

The indirect (systemic) action is secondary to the effects of the drug on metabolism in insulin-sensitive target tissues [256]. Insulin has mitogenic and pro-survival effects and tumor cells often express high levels of the insulin receptor [257]. The insulin-lowering effect of metformin may play a major role in anticancer activity, preventing insulin-mediated tumor growth and progression. Another potential antineoplastic mechanism is based on the capacity of metformin to inhibit chronic inflammation, a main contributory factor to cancer development and progression. In addition, metformin may participate in the reduction of tumor growth also through inhibition of neoplastic angiogenesis [208, 220].

The direct (insulin-independent) effects of metformin are mainly associated with the inhibition of the mammalian target of rapamycin complex 1 (mTORC1) signalling through regulation at multiple

levels [255]. mTOR is a key integrator of growth factors and nutrient signals and upregulates many energy consuming cellular processes. It plays a pivotal role in regulating metabolism, growth and proliferation of cancer cells by controlling mRNA translation and ribosome biogenesis [258].

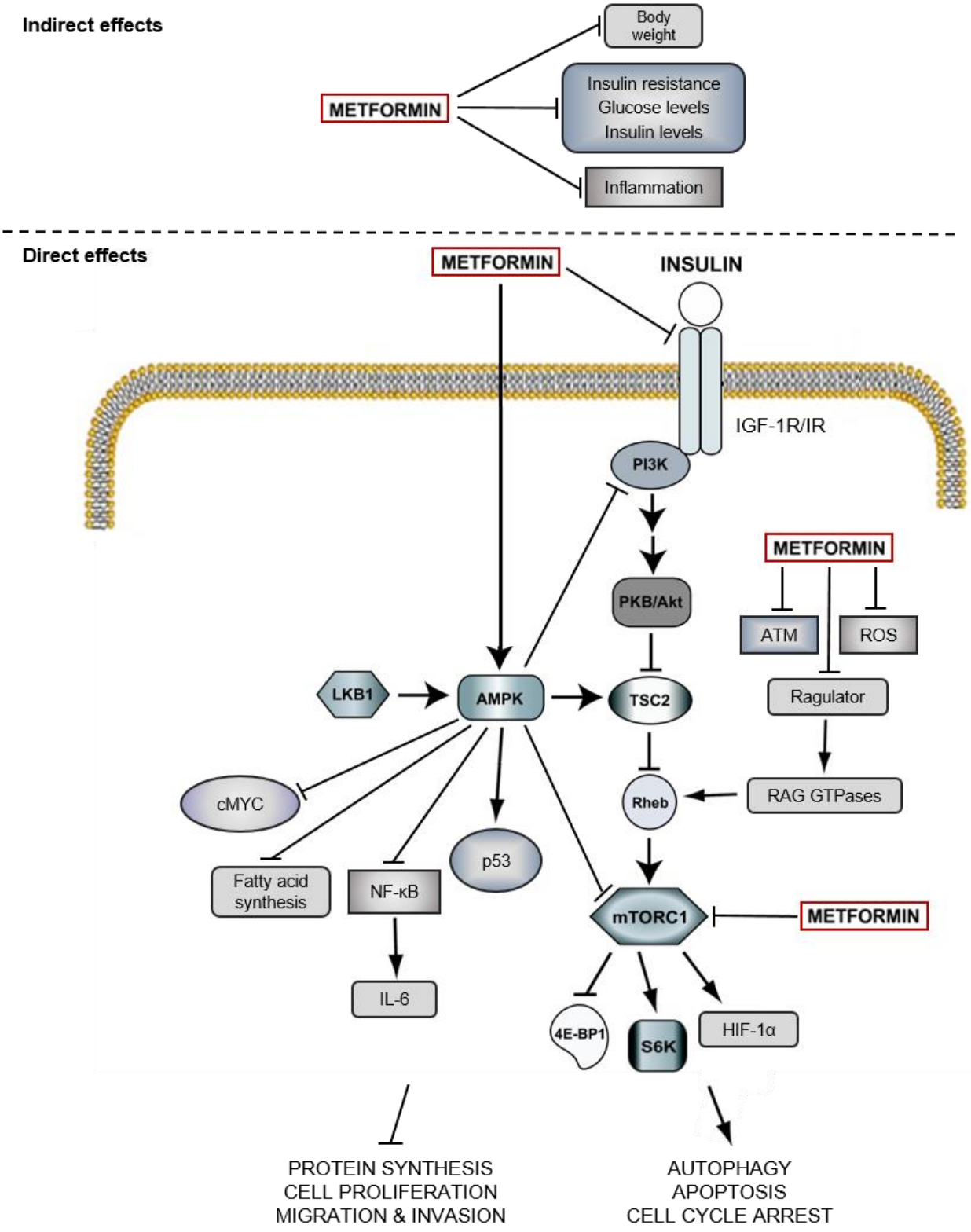


Figure 7. Proposed actions of metformin in cancer (adapted from Dowling R.J., Zakikhani M., et al. [255]).

Increased systemic level of insulin leads to increased liver production of IGF-1 that binds to IGF-1 receptor and insulin receptor. Then, through insulin receptor substrate (IRS), the signal is transmitted to phosphoinositide 3-kinase (PI3K) and Akt/protein kinase B (PKB) that indirectly activates mTORC1. PI3K/PKB/Akt signalling pathway is one of the most frequently deregulated molecular networks in human cancer [259]. Insulin receptor, through growth factor receptor-bound protein 2 (GRB2), propagates signal also to Ras/Raf/ERK pathway, that promotes cell growth [254] and plays an important role in changes of cellular metabolism that are typical features of tumor cells [260]. Increased levels of circulating insulin/IGF1 and upregulation of insulin/IGF receptor signalling pathways were demonstrated to be involved in the formation of many types of cancer. Metformin was found to reduce insulin level, inhibit insulin/IGF signalling pathways, and modify cellular metabolism in normal and cancer cells [261].

Different researches suggest that metformin could inhibit mTOR signalling through a pathway dependent or independent by AMP-activated protein kinase (AMPK) activation [254]. The energy-sensing kinase AMPK is inactive unless it has been phosphorylated by upstream kinases in response to cellular stresses that deplete cellular energy level and increase the AMP/ATP ratio [316]. AMPK, once activated by liver kinase B1 (LKB1), phosphorylates and activates tuberous sclerosis complex protein 2 (TSC2), resulting in the inhibition of the mTORC1 activator RHEB [262]. AMPK can also directly phosphorylate and inhibit RAPTOR, a member of the mTORC1 complex [208]. Metformin-mediated inhibition of mTOR signalling determinates a reduction in phosphorylation of its major downstream effectors, the eukaryotic initiation factor 4E-binding proteins (4E-BPs) and ribosomal protein S6 kinases (S6Ks), leading to an inhibition of global protein synthesis and cancer cell growth [255, 263]. Metformin can also downregulate mTORC1 signalling in an AMPK-independent manner via inactivation of the Ragulator complex, which results in inhibition of RAG GTPase and dissociation of mTORC1 from the activator RHEB [208, 264].

Another mode of action of metformin might be through the AMPK-mediated regulation of fatty acid synthesis and stimulation of fatty acid oxidation. Indeed, different tumor cells, including prostate and colon cancer cells, constitutively overexpress fatty acid synthase (FAS), which has been associated with a malignant phenotype. It has been observed that the AMPK-dependent reduction of FAS expression decreases the viability and growth of prostate cancer cells [220]. In addition, metformin has been proposed to hinder cancer cell growth through AMPK-dependent inhibition of the proto-oncogene c-MYC and the hypoxia-inducible factor-1α (HIF-1α), a transcription factor important for metabolic transformation in cancer. AMPK may also be a mediator of a reduced inflammatory feedback loop mediated by NF-κB/IL-6 pathway, restraining malignant transformation [208, 316]. Metformin also exerts its anticancer effect through induction of cell cycle arrest via a downregulation of cyclin D1 protein expression. Furthermore, metformin

has been shown to promote cell death of some cancer cells with the activation of apoptotic pathways [220, 316].

An antineoplastic action of metformin independent of AMPK activation may be the suppression of HER2 oncoprotein overexpression in breast cancer cells [220, 316]. Moreover, metformin has been found to be relevant to JNK/p38 MAPK pathway, influencing cancer cell growth, proliferation, differentiation, apoptosis and migration. Other possible AMPK-independent effects comprise inhibition of the kinase ATM and decrease of ROS levels, which confer mutagenesis risk [208].

The tumor suppressor protein p53 is involved in cell metabolism and control of cell cycle and its expression and phosphorylation are regulated by AMPK. Participation of p53 to metformin action is discussed: growing evidence revealed that in different tumors metformin blocked cell cycle in G0/G1 phase with a significant decreased expression of G1 cyclins and without changes in p53 status [265]. However, other researches indicated that inhibitory effect on cancer cell growth of metformin was associated with p53 activity [266].

Despite increasing preclinical studies confirm the efficacy of metformin to inhibit cancer cell proliferation *in vitro* and cancer growth *in vivo*, the effective anti-neoplastic properties against prostate and colon cancer are unclear and the molecular mechanisms underlying its action remain poorly understood [220].

Recently, our laboratory has shown that metformin induces anti-proliferative effects in glioblastoma (GBM) CSCs, recognizing CLIC1 channel as main molecular target [91].

It has been demonstrated that metformin interacts with CLIC1 channel from the external site of the membrane by binding the Arg29 inside the pore. Using its guanidinium motif, metformin may displace the side chain of Arg29 from the polar pocket, stabilizing the closed state and possibly obstructing the channel pore. It has been suggested that metformin can reach its binding site only when CLIC1 is in the open state. Thus, metformin effect directly depends on the probability of the channel to be in the open state [91].

# -3-

# MATERIALS AND METHODS

# 3.1 Cell cultures

The following prostate cell lines have been used: BPH1 cell line was kindly provided by Professor F. Montorsi's laboratory, Vita Salute San Raffaele University (Milan) and PC3 cell line was kindly provided by Professor A. Poletti's laboratory, University of Milan (Milan).

BPH1 and PC3 cells were grown in RPMI 1640 medium at 37°C in humidified air containing 5% of $CO_2$.

CCD841 CoN and SW620 colon cell lines were a kind gift of Dr. Costanza Giampietro, ETH Zurich, Zurich. CCD841 CoN (or CCD841) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in 5% $CO_2$-humidified atmosphere at 37°C, whereas SW620 cells were grown in Leibowitz's L-15 in incubator at 37°C without $CO_2$.

All the cell culture medium were supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (GIBCO) and Penicillin-Streptomycin (100 U/l) and Glutamine (2 mM) (Thermo Fisher Scientific).

For the culture, the cells were routinely passaged using trypsin/EDTA (0.05%/0.02% in PBS) (Sigma Aldrich) when they reached sub-confluence.

In all the experiments performed, the number of seeded cells was adjusted to a density that allowed the cells to grow exponentially for the full duration of the assay.

# 3.2 Reagents

Indanyloxyacetic acid 94 (IAA94) has been dissolved in absolute ethanol to make a 50 mM stock solution and stored at -20°C. It was used at final concentration of 100 µM. This concentration is known to completely, selectively and reversely block the chloride current of CLIC1 channel [59, 90].

4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS) has been dissolved in DMSO to form 50 mM stock solution and stored at −20°C. It was used at final concentration of 200 µM. This concentration inhibits the activity of the other chloride channels but it has no effect on CLIC1 conductance [67].

1,1-dimethylbiguanide hydrochloride (Metformin) has been dissolved at stock concentration 1 M in H2O. It was used at working concentration of 10 mM. This dose was previously observed being able to block CLIC1 channel activity as well as other intracellular targets [91].

All the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The treatments were performed by diluting the stock solution to the final concentration in fresh culture medium on the day of the experiment.

## 3.3 Protein extraction and Western blot analysis

Cells were plated at a density of $4\times10^5$ cells/well in p35 petri dishes. For the analysis of CLIC1 protein level, the cells were grown in complete medium. For MAPK activation and caspase-3 experiments cells were serum-starved (DMEM + 0.1% FBS for colon cells and RPMI + 0.1% FBS for prostate cells) for 24 hours and then were incubated in fresh serum-free medium with or without IAA94 or metformin for 48 hours.

At the end of the experimental time cells were directly lysed with the addition of 60 µl of hot lysis buffer (LB: 0.25 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol in $H_2O$) and scraped off the petri dishes. The lysates were then syringed and boiled for 10 minutes at 95°C to have a complete protein denaturation and centrifuged at 15,000 rpm for 15 min. Supernatants were collected and protein concentration was determinated by Pierce BCA assay (Thermo Fisher Scientific) reading the absorbance at the *EnSight* Multimode *Plate Reader* (PerkinElmer's).

For immunoblot analyses, equal amounts of protein (30–40 µg) for each sample was loaded onto a 12% or 15% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Separated proteins were then transferred onto nitrocellulose membranes (Amersham Protran, GE Healthcare) with 0.45 µm pore size into a transfer buffer (glycine 1%, tris-base 0.02 M, methanol 20%) for 1 h at 100 V constant or overnight at 30 V constant.

The membranes were blocked with 5% Bovine Serum Albumin (BSA) or 5% non-fat dry milk in PBS-0.2% Tween for 1 hour at room temperature and then incubated with primary antibodies against desired molecules overnight at 4°C. After washing in PBS-0.2% Tween, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive protein bands were detected with SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

For some experiments, membranes were stripped by incubation in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) for 20 min at 37 °C. Stripped blots were then rinsed extensively with TBST and hybridized again as described above.

Imagines were captured with *ChemiDoc Touch* Imaging System (Bio-Rad) and the intensity of the bands corresponding to the protein expression level was measured using Image Lab software (Bio-Rad). Results were normalized to levels of the housekeeping proteins.

The following antibodies were used: mouse monoclonal anti-CLIC1 (Santa Cruz Biotechnology), 1:750, rabbit monoclonal anti-p44/42 MAPK (Cell Signalling), 1:1000, rabbit monoclonal anti-phospho-p44/42 MAPK (Cell Signalling), 1:1000, rabbit monoclonal anti-cleaved caspase 3 (Cell Signalling), 1:1000, mouse monoclonal anti-β-Tubulin (Sigma Aldrich), 1:10000, mouse monoclonal anti-Vinculin (Sigma Aldrich) 1:2000, secondary anti-mouse and anti-rabbit antibody-HRP conjugated (Sigma Aldrich) 1:10000.

# 3.4 Immunofluorescence

Indirect immunofluorescence with an antibody direct against whole protein was used to evaluate CLIC1 localization.

Cells were seeded at a density of $5 \times 10^3$ cells for each 12 mm diameter cover glass and grown for 24 h. The cells were then rinsed with PBS $Ca^{2+}/Mg^{2+}$ and fixed with 2% paraformaldehyde in PBS for 12 min at room temperature. After some washing with PBS $Ca^{2+}/Mg^{2+}$ the samples are permeabilized with 0.05% Triton X-100, 1% Bovine Serum Albumin (BSA) in PBS $Ca^{2+}/Mg^{2+}$ for 5 minutes and non-specific binding sites are blocked through an 1 hour incubation with BSA 5 % in PBS $Ca^{2+}/Mg^{2+}$ at room temperature.
Cells are washed four times with PBS $Ca^{2+}/Mg^{2+}$ and then incubated with the primary mouse monoclonal anti-CLIC1 antibody (Santa Cruz Biotechnology) diluted 1:150 in 2% BSA-PBS $Ca^{2+}/Mg^{2+}$, for two hours at room temperature or overnight at 4°C. After different washes with PBS $Ca^{2+}/Mg^{2+}$, the samples are incubated with the secondary anti-mouse Alexa Fluor 488 antibody (Thermo Fisher Scientific) diluted 1:400 in 2% BSA-PBS $Ca^{2+}/Mg^{2+}$ for one hour in the dark.
To visualize cells nuclei the samples are incubated with 0.1 µg/ml DAPI (Sigma Aldrich) for 15 minutes in the dark and finally mounted on microscope slides using mowiol- mounting medium.

Samples were observed under a confocal microscope Leica TCS SP2 with a Leica HCX PL APO 63X/1.4 NA oil immersion objective. To acquire images Leica confocal software was used. Laser

lines 405 nm to see DAPI and 488 nm to see green fluorescence were used; the channels were acquired sequentially to avoid crosstalk problems. Images were analyzed using ImageJ software.

Only adjustments of brightness and contrast were used in the preparation of the figures. For comparison purposes, different sample images of the same antigen were acquired under constant acquisition settings.

The CLIC1 antibodies currently available are all directed against the C-terminal intracellular portion of the protein, limiting the possibility of a differential staining between the cytosolic and membrane CLIC1 fraction. Fixation and permeabilization steps required by the intracellular staining procedure, do not allow to perform live cell imaging.

A colocalization analysis with a cell membrane marker was not performed. The main technical limitations are due to the difficulty of distinguishing the few moleculecules of transmembrane CLIC1 from the large fraction of CLIC1 in cytoplasmic form and of staining an antigen that it is not stably expressed but dynamically in movement between cytosol and membrane.

# 3.5 Patch clamp

The patch clamp technique was used for the measurement of the ionic currents passing through CLIC1 channel.

The patch electrodes (BB150F-8P with filament, Science Products) with a diameter of 1.5 mm, were pulled from hard borosilicate glass on a Brown-Flaming P-87 puller (Sutter Instrument, Novato, CA) and fire-polished to a tip diameter of 1-1.5 µm and an electrical resistance of 3-4 MΩ. The cells were voltage-clamped using an Axopatch 200 B amplifier (Axon Instrument) in the perforated patch configuration. Pipette contained Gramicidin in a final concentration of 5 µg/ml. This antibiotic, forming in the membrane pores permeable only to monovalent cations, provides electrical access to the cell interior preserving the internal chloride concentration of the cells.

Ionic currents were digitized at 5 kHz and filtered at 1 kHz. Clampex 8 was used as the interface acquisition program. The voltage protocol consisted of 800 ms pulses from -40 mV to + 60 mV (20 mV voltage steps). The holding potential was set according to the resting potential of the single cell (between -40 and -80 mV); a 15 ms prepulse of -40 mV is applied before starting the voltage steps, in this way the different recordings are comparable despite the different holding potential.

CLIC1-mediated chloride currents were isolated from the other ionic currents in the cells by perfusing the specific inhibitor IAA94 (Indanyloxyacetic acid 94, 100 µM) dissolved in the bath solution.

The solutions used in the patch-clamp experiments of this thesis are the following: Bath solution (mM): 125 NaCl, 5.5 KCl, 24 HEPES, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 D-glucose, 10 NaOH, pH 7.4. Pipette solution (mM): 135 KCl, 10 HEPES, 10 NaCl, 1 $MgCl_2$, pH 7.2.

Offline analysis was performed using Clampfit 10.2 (Molecular Devices) and OriginPro 9.1. CLIC1-mediated current (IAA94-sensitive current, $I_{IAA94}$) was estimated by analytical subtraction of the residual ionic currents, after the addition of the inhibitor, from the total current ($I_{TOT}$) of the cell at each membrane potential tested. Current/voltage relationships (I/V curves) were constructed plotting the averaged current density of the last 100 ms of the pulse against the corresponding membrane potential. Current density (pA/pF) results from the ratio between the ionic current (pA) and the cell capacitance (pF). Error bars are the standard error of the mean in all plots. Statistical analysis was performed comparing the slopes (proportional to the channel conductance) of the I/V curves of the different groups.

# 3.6 Cell count assays

Cells were plated in complete medium into 24-well plates at a density of 2 x $10^4$ and 1.2 x $10^4$ cells/well for colon and prostate cells respectively. Once adhered, cells were incubated in the absence or presence of IAA94 100 µM, DIDS 200 µM or metformin 10 mM for 24, 48, 72 or 96 hours. At the indicated time points, cells were trypsinized, harvested by centrifugation and stained with Trypan Blue dye. Viable cells were then counted using *Countess* II FL *Automated Cell Counter* (Thermo Fisher Scientific, Inc.).

The doubling time of each cell line was calculated with the following formula: $DT = t*[\log(2)/\log(N_t/N_0)]$, in which t is the incubation time, $N_0$ is the cell number at the beginning of the incubation time, $N_t$ is the cell number at the end of the incubation time.

# 3.7 Lentiviral-mediated CLIC1 silencing

To achieve stable knockdown of CLIC1, were used lentiviral shRNA targeting CLIC1 or a scrambled control lentiviral shRNA in pMKO.1 puro vectors (Addgene plasmid # 8452).

PC3 and SW620 cells at about 50% of confluency were infected with viral supernatants in the presence of 8 μg/ml polybrene (Sigma Aldrich) for 20 hours at 37°C, 5% $CO_2$. After 24 hours of growth in complete medium, transduced cells were selected with 1 μg/mL puromycin for at least two weeks. Efficiency of CLIC1 knockdown was confirmed by Western blot analysis.

# 3.8 Viability assays

Cell viability was evaluated by measuring the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). Briefly, colon and prostate cells were seeded in 96-well culture plates respectively at a density of $5 \times 10^3$ and $3 \times 10^3$ per well. Cells were incubated in complete medium and let to attach, then they were treated with or without IAA94 (0.1, 10, 50, 100, 200 μM) or metformin (0.01, 1, 5, 10, 20 mM) for 96 hours.

At the end of the treatment, cells were incubated in MTT solution (0.5 mg/mL in PBS) at 37°C for 4 h. Then the medium was discarded and formazan crystals were dissolved in a solution of 4 mM HCl, 0.1% Nondet P-40 (NP40) in isopropanol.

Finally, the absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm using the *EnSight* Multimode *Plate Reader* (PerkinElmer's). Independent experiments were repeated in triplicate.

IC50 values were calculated using nonlinear regression curve fit analysis selecting the log(drug) vs. response-variable slope (four parameters) equation using GraphPad Prism 7 software (GraphPad Software, Inc., San Diego CA, USA).

# 3.9 Cell cycle analysis

Cell cycle analysis has been performed with a FACS Aria flow cytometer (BD Bioscience). Samples were analyzed at FACS using appropriate filter to visualize the fluorescence emitted by Propidium Iodide (PI) stoichiometrically intercalated into the DNA (Em=488 nm; Ex=617 nm) to evaluate the DNA content.

For each sample, $1x10^6$ cells were washed with 500 µl of PBS 1X, centrifuged for 10 minutes at 1000 rpm and fixed with 1 ml of cold ethanol 70%. After at least two hours at 4°C, cells were centrifuged for 5 minutes at 1000 rpm and washed twice with PBS 1X to remove all the ethanol. After the last wash cells were resuspended in 400 µl of staining mix (Propidium Iodide 20 µg/ml, RNAse 10 µg/ml, Triton X-100 0.1% in PBS 1X) and incubated 30 minutes at 37°C in the dark. Samples were syringed to break up cells clumps and directly analyzed on flow cytometer, acquiring at least 10000 events.

FACS data were analyzed using BD FACSDIVA software (BD Bioscience). The cell populations were previously selected on the basis of Forward-scattered light (FSC) and Side-scattered light (SSC) parameters and FL2-H values are used for cell cycle analysis.

# 3.10 Wound healing assays

The scratch assay (wound-healing assay) was performed for assessing 2D cell migration. Cells were seeded in 12-well plates ($1\times10^5$ cells per well) and grown to reach confluence. At this point, the cells in each well were scratched by a 200 µL pipette tip to create perpendicularly wound lines free of adherent cells. After washes with PBS, cells were incubated in complete growth medium with or without IAA94 100 µM or metformin 10 mM.

Gap closure was monitored with digital camera images taken in a phase-contrast Olympus IX81 microscope equipped with a digital camera and an OKOlab incubator to maintain samples at 37°C and with 5% $CO_2$. For each field images are taken with an Olympus 6X objective every 5 minutes for 24 hours for prostate cells or every 10 minutes for 48 hours for colon cells. Excellence software (Olympus) has been used to acquire images and images were analyzed using ImageJ software.

After measuring the gap area for each frame in the wound healing experiment and plotting gap area as a function of time, the cell migration rate ($v_{migration}$) and the $t_{1/2gap}$ values have been derived, accordingly with Jonkman J.E. and colleagues [267].

The $t_{1/2gap}$ (the time point at which the gap is half the original area) was calculated as: $t_{1/2gap}$ = Initial Gap Area/(2×|slope|). The $v_{migration}$ (the average velocity at which the cells collectively migrate into the gap) was measured as: $v_{migration}$ = |slope|/(2× gap length).

The percentage of wound healing at a fixed time point was quantified with the following formula: % wound closure = $[(A_{t0h}-A_{t\Delta h})/A_{t0h}]\times100\%$, in which $A_{t0h}$ is the initial area of the wound and $A_{t\Delta h}$ is the remaining cell-free area at determinate time point.

# 3.11 Transwell migration assays

Migration of cell lines was also quantified by 3D transwell chamber assays. Transwell with 8-µm pore membrane inserts in 24-well plates (Corning) were used. To promote response to the chemoattractant, cells were serum-starved overnight. $0.5x10^5$ prostate cells and $1\times10^5$ colon cells were then seeded in each upper chambers in serum-free medium (100 µl) additionated or not with IAA94 100 µM or metformin 10 mM. In the lower chambers complete medium (600 µl) with or without IAA94 or metformin was placed. After incubation for 24 h (for BPH1, PC3 and CCD841 cells) or 60 h (for SW620 cells), non-migrated cells on the upper surface of the chamber were removed with a cotton swab and the inserts were fixed in 3% paraformaldehyde in PBS. Migrated cells attached to the lower surface of the filters were mounted on cover-glass with Vectashield mounting medium with DAPI (Vector Laboratories).

Samples were observed under a widefield Zeiss Examiner A1 microscope with a Zeiss 40X/0.75 NA water immersion objective and DAPI fluorescence filter. Imagines were acquired using Zeiss software and cells in 10 randomly selected areas were counted using ImageJ software.

# 3.12 Statistical analysis

All the data that required statistical validation have been analyzed with the appropriate statistical test.

Statistical analyses were performed with GraphPad Prism software 7 (GraphPad Software, Inc., San Diego, CA). When comparing two groups of data an unpaired, two-tailed t-test was performed.

When comparing multiple groups a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test.

Differences between groups were considered to be significant at a P value of $<0.05$.

All data are reported as means ± S.E.M of at least three independent experiments.

# -4-

# AIMS OF THE THESIS

Colorectal cancer (CRC) and prostate cancer (PCa) are two of the most common malignancies throughout the world [114, 173]. Despite therapeutic advances, they remain a leading cause of cancer-related death [115, 174].

Therefore, the research of more effective pharmacological targets as well as biomarkers able to early detect the disease is essential to achieve a cure for these tumors. In this scenario, attention of our laboratory has been focused on CLIC1 protein.

CLIC1 is a metamorphic protein with the unique characteristic of being expressed both in a soluble globular form and as integral membrane protein with chloride channel function [51, 57, 60]. While in physiological conditions this protein is mostly cytoplasmic, during stress conditions, like an increase of the cellular oxidative level, it transiently translocates into the cell membranes [51, 61, 62].

In the last years, CLIC1 protein has gained a growing importance in carcinogenesis [1]. It is found overexpressed in a variety of human solid tumors, including prostate [113] and colon cancer [112] and it seems to be correlated with a poor prognosis [109, 111].

In conditions of persistent oxidative stress, as occurs for CRC and PCa [96, 97], CLIC1 can undergo to a chronic translocation into the plasma membrane [102]. Once inserted in the membrane and working as active ion channel, CLIC1 modulates different processes important for tumorigenesis, as cell volume regulation [157], cell cycle progression [67], cell migration and invasion [157, 158, 205] and tumor angiogenesis [49].

Despite the findings reported in literature suggest a potential role of CLIC1 in supporting tumor progression, the exact function performed by this protein remains still unclear.

It has been recently proposed that metformin, the widely used anti-diabetic drug, exerts direct antitumor activity [227, 228] and recognizes CLIC1 as a target [91].

The experimental work carried on during my Ph.D. was performed using *in vitro* model of colon and prostate tissue.

The research has been developed as follow:

1. assess wheatear malignant cells could be discriminated by non-tumor cells for a different level of expression and/or a different localization of CLIC1
2. elucidate the role played by CLIC1 channel in regulating proliferation, cell cycle progression and migration abilities of PCa and CRC cells,
3. explore the ability of metformin to inhibit the malignant abilities of CRC and PCa cells

The general aims of my project were: 1) to understand the contribution of CLIC1 to the tumorigenic process of PCa and CRC and in particular to the progression from a non-invasive phenotype to a more aggressive form, 2) to evaluate whether CLIC1 could represent a valid biomarker and/or a suitable pharmacological target for PCa and CRC and 3) to investigate the potential benefit of metformin as antitumor compound with CLIC1 channel blocker activity.

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# RESULTS

All the experiments performed in my experimental project have been carried on in prostate and colon human cell lines.

The colon cell lines used are CCD841 CoN (or CCD841) cells, a normal human colon epithelial cell line derived from a 21 weeks gestation foetus [268] and SW620 cells, a colorectal adenocarcinoma epithelial cell line, established from the lymph node metastasis. These cells are hyperdiploid, highly tumorigenic and metastatic in nude mice [269].

The prostate cell lines used are BPH1 cells and PC3 cells. BPH1 are a non-tumorigenic prostatic hyperplasia epithelial cell line, derived from benign hypertrophic prostate tissue and immortalized with SV-40 large T antigen [270]. These cells are viable but non-tumorigenic after injection in nude mice and fail to form colonies in soft agar [270, 271]. PC3 are an androgen-independent prostatic adenocarcinoma epithelial cell line, originally isolated from a metastatic lesion to bone [272]. They are able to form colonies in soft agar suspension culture and produce subcutaneous tumors in nude mice [273].

# 5.1 CLIC1 expression level in colon and prostate cells

As CLIC1 is found to be overexpressed in a variety of human solid tumors [104-111], including colon [112] and prostate cancer [113], it has been primarily investigated the level of expression of this protein in the *in vitro* models used in this work.

To this purpose it has been evaluated the extent of CLIC1 protein expression in cultured colon and prostate cell lines through Western Blot analysis.

The analysis of whole cell lysates from normal colon cells (CCD841) and tumor colon cells (SW620) has demonstrated that CLIC1 is expressed both in CCD841 than in SW620 cells. Opposite to what reported in literature for several solid tumors [104-111], no significant differences in total CLIC1 protein level present in carcinoma cells compared to normal epithelial cells have been found (figure 8 A-B).

Comparing the expression of CLIC1 on extracts of benign prostate cells (BPH1) and metastatic prostate cells (PC3) it has been observed that CLIC1 protein is expressed also in the two prostate cell lines, without significant differences in the total amount of CLIC1 between benign hyperplasia cells and malignant cancer cells (figure 8 C-D).

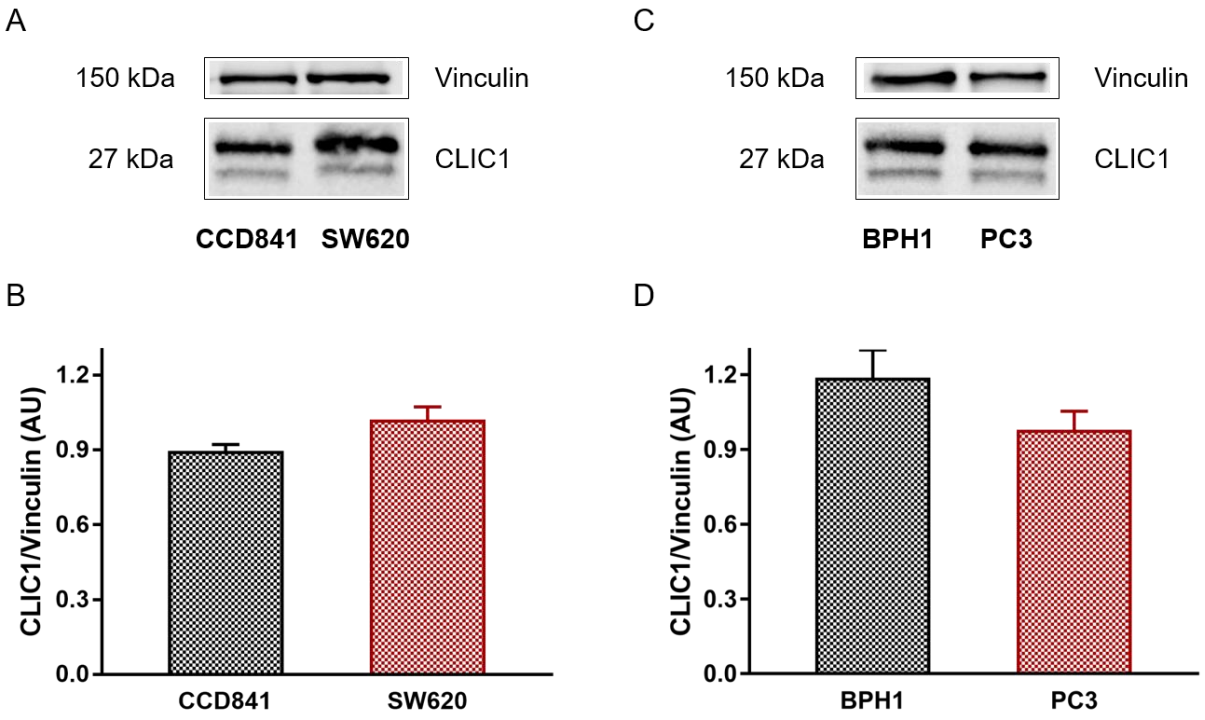



Figure 8. (A-B) Representative Western blot analysis and quantification of CLIC1 protein expression in whole-cell lysates from normal CCD841 and tumor SW620 cells. CLIC1 levels are not significant different between normal colon and tumor colon cell line (n=6, One-sample t test, n.s.). (C-D) Representative Western blot analysis and quantification of CLIC1 protein levels in total extract from benign BPH1 and metastatic PC3 cells. CLIC1 expression does not significantly differ between the two prostate cell lines (n=5, One-sample t test, n.s.). Vinculin was used as loading control.

# 5.2 CLIC1 localization in colon and prostate cells

CLIC1 can exist both as hydrophilic cytosolic protein as integral transmembrane protein with ion channel activity [51, 59]. As CLIC1 is a metamorphic protein, it has been evaluated whether there were any differences in the localization of this protein between normal and malignant cells.

To this purpose immunofluorescence analysis were performed. Indirect immunofluorescent staining of permeabilized cells with an antibody direct against CLIC1 and with the nuclear-specific marker DAPI have shown the intracellular distribution of CLIC1 protein in the different cell lines studied.

Confocal analysis reported in figure 9 confirmed that CLIC1 level is comparable in both colon and prostate cells. Interestingly, CLIC1 immunoreactivity was observed both in the cytoplasm and in the nuclei of all the cell types, whereas plasma membrane staining was detected only in tumor cells.

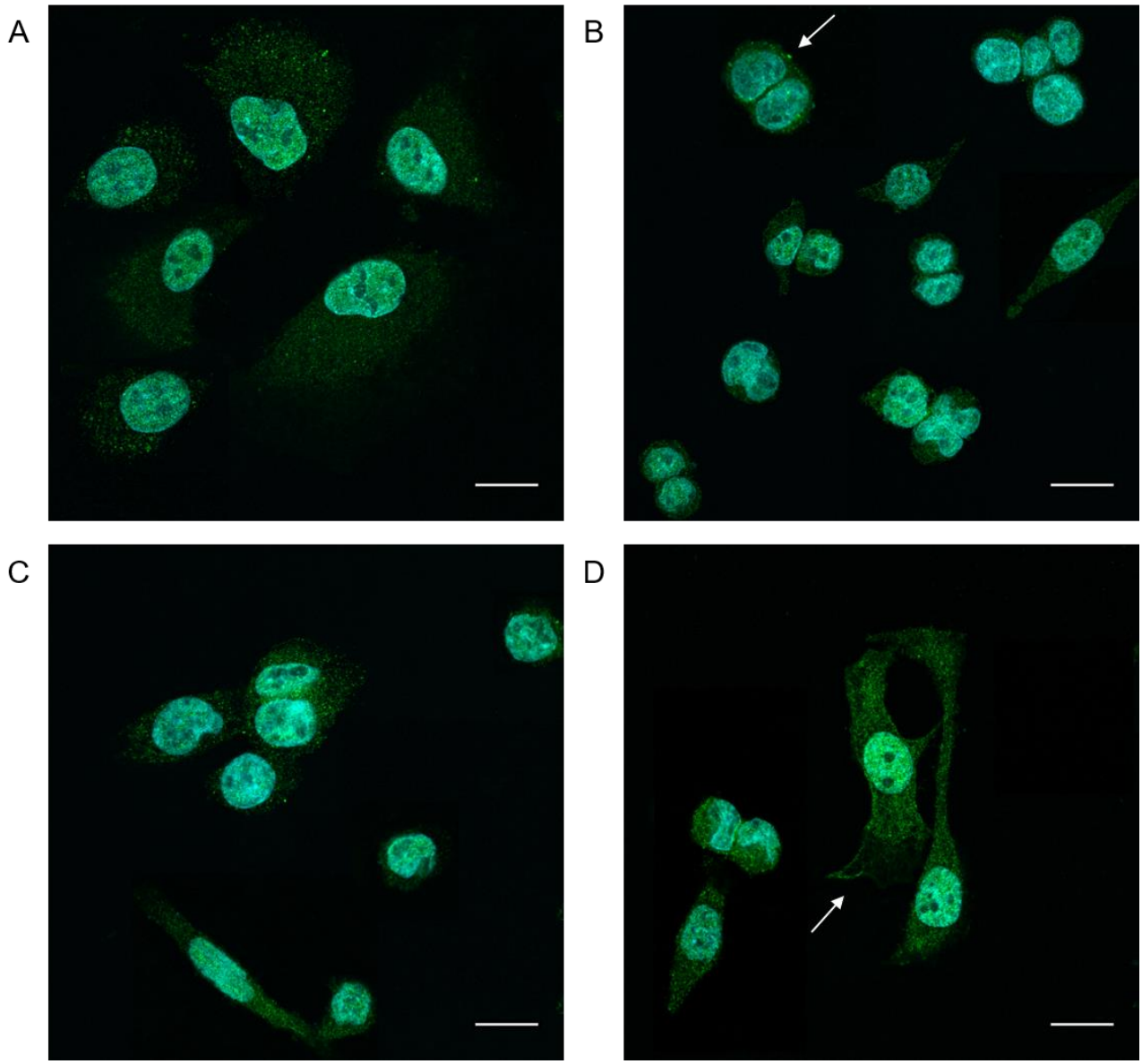



Figure 9. Representative confocal microscope imagines of CLIC1 immunostaining in CCD841 (A), SW620 (B), BPH1 (C) and PC3 cells (D). The pattern of CLIC1 (green) is widely localized in the cytoplasm and in the nuclei (blue) of all the different cell types. Plasma membrane staining is detectable in tumor cells, whereas is absent in CCD841 and BPH1 cells. Scale bars: 25 μm.

To further confirm that CLIC1 localization on cell plasma membrane was associated with the channel activity it has been performed perforated patch clamp electrophysiology experiments.

CLIC1 mediated chloride current was isolated using the specific inhibitor indanyloxyacetic acid 94 (IAA94) and normalized to the total current in the corresponding cell. Steady state currents amplitude at different membrane potentials has been used to build the current/voltage relation showed in figure 10.

Notably, we found that CLIC1-mediated current (IAA94-sensitive current) recorded in tumor SW620 cells is significantly higher than in normal counterpart CCD841 (fig. 10 A). Similar results were obtained in prostate cells with CLIC1 current more represented in malignant PC3 cells compared to benign BPH1 cells (fig. 10 B).

Taken together these results demonstrate that, although CLIC1 is expressed in similar level in normal cells as well as in tumor cell lines, it is present in the membrane as active channel exclusively in the malignant cells.

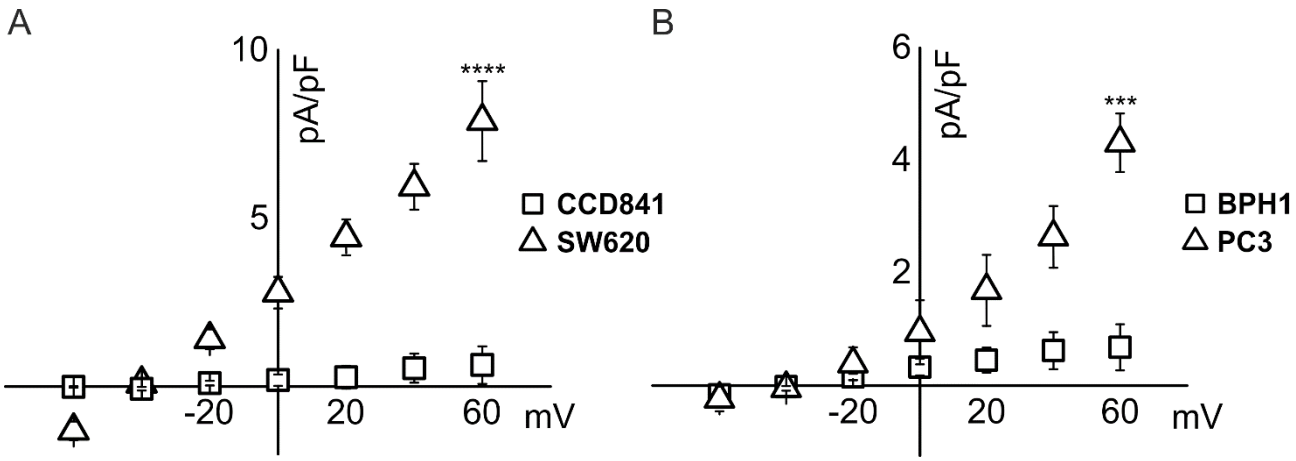



Figure 10. Current density/voltage relationships of CLIC1 mediated chloride current measured by perforated patch clamp technique in colon (A) and prostate (B) cell lines. SW620 showed a significantly higher current compared to CCD841 cells (n=7, One-sample t test, $p<0.0001$). In a similar manner CLIC1-mediated currents are significantly higher in PC3 than in BPH1 cells (n=7, One-sample t test, $p<0.001$).

# 5.3 CLIC1 promotes tumor cell proliferation

Our laboratory has previously demonstrated that CLIC1 is involved in the cell cycle regulation of Chinese hamster ovary (CHO-K1) cells [67] and it is required to maintain *in vitro* and *in vivo* proliferation capacity of glioblastoma cancer stem cells [102]. Furthermore, a recent study has reported that the intracellular chloride concentration affects cell growth and cell cycle progression of PC3 cells [274].

This evidence has suggested exploring the possible role played by CLIC1 activity in the growth of prostate and colon tumor cells.

First of all, cell count assays on the different cell lines studied were performed. Equal numbers of cells were seeded, then the cells were counted for the following 4 days and growth curves were generated to determine the doubling time of each cell population.

Data obtained have shown that tumor SW620 cells, with a doubling time of 24.5 h, proliferate significantly faster than CCD841 normal cells, which are characterized by a doubling time of 53 h (figure 11 A).

Conversely, results obtained for prostate cell lines indicated that malignant PC3 cells display a significantly slower growth compared to benign BPH1 cells. The doubling time was 23 h and 32.3 h for BPH1 for PC3 cells respectively (figure 11 B).

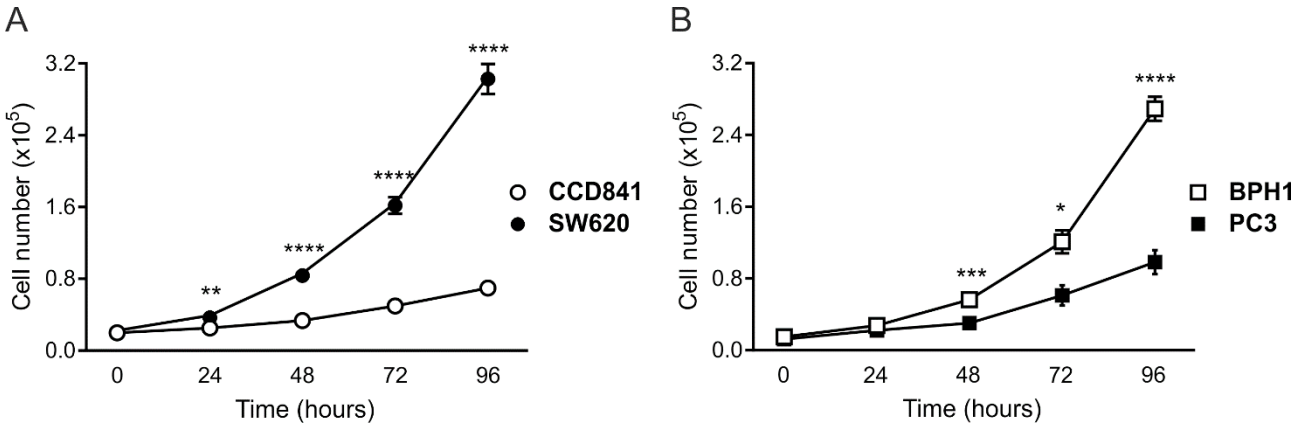



Figure 11. Growth curves of colon cell lines (A) and prostate cell lines (B) at the indicated time points. The number of SW620 cells (n=6) was significantly higher compared to that of their normal counterpart (n=3), both at 24 h ($P<0.01$), 48 h ($P<0.0001$), 72h ($P<0.0001$) and 96 h ($P<0.0001$) (One sample t-test). The number of BPH1 cells (n=4) was significantly higher respect to that of PC3 cell line (n=5), both at 48 h ($P<0.001$), 72 h ($P<0.05$) and 96 h ($P<0.0001$) (One-sample t test).

To verify that CLIC1 channel plays a functional role in the regulation of tumorigenic properties of colon and prostate cancer cells different loss-of-function approaches were applied.

Using lentiviral vectors carrying CLIC1-targeting shRNA it has been stably knocked down CLIC1 expression in the cells that had previously demonstrated to have active CLIC1 channel (SW620 and PC3 cells). CLIC1 expression level following infection with the specific CLIC1-shRNA compared with scramble-shRNA vector was verified by Western Blotting analysis. As shown in figure 12, it has been obtained a reduction of CLIC1 expression of about 87% in PC3 cells and of about 72% in SW620 cells.

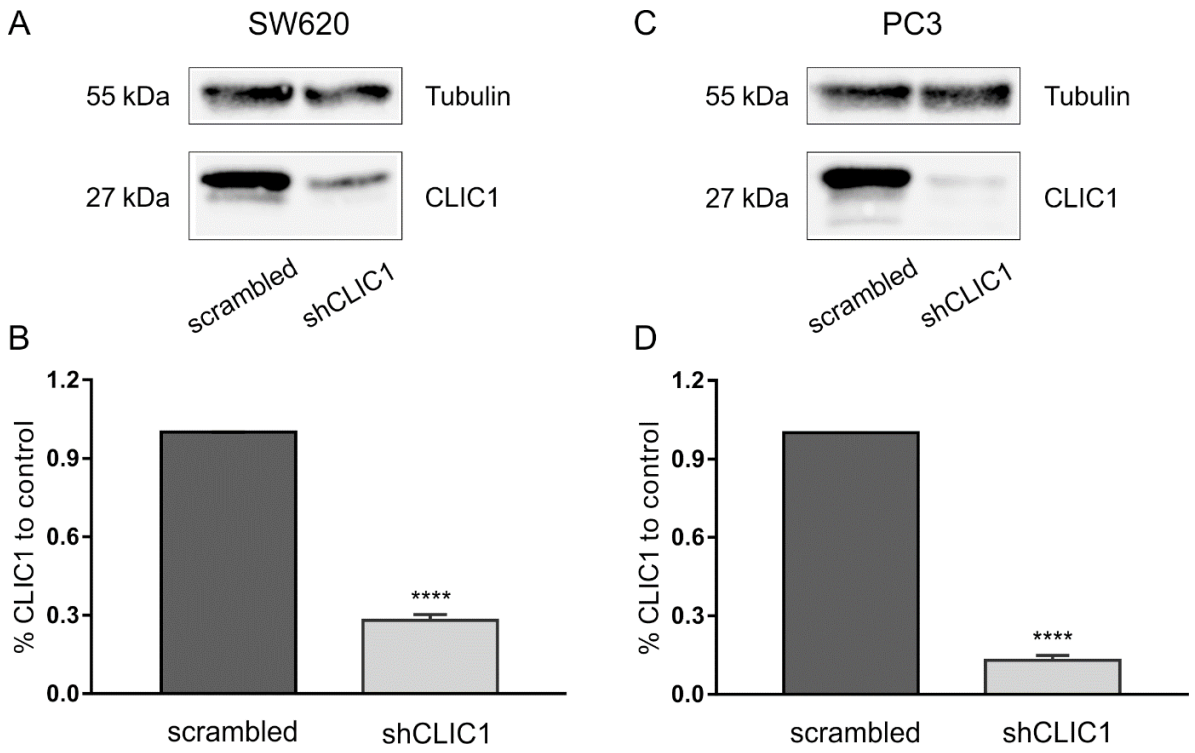



Figure 12. Representative immunoblotting and densitometric quantitative analysis of CLIC1 expression in SW620 (A-B) and PC3 (C-D) cells stably transfected with control scrambled siRNA (scrambled) or CLIC1-specific siRNA (shCLIC1). CLIC1 expression levels were significantly down-regulated in shCLIC1 cells compared to scrambled cells in both SW620 (n=3, One-sample t test, P<0.0001) and PC3 cells (n=3, One-sample t test, P<0.0001).

By cell count experiments was assessed the effect of CLIC1 silencing on the growth rate of colon and prostate cancer cells.

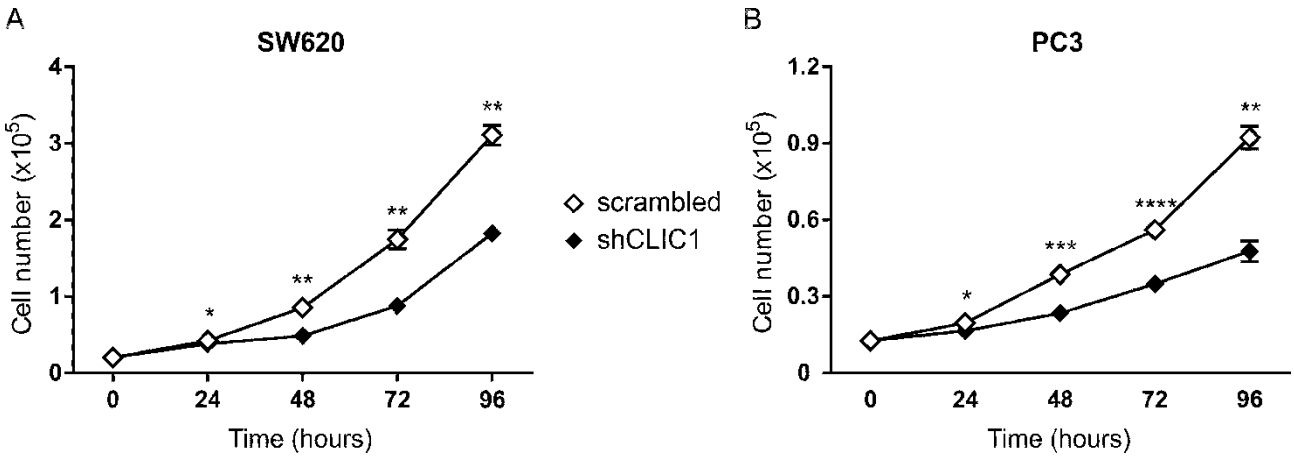



Figure 13. (A) Growth curve of stably transfected SW620 cells. The number of cells in the scrambled shRNA group was significantly increased starting from 24 h and arriving at more than 40% at 96 h (P<0.01) compared to the CLIC1 shRNA group (n=3, One-sample t test). (B) Growth curve of stably transfected PC3 cells. Compared to the scrambled shRNA cells, the number of CLIC1 shRNA population was significantly higher at each time point and reached a 48% of increase at 96 h (P<0.01) (n=3, One-sample t test).

As shown in figure 13 A, CLIC1 knockdown led to a significant decline in SW620 cell proliferation. CLIC1-shRNA population grew more slowly than scrambled shRNA population and the doubling time was increased of 2.1- fold.
Similar results were obtained in PC3 cells: compared with the scrambled shRNA cells, the proliferation of CLIC1-shRNA cells was significantly reduced with a doubling time 1.5 fold higher (figure 13 B).

To evaluate the specificity of CLIC1 channel activity in the proliferation of malignant cells compared to the benign or non-tumor counterpart, cell growth curves were performed. The cells were daily counted following the incubation with different compounds: IAA94, the specific CLIC1 channel blocker, DIDS, a blocker of the other chloride channels with no effect on CLIC1, and metformin, a drug able to inhibit CLIC1 together with other intracellular targets.

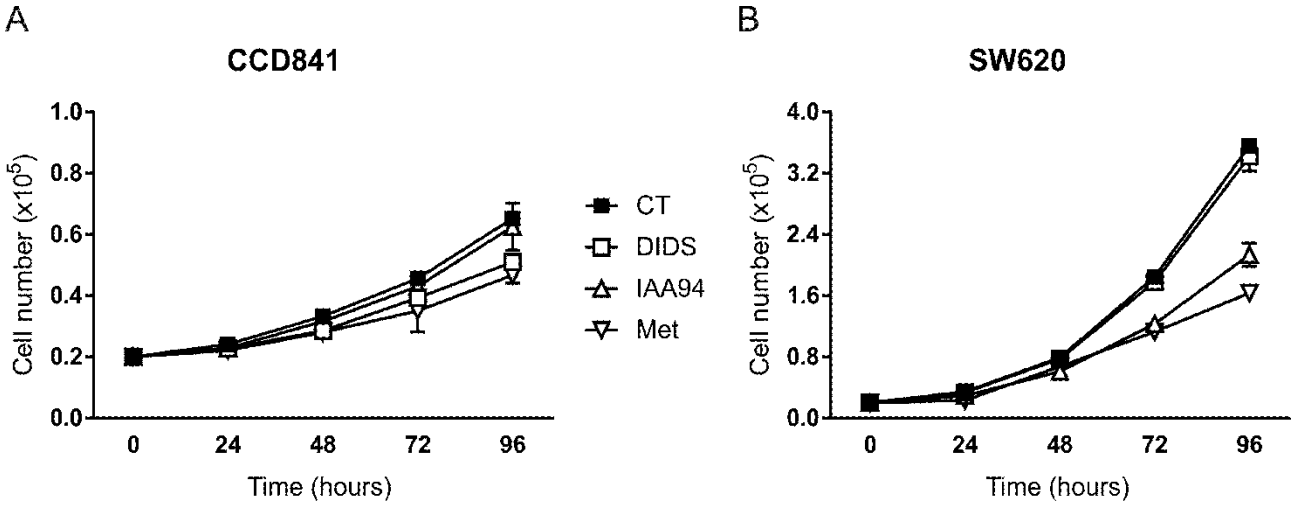



Figure 14. Growth curves of colon cells after treatment with or without DIDS 200 µM, IAA94 100 µM and metformin 10 mM for the indicated time points. A. In CCD841 cell line IAA94 had no significant effect on cell growth (n.s), whereas metformin ($P<0.001$) and DIDS ($P<0.01$) significantly decreased cell proliferation at 96 h compared with the untreated cells (n=3, One-way ANOVA, Dunnett's test). B. In SW620 cell line DIDS did not alter cell proliferation (n.s.), while metformin ($P<0.0001$) and IAA94 ($P<0.0001$) strongly inhibited cell growth at both 72 and 96 h (n=3, One-way ANOVA, Dunnett's test).

Growth curves reported in figure 14 shows the effect of these treatments on colon cell growth rate. IAA94 led to a time-dependent decrease of SW620 cells proliferation, starting from about 23% at 48 h and reaching 40% of growth reduction at 96 h (fig. 14 B).
Consistently with the results reported in figure 10 showing that CCD481 cells did not have CLIC1-mediated current, treatment with IAA94 did not reduce the proliferation of these cells (fig. 14 A).
On the contrary, DIDS did not affect the proliferation rate of SW620 cells, but it significantly reduced of 22% the growth of CCD841 cells at 96 h (fig. 14 A-B).

Compared with the untreated control, metformin significantly suppressed the proliferation of SW620 cells in a time dependent manner, causing a reduction of 39% at 72 h and of more than 50% at 96 h (fig. 14 B). Metformin treatment had a reduced anti-proliferative effect on CCD841 cells, decreasing cell growth of less than 30% at 96 h (fig. 14 A).

These results suggested that CLIC1 plays an important role in the regulation of colon tumor cell proliferation. Furthermore, pharmacological blockage of this channel determinates a strong growth inhibition selectively of tumor cells, without altering that of normal cells.

Growth curves of prostate cells reported in figure 15 have demonstrated that BPH1 cells are highly sensitive to both IAA94 and metformin treatments, undergoing a completely inhibition of cell proliferation (fig. 15 A).
In malignant PC3 cells, IAA94 significantly reduced the proliferation rate of about 18% and 23% respectively at 72 and 96 h. Metformin determined a further 10% reduction in the proliferation rate (fig. 15 B).
On the contrary, DIDS treatment did not influence cell growth of both cell lines (fig. 15 A-B).
These data show that CLIC1 is also involved in regulation of prostate cells proliferation, but with a minor extent than that of colon tumor cells.

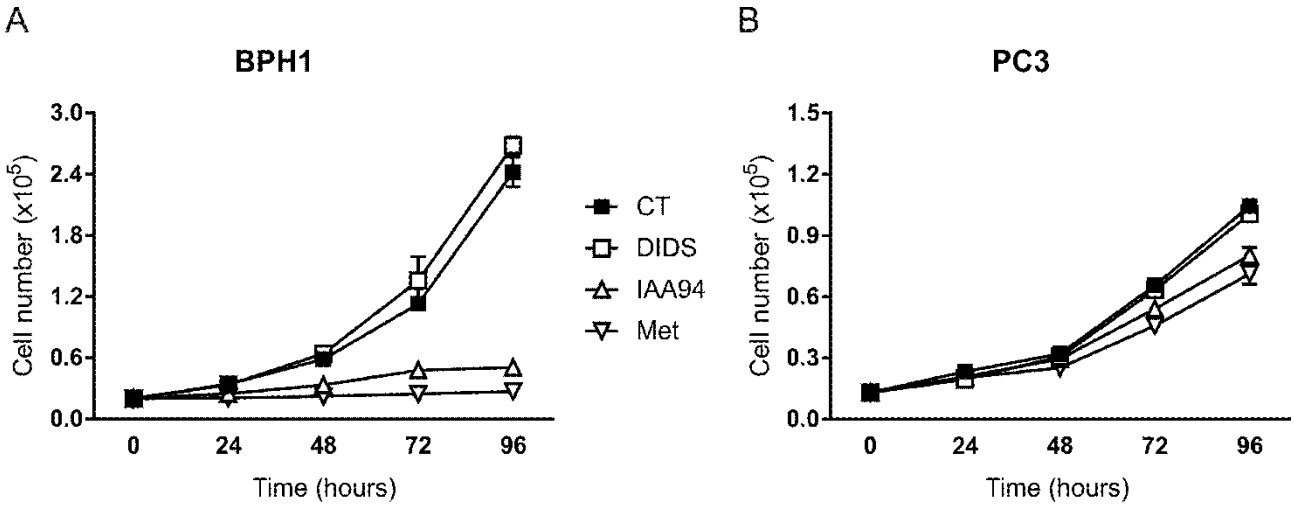



Figure 15. Growth curves of prostate cells after incubation in presence or absence of DIDS 200 µM, IAA94 100 µM and metformin 10 mM for the indicated time points. A. In BPH1 cell line IAA94 strongly inhibited cell growth at both 24 h ($P<0.001$), 48 h ($P<0.001$), 72 h ($P<0.05$) and 96 h ($P<0.0001$) compared to control. Metformin also significantly inhibited cell growth at 24 h ($P<0.0001$), 48 h ($P<0.0001$), 72 h ($P<0.01$) and 96 h ($P<0.0001$) compared to control, whereas DIDS did not alter the proliferative rate (n.s.) (n=3, One-way ANOVA, Dunnett's test). B. In PC3 cells IAA94 significantly decreased cell proliferation at 72 h ($P<0.01$) and 96 h ($P<0.01$), as well as metformin ($P<0.0001$ at 72 h and $P<0.001$ at 96 h). Conversely, DIDS had no significant effect on cell growth (n.s.) compared to control (n=3, One-way ANOVA, Dunnett's test).

The effect of CLIC1 pharmacological inhibition on cell viability was measured using the MTT-viability assay. Cells were treated for 96 hours with increasing concentrations of metformin (from 0.1 mM to 20 mM) or IAA94 (from 1 µM to 200 µM) and a dose-response curve was obtained.

IAA94 produced a dose-dependent decrease of viability of SW620 cells starting from an inhibition of 5% with 10 µM concentration and reaching -80% at 200 µM (IC50 of 148.3 µM). On the contrary, CCD841 cells started to display a little decrease of cell viability only when the concentration of treatment reached the maximal doses (IC50 of 302.2 µM), suggesting that CLIC1 channel inhibition do not have any effect on the proliferation of these cells (fig. 16 A).

IAA94 had a modest effect on viability of PC3 cells and started to significantly decrease cell viability at 100 µM concentration. In BPH1 cells, instead, IAA94 strongly affected cell viability, significantly decreasing cell viability already at the lower dose, suggesting that in these cells the treatment produce a toxic effect (fig. 16 B).

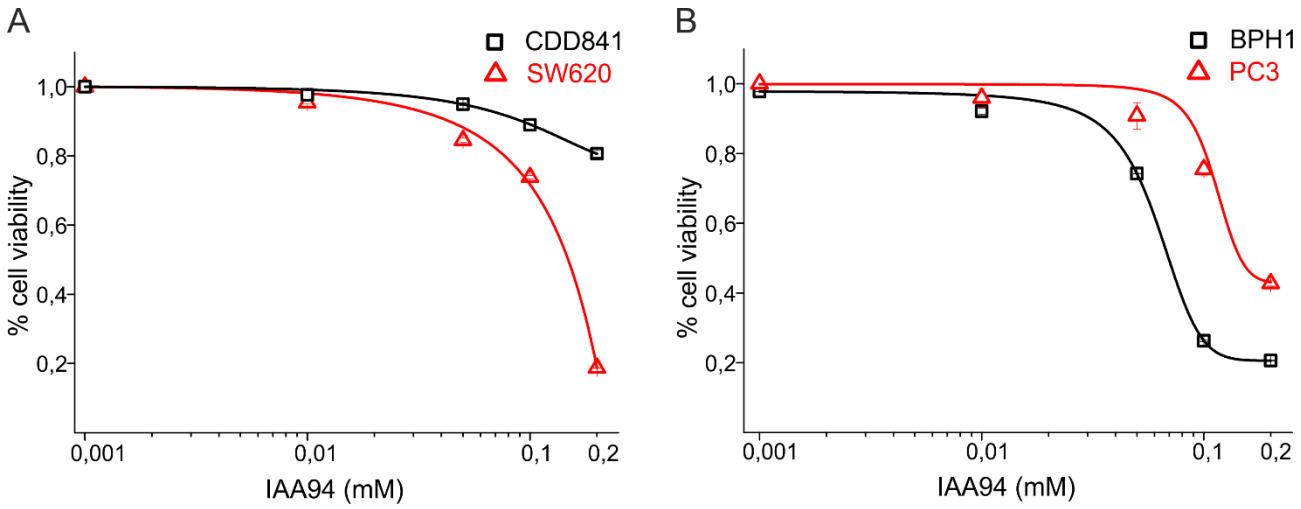



Figure 16. Dose-response curve for colon (A) and prostate (B) cell lines treated for 96 h with increasing concentration of IAA94. In SW620 cells IAA94 treatment led to a significant decrease of cell viability starting from -5% at 10 µM concentration and reaching -80% at 200 µM (n=3, One way ANOVA, Dunnett's test, P<0.0001 for each concentration). In CCD841 cells IAA94 was able to little decrease cell viability only at concentrations of 100 µM (P<0.001) and 200 µM (P<0.0001) (n=3, One way ANOVA, Dunnett's test, p<0.0001). In PC3 cells IAA94 started to significantly decrease cell viability at 100 µM (P<0.001) and reached -57% at 200 µM concentration (P<0.0001) (n=3, One way ANOVA, Dunnett's test). Conversely, in BPH1 cells was observed a significantly decrease of cell viability already at the lower dose (n=3, One-Way ANOVA, Dunnett's test, p<0.0001 for each concentration).

Metformin is effective in decrease viability of SW620 cells at low concentrations (IC50: 3.34 mM) determining a 44% reduction already at 5 mM and reaching a 60% decrease with 20 mM treatment. Conversely, metformin has a reduced effect on CCD841 cells (IC50: 30.22 mM) and the maximum concentration led to a reduction of cell viability of only 35% (fig.17 A).

In PC3 cells metformin produced a dose-dependent decrease of viability that started to be significant at 10 mM and reached 55% at 20 mM (IC50: 64.5 mM). Metformin instead strongly affected BPH1 cells cell viability already at low dose (IC50: 13.76 mM), suggesting that in these cells the treatment produced a toxic effect (fig. 17 B).

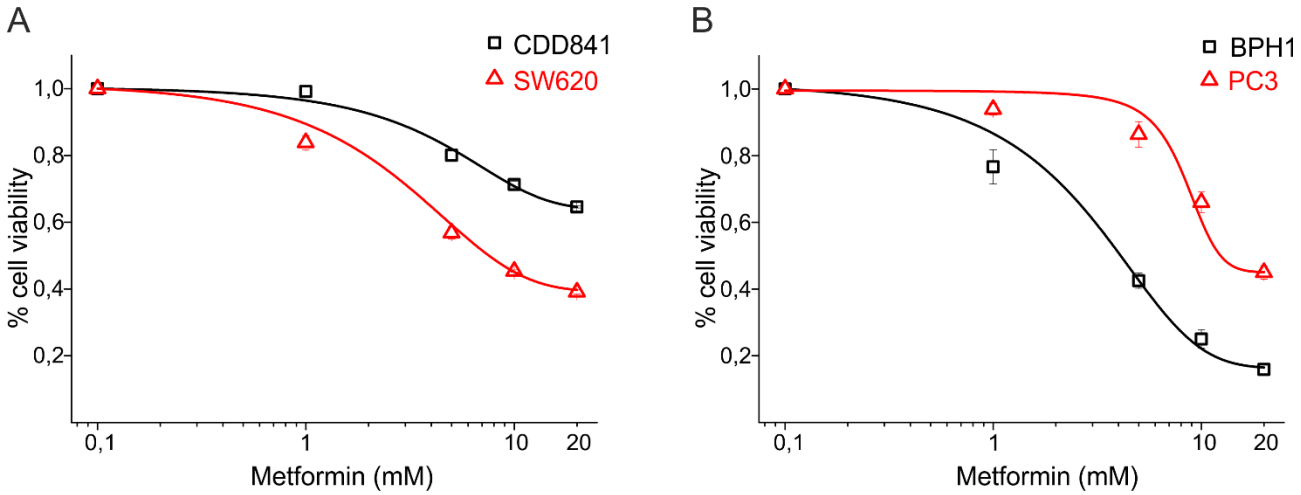



Figure 17. Dose-response curve for colon (A) and prostate (B) cell lines following 96 h treatment with increasing concentration of metformin. In SW620 cells metformin reduced cell viability already at the lower concentration and reaching -60% at 20 mM (n=3, One way ANOVA, Dunnett's test $p<0.0001$ for each concentration). In CCD841 cells metformin led to a significant inhibition of cell viability that was -21% with 5 mM and only -35% with the maximal concentration (n=3, One-Way ANOVA, Dunnett's test, $p<0.0001$). In PC3 cells metformin reduced cell viability of 34% at 10 mM concentration ($P<0.0001$) and of 55% at 20 mM ($P<0.0001$) (n=3, One-way ANOVA, Dunnett's test). In BPH1 cells was observed a significant decrease of cell viability that already started at 1 mM concentration and reached 84% with 20 mM concentration (n=3, One-way ANOVA, Dunnett's test, $p<0.0001$ for each concentration).

It has been previously observed that IAA94 and metformin induced a dose and time-dependent reduction of tumor cells proliferation. To elucidate if the anti-proliferative activity of metformin was dependent by CLIC1 inhibition was evaluated the effect of IAA94 and metformin treatments on growth curve of cells stably expressing CLIC1 shRNA or shRNA vector.

As expected, in SW620 cell line treatment with IAA94 100 µM did not affect proliferation of CLIC1-shRNA cells, whereas significantly reduced proliferation of scrRNA cells of about 28% at 72 h and 34% at 96 h (figure 18).

A similar result was obtained in PC3 cell line: following IAA94 treatment the growth of CLIC1-shRNA cells was not significantly altered, while in scrRNA cells was reduced of 20% and 35% respectively at 72 h and 96 h (figure 19).

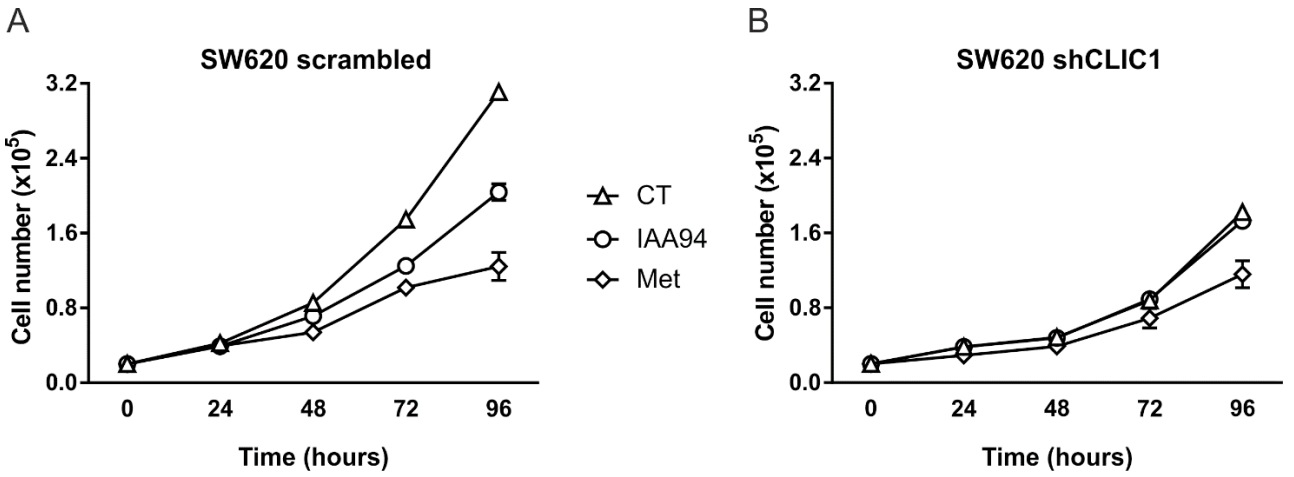



Figure 18. Growth curve of SW620 cells stably transfected with control scramble shRNA (A) or specific CLIC1 shRNA (B). Compared to control, IAA94 significantly decreased the number of cells in scrambled shRNA group starting from 48 h ($P<0.01$) and continuing at 72 h ($P<0.01$) and 96 h ($P<0.001$) (n=3, One-way ANOVA, Dunnett's test). Conversely, the number of cells in the CLIC1 shRNA group was not significantly affected by IAA94 (n=3, One-way ANOVA, Dunnett's test, n.s.). Metformin strongly reduced proliferation of scrambled shRNA cells at 48 h ($P<0.0001$), 72 h ($P<0.01$) and 96 h ($P<0.0001$) compared to control (n=3, One-way ANOVA, Dunnett's test). In CLIC1 shRNA group metformin significantly reduced cell growth only at 96 h (n=3, One-way ANOVA, Dunnett's test, $P<0.01$).

Following treatment with 10 mM metformin a significant decrease of CLIC1-shRNA cell proliferation it has been observed both in SW620 cells and in PC3 cells. However, the reduction of proliferation induced by metformin was of lesser extent than one observed in scrRNA cells.

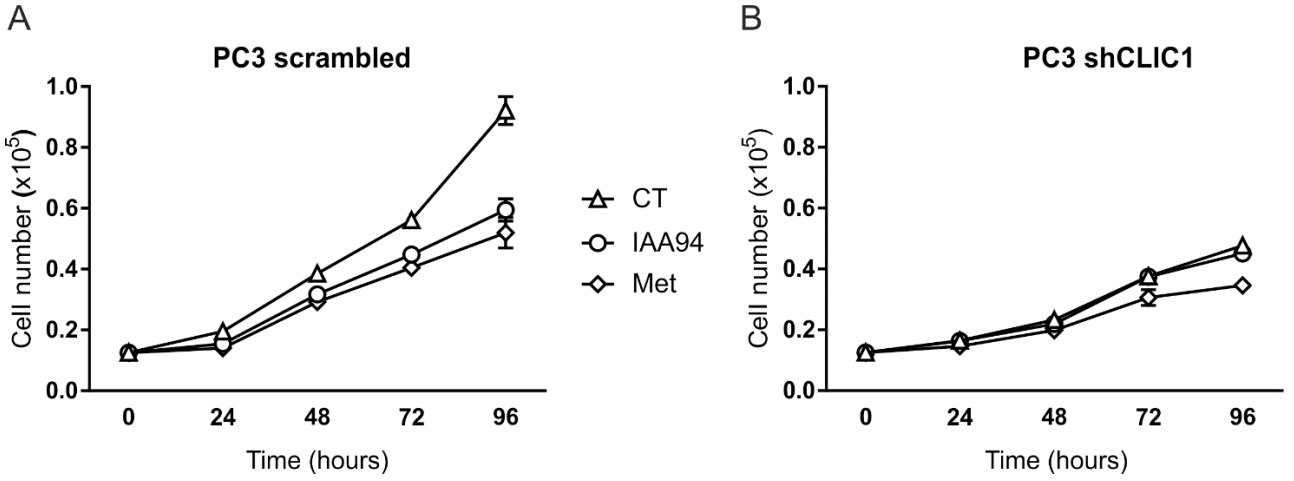



Figure 19. Growth curve of PC3 cells stably transfected with control scramble shRNA (A) or specific CLIC1 shRNA (B). IAA94 treatment was able to reduce the number of scrambled shRNA cells at 48 h ($P<0.05$), 72 h ($P<0.01$) and 96 h ($P<0.01$) compared to control (n=3, One-way ANOVA, Dunnett's test). Conversely, the number of CLIC1 shRNA cells was not significantly different compared to untreated cells (n=3, One-way ANOVA, Dunnett's test, n.s.). Following metformin treatment was observed a significant reduction of scrambled shRNA cells both at 48 h ($P<0.01$), 72 h ($P<0.001$) and 96 h ($P<0.01$) (n=3, One-way ANOVA, Dunnett's test vs control), but a significant decrease of CLIC1 shRNA cells only at 96 h (n=3, One-way ANOVA, Dunnett's test, $P<0.001$ vs control).

In scrRNA SW620 group metformin led to a 60% of growth inhibition at 96 h, whereas in CLIC1-shRNA SW620 group induced a decrease of about 36% (fig. 18). The growth inhibition induced by metformin at 96 h was 44% in PC3 scrRNA cells and 27% in CLIC1-shRNA cells, respectively (fig. 19).

Since knockdown of CLIC1 by RNA interference completely abolish the effect of growth inhibition of IAA94 and strongly attenuate that of metformin, was confirmed that CLIC1 mediates IAA94 and metformin's anti-proliferative effects.

In order to evaluate wheter the decrease in cell number observed after exposure to IAA94 and metformin was partially due to apoptosis activation, a western blot analysis of cleaved caspase 3 level has been performed.

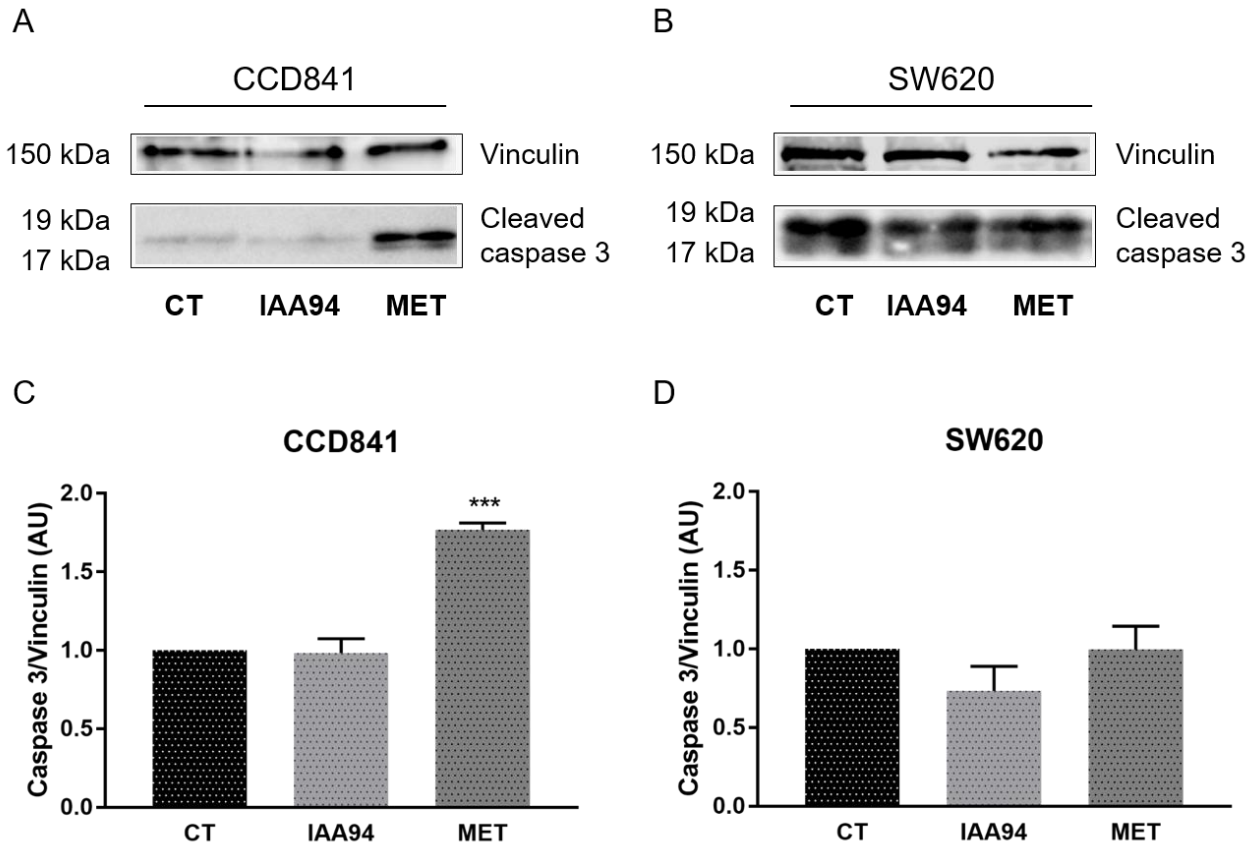



Figure 20. Representative immunoblotting and densitometric quantitative analysis of cleaved caspase 3 expression in CCD841 cells (A-B) and SW620 cells (C-D) following treatment with or without IAA94 or metformin for 48 h. IAA94 had no effect on apoptosis activation in both normal cells (n=3, n.s.) and tumor cells (n=4, n.s.). In CCD841 cells, caspase 3 expression level was significantly up-regulated after treatment with metformin (n=3, $P<0.001$), whereas in SW620 cells no significant changes were observed compared to untreated control (n=4, n.s.) (One-way ANOVA, Dunnett's test vs control).

As shown in figure 20, treatment with IAA94 100 μM did not lead to a significant increased activation of apoptosis neither in CCD841 nor in SW620 cells, suggesting that the reduced number

of cells depended on the anti-proliferative effect of these treatments and was not due to apoptosis induction.

On the contrary, in CCD841 cells metformin 10 mM caused a significant increase in the activation of apoptotic pathway compared to untreated condition.

In PC3 cells it has not been observed a significant induction of apoptosis upon treatment with IAA94, whereas metformin led to an increase of 20% of apoptosis (figure 21 B-D). In BPH1 cells, both IAA94 and metformin strongly activated apoptosis with a fold increase respectively of 1.2 and 0.65 compared to control (figure 21 A-B).

These data suggest that the drastic decrease of BPH1 cell number observed also at low concentration of treatments was independent by CLIC1 inhibition and was mediated by apoptosis activation.

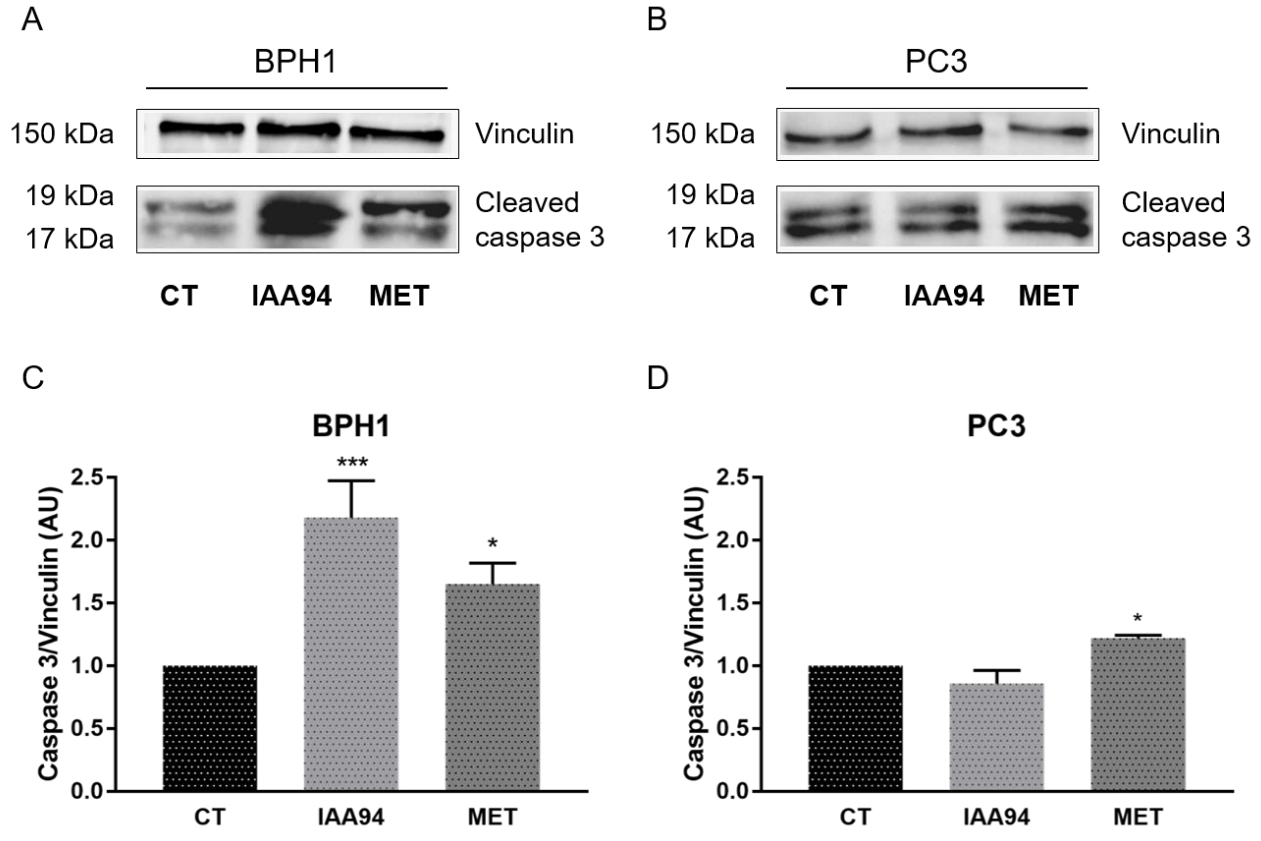



Figure 21. Representative immunoblotting and densitometric quantitative analysis of cleaved caspase 3 expression in BPH1 cells (A-B) and PC3 cells (C-D) upon incubation in presence or absence of IAA94 or metformin for 48 h. IAA94 had no effect on apoptosis activation of malignant cells (n=5, n.s.), whereas strongly induced apoptosis in benign cell line (n=6, n.s.). Metformin treatment led to a significant increase of caspase 3 expression level in PC3 cells (n=5, $P<0.05$) as well as in BPH1 cells (n=6, One-way ANOVA, Dunnett's test, $P<0.05$ versus control).

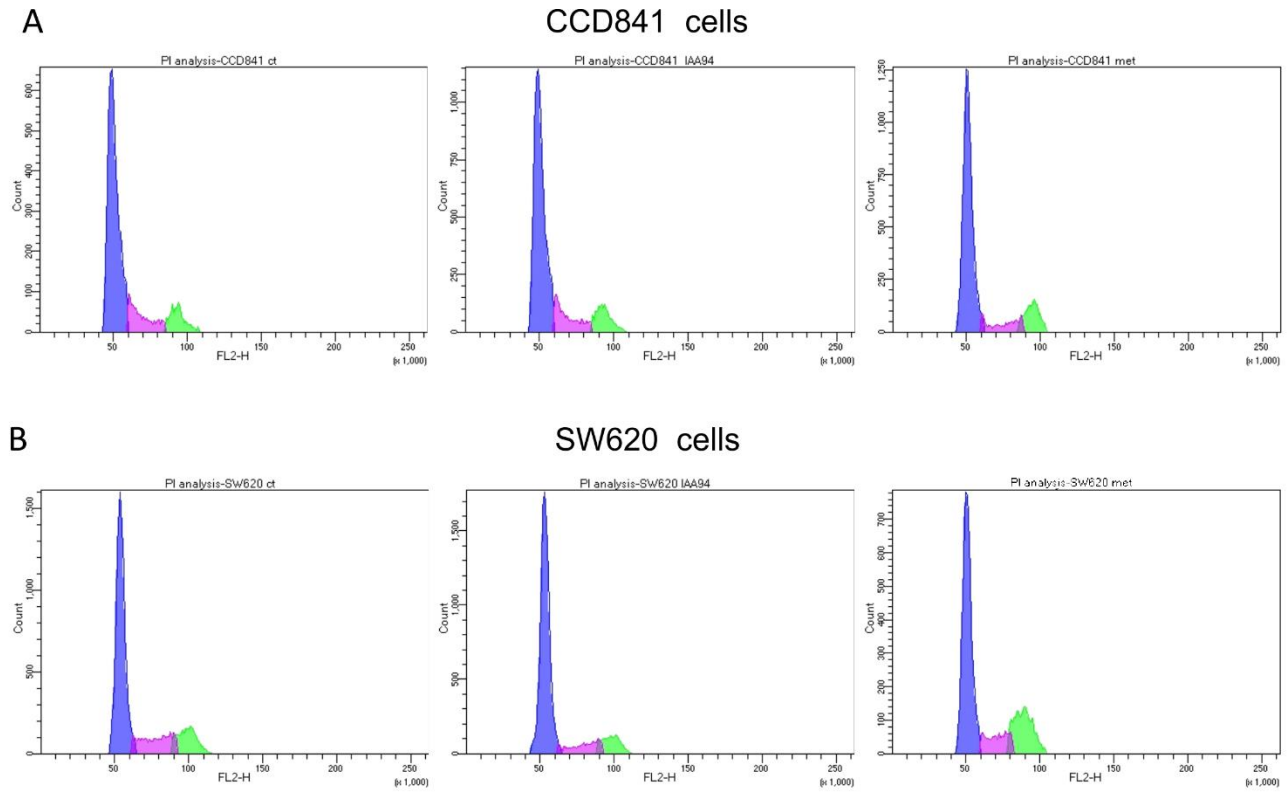
# 5.4 CLIC1 promotes tumor cell cycle progression

Chloride channels are key factors in regulation of the cell cycle and cell proliferation [65-67, 274] since ion transport across the cell membrane participates in regulation of cell volume, which would be indispensable in cell cycle progression [275-278].

Therefore, it has been hypothesized that CLIC1 protein could support the aberrant cell division of cancer cells, promoting an inappropriate cell cycle progression.

To investigate whether cell cycle changes are partially responsible for metformin and IAA94-induced cell growth inhibition, cell cycle analysis with PI staining has been performed. Cells were treated with or without IAA94 100 µM or metformin 10 mM for 72 h and were then analyzed by flow cytometry.

Results have shown that, in SW620 cells, treatment with IAA94 led to a significant G1/S cell cycle arrest, simultaneously with a decreasing in the proportion of cells in both the S and the G2/M phase. Metformin also modified cell cycle progression of SW620 cells, decreasing the fraction of cells in G0/G1 phase, and determining a significant accumulation in the G2/M phase (figure 22 B-D).
On the other hand, no change in cell cycle distribution was observed following the treatment of CCD841 cells with either IAA94 or metformin (figure 22 A-C).



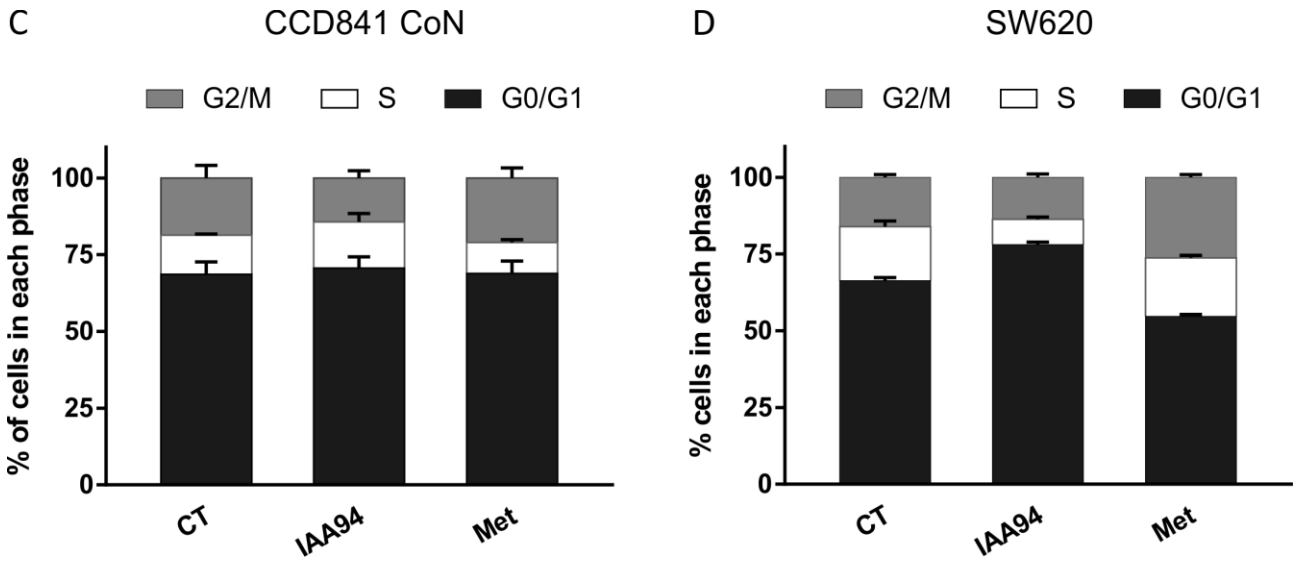



Figure 22. Representative flow cytometric profiles (A) and quantification of cell cycle distribution (C) of CCD841 cells treated with or without IAA94 or metformin. No significant alteration in cell cycle progression was observed after both IAA94 and metformin treatment compared to control (n=3, One-way ANOVA, n.s.) Representative flow cytometric profiles (B) and quantification of cell cycle distribution (D) of SW620 cells treated with or without IAA94 or metformin. Compared to untreated cells, IAA94 significantly increased G0/G1 population from ~66% to ~78% (n=5, One-way ANOVA, $P<0.05$) and metformin led to a significant increase in G2/M fraction from ~16% to ~26% (n=5, One-way ANOVA, $P<0.0001$).

In figure 23 are reported the data obtained in prostate cell lines. In PC3 cells, IAA94 treatment induced a cell cycle arrest in the G0/G1 phase with a concomitant reduction of cell percentage in the S and G2/M phases. At the same time, IAA94 did not significantly affect cell cycle progression of BPH1 cells. Conversely, following treatment with metformin no significant changes in cell cycle distribution were observed neither in PC3 nor in BPH1 cells.

Overall these data show that anti-proliferative effect induced by IAA94 treatment on both colon and prostate cancer cells was mediated by a cell cycle arrest in the G0/G1 phase. Thus, in line with an our recent publication [91], CLIC1 channel appears to be involved in regulation of cell cycle progression of malignant cells, promoting the transition between G0/G1 and S phase.

Metformin treatment instead induced a growth inhibitory effect leading to a cell cycle arrest in the G2/M phase in colon cancer cells or without affecting cell cycle progression in prostate cancer cells, respectively. As previously observed for cell proliferation, the effect of metformin on cell cycle was not completely comparable to that of IAA94. This discrepancy suggests that metformin may act not only on CLIC1 channel but also on its other targets, inducing additional cellular effects.

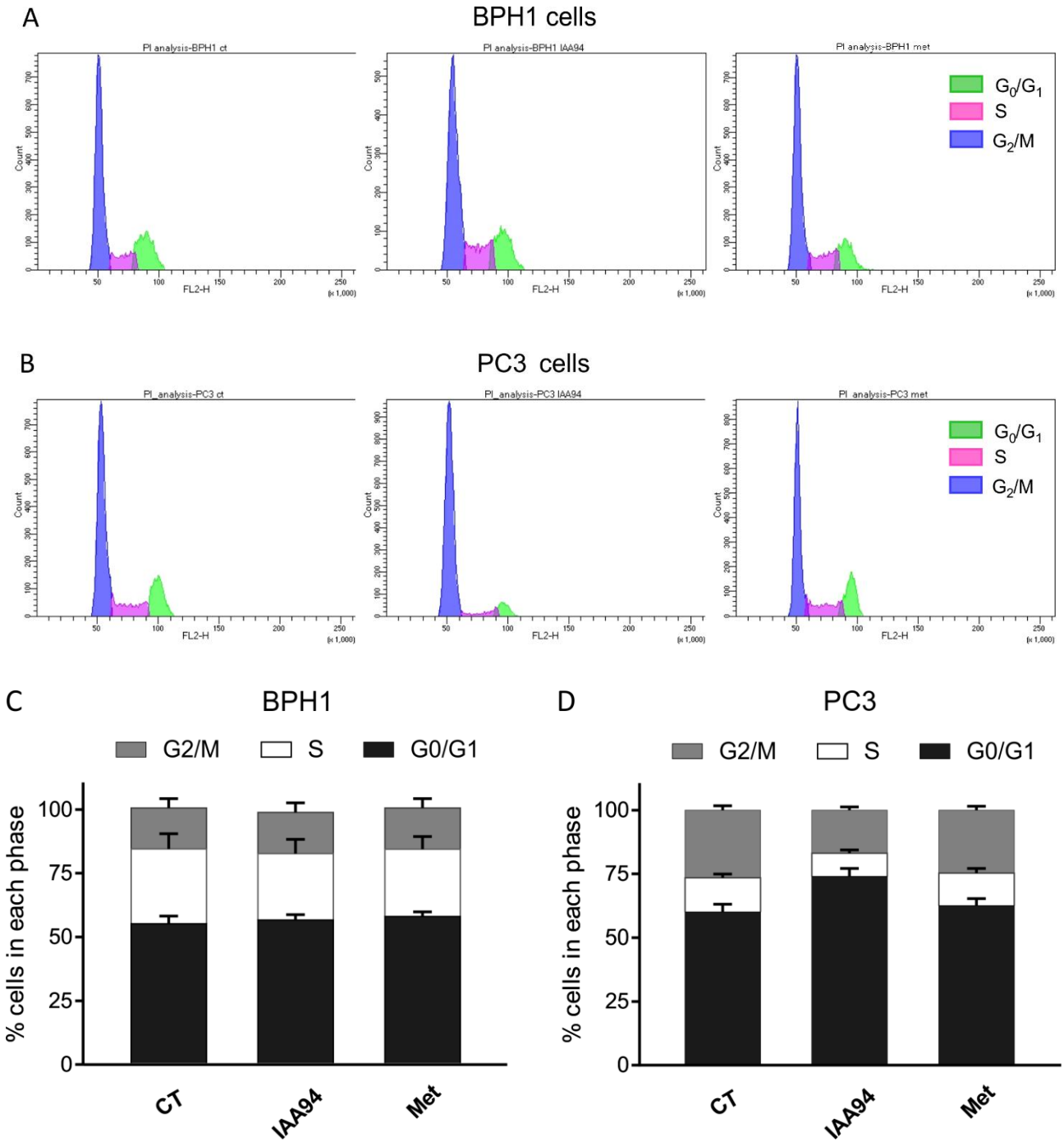



Figure 23. Representative flow cytometric histograms (A) and analysis of cell cycle phases (C) of BPH1 cells after treatment with or without IAA94 or metformin. IAA94 as well as metformin did not significantly altered cell cycle distribution (n=4, One-way ANOVA, n.s.). Representative flow cytometric histograms (B) and analysis of cell cycle phases (D) of PC3 cells following treatment with or without IAA94 or metformin. IAA94 significantly increased G0/G1 population from ~60% to ~74% (n=4, One-way ANOVA, $P<0.01$), whereas metformin did not significantly perturbed cell cycle (n=4, One-way ANOVA, n.s.).

To further demonstrate the role of CLIC1 in regulating cell cycle of cancer cells it has been examined the cell cycle profiles of SW620 and PC3 cells infected with scrambled siRNA or CLIC1-specific siRNA.

It has been observed that SW620 CLIC1-siRNA cells displayed a significantly increased proportion of G0/G1 phase and a reduced proportion of the S and G2/M phases compared with the scrambled siRNA cells (figure 24).

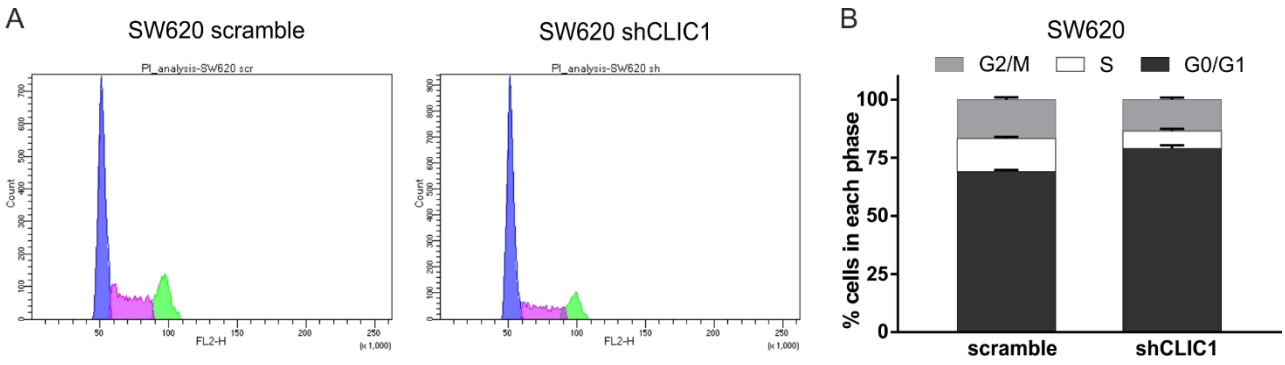



Figure 24. Representative FACS analyses (A) and quantification of cell cycle distribution (B) of SW620 scrambled siRNA and CLIC1-siRNA cells. The percentage of cells in G0/G1 phase was significantly increase to ~79% in SW620 shCLIC1 cells respect to ~68% of SW620 scrambled cells (n=3, One-sample t test, $P<0.01$).

Similarly to what observed in colon cells, in PC3 CLIC1-siRNA cells there was a significant increase in the G0/G1 cell population with a simultaneous decrease in S and G2/M fractions compared to scrambled siRNA cells (figure 25).

These results revealed that the inhibition of cancer cell proliferation upon CLIC1 silencing corresponded to a cell cycle arrest in G0/G1 phase.

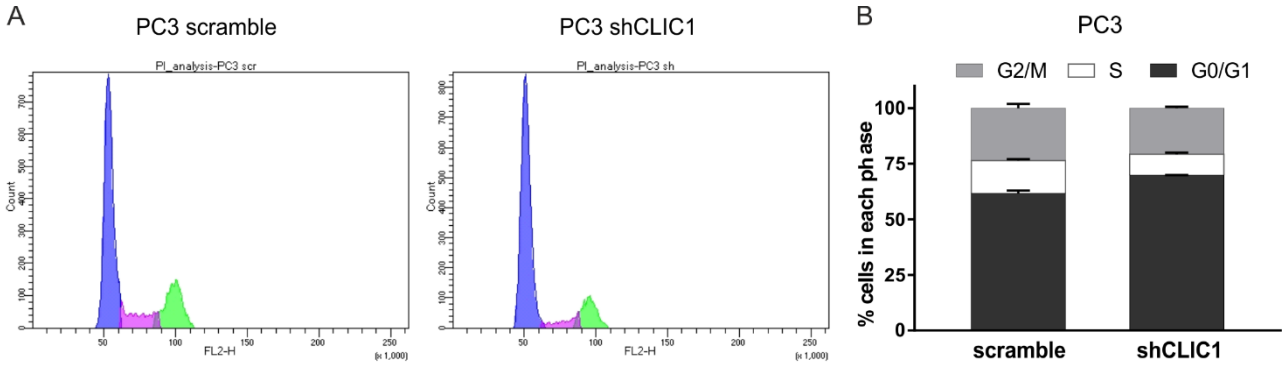



Figure 25. Representative FACS analyses (A) and quantification of cell cycle distribution (B) of PC3 scrambled siRNA and CLIC1-siRNA cells. The percentage of cells in G0/G1 phase was significantly increase in PC3 shCLIC1 cells compared to PC3 scrambled cells, increasing from ~62% to ~70% (n=3, One-sample t test, $P<0.05$).

# 5.5 CLIC1 promotes tumor cell migration

It is known that for CRC and PCa, as well as for the most tumors, metastasis represent the main cause of cancer-related fatality [148, 149, 203]. The ability to migrate and invade other tissues is of primary importance for prostate and colon cancer cells, as these tumors has an high tendency to metastasize [140, 141, 198]. Chloride channel have been demonstrated to play an important role in regulating cell migration [49, 70, 71].
To elucidate whether CLIC1 is involved in additional malignant properties of cancer cells, it has been investigated whether CLIC1 is able to regulate cellular migration in prostate and colon cancer cells.

Firstly, it has been observed the effect of pharmacological inhibition of CLIC1 activity on cell migration through wound healing assay.

CCD841 cells have shown a high migration rate, displaying a $v_{migration}$ of 15.9 µm/h and reaching about 80% of wound closure at 24 h. As reported in figure 26, the migration ability of CCD841 cells was not significantly decreased after treatments with both IAA94 and metformin, compared to that of the control.

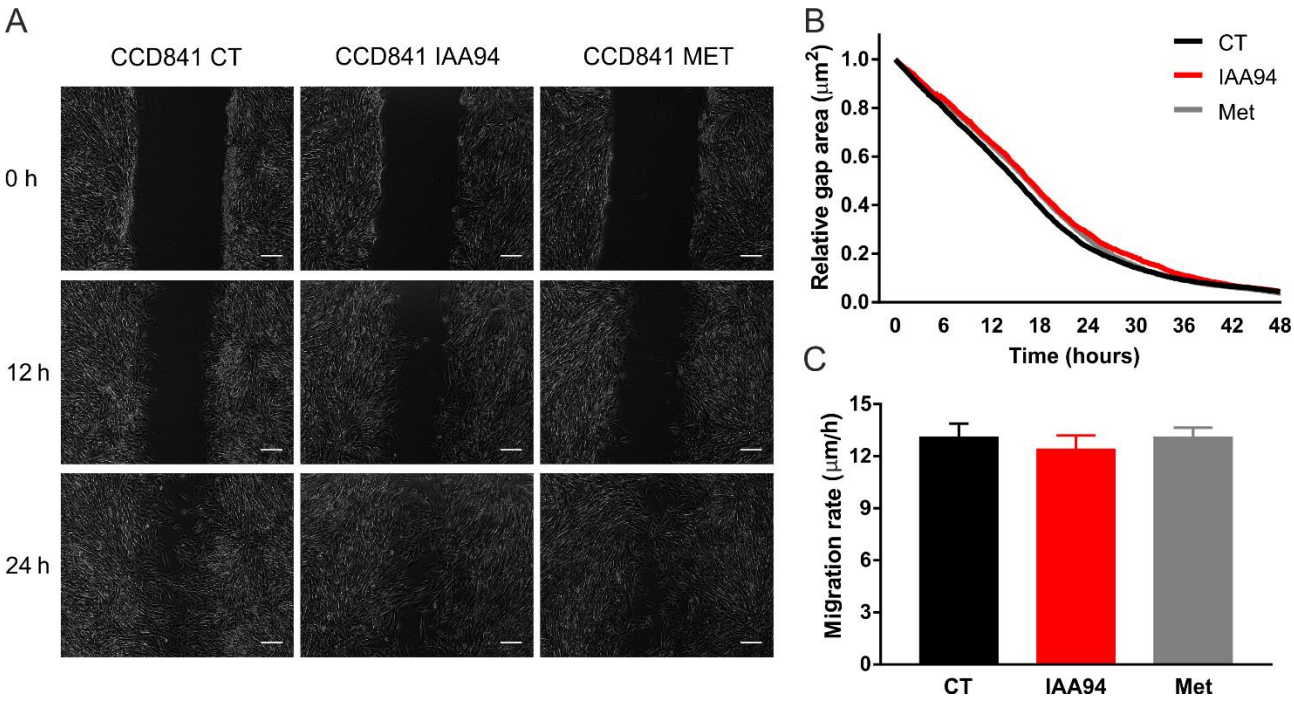



Figure 26. Representative imagines (A) and quantification of wound area closure (B) and migration rate (C) of CCD841 cells incubated with or without IAA94 100 µM or metformin 10 mM for 48 h. The migration rate of both IAA94 and metformin treated cells was not significantly changed compared to control (n=12, One-way ANOVA, Dunnett's test, n.s.). Scale bar: 200 µm.

SW620 cells exhibited a slowly migration rate, with a $v_{migration}$ of 6 µm/h and a percentage of gap closure of about 38% at 48 h.

As shown in figure 27, treatment with IAA94 resulted in a significant reduction of cell migration ability: the $v_{migration}$ was decrease at 4 µm/h and the $t_{1/2\ gap}$ was increase of 38% compared to control (36.7 h). Metformin also caused a less but significant reduction of SW620 cell migration ($v_{migration}$: 5 µm/h and $t_{1/2\ gap}$: 31.5 hours).

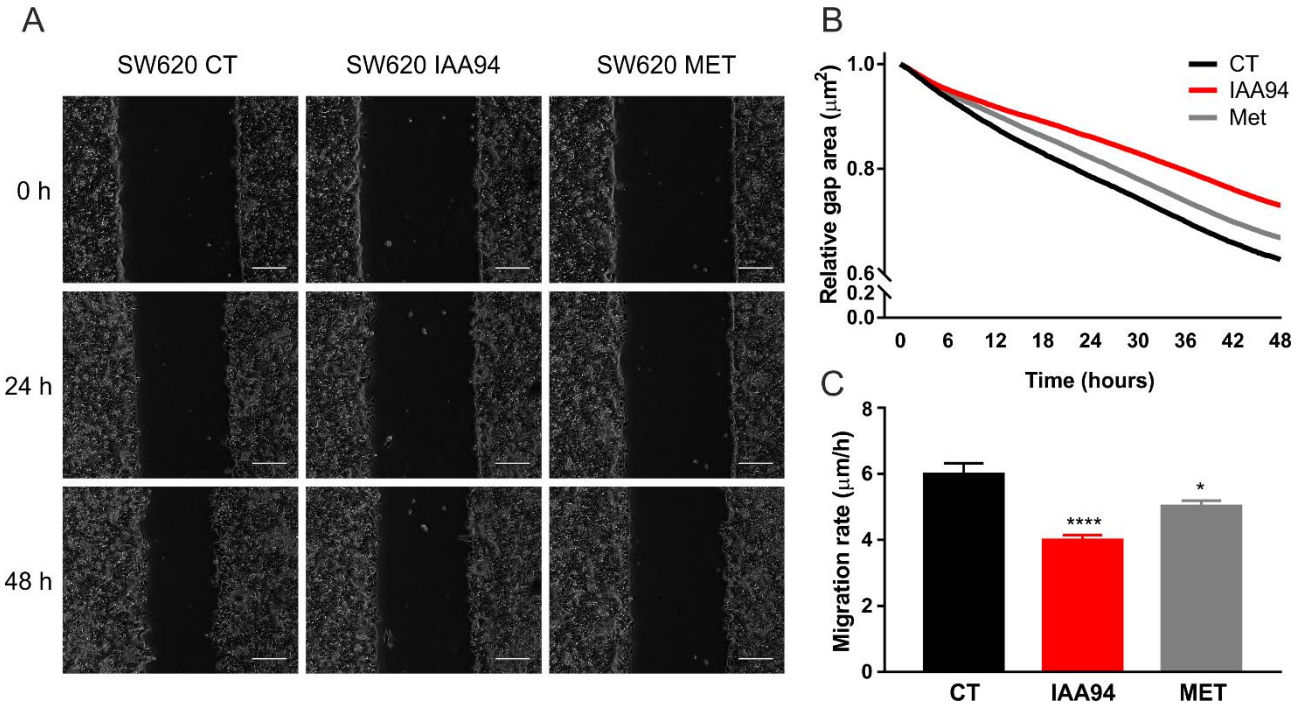



Figure 27. Representative imagines (A) and quantification of wound gap closure (B) and migration rate (C) of SW620 cells incubated with or without IAA94 100 µM or metformin 10 mM for 48 h. Compared to control, the migration rate was significantly reduce of 33% after treatment with IAA94 ($P<0.001$) and of 17% after treatment with metformin ($P<0.05$) (n=14, One-way ANOVA, Dunnett's test, n.s.). Scale bar: 200 µm.

It has been observed that BPH1 cells were able to migrate with a high rate ($v_{migration}$ 17.4 µm/h). Interestingly, treatment with IAA94 did not determinate any effect on the migration ability of these cells, whereas metformin led to a 58% decrease of the $v_{migration}$.

As shown in figure 28, control cells and IAA94 treated cells reached an almost completely closure of the wound area 12 hours after the scratch, conversely in metformin treated cells the percentage of wound healing was stopped at 40%.

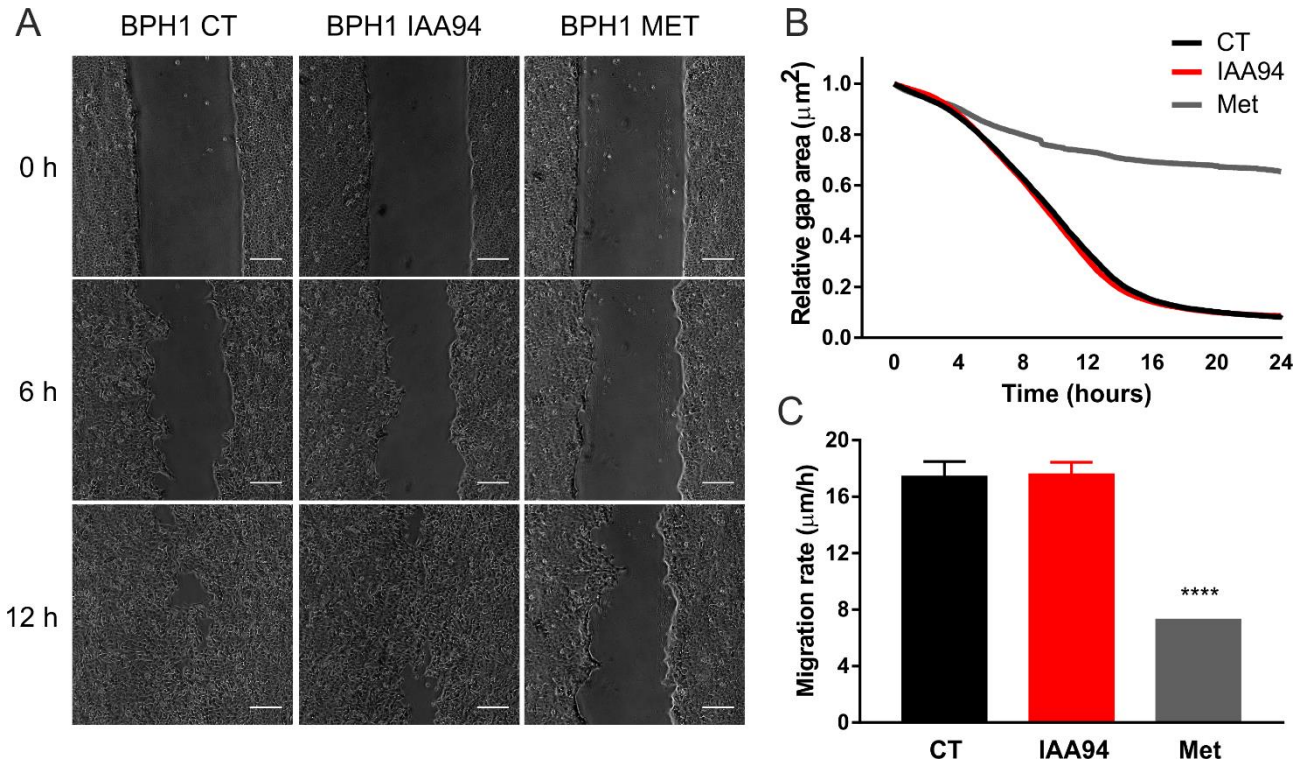



Figure 28. Representative imagines (A) and quantification of wound healing (B) and migration rate (C) of BPH1 cells treated with or without IAA94 100 µM or metformin 10 mM for 24 h. IAA94 treated cells did not shown any decrease in the migratory ability (n=18, n.s.), whereas metformin incubated cells displayed a significant reduced migration rate (n=19, P<0.0001) compared to control (n=18) (One-way ANOVA, Dunnett's test). Scale bar: 200 µm.

In PC3 cells, characterized by high migration ability ($v_{migration}$: 16.9 µm/h and $t_{1/2\ gap}$: 7.3 hours), the pharmacological inhibition of CLIC1 channel activity determined strong effects on cell migration: IAA94 and metformin significantly reduce migration rate of 46% and 60% respectively compared to control (figure 29).

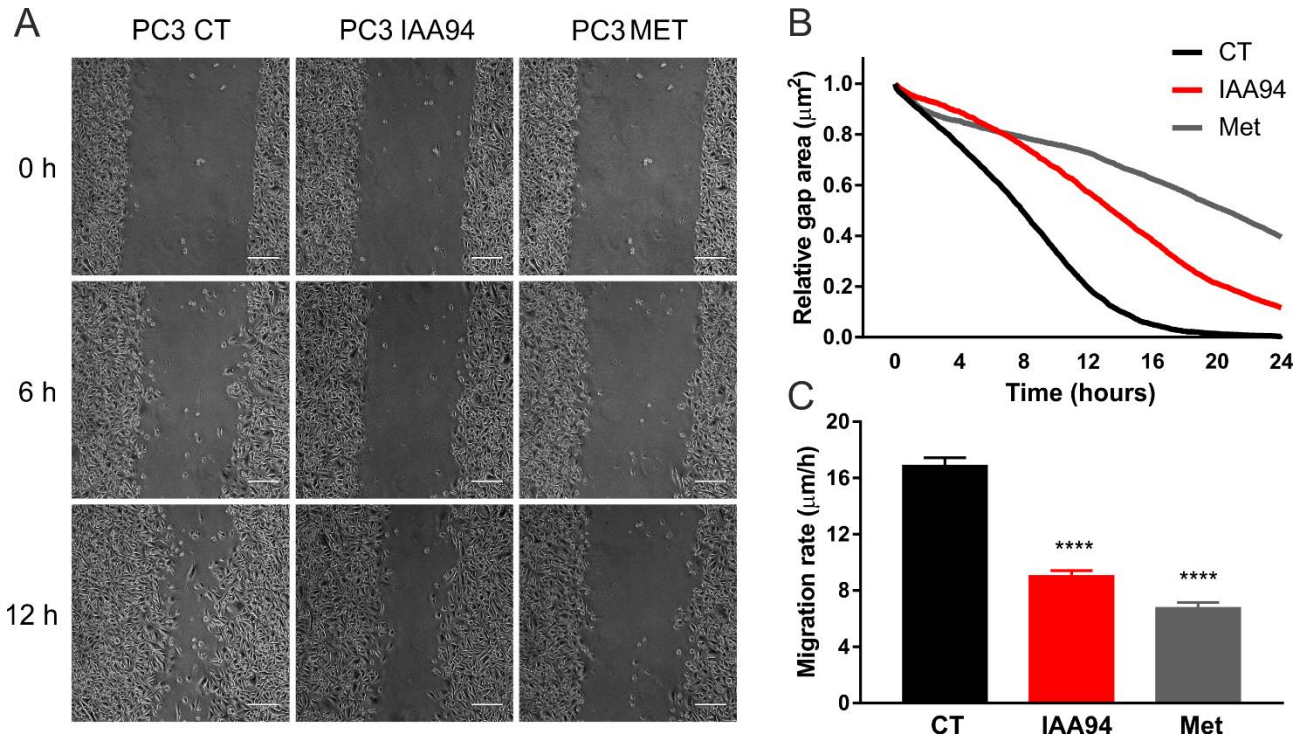



Figure 29. Representative imagines (A) and quantification of wound healing (B) and migration rate (C) of PC3 cells treated with or without IAA94 100 µM or metformin 10 mM for 24 h. Compared to control cells (n=17), IAA94 (n=14, P<0.0001) and metformin (n=14, P<0.0001) led to a significantly reduction of migration rate (One-way ANOVA, Dunnett'st). Scale bar: 200 µm.

All together, these findings demonstrate that CLIC1 activity promotes tumor cell migration with a major effect in tumor prostate cell line.

To further corroborate the involvement of CLIC1 in cell migration, 3D transwell migration assays were performed.

Accordingly with the data already observed in wound healing assay, IAA94 and metformin did not determinate a significant inhibition of migration in CCD841 cells (fig. 30 A-C), whereas they significantly reduced migration of SW620 cells respectively of 45% and 29% (fig. 30 B-D).

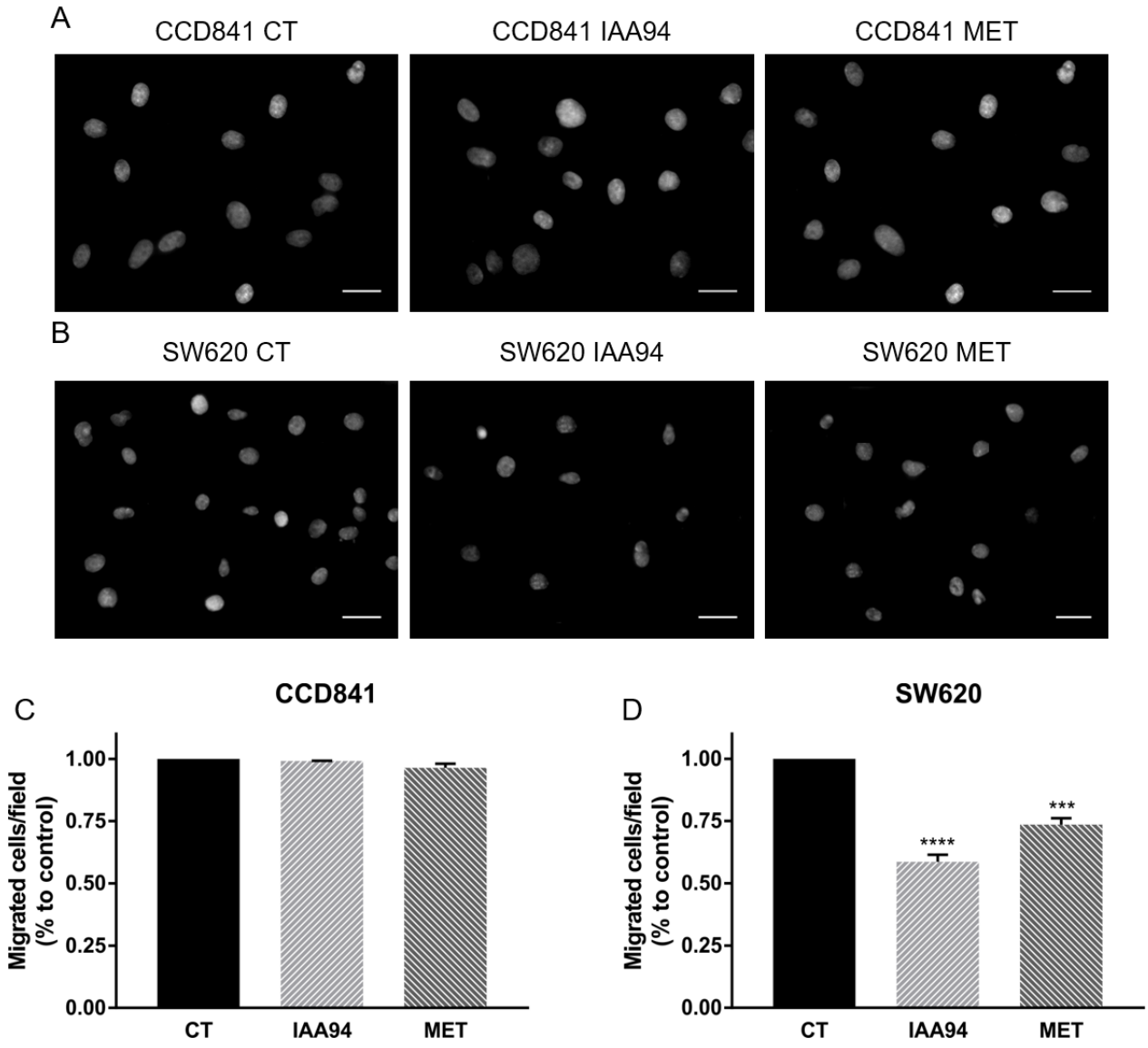



Figure 30. Representative imagines (A-B) and quantification (C-D) of 3D transwell migration assay of CCD841 cells and SW620 cells treated with or without IAA94 100 µM or metformin 10 mM respectively for 24 h and 60 h. The percentage of migrated CCD841 cells was not affected by IAA94 or metformin respect to control (n=3, One-way ANOVA, Dunnett's test, n.s.). Indeed, the number of migrated SW620 cells was significantly decrease after treatment with both IAA94 ($P<0.0001$) and metformin ($P<0.001$) relatively to control (n=3, One-way ANOVA, Dunnett's test). Scale bar: 25 µm.

The strong inhibition of cell migration upon CLIC1 pharmacological inhibition was also confirmed in PC3 cells. IAA94 and metformin were able to significantly reduce the percentage of migrated cells of about 57% and 39% respectively, compared to control (figure 31 B-D). Moreover, IAA94 had no significantly effect on the migration of BPH1 cells, whereas metformin led to a reduction of about 40% (figure 31 A-C).

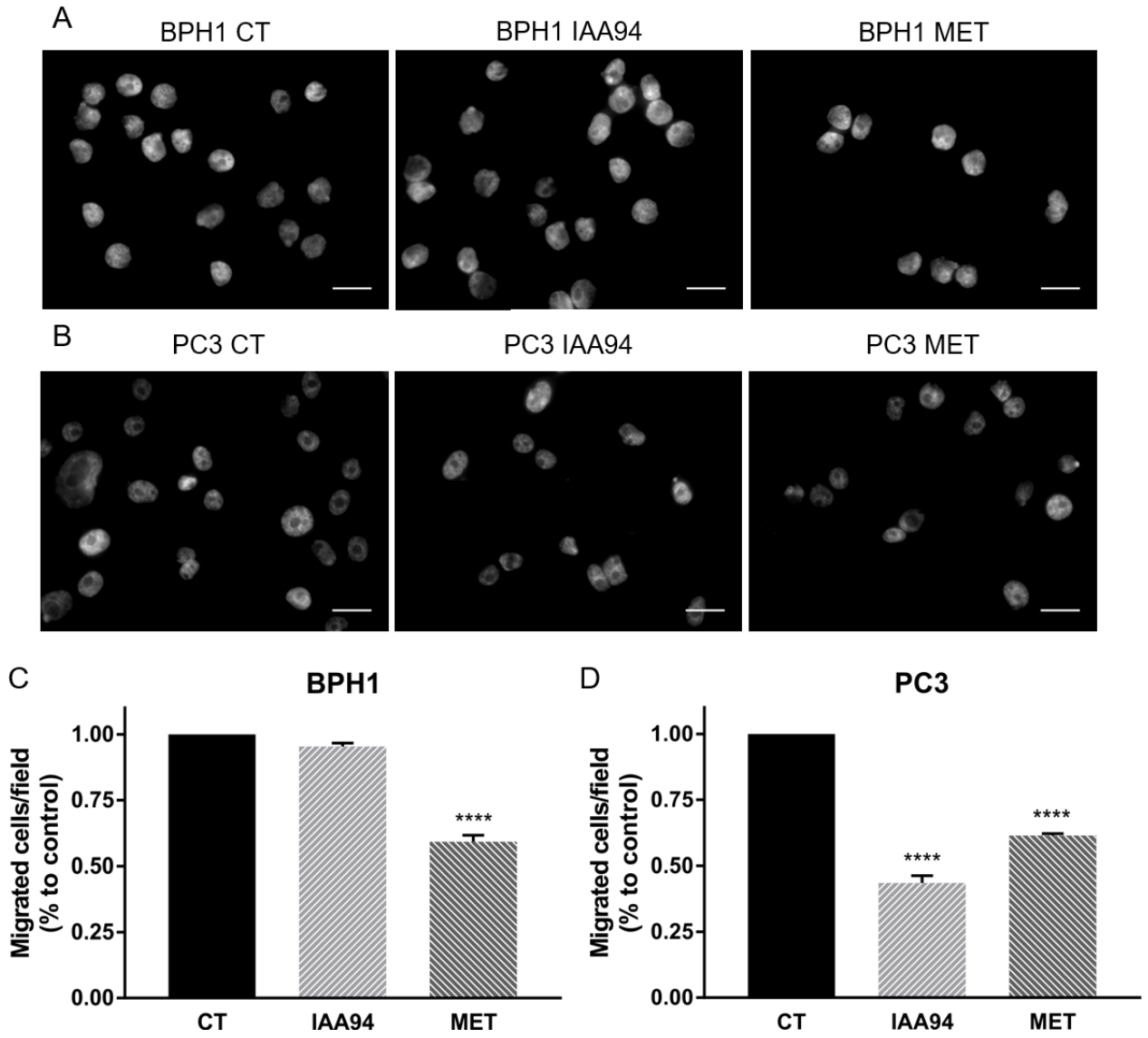



Figure 31. Representative imagines (A-B) and quantification (C-D) of Boyden chamber migration assay of BPH1 cells and PC3 cells treated with or without IAA94 100 µM or metformin 10 mM for 24 h. Compared to control, the percentage of migrated BPH1 cells was not affected by IAA94 (n.s.), whereas it was significantly decreased by metformin ($P<0.0001$) (n=3, One-way ANOVA, Dunnett's test). The number of migrated PC3 cells was significantly reduced after treatment with both IAA94 and metformin relatively to control (n=3, One-way ANOVA, Dunnett's test, $P<0.0001$). Scale bar: 25 µm.

Next it has been observed the effect of CLIC1 knockdown on cell migration through wound healing assay.

The stable knockdown of CLIC1 resulted in a significant inhibition of the migration rate both in colon and in prostate cancer cells. As shown in figure 32, shCLIC1 SW620 cells displayed a 30% slowdown of the migration rate, compared to that of scrambled shRNA SW620 cells.

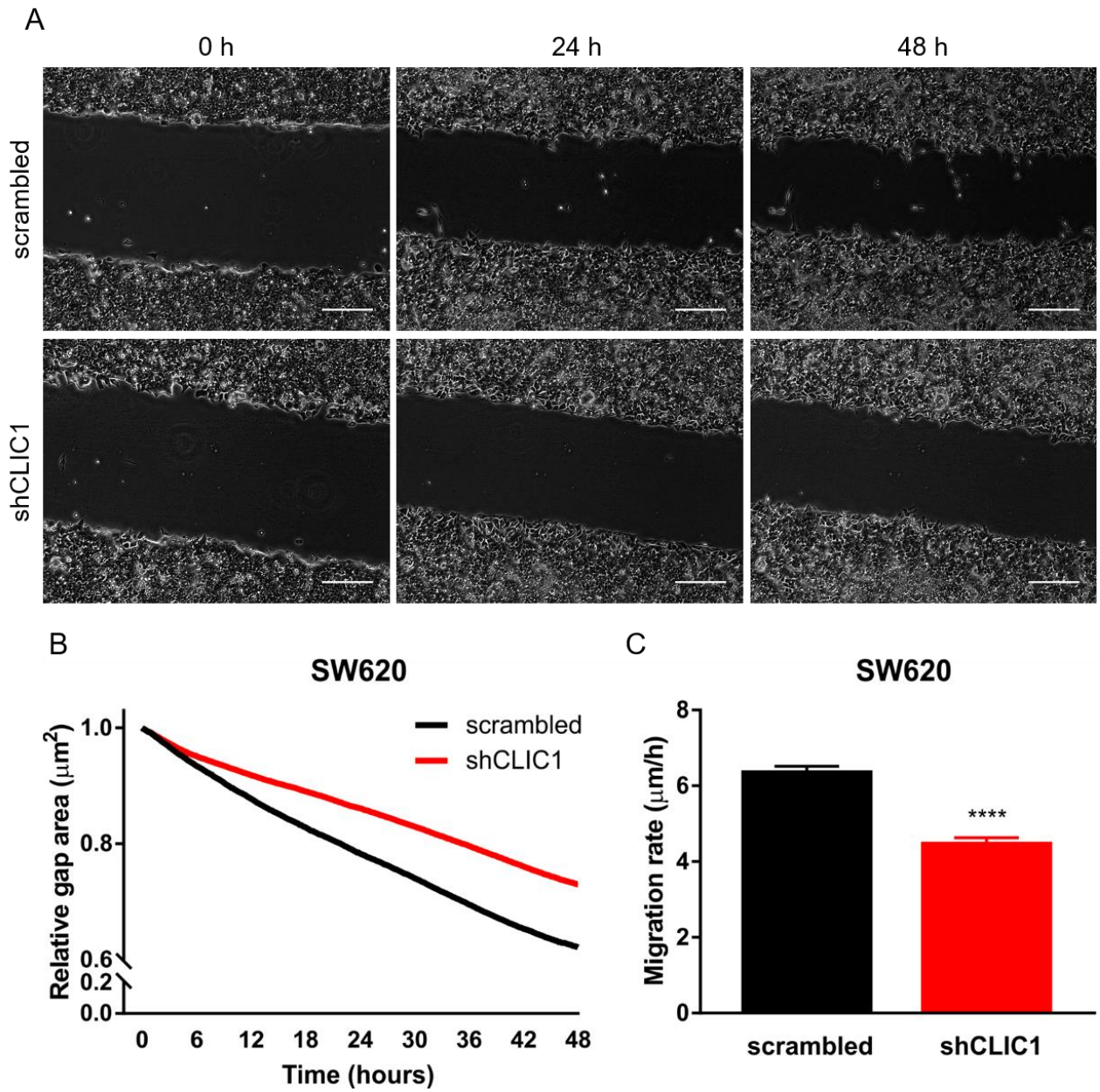



Figure 32. Representative images (A) and quantification of wound healing (B) and migration rate (C) of SW620 cells infected with scrambled shRNA or specific CLIC1 shRNA. In shCLIC1 SW620 cells (n=17) was observed a significant reduction of migration rate compared to scramble shRNA SW620 cells (n=16) (One-sample t test, P<0.0001). Scale bar: 200 μm.

Similarly, shCLIC1 PC3 cells migrate significantly slower than scrambled shRNA PC3 cells, presenting a 28% reduction of the $v_{migration}$ (figure 33).

These findings taken together clearly demonstrate that CLIC1, when localized at the plasma membrane with ion channel function, actively promotes cell migration.

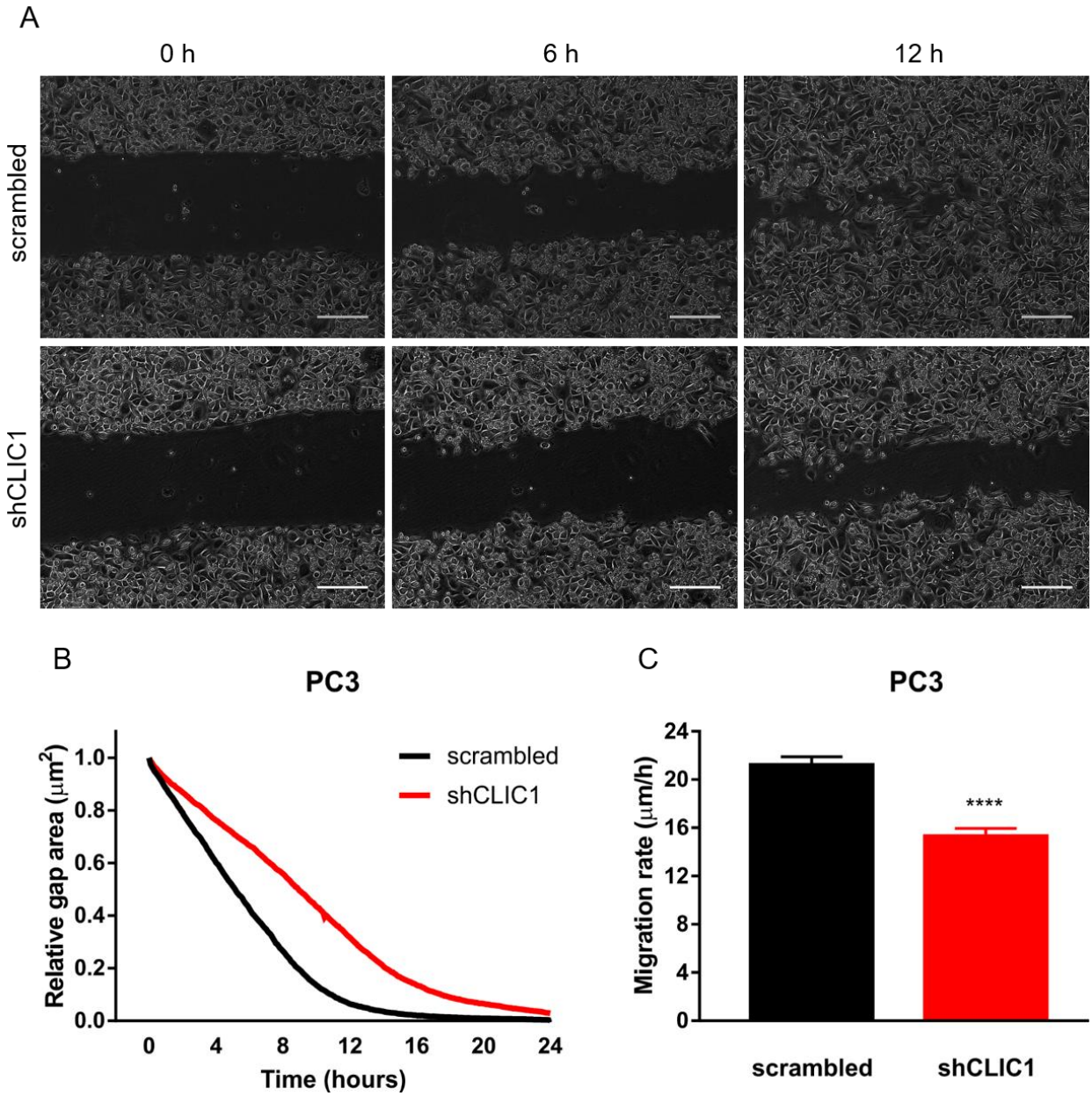



Figure 33. Representative imagines (A) and quantification of wound healing (B) and migration rate (C) of PC3 cells infected with scrambled shRNA or specific CLIC1 shRNA. shCLIC1 PC3 cells (n=26) migrated with a significantly slower migration rate compared to scramble shRNA control cells (n=17) (One-sample t test, $P<0.0001$). Scale bar: 200 μm.

It has been shown that IAA94 has no effect on the migration ability of shCLIC1 SW620 cells, whereas it significantly decreased of about 32% the migration rate of scrambled SW620 cells. On the other hand, metformin led to a 17% decrease of the $v_{migration}$ both in scramble SW620 cells than in shCLIC1 SW620, compared to control (figure 34).

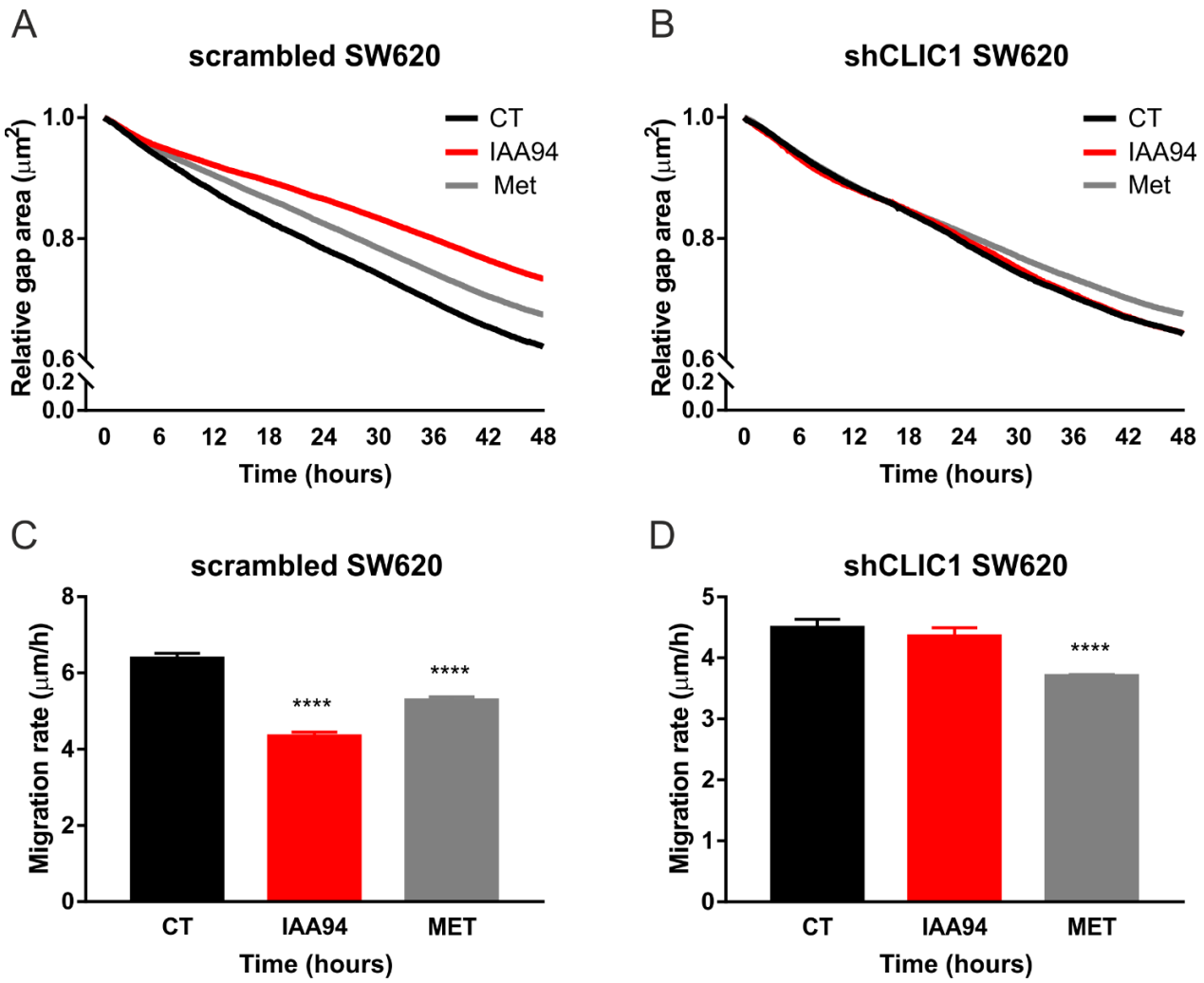



Figure 34. Quantification of wound area closure and migration rate of scramble shRNA SW620 cells (A, B) and CLIC1 shRNA SW620 cells (C, D) treated with or without IAA94 100 µM or metformin 10 mM for 48 h. Compared to control (n=16), IAA94 (n=18) and metformin (n=16) led to a significantly reduction in the migration rate of scramble shRNA SW620 cells (One-way ANOVA, Dunnett's test, P<0.0001). In CLIC1 shRNA SW620 cells metformin led to a significantly reduction of migration rate (n=18, P<0.0001), respect to control (n=26), whereas IAA94 had no effect on migration ability (n=28, n.s.) (One-way ANOVA, Dunnett's test).

It has been observed that IAA94 significantly decreased of 23% the migration rate of scrambled PC3 cells, whereas it did not affect the migration of shCLIC1 PC3 cells. Metformin instead was able to strongly reduce of 41% the $v_{migration}$ of scramble PC3 cells, while it reduce of only 28% the migration rate of shCLIC1 PC3 cells (figure 35).

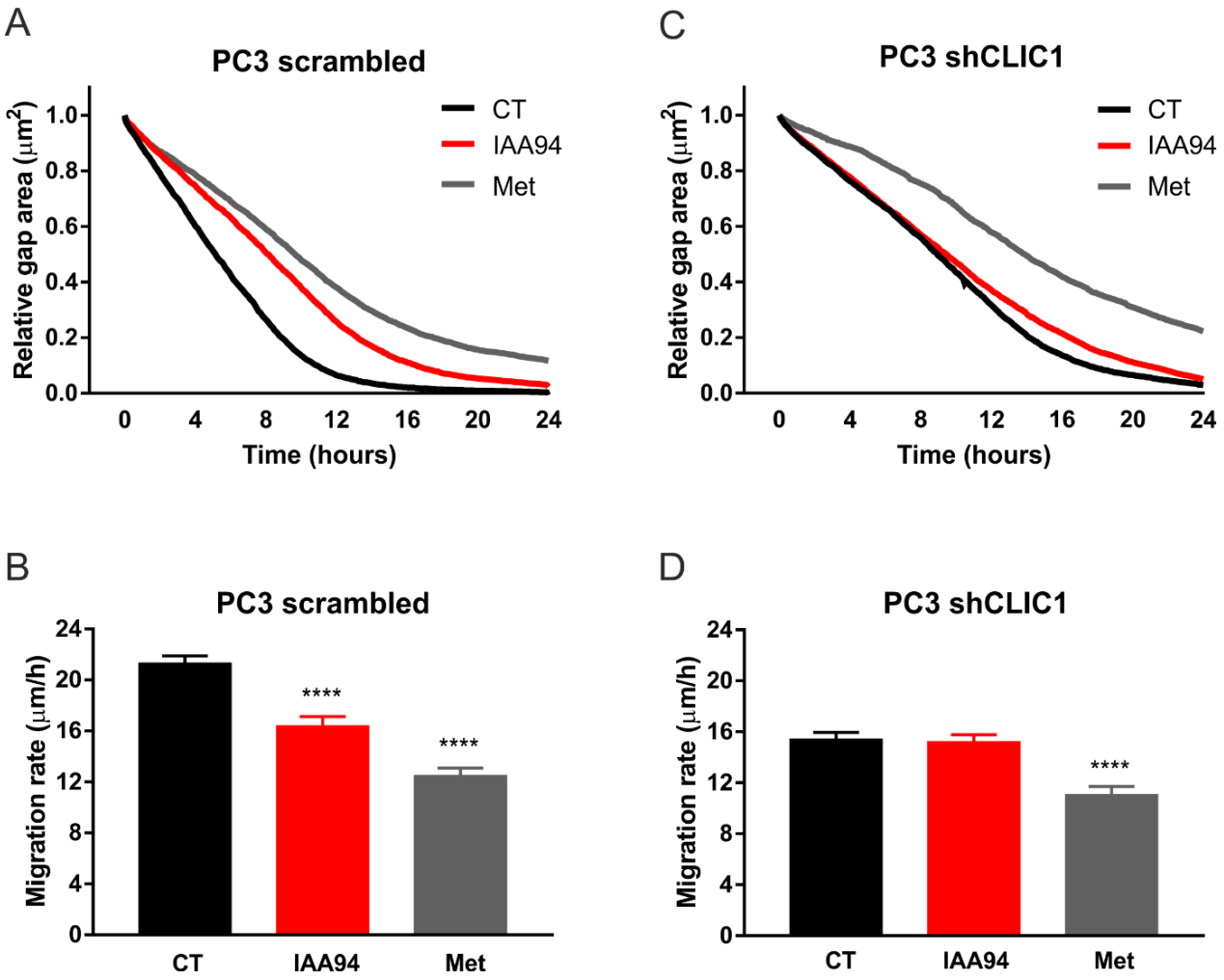



Figure 35. Quantification of wound area closure and migration rate of scramble shRNA PC3 cells (A, B) or CLIC1 shRNA PC3 cells (C, D) treated with or without IAA94 µM or metformin 10 mM for 24 h. In scramble shRNA PC3 cells, IAA94 (n=17) and metformin (n=15) led to a significantly reduction of migration rate, compared to control condition (n=17) (One-way ANOVA, Dunnett's test, $P<0.0001$). Compared to control cells (n=26), metformin (n=18, $P<0.0001$) led to a significantly reduction in the migration rate of CLIC1 shRNA PC3 cells, whereas IAA94 had no effect on migration (n=28, n.s.) of these cells (One-way ANOVA, Dunnett's test).

Therefore, it has been shown that CLIC1 knockdown completely abolished the anti-migratory effect of IAA94 but only partially reduced that of metformin. These results clearly demonstrate that the action of IAA94 treatment on cell migration was specifically mediated by CLIC1 inhibition, while the effect of metformin was performed also through inhibition of its other targets. Thus, the hypothesis that CLIC1 plays a pivotal role in the regulation of tumor cell migration was supported.

# 5.6 CLIC1 regulates tumor cell migration through MAPK/ERK pathway

The MAPK signalling pathway is frequently up-regulated in tumors, including colon cancer [135] and prostate cancer [279]. This pathway plays a critical role in the tumorigenesis process, promoting proliferation and migration of cancer cells [280]. A previous study has highlighted that CLIC1 promotes migration and invasion of colon cancer LOVO cells via regulating the ROS/ERK pathway under hypoxia-reoxigenation conditions [158]. Another study has reported that the downregulation of CLIC1 by siRNA inhibits MAPK/ERK signalling pathway in PC3 cells [205].

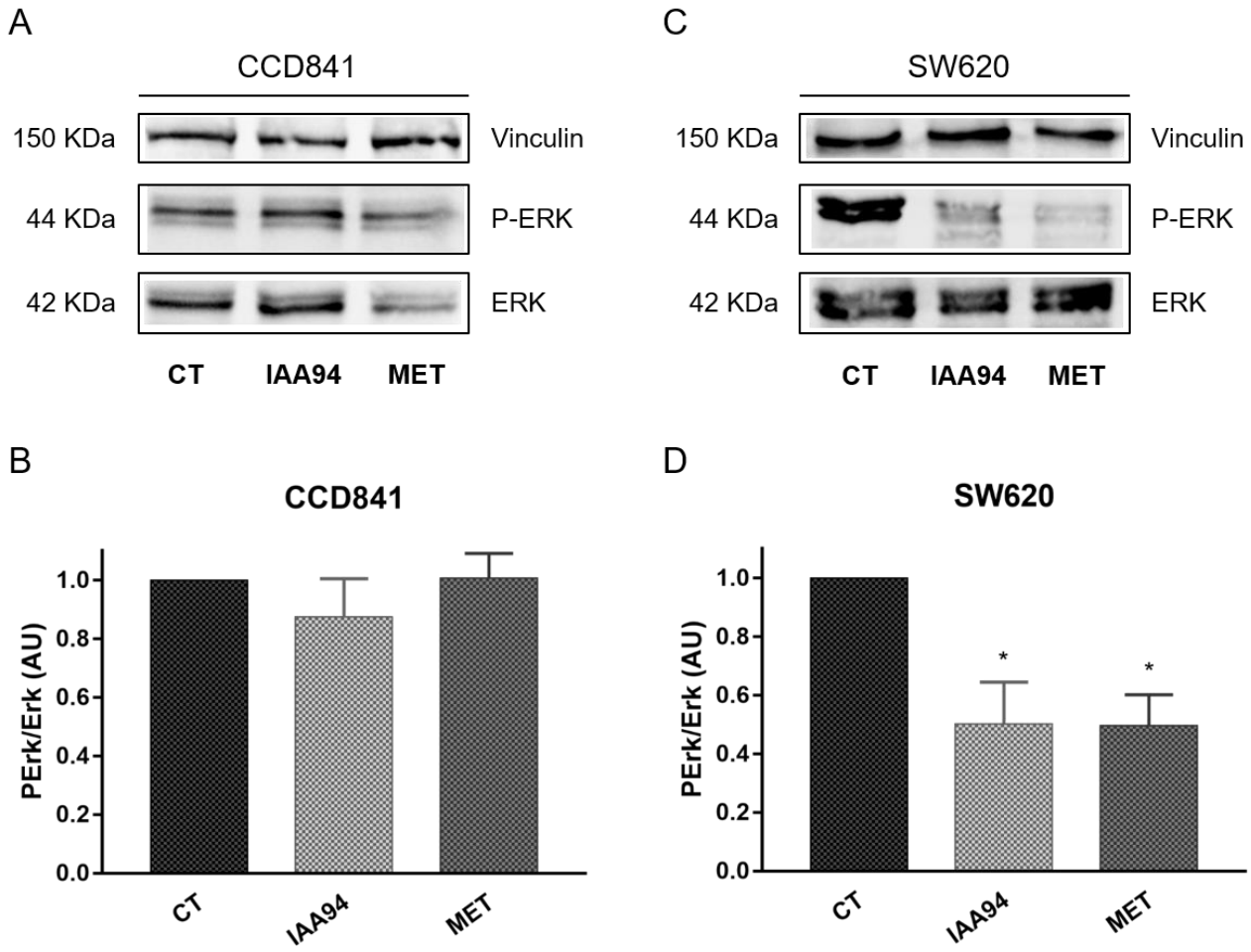



Figure 36. (A, B) Representative western blots showing levels of phospho-ERK, total ERK, and vinculin, used as loading control. (C, D) Quantification of pERK/ERK ratio in whole cell lysates of CCD841 and SW620 cells treated with or without IAA94 and metformin for 48 h. Compared to control, both IAA94 and metformin significantly decreased ERK activation in SW620 cells (n=3, One-way ANOVA, Dunnett's test, $P<0.05$), whereas in CCD841 cells they did not significantly change the pERK/ERK ratio (n=3, One-way ANOVA, Dunnett's test, n.s. versus control).

To investigate the molecular mechanisms through which CLIC1 activation induces cell migration, the levels of phosphorylated (active) ERK1/2 were examined through western blot analysis.

As shown in figure 36, and consistently with migration data, both IAA94 and metformin significantly decreased ERK activation in SW620 cells of about 50%. The same treatments did not significantly modify ERK activation in CCD841 cells.

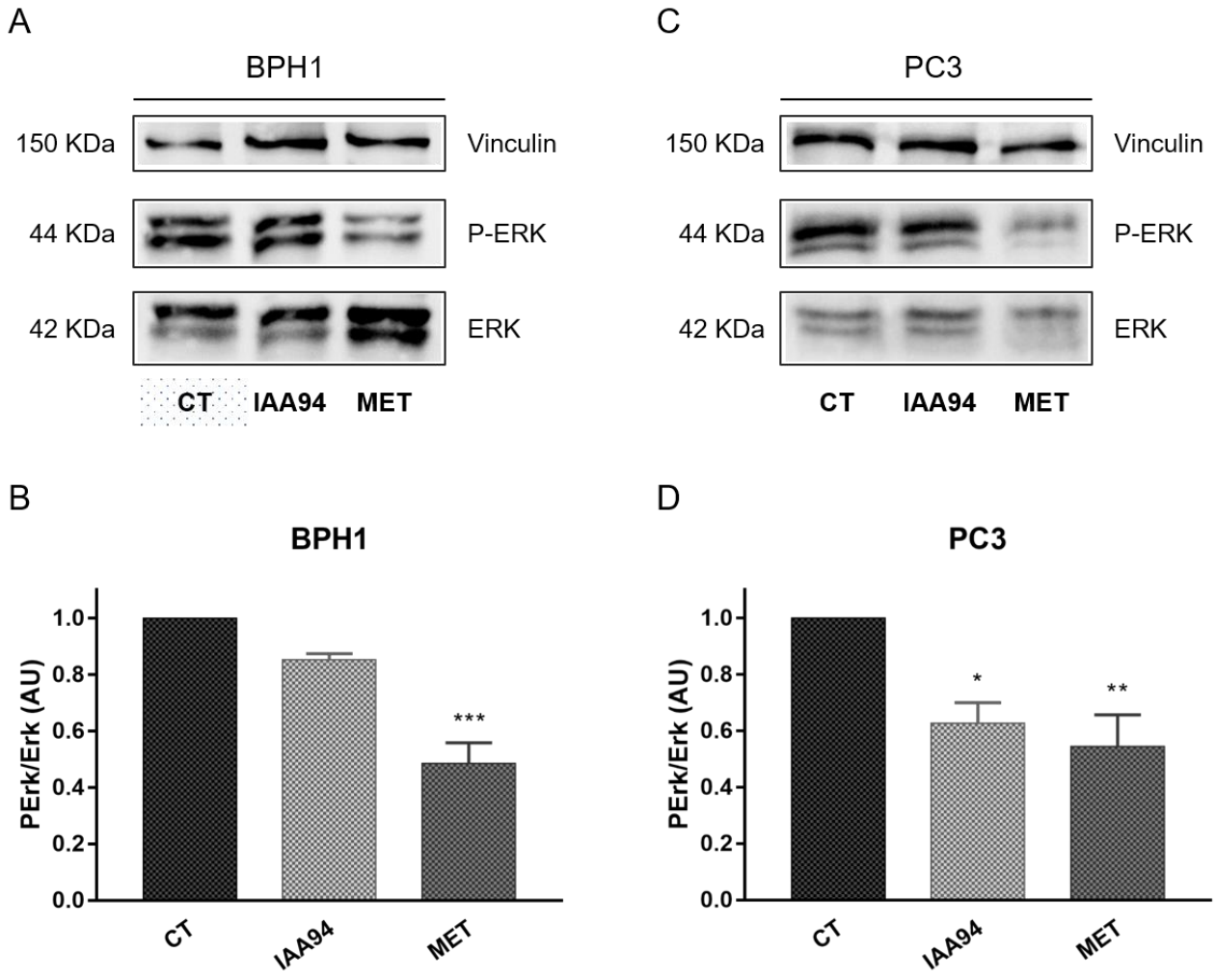



Figure 37. (A, B) Representative western blots showing levels of phospho-ERK, total ERK, and vinculin, used as loading control. (C, D) Quantification of pERK/ERK ratio in whole cell lysates of BPH1 and PC3 cells treated with or without IAA94 and metformin for 48 h. Compared to control, IAA94 significantly decreased ERK activation in PC3 cells ($P<0.05$) whereas had no effect on ERK phosphorylation in BPH1 (n.s.) (n=4, One-way ANOVA, Dunnett's test). Metformin also significantly reduced ERK activation in BPH1 cells ($P<0.001$) and in PC3 cells ($P<0.01$) (n=4, One-way ANOVA, Dunnett's test versus control).

In line with the results obtained in the migration assays, IAA94 significantly decreased ERK activation of about 37% in PC3 cells, whereas it did not significantly modify the activation of this

pathway in BPH1 cells. Metformin significantly reduced ERK phosphorylation of 51% in BPH1 cells and 45% in PC3 cells (figure 37).

Taken together these results demonstrate that CLIC1could plays a role in the promoting colon and prostate cancer cells proliferation and migration through the activation of the MAPK pathway.

# -6-

# DISCUSSION

Under physiologic conditions, there is a fine balance between the generation and removal of reactive oxygen species (ROS) in order to maintain cell homeostasis [93, 94]. The redox balance ensures the normal function of redox-sensitive proteins, allowing the cells to properly respond to endogenous and external stimuli. An increase in ROS production or a decrease in ROS-scavenging capacity (or both) can disrupt cell redox equilibrium, leading to a condition of oxidative stress [281].

ROS are persistently elevated in many types of tumors, where they contribute to all the stages of carcinogenesis both promoting genomic instability and functioning as a second messenger [95, 98, 282]. In particular, ROS-sensitive signalling pathways have been involved in cell growth/proliferation, apoptosis, differentiation, protein synthesis, migration and invasion [96-98, 282]. Among the other tumors, high ROS level plays a broader role in the development and progression of prostate (PCa) [283, 284] and colorectal cancer (CRC) [285, 286].

CRC and PCa are two of the most commonly diagnosed malignancies throughout the entire planet [114, 173]. Over the last years, significant progress has been made in the early diagnosis and in the systemic treatment of these malignant conditions [150]. However, the current therapies are far from satisfactory and both colorectal and prostate tumors remain among the leading causes of cancer fatality [115, 116, 174, 175].

In case of a localized tumor mass CRC and PCa can be effectively eradicated by surgical procedure and/or radiotherapy and chemotherapy [148, 149, 188]. However, the chances of survival drop abruptly the more advanced is the cancer stage [147, 201, 202]. Late diagnoses are usually accompanied by tumor metastasis that are then the main causes of death [124, 203]. Therefore, the research of new strategies to replace or complement actual standard therapies are urgently needed. Moreover, the direct correlation between clinical prognosis and tumor development at the time of the first diagnosis, make the discovery of more specific biomarkers for early detection of CRC and PCa necessary.

One of the main problem that traditional chemotherapic drugs are facing concerns their inability to selectively recognize normal non-cancerous cells from malignant tumor cells, resulting in a high systemic toxicity [287, 288]. Indeed, practically all the known proteins that are deregulated in the tumorigenic process are also implicated in important physiological functions. Targeting these fundamental proteins makes the effective eradication of tumor cells without producing a serious collateral damage very difficult [1, 287, 289]. Finding a target(s) that is not only important for neoplastic progression but also specific for malignant cells could offer the possibility to perform a successful therapy, minimizing side effects [289-291].

The main aim of my thesis points the attention on CLIC1 protein as a promising candidate to satisfy these requests.

CLIC1 is a metamorphic protein able to shuttle between a cytosolic soluble form and a membrane-integrated form with ion channel activity [51, 59].

As a consequence of perturbations in their redox homeostasis, cells can modulate the rate of translocation of CLIC1 in the membranes as well as its channel activity [51, 61, 62]. The hypothesis underlying this research project is based on the idea that during neoplastic process, PCa and CRC cells leave homeostatic condition to enter in a new steady state, characterized by sustained high ROS levels and up-regulation of several redox-sensitive signalling pathways. In this allostatic condition the functional insertion of CLIC1 in the plasma membrane, that in a physiological context is a transient event, becomes chronic, thus contributing to the neoplastic transformation (or progression).

Our laboratory had previously demonstrated that CLIC1 was overexpressed in glioblastoma (GBM) and was essential for proliferation of GBM cancer stem cells (CSCs) [102]. Moreover, CLIC1 was found to be up-regulated in a variety of human tumors compared with normal tissue, including colon cancer [112] and prostate cancer [113]. Therefore, the first question that arises legitimately is: what is the role of CLIC1 in the proliferation of colon and prostate cancer cells?

Differently from the data reported in literature, it has not been observed a significant difference in the level of CLIC1 protein expression between normal/benign cells and malignant cells of both CRC and PCa (fig. 8). As CLIC1 is a metamorphic protein, we asked whether there were differences in the protein localization and channel activity. Indeed, immunolocalization of the protein and electrophysiological measurements of the chloride current have demonstrated the presence of CLIC1 in a transmembrane functionally active form, exclusively in malignant cells (fig. 9 - 10). Therefore, the presence of CLIC1 as active ion channel, and not the total amount of protein expressed, is the discriminating factor between normal/benign cells and malignant cells.

By different loss-of-function approaches, we demonstrated that the protein is extremely important for *in vitro* proliferation.

Silencing CLIC1 in CRC and PCa cells reduced cell proliferation and impaired cell cycle progression, elongating the G1/S transition time (fig. 13, 24, 25), as previously described for other model systems [67, 91].

Pharmacological inhibition of CLIC1 activity with the specific inhibitor IAA94 has confirmed that CLIC1 positively regulates the proliferation of CRC cells (which display higher amount of CLIC1 mediated current), and in lesser extent in that of PCa cells (which have lower amount of CLIC1 mediated current). The absence of anti-proliferative effect upon inhibition of the other chloride channels is a direct demonstration about the specific role of CLIC1 ion channel in modulating CRC and PCa cell growth (fig. 14 B and 15 B). Interestingly, CLIC1 activity is not involved in the proliferation of normal epithelial colon cells (fig. 14 A).

The implication of CLIC1 in PCa and CRC cell proliferation was further confirmed by the observation that CLIC1 silencing completely abolish the anti-proliferative effect of IAA94 (fig. 18, 19). At the same time, active caspase 3 analysis has shown that IAA94 treatment is toxic for prostatic hyperplasia cells, while it does not produce significant changes in apoptosis activation of the other cells (fig. 20, 21). Downregulation of CLIC1 by specific shRNA as well as pharmacological inhibition of CLIC1 by IAA94 impaired cell cycle progression of the malignant cells, leading to an accumulation in G1 phase (fig. 22, 23). These results suggest the need of CLIC1 mediated chloride current for the transition from G1 to S phase of the cell cycle, as previously described for GBM CSCs [91].

All data presented so far confirm the involvement of CLIC1 in proliferation and cell cycle progression of cancer cells, in line with previous studies [49, 67, 91]. Thus, it is reasonable to think that the localization of CLIC1 in the plasma membrane confers a proliferative advantage to tumor cells and therefore promotes the growth of tumor mass.

One of the main factors known to regulate CLIC1 activity is the oxidative level of the cells [51, 61]. We have previously reported that CLIC1 translocates to the membrane following an increase in cytosolic oxidative state [51, 59, 61, 62]. It has also been demonstrated that an increase in ROS level is fundamental for the progression from G1 to S phase of the cell cycle [103, 292]. In addition, it has been shown that increased ROS levels are crucial for the regulation of gene expression related to cancer cell survival, growth and cell cycle progression [96-98].

PCa and CRC cells are characterized by defect in redox system and produce persistently elevated amount of ROS [283-286]. Among the main sources of the oxidative stress in tumors there are NADPH oxidases (NOX), transmembrane proteins that transport electrons across biological membranes thereby generating ROS [293]. In particular, NOX1 and NOX5 are overexpressed in colon and prostate cancer and are considered responsible for sustained ROS production in tumor microenvironment [294-303]. Furthermore, our laboratory has previously reported the presence of a positive feed-forward mechanism between ROS production and CLIC1 functional expression as an ion channel in the activated microglia cells membranes [81, 82]. Activated microglia is in an allostatic state in which CLIC1 and NADPH oxidase are hyper-activated and they are part of a self-sustain mechanism. During ROS production, NOX generates also an outward negative charge flow that requires to be compensated. CLIC1 mediated chloride current balances the excess of positive charges produced by the enzyme and plays therefore a major role in supporting NOX activity [82].
On this basis, it is possible to hypothesize that activated microglia cells and tumor cells share a similar mechanism. In this scenario, tumor cells would be in an allostatic state and would

continuously produce high ROS amount. The elevated ROS level accelerates the G1/S phase transition of cell cycle, promoting the aberrant hyperproliferation of tumor cells. In response to the peak of ROS, CLIC1 translocates to the plasma membrane, where it acts as chloride channel. CLIC1 activity generates a flux of anions, which in turn counterbalances the negative charge flow extruded by NADPH oxidase. In this manner, CLIC1 in its transmebrane form supports the prolonged generation of ROS by NADPH-oxidase, driving the volume rearrangements necessary for cell proliferation.

Tumor metastasis are incurable and in patients with PCa and CRC, similar to those with other malignancies, represent the main cause of cancer-related morbidity and mortality [124, 146, 202, 203]. The metastatic cascade is a sequential multi-step process, which depends on different properties of cancer cells, including their migration ability [304]. ROS up-regulation in tumor microenvironment is considered a key promoter of the metastatic cascade, resulting in increased cell motility and invasiveness of cancer cells and sustained angiogenesis [96, 305-307]. CLIC1 in turn has been reported to be implicated in tumor cell migration and invasion and in angiogenesis [46, 49, 157, 158, 205]. Thus, another question that is worth asking is: what is the role of CLIC1 in PC and CRC cell migration?

Scratch and transwell migration assay experiments have unequivocally shown that pharmacological inhibition of CLIC1 activity by IAA94 compromises the migration ability of malignant cells (fig. 27, 29, 30 B, 31 B) but not that of normal/benign cells (fig. 26, 28, 30 A, 31 A). It has been also observed that CLIC1 downregulation by shRNA decreases the migration rate of malignant cells and abolishes the anti-migratory effect of IAA94 (fig. 32-35). These findings further corroborate an effective and specific involvement of CLIC1 in cancer cell migration.

Contrary to what has been observed for proliferation, the impact of CLIC1 on cell migration seems to be stronger in PCa cells, which display lower CLIC1-mediated current, respect to CRC cells, which are characterized by higher channel mediated current. This result suggests that CLIC1 may behave in different way depending on cell contexts.

Now a spontaneous question arises: what is the molecular mechanism through which CLIC1 modulates migration of CRC and PCa cells?

The MAPK/ERK pathway is frequently up-regulated in a variety of cancers, including colon [135] and prostate cancer [279] and it can be activated by ROS [280, 311-313]. It is known that MAPK/ERK pathway is involved both in cell growth and in cell death, and can promote abnormal proliferation, migration and invasion of cancer cells [280].

In line with previous studies [158, 205, 308] our experiments have shown that CLIC1 inhibition decreased ERK activation in malignant cells, whereas it has no effect on the normal/benign cells

(fig. 36 and 37). In light of the results obtained, it is reasonable to think that CLIC1 promotes the motility of PCa and CRC cells by a specific activation of the MAPK/ERK pathway.
We have postulated two hypothesis to explain the possible mechanism by which CLIC1 regulate MAPK/ERK pathway. On one hand, CLIC1 in transmembrane channel form could indirectly sustain the activation of ERK pathway supporting the generation of high ROS level by NADPH oxidase, as previously explained. On the other hand, it is also possible that CLIC1 in cytoplasmic soluble form could directly interplay with MAPK/ERK pathway. The intracellular cascade mediated by CLIC1 and responsible for MAPK/ERK signalling activation has not yet been elucidated. It is likely that CLIC1, in response to oxidative intracellular environment, will release glutathione from its binding site and interact with the cytoskeletal proteins [309]. Then it promotes cell volume regulation [157, 310], may activate intracellular kinases and ultimately affects cell motility via MAPK/ERK pathway.
The final effect of CLIC1 in a certain type of cell may be greater on migration than on proliferation or vice versa, depending on which of the possible CLIC1 mechanisms is prevalent.
By virtue of its action on cell migration, CLIC1 could confer an increased migration ability to tumor cells and therefore it could be a primary actor in the progression of PCa and CRC towards a more aggressive disease. This observation appears of primary importance considering that metastasis are estimated to be responsible for about 90% of cancer deaths [311].
As future research plan, invasion assays will be performed in order to explore the possible role of CLIC1 in promoting invasion of PCs and CRC cells.

Overall experiments performed in this project suggested a role of CLIC1 in promoting the development and progression of PCa and CRC. However, these characteristics do not represent *per se* a valid approach for antitumoral therapies. What makes CLIC1 particularly attractive is that, unlike the plethora of different proteins involved in tumorigenesis, CLIC1 is exclusively present in the membrane of tumor cells. The change in its localization and function, uniquely associated with neoplastic transformation, could permit to specifically hit malignant cells and save their normal counterparts. In this scenario, CLIC1 could represent not only a promising pharmacological target, but also a possible biomarker in monitoring tumor progression.

IAA94, the only specific CLIC1 channel blocker so far identified [52, 59, 63], is highly toxic in vivo, making impossible a potential clinical use. In this context, the research of other compounds which prove to be able to inhibit CLIC1 but exhibit reduced collateral effects has a primary importance.
Recently, several studies have demonstrated that metformin, the most used anti-diabetic drug, exhibits *in vitro* and *in vivo* antineoplastic activity against different human tumors [209, 212, 221-

225], including PCa [227, 229, 234-236] and CRC [230, 240-243, 246]. The antineoplastic activity of metformin is mainly mediated by AMPK activation, which results in downregulation of mTORC1 and the IGF-1/AKT pathways and inhibition of cellular metabolism. These mechanisms promote, according to the cell type and the extent of stress, additional suppressive mechanisms leading to inhibition of cancer cell proliferation, cell-cycle arrest or cell death [315, 316].

In addition to these intracellular targets, our laboratory has recently proposed CLIC1 channel as molecular target of metformin action [91]. It has been demonstrated that metformin interacts with CLIC1 from the external site of the membrane by binding the Arg29 inside the pore only when the channel is in the open state. We found that in GBM CSCs metformin inhibits cell proliferation and blocks CLIC1 mediated current in a very similar manner to IAA94 [91].

In accordance with our initial hypothesis that CLIC1 is differentially localized and activate in tumor cells compared to normal cells, it is reasonable to suppose a preferential activity of metformin towards tumor cells.

Thus, the following question arises: what is the effect of metformin on proliferation, cell cycle progression and migration abilities of PCa ad CRC cells?

The reduced effect of metformin on proliferation and migration in CLIC1-silenced cells provides a clearly demonstration that metformin activity should be, at least partially, dependent by CLIC1 inhibition. Moreover, experimental results have shown that metformin effect is not completely superimposable to that of the specific CLIC1 channel blocker (IAA94), suggesting that metformin acts not only on CLIC1, but also on its other intracellular targets.

Comparing the effects of metformin and IAA94, some differences in efficacy, cell sensitivity and activated pathways are evident in prostate and colon cells. In CRC and PCa cells as well as in prostatic hyperplasia cells metformin displays an anti-proliferative and anti-migratory activity that is, at least in part, mediated by MAPK/ERK pathway inhibition. Metformin is also associated with apoptosis induction in prostate cells and cell cycle arrest in G2/M in CRC cells. Interestingly, in normal colon cells metformin has only a weak anti-proliferative effect, due to apoptosis activation. These findings suggest that metformin may act differently depending on cell type.

Our study reinforces the potential benefit of metformin in cancer treatment, accordingly with the recent publications, and it provides new insight about the possible mechanism of action of this anticancer drug.

However, it has been shown that metformin is not the ideal CLIC1 blocker either because is not highly specific for CLIC1 and either because its antitumor effects occur at concentration in the millimolar range. Anyway, chronic treatment with metformin has been demonstrated to be very well tolerated in patients and produce only negligible side effects.

The future research line will be orientated towards the discovering and testing of new pharmacological compounds that have the ability to target CLIC1 channel with high specificity, in order to improve the inhibiting efficacy and reduce the toxicity.

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