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New DAG-dependent mechanisms modulate cell cycle progression

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Introduction

1.1 CELL CYCLE OF MAMMALIAN CELLS

Mammalian cell cycle consists in a series of events that leads to DNA duplication and chromosomes segregation into two new daughter cells (Figure 1) [1] [2] [3]. These events can be divided in two steps: the first, known as interphase, consists in a transition phase through which cells prepare to divide; a second, called mitosis, consisting in the dividing process in two new daughter cells. In particular, the interphase can be separated in three more stages: a G1 phase where cells start to prepare for DNA synthesis, which takes place during the next phase, named S; finally, a G2 phase where cells prepare to divide. On the other hand, also the mitotic process can be divided itself in different steps: prophase, in which chromatin starts to condensate in chromosomes and two structures, called centrosomes, coordinate the microtubule synthesis and disposition; prometaphase where nuclear envelope disappears after phosphorylation of lamins and microtubules attach the chromosomes at the kinetochores; metaphase, through which the alignment of the chromosomes along the metaphase plate (equatorial plane) takes place; anaphase, where the chromosomes are dragged to the two extremities of the cell; telophase, during which decondensing cromosomes are surrounded by a new nuclear envelope; cytokinesis, already begun in late telophase, where the cell splits in two new daughter cells [1] [2] [3].

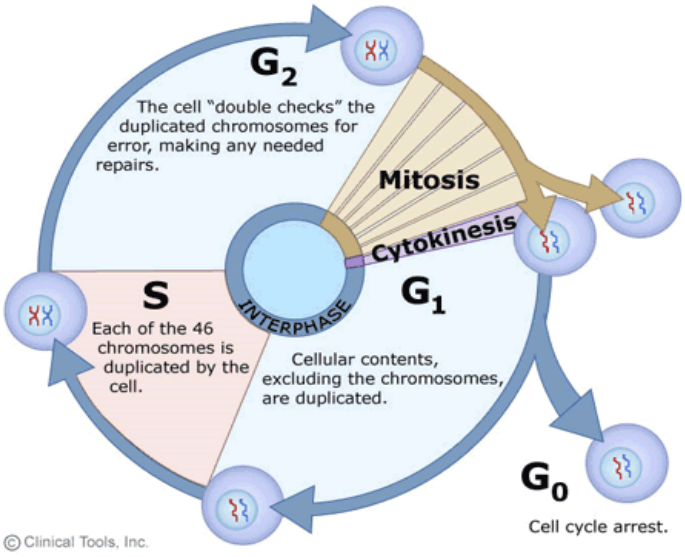

Figure 1 : Schematic representation of the cell cycle stages. *G0: cell cycle is blocked and cells does not proliferate. G1: cells increase their contents and their size. S: duplication of the genetic chromosomes. G2: cells check the right chromosomes duplication and prepare for mitosis. Mitosis/Cytokinesis: several steps through which cells undergo division creating two new daughter cells.*

1.1.1 Cyclins, Cyclin-Dependent Kinases and cell cycle inhibitors

All the steps through which a cell divides are strictly controlled and regulated by different proteins [4] [5] [6] [7] [8] [3]. Among these enzymes we can find the Cyclins, discovered for the first time in 1982 thanks to pioneering studies on sea urchin oocytes [9]. Cyclins are molecules characterized by oscillations strictly connected to the different phases of cell cycle progression, given by real fluctuations of their expression and degradation generally induced by the ubiquitin mediated system . Several isoforms of Cyclins are known to play a fundamental role in cell cycle regulation, such as Cyclins D, Cyclins E, Cyclins A and Cyclins B. The role of these proteins has been described to be regulatory: indeed, Cyclins are the regulatory molecules of the complex that they form with the Cyclin-Dependent Kinases (Cdk) [10, 11]. The expression of these phosphotransferases does not change along the cell cycle progression, but their activation is dependent by different mechanisms such as phosphorylations [10-12]. The complexes Cyclin-Cdk are activated in specific phases of the cell cycle regulating its progression until mitosis. Other proteins are involved in these processes. Among them, different kinases (Polo-Like Kinase 1, Cdk Activating Kinases, Wee1) and phosphatases (cdc25a/b/c) are known to play fundamental roles through cell cycle progression. In addition, it has been reported through the years the important role of Cyclin/Cdk inhibitors including the Cip/Kip (p21 and p27) and Ink4 Cdk (p15, p16) [4].

1.2 SPECIFIC MODULATION OF CELL CYCLE CHECKPOINTS

1.2.1 G1/S phase

In this phase of cell cycle the D and E isoforms of Cyclins are involved (Figure 2) [13-15]. In particular, Cyclins D interact with Cdk4/6, fundamental complex for G1 entry [14]; once activated, Cdks phosphorylate the Retinoblastoma Protein (pRB), which, in its dephosphorylated form, is bound with the Elongation Factor 2 (E2F) [16] [17] [18]. Until the complex pRB-E2F is present, E2F cannot promote the G1/S transition of the cell cycle. The phosphorylations by Cdk4/6 on pRB allow the complex to split and the E2F to become active and affects its related targets [19]. Here, Cyclins E, interacting with Cdk2, starts to be highly synthesized, playing a fundamental role in the entry into the S phase [20, 21]. Notably, these events can be inhibited by the action of some

molecules which inhibit the formation of the complexes between Cyclins and Cdks [22, 23] . In particular, at these stages of the cell cycle, the most important inhibitors result to be the Cip/Kip p21, p27 and Ink4 Cdk p16.

1.2.2 G2/M phase

At the end of the S phase, Cyclin A/Cdk2/1 complexes lead the cells into early G2 phase. Next, as widely described in literature [24-26], the formation of the Mitosis-Promoting-Factor (MPF) , represented by Cyclin B1/Cdk1 interaction, leads the cells to enter into the mitotic process (Figure 2) [27]. MPF becomes active when Cdk1 is phosphorylated at Thr161 by Cdk activating kinases (CAK) and de-phosphorylated by Cdc25c at Thr14/Thr15 [28]. In addition, also Cyclin B1 has to be phosphorylated to promote the activation of the complex. Indeed, different kinases, such as Cdk1 and Polo-like kinase 1 (PLK1), add phosphates on a group of five Ser, the so called cytoplasmic retention signal (CRS) domain of Cyclin B1, which regulates its nuclear translocation at late prophase [29, 30]. The nuclear import of Cyclin B1/Cdk1 has been deeply described, but it remains not completely understood for the lack of a canonical nuclear localization signal (NLS) in Cyclin B1 structure, usually necessary for nuclear import of proteins through the karyopherins/importins system [31]. However, once in the nuclei, Cyclin B1/Cdk1 phosphorylates a wide number of substrates, such as Lamins, driving the cells into mitosis [27]. Finally, at the end of the mitotic process, Cyclin B1 begins to be degraded by the APC/C complex and Cdk1 undergoes inactivation leading cells to mitotic exit and cytokinesis [32, 33]. As well as for the G1/S transition, also the G2/M progression can be inhibited by several molecules. Indeed, the action of p21, Cip/Kip inhibitor, has been reported to be involved in the degradation of Cyclin B1 and, then, in the inhibition of the activity of the complex Cyclin B1/Cdk1 [34].

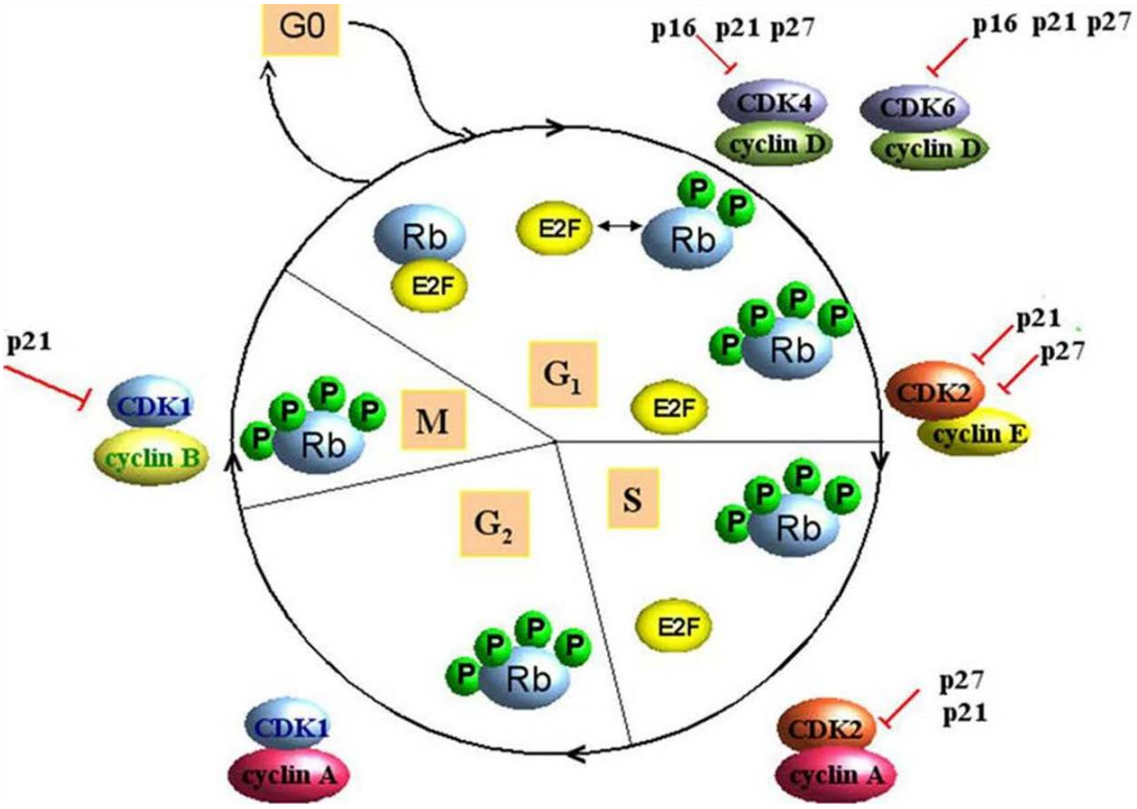

Figure 2: Cell cycle and cell cycle related proteins. *G0/G1: Cyclin D/Cdk4/6 are involved in the phosphorylations of pRB, which in turn separates from the E2F leaving it able to trigger the transition to S phase. G1/S: Cyclins D begin to decrease while Cyclin E/Cdk2 complexes vehicle the cells through S phase. In late S phase, Cyclin A/Cdk2/1 regulate the entry in G2/M. G2/M: Cyclin B1/Cdk1, once activated, translocates into the nucleus and stimulates the events necessary for the start of the mitotic process. Ink4 (p16) and Cip/Kip (p21/p27) proteins inhibit the complexes Cyclin/Cdk along different phases, downregulating the cell cycle progression. Ref. [35]*

1.3 LIPID SIGNALLING

Cell signalling is part of a complex network through which cells respond to different stimuli . The commonly known cell signalling can be represented in three steps: firstly, an extracellular molecule (grow factors, hormones, proteins) binds specific receptors situated on the membrane of the cells; second, these extracellular stimuli trigger a cascade of events within the cells, called signal transduction, which lead to the synthesis of second messengers; third, second messengers either activate or deactivate different cellular responses to which they are connected to. Among the molecules involved in the signal transduction system, lipids have been reported to be very important in the mediation of multiple cell activities such as proliferation, survival, differentiation, migration, cell cycle progression, metabolism and many others [36-38]. In particular, the production of second messengers starting from Phosphatidylinositol (PI) is the basement of multiple cell processes (Figure 3) [36-38]. PI consists of a hydrophilic inositol head group linked to glycerol by a phospho-diester bond, which in turn is coupled to two fatty acyl chains. One of the chains is often a saturated fatty acid (stearate), while the other is predominantly an unsaturated fatty acid (arachidonate).

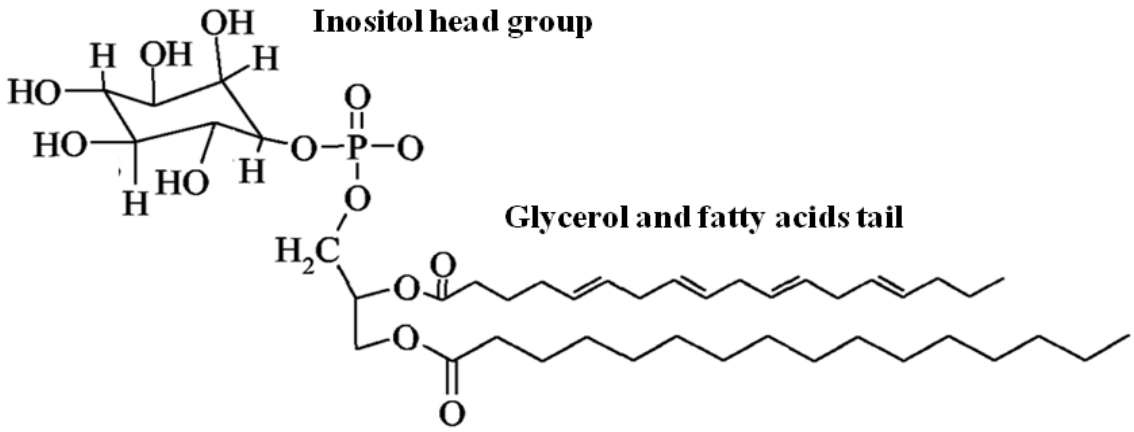

Figure 3 : Structure of Phosphatidylinositol (PI). *The common PI structure is characterized by the presence of a hydrophilic inositol head group linked to a hydrophobic tail formed by glycerol through a phospho-diester bound. Glycerol, in turn, is connected with two fatty acids, often a saturated (stearate) and an unsaturated (arachidonate) acid. Ref. [39]*

1.3.1 PHOSPHATIDYLINOSITOL (PI) METABOLISM

The metabolism of PI involves multiple enzymes such as kinases, phosphatases and lipases, which, in turn, modulate the pool of PI adding or removing phosphate groups on the inositol ring. These events lead to the production of 7 Polyphosphoinositides (PPIs), used as second messengers in different signalling cascades: Phosphatidylinositol 3-Phosphate (PI3P), PI4P, PI5P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3. See Figure 4. As previously indicated, a large number of enzymes act at different levels along the PI metabolism and are involved in synthesis of new second messengers. Many kinases add phosphate groups on the inositol ring at different positions, such as Phosphatidyl-4-kinase (PI4K), Phosphatidylinositol-3-kinase (PI3K), Phosphatidylinositol-phosphate 4-kinase (PIP4K), PIP5K, PIP(4,5)K. On the other hand, some phosphatases, including phosphatase and tension homologue deleted on chromosome 10 (PTEN) and SH_2-domain containing inositol phosphatase 2 (SHIP2), act in the opposite way removing Phosphates [36-38]. Another class of enzymes involved in these processes is represented by the Phospholipases C (PLC), able to hydrolyze PI(4,5)P_2 and produce DAG and Inositol-1,4,5-P_3 (IP_3). These two products are very important second messengers: DAG is fundamental for the activation of Protein Kinases C (PKC), while IP_3 can either promote Ca^{2+} release from the ER stores, which in turn concurs in the activation of PKC, or be phosphorylated itself by several kinases to generate Inositol polyphosphates, some of which have been found to be involved in different signalling cascades [36-38].

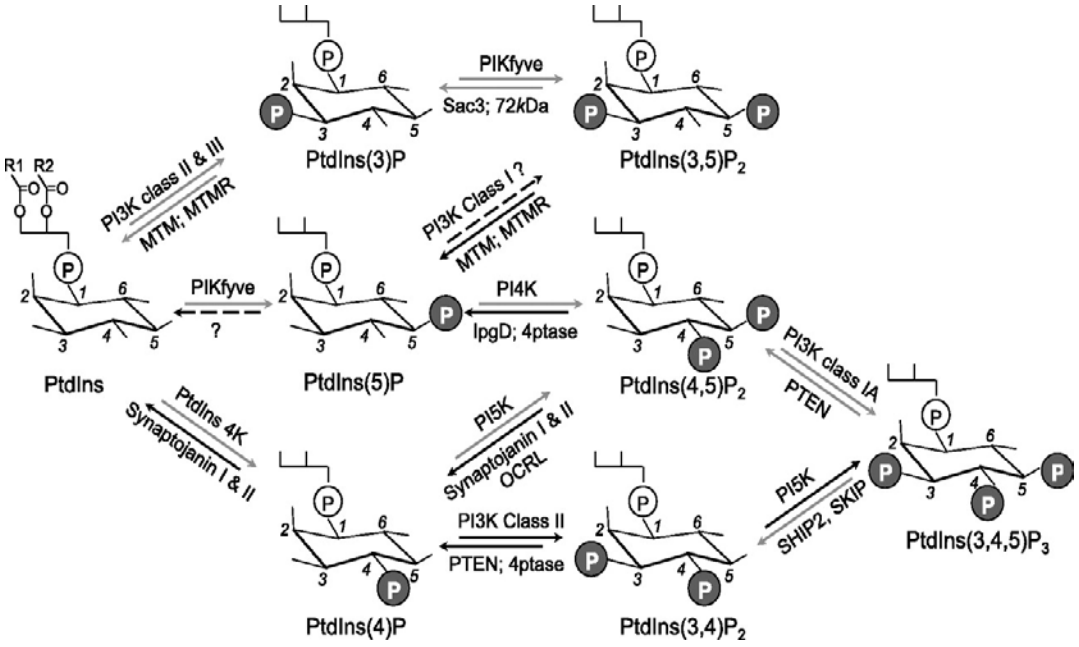

Figure 4 : A schematic representation of the PI metabolism. *Starting from PI, different kinases add on the inositol ring phosphate groups in the different positions. The production of different Polyphosphoinositides (PPIs), used as second messengers, resulted fundamental for several signalling cascades. Other than kinases, some important phosphatases are involved in this network, characterized by the property to remove the phosphate groups from different positions on the inositol head group. Ref. [40]*

1.3.2 NUCLEAR LIPID SIGNALLING

Although the common lipid signalling have been discovered half a century ago, over the past twenty years, many evidences reported the presence of different phospholipids in the nuclei of eucaryotic cells [39, 41, 42]. As expected, cell fractionation and isolation of nuclei indicated that lipids were present due to the presence of the nuclear envelope, which in eucaryotic cells is a double membrane composed by proteins and phospholipids, partly connected with the Endoplasmic Reticulum (ER) [43]. However, different studies showed that, after nuclear membrane removal through detergents, a significant amount of lipids was still detectable inside the nuclei [44, 45]. The first evidences about a proper nuclear lipid signalling separated by the cytoplasmic one were reported in mouse erythroleukemia cells (MEL), where nuclear Phosphatidylinositol-4-

phosphate (PI4P) and Phosphatidylinositol-4,5-biphosphate ($PI(4,5)P_2$) were synthesized differently from the cytoplasmic counterpart [46]. Indeed, highly purified nuclei extracted from differentiating cells via Dimethyl sulfoxide (DMSO) showed the appearance of PI, PIP and $PI(4,5)P_2$ instead of growing cells. Other studies performed on Swiss 3T3 cells showed that, upon stimulation of the cells with Insulin-like growth factor (IGF-1), the nuclear pool of PI4P and $PI(4,5)P_2$ decreased with a concomitant increase of nuclear Diacylglycerol (DAG). On the other hand, Bombesin, which affected only the classical phosphatidylinositol signalling, did not lead to the same effects in the nuclear compartment [47-50]. These data confirmed the presence of two distinct lipid signalling in eucaryotic cells, one located in the cytoplasms and one located within the nuclei. Changes in the nuclear pool of PIs have been found during cell cycle progression, differentiation, DNA damage, oxidative stress and many others stimuli (Figure 5).

1.3.2.1 Nuclear DAG accumulation during cell cycle progression

As already reported, the pool of PIs changes in the nuclei upon different stimuli. In particular, several studies showed changes in the production of DAG and IP_3, related to the activity of specific PLCs located in the nuclear compartment [47-50]. Evidences collected in regenerating liver indicated an increased DAG production during cell proliferation [51]. Moreover, data collected on different leukemia cell lines, U937 and HL60, synchronized at G2/M, indicated that the amount of DAG raised in this phase of the cell cycle, particularly in the nuclei [52, 53]. This higher production of DAG was linked with the activity of a nuclear PLC isoform; indeed, experiments act to inhibit PLC activity led to a minor production of DAG. Finally, as consequence of these events, nuclear translocation of DAG-dependent PKC isoforms was detectable. Here, these phosphotransferases were found able to phosphorylate different targets, such as Lamins, and promote the G2/M progression [49, 54, 55].

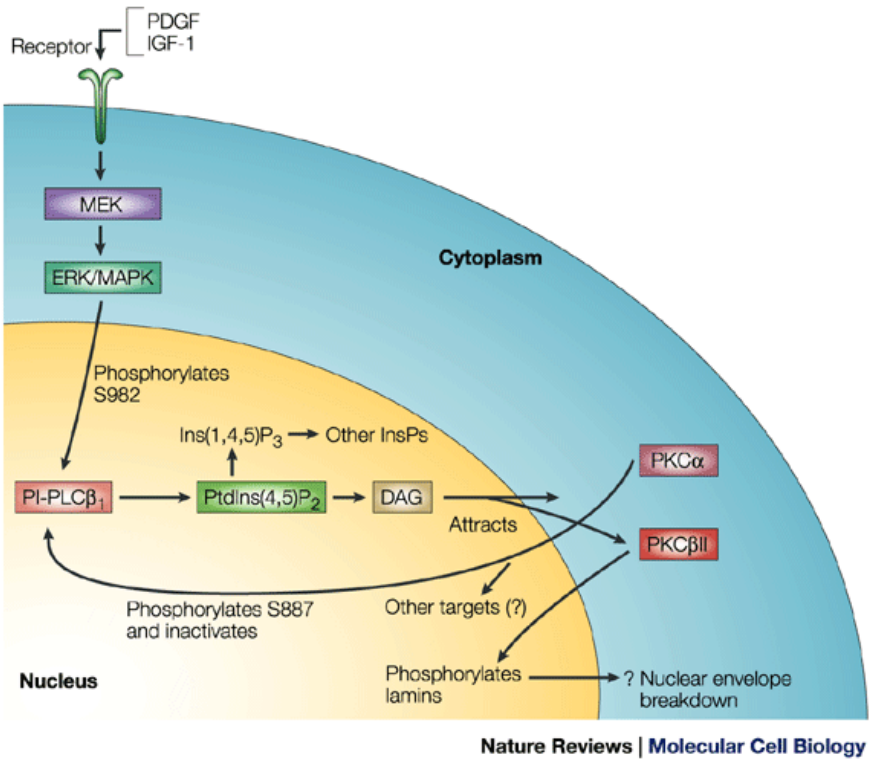

Figure 5: Nuclear Lipid Signalling: focus on PLCβ1 activation. *Different stimuli external at the cell, such as the binding of grow-factors to specific membranes receptors, activate several signalling cascades which, using second messengers, transduce the signal in order to obtain a cellular response. Nuclear cell signalling is characterized by different activation stimuli. Indeed, activation of PLCβ1 is different between the nucleus and the cytoplasm. Once activated, PLCβ1 produces DAG through the nuclear PIP_2 hydrolysis. This event is particularly connected with the different stages of cell cycle and leads to a nuclear translocation of some DAG-dependent PKC isoforms, including PKCα and PKCβII, capable to phosphorylate Lamins and trigger nuclear envelope breakdown during mitosis. Ref. [39]*

1.4 PHOSPHOLIPASES C (PLC) FAMILY

The Phospholipases C represent a family of enzymes involved in the PI metabolism [56] [57]. The substrate of this class of proteins is PIP_2, which is hydrolyzed to generate DAG and IP_3. The first discover of PLC activity was reported in pigeon's pancreas slices, where hydrolysis of phospholipids took place after cholinergic stimulation [58]. In 1981, the first PLC isozyme was purified with a molecular weight of 68 kDa [59]. Through the years, many PLC were found and now the PLC family comprehends 13 isoforms divided in different classes, due to their aminoacidic composition (Figure 6) [60].

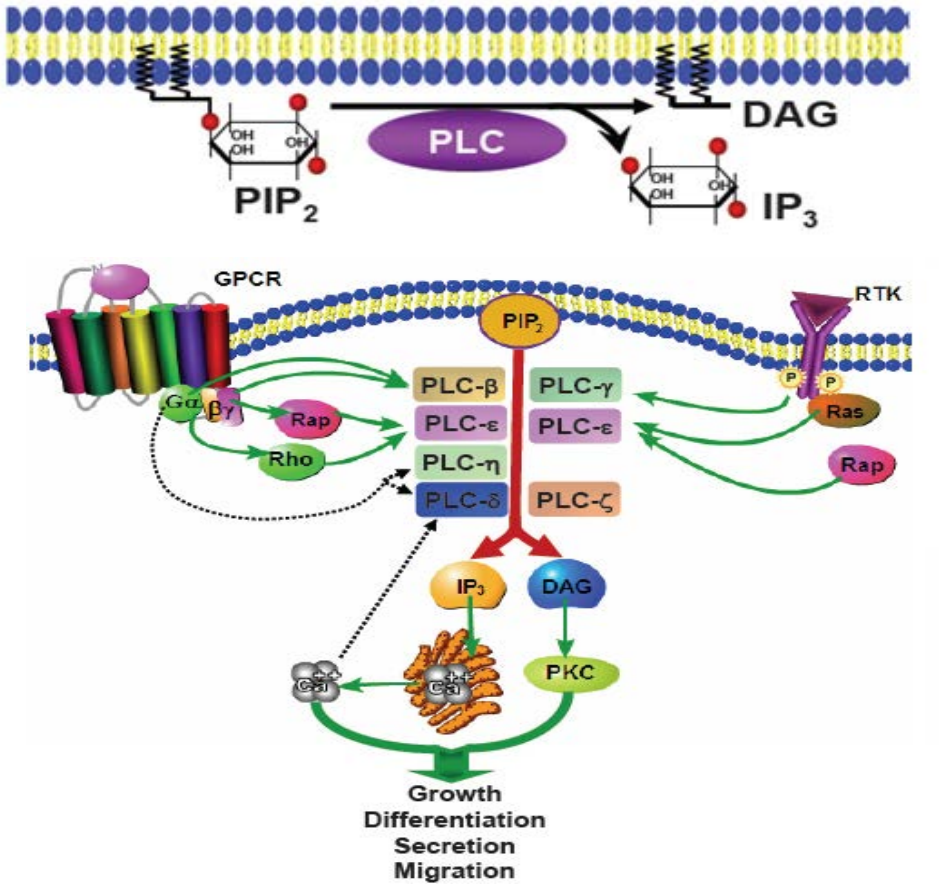

Figure 6 : Phospholipase C family of proteins. *PLC enzymes are involved in the PI metabolism through the PIP_2 hydrolysis. The activation of the PLC class of proteins is linked to various external stimuli: if the class γ is activated by the RTK activation, PLCβ isoforms are linked to the GPCR receptors. The lipase activity of these proteins results fundamental for the production of DAG and IP_3, which in turn triggers Ca^{2+} release from the Endoplasmic Reticulum. These two second messengers are known to be very important for the attraction to the membrane and the activation of a class of phosphotransferases, PKC, involved in many cell functions. Ref. [57]*

1.4.1 DOMAIN STRUCTURE

1.4.1.1 Catalytic domains

The domain composition of these enzymes is characterized by the presence of two catalytic domains, X and Y, where some fundamental amino-acidic residues for the activity of PLC reside (Figure 7) . Evidences reported on the structural analysis of PLCδ1 indicated Lys^{438}, Lys^{440}, Ser^{522} and Arg^{549} residues very important for the interactions of these enzymes with 4- or 5-phosphate of the substrate headgroup ($PI(4,5)P_2$) [61]. Moreover, in mammalian PLC-δ_1 two fundamental aminoacids were indicated as the catalytic residues, His^{311} and His^{356} [62, 63]. In particular during phosphoinositide hydrolysis, His^{311} participates to the stabilization of the highly negative charged transition state together with calcium, while His^{356} takes part both in diacylglycerol protonation and in the hydrolysis of the cyclic intermediate [61] [62, 63]. In particular, a study performed on myogenic differentiation of C2C12 cell line showed that mutation of the His^{331} and His^{378} of PLCβ1 could inhibit its catalytic activity as well as for PLCδ1. This was confirmed by the fact that Cyclin D3 promoter expression, found to be positively modulated by PLCβ1 action, was not affected by the catalytic inactive mutant of the protein characterized by substitution of these two residues with two Ala [64].

1.4.1.2 PH domains

The Pleckstrin-Homology domains contained in PLC mediate their binding with different molecules; in PLCδ1, PH domain is fundamental for the binding of PIP_2 and the subsequent access to the membrane surface [65]. On the other hand, PH domains of PLCβ2 and PLCβ3 bind directly to the heterotrimeric G protein subunit, Gβγ [66]. Moreover, PH domain of PLCγ has been reported to interact PIP_3, important for the PI3K-dependent PLCγ activation [67]. This PLC class is characterized by the presence of two PH domains, divided by two Src Homology domains (SH_2) and a Src Homology (SH_3) domain (Figure 7) [68].

1.4.1.3 C2 and EF-hands domains

The C2 domain of PLC contains some aminoacidic residues which allow the protein to bind Ca^{2+} in order to enhance the lipase activity of the enzyme [69]. Otherwise, EF-hands motifs can be very important for the control of PLC activity; indeed, mutations on this domain decreases PLC activity, in a Ca^{2+} independent way (Figure 7) [70, 71].

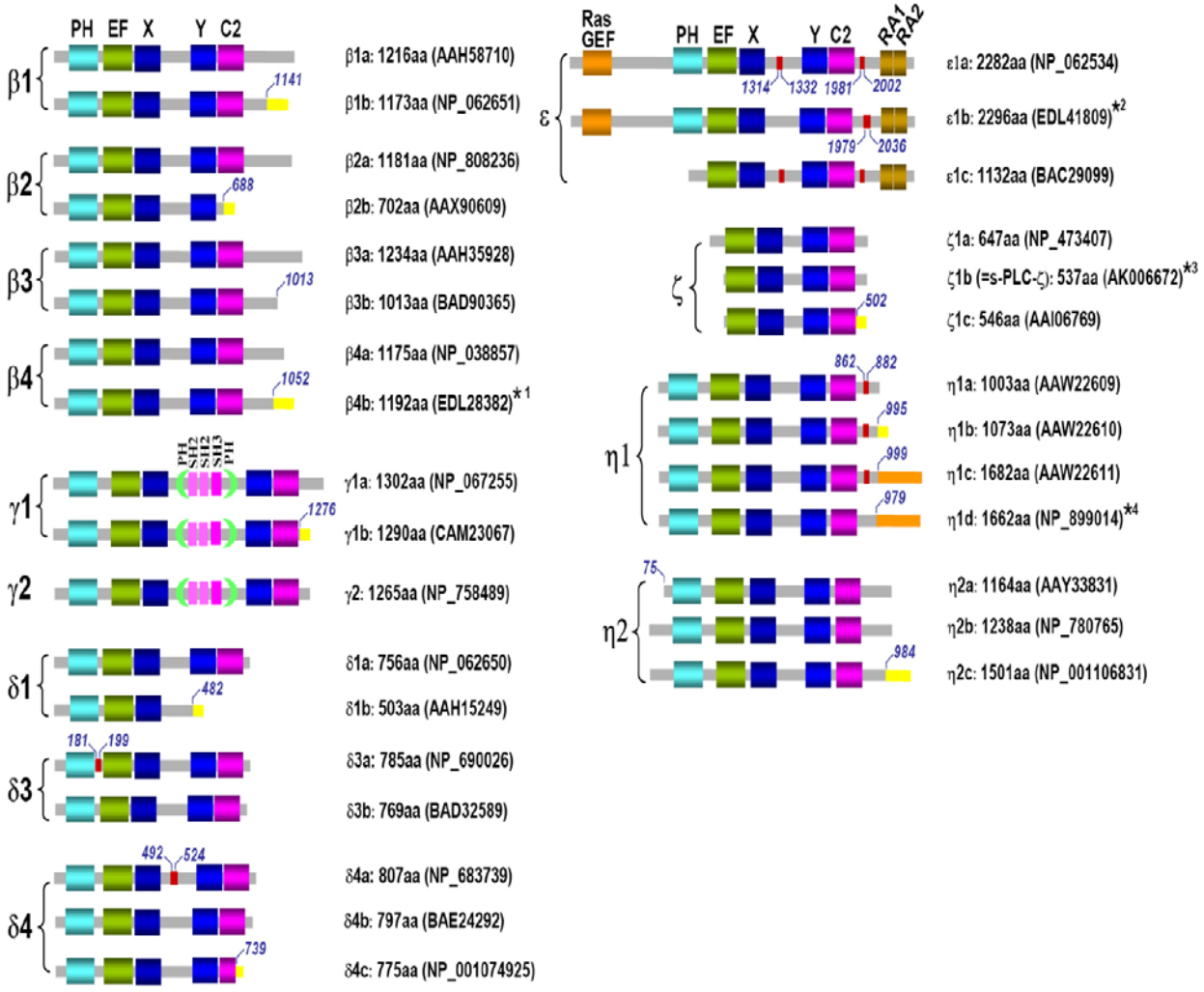

Figure 7 : Schematic representation of the PLC classes and domains. *13 PLC isoforms are known in eucaryotic cells. Every PLC isozyme shares some important domains with the others such as X and Y catalytic domains, PH domains (PLCγ is characterized by two PH of them), C2 (Ca^{2+} sensitive) and EF-hands regulatory domains. All the classes of PLC are represented by more than one isoforms, which are characterized by different splicing variants (such as PLCβ1 with PLCβ1a and PLCβ1b). Ref. [57]*

1.4.2 PLC ISOFORMS: FOCUS ON PLCβ

Thirteen PLC isoforms, divided in 6 classes, are known to be present in eucaryotic cells. The different members of the classes are commonly present in different forms; indeed, splicing variants of every gene that encodes PLC isozymes are present in the cells and influence their localization in the tissues and in the cellular compartments [72, 73]. In particular, two spicing variants of the gene encoding for PLCβ1 are known as PLCβ1a and PLCβ1b, which differ each other in the C terminal sequence [74, 75]. PLCβ1 is member of the PLCβ class, which comprehends PLCβ1~4 [76]. These enzymes share with the other classes part of the characteristic structure of PLC: they possess a conserved catalytic domain, split in X and Y regions, as well as two membrane-phospholipid binding domains such as C2 and PH domains [77, 78]. However, PLCβ isozymes are distinguished by differences in the C-terminal sequence of their structure. Indeed, these molecules are characterized by an elongated C-terminus consisting in about -450 aminoacids, which contain different important residues for the interaction with membranes and for their localization in the cells [76-78]. PLCβs are regulated by G-protein-coupled-receptors (GPCR), which activate G-protein mediated signal transduction [76]. The G-proteins form a heterotrimeric structure in the inactive GDP-binding state comprehending Gα, Gβ and Gγ subunits. When GPCR is stimulated, Gα, which binds GDP (Guanosine Di-Phosphate) in the inactive state, exchanges GDP with GTP (Guanosine Tri-Phosphate) causing its dissociation from Gβγ dimer [79, 80]. Both the complexes GTP-Gα and Gβγ dimers are important for signal transduction which leads to PLCβ activation [76]. Moreover, the Gα-GTPase activity is also involved in the inactivation of the signalling GPCR-dependent through the hydrolysis of GTP in GDP and the return of the heterotrimeric structure, Gα-β-γ binding GDP [79, 80]. However, several studies showed the PLCβ isoforms are characterized by different responsiveness to the GPCR mediated signalling: indeed, PLCβ1 results to be the least sensitive to the Gβγ-dependent activation, while PLCβ4 is completely insensitive to Gβγ [81–83]. Moreover, the Gα subunit can be divided in 4 isoforms: $G_s\alpha$, $G_i\alpha$, $G_q\alpha$ e $G_{12}\alpha$. Among them, G_q subtype comprehends four members (α_q, α_{11}, α_{14} e α_{16}), all able to activate PLCβ1 [84]. $G_q\alpha$ activation site has been indicated to reside in the C-terminus of PLCβ1. On the other hand, the activation mediated by Gβγ dimers is due to the binding at the N-terminus of the PH domain [85].

1.4.2.1 PLCβ1 splicing variants: PLCβ1a and PLCβ1b

The gene encoding PLCβ1 is located on chromosome 20 and encodes two different splicing variants of the proteins, PLCβ1a (150kDa) and PLCβ1b (140kDa) [74]. These two isoforms of PLCβ1 conserve the typical structure of the PLCβ class of PLCs, but differ each other for the C-terminus. Indeed, PLCβ1b replaces 75 residues in the C-terminal sequence with a sequence of 43 aminoacids [86, 87]. This difference resulted to be very important for the distribution of the enzyme among the different cell compartment. Indeed, PLCβ1 is known to be one of the pivotal enzymes involved in nuclear cell signalling. Several studies indicated this PLC as completely localized in the nucleus, others showed it as more cytoplasmic. The differences in these reports can be linked to the existence of two splicing variants of PLCβ1. The 1b isoform is characterized for the lack of a common Nuclear Exportation Signal (NES), located in the C-terminus of the protein, which, on the contrary, is contained in PLCβ1a structure. Moreover, both the variants possess a Nuclear Localization Signal (NLS) (Figure 8) [86]. This NLS has been described to be located at the C-terminus. Indeed, the overexpression of a mutant of PLCβ1 (M2b), characterized by the substitution of Lysine$^{1056,\ 1063}$ and ^{1070} at C-terminal, markedly reduced the nuclear localization of the enzyme in various cell lines [88]. Particularly, PLCβ1 resides in specific nuclear structures called nuclear speckles, small subnuclear membraneless organelles or structure corresponding to nuclear domains located in interchromatin regions of the nucleoplasm of mammalian cells [45, 89-92]. Together with PLCβ1, several other molecules and enzymes are known to be located in these nuclear compartments, such as PIP kinase, PI3KC2α, PIP_2, diacylglycerol kinase θ (DGK θ), PLC-δ4, phosphatase and tension homologue deleted on chromosome 10 (PTEN) and SH_2-domain containing inositol phosphatase 2 (SHIP2) [45, 89, 90].

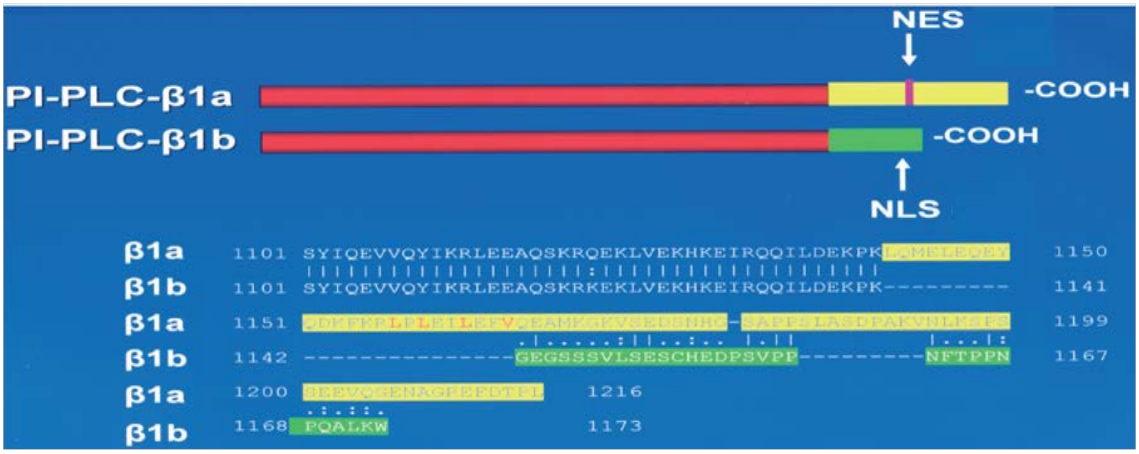

Figure 8 : PLCβ1 splicing variants, PLCβ1a and PLCβ1b. *Both the isoforms present in their C-terminal a sequence fundamental for the nuclear import called Nuclear Localization Signal (NLS). The main differences in their structure are found always in C-terminal: PLCβ1b lacks a series of residues where in PLCβ1a the Nuclear Exportation Signal (NES) is localized. Then, PLCβ1b results to be mainly nuclear, while PLCβ1a is situated in both nuclear and cytoplasmic compartment. Ref. [86]*

1.4.2.2 PLCβ1 mediated nuclear signalling

The nuclear production of IP_3 and DAG mediated by the activity of PLCβ1 has been widely described in literature [47] [49, 93]. However, how PLCβ1 is activated at nuclear level remains unclear. Indeed, the mechanism which triggers PLC signalling in the nucleus resulted different from the one located in the cytoplasm. Some evidences reported the possibility for the $G_i\alpha$ subunit to translocate in the nucleus, while others indicated, as the main candidate for PLCβ1 activation, p42/44 MAPK (Mitogen Activated Protein Kinase). Indeed, experiments performed in order to inhibit p42/44 activity or its nuclear translocation showed a minor activation of nuclear PLCβ1 in different cell lines [94-96]. In particular, it was also reported that PLCβ1 possesses a phosphorylation site for p42/44 in the C-terminus, the residue Ser^{982} [97]. However, this phosphorylation cannot activate PLCβ1 but is thought to be important for the recruitment of other regulatory elements which stimulate the lipase activity of the enzyme. On the other hand, the inactivation of the PLCβ1 dependent nuclear signalling

seems to be related to the phosphorylation by PKCα of the residue Ser^{887}, which seems to modulate the capacity of PLCβ1 to bind Gβγ dimers in vitro [98].

1.4.2.3 PLCβ1 involvement in cell cycle and cell differentiation

Several reports indicated cell cycle and cell differentiation as highly connected with PLCβ1 mediated metabolism. Studies on MEL cells suggested an involvement of this lipase in these two important processes. Indeed, experiments on MEL, inducted to differentiate by Dimethyl-sulfoxide (DMSO) treatment, indicated a high decrease of PLCβ1 amount in the cells, which was followed by inhibition of its nuclear activity [39, 46, 93, 99]. In particular, it has been found that the nuclear localization and activity of the enzyme was important during differentiation . In order to study that, PLCβ1 and M2b mutant (only cytoplasmic) were overexpressed in differentiating MEL. The result was a minor expression of the differentiating marker of this cell line, β-globin, which followed to the increase of wild type PLCβ1, while in M2b overexpressing cells no changes in differentiation were encountered [100, 101]. Moreover, studies on PLCβ1 involvement in cell cycle regulation showed its importance for both G1/S and G2/M checkpoints. Indeed, always using MEL cells, PLCβ1 has been found to have a positive correlation with Cyclin D3 expression, which led to an increase in Retinoblastoma Protein (pRB) phosphorylation and, then, to a consequent elongation of the S phase of the cell cycle [102]. Otherwise, PLCβ1 resulted also involved in the early stages of the mitotic process. Its activity was fundamental to induce nuclear translocation of some isoforms of PKC, which, in turn, were able to phosphorylate Lamin B1 driving the disassembly of the nuclear envelope and the progression through the G2/M phase [103].

1.5 PROTEIN KINASES C (PKC) FAMILY

Protein kinases C (PKC) are serine/threonine phosphotransferases which belong to the AGC family of protein kinases (cAMP-dependent, cGMP-dependent, and protein kinase C) [104-106]. The first PKC was discovered in 1977 in old bovine brains as a proteolitically activated protein kinase able to phosphorylate Histones [107]. Then, in the early 1980s, these enzymes were found to be receptors of the tumor promoting phorbol esters, which raised their importance in signal transduction researches. Indeed, first PKC was cloned in the middle of 1980s and it was found to possess a C1 domain specific for phorbol esters binding [108-110]. Through the years, it became clear that these kinases can be involved in many cellular processes such as proliferation and cell cycle progression, differentiation, tumorigenesis, apoptosis and autophagy. The canonic dogma about their activation indicates that these proteins are dependent by PIP_2 hydrolysis mediated by the PLC family leading to the production of DAG and IP_3, which in turn triggers Ca^{2+} release from the Endoplasmic Reticulum (ER) [104-106].

1.5.1 PKC CLASSES

Nowadays, ten PKC enzymes are known in mammalian cells and are subdivided in three classes based on the differences in their domain composition, which indicate what cofactors are needed for their activation (Figure 9) [104, 105]. First, the conventional class is composed by four isoforms: PKCα, the two splicing variants PKCβI and PKCβII (which differ in their C-terminus for 43 amino acids) and PKCγ; next are the four novel isoforms, PKCδ, PKCε, PKCη and PKC θ; finally, the atypical class refers to PKCζ and PKCι/λ (ι human; murine isozyme is PKCλ). In addition, two more PKC isozymes are known, PKC μ and ν, which are considered either to belong to a fourth class of PKCs or to be member of a different family called Protein Kinases D [111].

1.5.2 DOMAIN STRUCTURE

All the members of the PKC family share a common structure characterized by conserved (C1–C4) and variable regions (V1–V5) split in two main domains, the regulatory and the catalytic moieties connected each other by a hinge region. The regulatory domain of PKC isozymes consists in two main modules, C1 and C2 domains, which regulate the binding of DAG/Phorbol esters and Ca^{2+} respectively. In addition, aminoacidic residues close to the C1 domain are reported to act as an autoinhibitory

sequence indicated as pseudosubstrate. On the other hand, the catalytic domain is represented by the ATP-binding site sequence and, at C-terminus, by some important aminoacids which, once phosphorylated, influence the maturation and, in turn, the activation of these phosphotransferases (Figure 9) [104-106].

1.5.2.1 Regulatory domain

1.5.2.1.1 Pseudosubstrate

The pseudosubstrate domain of PKC is a stretch of aminoacids between residues 19 and 36 which is responsible for maintaining the enzyme in the inactive form in absence of allosteric activators such as phospholipids [112]. Studies on PKC structure showed that this sequence was very similar to a common sequence substrate for PKC except an Alanine (Ala^{25}) that occupied the phosphoacceptor Ser/Thr position. Synthetic peptides based on this sequence resulted good inhibitors for PKC, while substitution of Ala^{25} with a Serine transformed the pseudosubstrate into a potent substrate [113].

1.5.2.1.2 C1 domain

Although the C1 domain is present in all PKC isoforms, where it results to be the Phorbol Ester/DAG binding site, atypical class is characterized by a modified C1 domain, not sensitive to these molecules. However, C1 domain is present in PKC as a tandem, C1A and C1B, which link DAG and Phorbol Esters respectively. It is a Cystein-rich sequence of approximately 50 aminoacids and it can be found also in other proteins not related to PKC family [114, 115]. Tri-dimensional studies showed a globular structure characterized by two β-sheets which constitute the ligand-binding pocket for DAG or Phorbol Esters. The binding of these molecules to the C1 domain results fundamental for the recruitment of PKC to the membranes, due to the changes in the surface properties of the module which becomes hydrophobic, a necessary step for membrane interaction [115].

1.5.2.1.3 C2 domain

The C2 domain is the part of the regulatory moiety sensitive to Ca^{2+}. It is present only in conventional and novel classes of PKC, while atypical one lacks it. However, novel isoforms are characterized by a C2 domain not sensitive to Ca^{2+} and, then, they are

activated only by DAG or Phorbol Esters. Moreover, as C1 domain, C2 domain has been found in several molecules other than PKC. The localization of this module along the aminoacidic sequence changes within different PKC classes: conventional isoforms present it after the C1 domain, while in the novel is true the opposite. In particular, C2 domain results to be a β-strand-rich globular domain that binds Ca^{2+} increasing the affinity of PKC for anionic phospholipids located at membrane levels, such as Phosphatidylserine (PS) or PIP_2 [116-118].

1.5.2.2. Catalytic domain

1.5.2.2.1 ATP-binding site

The catalytic domain of ABC kinases (PKA, PKCB/Akt and PKC) is highly conserved, with more than 40% of homology among the sequences. In particular, the kinase domain of PKC has been remained not well understood for years due to its refraction to crystallization. However, studies on PKCβII, θ and ι isoforms reported new information about the structure of the catalytic domain of this class of proteins which resulted to be a bilobal structure with a β-sheet comprising the N-terminal lobe and an α-helix constituting the C-terminal lobe. Both the ATP-(C3) and substrate-binding (C4) sites are located in the cleft formed by these two lobes [119-121].

1.5.2.2.2. C-terminus phosphorylation sites

All the ABC kinases share three conserved phosphorylation motifs in the C-terminus (C4) which modulate their maturation and activation. These sites can be considered as switches that trigger changes in the molecular conformation of the enzyme and mediate the substrate phosphorylation [104-106].

1.5.2.2.2.1. Activation loop

This site is represented by Thr^{500} in the conventional PKCβII, Thr^{197} in PKA and Thr^{308} in PKB/Akt. The kinase responsible for the phosphorylation on these residues resulted to be the 3-Phosphoinositide-dependent protein kinase-1 (PDK1) [122]. In particular, this is the first phosphorylation for PKC and it results to be constitutive for conventional and novel PKC, while it is agonist dependent for atypical isoforms [104, 105].

1.5.2.2.2.2. Turn motif

The phosphorylation on the turn motif is essential for the stabilization of the structure of mature PKC by anchoring the C-terminal tail on the upper lobe of the kinase. It takes place on Thr^{641} as indicated in studies performed on PKCβII. Substitution of this residue with Ala triggers compensating phosphorylations on adjacent Thr that accompany the maturation process of PKC. However, further mutations on these Thr inhibit these compensating events. The enzyme responsible of phosphorylation on the turn motif resulted to be the mammalian target of rapamycin complex 2 (mTORC2) [123-125].

1.5.2.2.2.3. Hydrophobic motif

The hydrophobic motif phosphorylation is the third and last phosphorylation which takes place on PKC structure leading to their activation. The residue where a phosphate group is added is Ser^{660} as reported by studies on conventional PKCβII. Kinetic analyses found that PKC are able to autophosphorylate themselves on this motif, while other studies indicated mTORC2 as the responsible for this event. Indeed, PKCδ hydrophobic motif phosphorylation is inhibited by treatment with rapamycin, while mTOR-deficient cells show no phosphorylation at all [123-126].

1.5.2.3. *Atypical PKC domains*

As previously reported, this class of PKC is not activated by neither DAG nor Ca^{2+}, but it needs protein-protein interactions. This is mediated by the two regions located in the regulatory and catalytic domains respectively: Phox/Bem1p (PB1) domain and postsynaptic density protein 95 (PSD95), *Drosophila* discs large tumor suppressor (Dlg1) and zonula occludens 1 (ZO-1) (PDZ) domains [104-106].

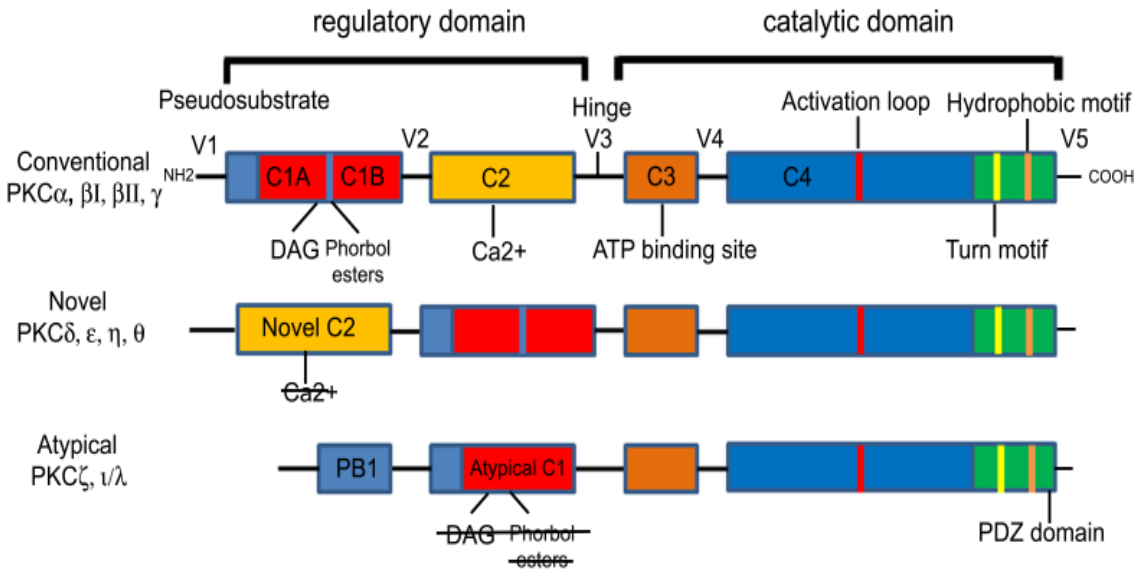

Figure 9 : Classes and domain compositions of PKC family. *PKC are divided in three different classes due to their domain composition: conventional (PKCα, βI, βII and γ), novel (PKCδ, ε, ν, η) and atypical (ζ, ι/λ). The structure of these enzymes is characterized by two principal moieties: one regulatory and one catalytic. The regulatory one of conventional isoforms is represented by a C1 (present in tandem, C1A and C1B) domain, able to bind DAG and Phorbol-Esters, and a C2 domain sensitive to Ca^{2+}. Every enzyme presents a pseudosubstrate region, fundamental for autoinhibition of the enzymes. Novels possess a C2 domain not sensitive to Ca^{2+}, while atypical class has an atypical C1 domain not able to bind Ca^{2+} and completely lacks the C2 domain. The catalytic domain of PKC is composed by the ATP-binding site and a C-terminus sequence where some important aminoacidic residues for maturation and activation reside. Atypical PKC present a PB1 and a PDZ domains which mediate protein/protein interactions fundamental for their activation. Ref. [106]*

1.5.3 PKC LIFE CYCLE

As explained, PKC undergo three subsequent phosphorylations, fundamental for their maturation and activation (Figure 10) [104-106]. However, this process results to be influenced also by others molecules. Newly-synthesized PKC are linked to a fraction of the cell membrane due to several weak interactions between C1 and C2 domains with anionic lipids. Here, PKC interact with chaperone Heat-Shock protein 90 (HSP90) and the co-chaperone Cdc37, a necessary event for the phosphorylation of conventional and novel PKC. Experiments of inhibition of HSP90 and/or its mutation decreased the possibility of PKC to be phosphorylated [127]. Next, the three phosphorylation events take place thanks to the activity of PDK1 and mTORC2. The fully mature PKC is now located in the cytosolic fraction [104-106]. The PLC-dependent production of the two second messengers DAG and Ca^{2+}, through PIP_2 hydrolysis, is fundamental for the continuation of the process [56, 57]. Ca^{2+}, bound by the C2 domain, is responsible for the attraction of PKC to the cell membrane where they tie anionic lipids as PS or PIP_2. Moreover, C1 domain binds its membrane-ligand DAG. For novel isozymes, which lack a common C2 domain, the interaction between DAG and C1 domain results to be sufficient for their recruitment to the membrane [104-106]. These events provide the energy to release the autoinhibitory pseudosubstrate from the substrate-binding cavity, allowing the enzyme to recruit and phosphorylate its targets. The "open" conformation that PKC adopt once they are bound to membranes renders these enzymes sensitive to dephosphorylation on the hydrophobic motif mediated by the PH domain leucine-rich repeat protein phosphatase (PHLPP) [128]. This leads to the shift of PKC to detergent-insoluble fraction where they are further dephosphorylated on the others motifs and degraded. Notably, PKC which present a dephosphorylated turn motif can bind HSP70, an event that promotes rephosphorylation of the molecule and, in turn, a longer lifetime [129]. Other scaffold proteins can regulate PKC life cycle, such as receptors for activated C kinase (RACKs), maintaining the proteins in autoinhibited form which avoids their dephosphorylation and subsequent degradation [130]. Interestingly, the inactive PKC are characterized by a quite long half-life, while a chronic stimulation that changes their conformation in open, as occurs upon Phorbol Esters treatment, leads to a rapid degradation of these isozymes. Indeed, open PKC undergo higher dephosphorylation than inactive/close, which makes them unstable and shunted to degradation [131, 132].

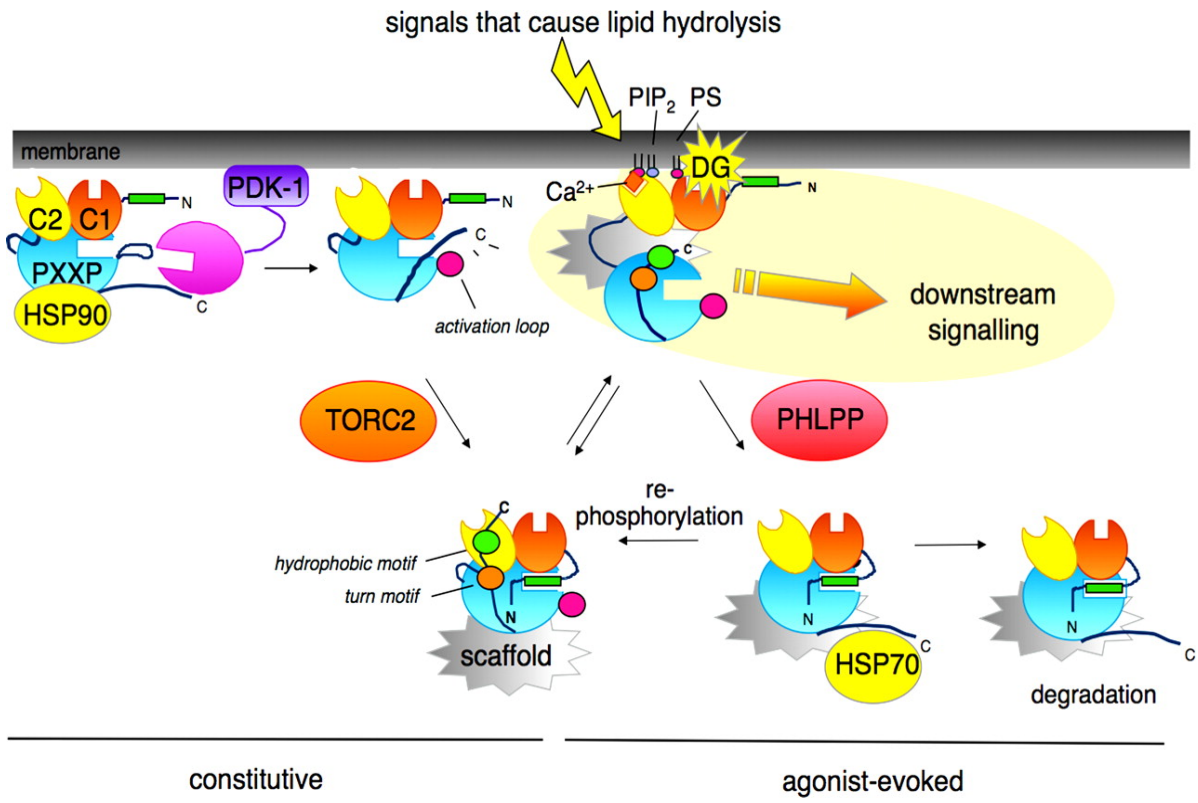

Figure 10 : PKC life cycle: the steps for PKC activation and degradation. *PKC maturation and activation take place trough different and subsequent phosphorylations: first, novel-synthesized PKC bind the membrane through C1 and C2 domains. Here, the interaction with HSP90 modulates the first phosphorylation by PDK1 on the activation loop at the C-terminus of the proteins. Then, PKC leave the membrane and are locate in the cytosol, where they are again phosphorylated on the Turn and Hydrophobic motives by mTORC2. Here, production of the second messengers DAG and IP_3 (and Ca^{2+}) leads to the recruitment of PKC to the plasma membrane where they bind PIP_2 and/or PS. Next, dephosphorylation of the hydrophobic motif by PHLPP inhibits their activity and leads them to degradation. However, binding with HSP70 can start again PKC phosphorylation and reactivation, prolonging their life cycle. Ref. [105]*

1.5.4 PKC SIGNALLING AND CELL CYCLE REGULATION

The importance of PIP_2 hydrolysis related signalling in cell cycle progression has been widely discussed through the years [55, 106, 133] [100-102, 134]. In particular, the involvement of PKC in cell proliferation and differentiation has been found highly connected with the progression through the different checkpoints of cell cycle (Figure 11). What has emerged by many reports is that the effects mediated by PKC are mostly context-dependent. As a matter of fact, different roles for PKCs were described in cell cycle machinery both as anti-proliferative and growth-stimulatory enzymes. Indeed, single PKC isoforms can affect more molecules, such as Cyclins, Cdk, cell cycle inhibitors, depending by cell models, signalling environment and cell cycle phase [106] [133]. Both in vitro and in vivo experiments indicated specific effects of PKC isozymes during both G1/S transition and in G2/M progression [135]. In particular, the activation of PKC has been negatively correlated with Cyclins D expression in intestinal crypt cells, by different modulation of the cell cycle inhibitors p21 and p27 [136]. Another study indicated PKCα as capable to phosphorylate p27 in vitro [137]. Moreover, other isoforms, such as PKCδ and η, have been reported to regulate Cyclin D1 and Cyclin E [138-140]. Other functions of PKC have been found during G2/M progression where the nuclear translocation of these molecules, due to an increase in nuclear DAG production, resulted fundamental for their activity [141]. Indeed, reports on leukemia cell lines, such as HL60 and MEL, showed that the nuclear translocation of PKCβII and PKCα at G2/M checkpoint led to phosphorylation of Lamins, necessary for the disassembly of the nuclear envelope and progression through mitosis [103, 142]. Another possible mechanism of modulation of cell cycle mediated by PKC is the regulation of nuclear lipid signalling. Indeed, as cell cycle progression has been widely described to be connected to the signal transduction based on PIP_2 hydrolysis, it has been reported that nuclear translocation of PKCα can downregulate it through the phosphorylation of PLCβ1 on Ser^{887}, which inhibits the activity of this enzyme and, in turn, the production of nuclear DAG [98].

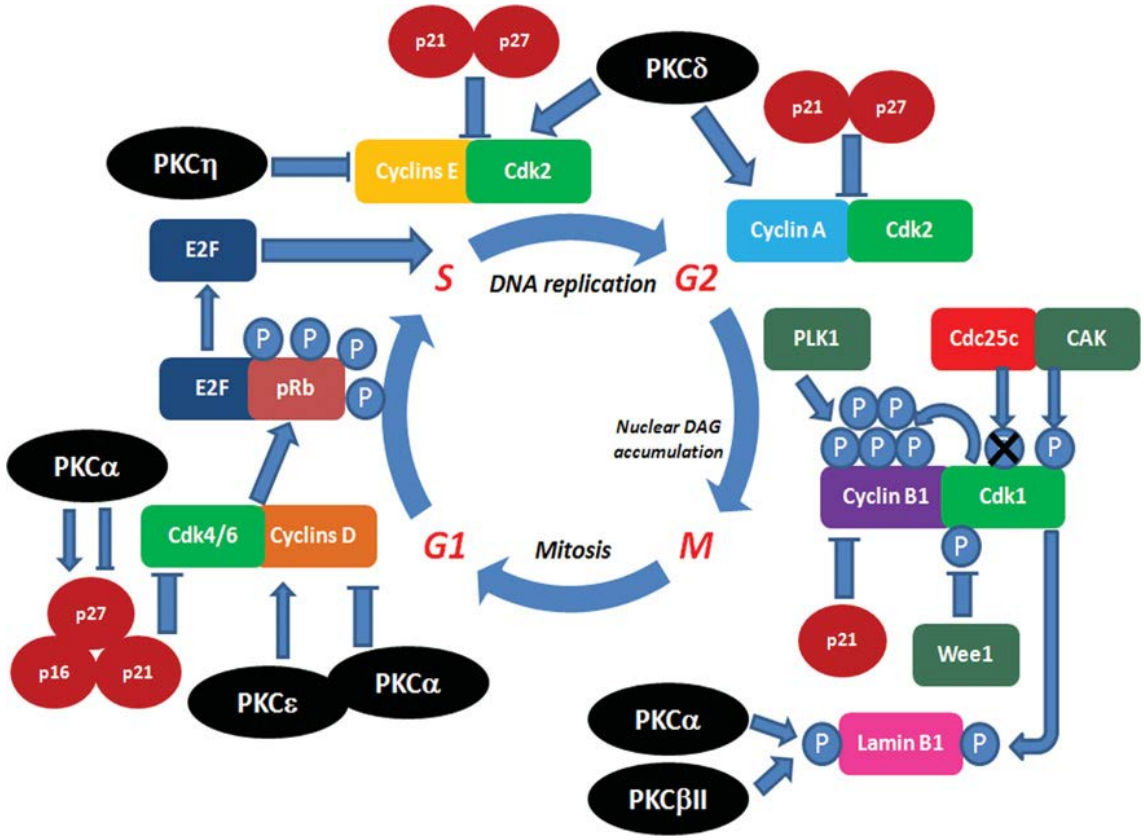

Figure 11 : PKC involvement in cell cycle modulation. PKC have been reported to be highly connected with cell cycle at multiple levels. *Different PKC isoforms (PKCα, PKCδ, PKCε and PKCη) can regulate G1/S transition targeting Cyclins D/E /Cdk4/6/2 complexes or INK4-Cip/Kip inhibitors. G2/M progression: less information are reported about this phase of cell cycle. PKC can modulate Cyclin A/Cdk2 or phosphorylations of nuclear Lamins. Ref. [106]*

Aim

As inositol lipid signalling has been found fundamental in cell cycle and cell differentiation of several murine models, we decided to study its role also in human models. In particular, we focused our attention on signal transduction mediated by the activity of nuclear PLCβ1, member of the PLC family, enzymes able to cleave PIP_2 creating DAG and IP_3. For our purpose, we decided to use a human erythroleukemia cell line (K562), characterized by a high accumulation of this PLC isoform in their nuclear compartment. First, we started seeking possible involvements of this protein in the G1/S phase of cell cycle. Considering previous findings on MEL cells, we studied the possible connection between PLCβ1 and Cyclin D3. We found that the activity of PLCβ1 was fundamental for Cyclin D3 expression. Indeed, overexpression of the enzyme led to a positive modulation of its expression and to a prolonged S phase of the cell cycle. Then, we decided to better elucidate which mechanism could be responsible of these effects. We focused on the possible involvement of the direct targets of PLC signalling, the PKC family. As K562 cells express only two DAG-dependent PKC isoforms, PKCα and PKCβII, we found that an overexpression of PLCβ1 was able to decrease the levels of PKCα in our model [143]. Mimicking the effects of PLCβ1 overexpression, we directly silenced PKCα finding a concomitant upmodulation of Cyclin D3 and a very similar cell proliferation of the cells. These evidences indicated that the PLCβ1/PKCα pathway could be very important in G1/S transition of cell cycle. Moreover, as PIP_2 related signalling can be involved also in G2/M checkpoint, we investigated new possible targets for PKC during this phase. Interestingly, we found that PKCα was able to positively affect Cyclin B1 expression, independently by its kinase activity. Indeed, PKCα was found to act as a scaffold protein for Cyclin B1 avoiding its degradation along cell cycle progression. Moreover, both the proteins accumulated into the nucleus at G2/M checkpoint. The main factor responsible of this event was nuclear DAG accumulation during this phase, which was connected to the increased activity of nuclear PLCβ1 during mitosis. These evidences indicated new roles for PLCβ1 and PKCα during G2/M progression. Then, in this thesis, new pathways and signalling cascades linked to nuclear PIP_2 hydrolysis are described as very important cell cycle modulators both at G1/S and G2/M. The manuscript will be split in two parts: a first part where the involvement of PLCβ1 and PKCα in G1/S will be elucidated; a second that will show how these two enzymes could regulate also the G2/M progression of cell cycle of human erythroleukemia cell line, K562.

Materials and methods

2.1 Cell culture

Human erythroleukemia cells (K562) were grown in RPMI 1640 (Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and L-glutamine/streptomycin (1X), at 5% CO_2 and 37°C.

2.2 Cell treatments

Cells were treated with the following compounds: Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) at a final concentration of 50-100nM for 30 minutes or 16 hours (in order to stimulate nuclear translocation of PKC and their degradation respectively), U73122 (Sigma Aldrich, PLC inhibitor) at 10μM for 16 hours, with Go6976 and Go6983 at 1μM (Sigma Aldrich, PKC inhibitor) and with 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione-anilinomonoindolylmaleimide (Calbiochem, PKC inhibitor) at 1μM for 24 hours [144]. Finally, MG-132 (Sigma Aldrich, proteasome inhibitor) was used at a final concentration of 15μM for 2 hours.

2.3 Cell synchronization

Cells were collected as described below for the experiments: (1) G1/S cells: proliferating cells were cultured in complete RPMI 1640 FBS 10%; (2) G2/M blocked cells: growing cells were treated with Nocodazole (Sigma Aldrich) at a final concentration of 20ng/ml for 16h; (3) G0/G1 cells: growing cells were starved in Hank's Balanced Salt Solution (HBSS, Sigma Aldrich) for 16 hours. After synchronization, cells were seeded again in complete RPMI 1640 FBS 10% and left to grow for experiments of cell cycle analysis and cell counting. In experiments where PKCα was overexpressed, cells were partially stimulated to undergo G2/M accumulation adding Nocodazole for 6 hours at a final concentration of 20ng/ml.

2.4 Protein extraction and Western Blotting Analysis

Cells were collected by centrifugation, washed in PBS and lysed in mPER lysis buffer (BIORAD) containing protease inhibitors (Thema Ricerca) for at least 30 minutes at 4°C. Next, homogenates were centrifuged for 5 minutes at 13000 rpm at 4°C in order to remove any insoluble elements and the surnatant moved in new vials. The protein concentration of the samples was detected via Spectrophotometer analysis using a

Bradford protein assay (Bio-Rad). Briefly, empty vials were filled with 800μl of H2O plus 200μl of Bradford reagent. Then, 1μl of every lysate was added to the mix while 1μl of mPER lysis buffer was used as the white sample to set the instrument. Note that every measurement was made in triplicate. The assorbance of the samples at 595nm was measured using a spectrophotometer and the protein concentration was detected comparing the values of the lysates with the values of a standard BSA curve already prepared. Next, samples for western blotting analysis were prepared as follows: every lysate was added of Laemli Sample Buffer 4X and water in order to have the same volume and same protein amount. Then, 50μg of samples were separated on SDS-PAGE and electro-transferred to nitrocellulose membranes at 400mA for 1:30h. Ponceau Red staining was used to control the occurred transfer of proteins. Membranes were washed in PBS-0.1%/Tween-20 (PBS/T) and nonspecific binding sites were blocked by incubation in blocking buffer (PBS/T with 5% non-fat dry milk) for 1 h at room temperature. After several PBS/T-washes, membranes were incubated with specific primary antibodies overnight at 4°C. The day after, membranes were washed again at least 3 times, then incubated with peroxidase conjugated secondary antibodies diluted in PBS/T for 1 h at room temperature. After several washing passages, proteins were detected by incubating membranes in enhanced chemiluminescence detection system (ECL, Thema Ricerca). Antibodies were as follows: PLCβ1, Cyclin D3, Cyclin B1, PKCα, PKCβII, PKCζ, Lamin A/C from Santa Cruz, PKCα, Cyclin B1, Cyclin A, Cyclin E, phospho-Cyclin B1 Ser133, phospho-Cyclin B1 Ser147, Cdc25c, Cdk1/cdc2, phospho-Cdk1/cdc2 Tyr15, Cdk4, Cdk6, Cdk7 from Cell Signaling technology and β-tubulin from Sigma Aldrich. Analysis with an antibody for β-tubulin demonstrated equal protein loading. Every antibody was diluted as indicated by the manufacturer's instructions.

2.5 Flow Cytometric analysis of cell cycle

For FACS analysis 1×10^6 cells were collected by centrifugation at 1,200 rpm for 5 min at 4°C and washed twice in ice-cold PBS. Cells were fixed (and permeabilized) with −20°C cold 70% ethanol overnight at 4°C. Fixed cells were, then, washed in PBS twice and resuspended in 1 ml of staining solution (40 μg/ml propidium iodide and 100 μg/ml RNase A in PBS). The samples were incubated for at least 30 min at room temperature in the dark. FACS analysis was performed, and the percentage of cells in different

phases of the cell cycle was assessed using a FC500 flow cytometer equipped with cxp software (Beckman Coulter Inc.). At least 10,000 events per sample were acquired.

2.6 Cell transfections and Isolation of stable clones

Cells were transfected with full-length DNA vectors for human PKCα (Addgene, plasmid number 21232 and 21235, [145]), PLCβ1a or PLCβ1b using empty pcDNA/2.1 plasmid (Invitrogen) as control. Overexpressions were performed using Lipofectamine 2000 from Invitrogen. Cells were seeded at a cell density of 5×10^5/ml in 6-well plates, to which was added the mix of Lipofectamine 2000 (Life Technologies) and right vectors, following manufacturer's instructions. In order to obtain stable clones overexpressing PLCβ1a and PLCβ1b, cells were selected by limiting dilution in complete RPMI 1640 10% FBS containing Geneticin (G418 from Sigma Aldrich) at a concentration of 1000μg/ml starting 72h after the transfection, then expanded and kept always in selection with G418. The expression of PKCα, PKCβII and PLCβ1 was silenced by RNAi at a final concentration of 50nM using the electroporation assay kit by Thema Ricerca: cells, plated at a cell density of 2 x 10^6/ml before the transfection, were resuspended in 100μl of Mirrus Solution (pre-warmed at 37°C); specific siRNAs were added at the suspension and the mix moved in proper cuvettes. Using program T-16, cells were electroporated in Nucleofector I (Amaxa). Afterwards, cells were plated in 25ml flasks with 5ml of complete RPMI 1640 10% FBS. The following siRNAs were used: in order to silence PLCβ1 s23358 and s23359 (Applied Biosystems), to silence PKCα s11092, s11093 and s11094 (Applied Biosystems), to silence PKCβII s11095 (Applied Biosystems). As negative control a mix of Silencer Select Negative Control #1 and #2 siRNAs (Applied Biosystems).

2.7 Mutagenesis

For our purpose, we used the catalytic inactive mutants of PLCβ1a and PLCβ1b created by mutations of His^{331} and His^{378} in the putative active site of the enzymes. The substitution of the two residues with two Ala was performed on the plasmids encoding PLCβ1a and PLCβ1b using the QuickChange XL II site-directed mutagenesis kit from Stratagene (La Jolla, CA). Two subsequent mutagenesis reactions were performed: the first reaction using primer I (5′-CTA TTT CAT CAA TTC CTC A*GC* CAA CAC CTA CCT CAC AGC TG-3′) and primer II (5′-CAG CTG TGA GGT AGG TGT TG*G C*TG

AGG AAT TGA TGA AAT AG-3′) to obtain $His^{331} \rightarrow Ala$, was followed by a second reaction performed on the resulting mutated vector using primer III (5′-GAA GAG CCT GTC ATC ACC *GC*T GGA TTC ACC ATG ACA AC-3′) and primer IV (5′-GTT GTC ATG GTG AAT CCA *GC*G GTG ATG ACA GGC TCT TC-3′) to obtain $His^{378} \rightarrow Ala$. All mutations were verified by DNA sequencing [64].

2.8 Nuclear/Cytoplasmic separation

Cells were collected, centrifuged and washed in PBS 1X. PBS 1X was accurately removed and the pellets were resuspended in 1ml of TM2 buffer (hypotonic buffer containing 10 mM Tris-HCl pH 7.4 and 2 mM $MgCl_2$) for 2 minutes. Then, Triton 0.6% was added and everything was passed twice through a syringe with a 22 ½ gauge needle. Preliminary controls of nuclear purity have been performed through microscope analysis. Next, MgCl2 3mM was added to the solution, which was centrifuged for 10 mins at 0.8 rpm. The nuclear pellets were washed twice in TM5 buffer (isotonic buffer containing 10 mM Tris-HCl pH 7.4 and 5 mM $MgCl_2$), while the supernatant was transferred in a new vial and used like a cytoplasmic control. The nuclei were lysed using mPER (as total lysates) to which DNAse (Invitrogen) was added to remove the possible chromatin contamination.

2.9 Immunoprecipitation

Cells were lysed in mPER lysis buffer (BIORAD) containing protease inhibitors (Thema Ricerca). 500μg of proteins were pre-cleared adding 20μl of Protein A/G PLUS-Agarose (Santa Cruz) for 1 hour at 4^o C. Then, beads were centrifuged and the supernatant transferred in another vial. 500 μl of protein lysates were incubated over/night with the appropriate primary antibodies on wheels at 4^o C. The day after, 25μl of Protein A/G PLUS-Agarose were added for 1h at 4^o C. Beads were centrifuged and pellets washed 3 times with mPER. Immuno-complexes were resuspended in loading buffer (4X), boiled at 95°C for 5 minutes, resolved on SDS/PAGE and transferred on nitrocellulose. After incubation with the indicated antibodies, antibody–protein interactions were detected with enhanced chemiluminescence detection system (ECL, Thema Ricerca). As positive control 50μg of proteins from total lysates of K562 were loaded on SDS/PAGE gel.

2.10 Immunofluorescence microscopy

Cells were seeded on electrostatically charged glass slides using a Shandon Cytospin (Thermo Electron Corporation, Pittsburgh, PA, USA) at low acceleration and 200 rpm for 5 min. Slides were fixed in 4% paraformaldehyde at 37°C for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 8 min. Then slides were blocked with PBS containing 5% BSA for 1h. Incubation with monoclonal anti-PLCβ1, PKCα or Cyclin B1 (1:100) (Santa Cruz) was performed overnight at 4°C in blocking medium and then with FITC-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:200) or Cy3-conjugated anti-rabbit IgG antibody (1:100) (Sigma-Aldrich) for 1 h at room temperature. Slides were washed 3 times for 10 min at room temperature with PBS 1X / Tween 0,1% and mounted with a DAPI anti-fade reagent in glycerol (Molecular Probes, Eugene, OR, USA). Images were taken on a Zeiss Axio Imager Z1 microscope, equipped with 60X/NA 1.4 optics and Apotome apparatus, coupled to a computer driven Zeiss AxioCam digital camera (MRm), using Zeiss Axio Vision 4.4 software (Carl Zeiss, Oberkochen, Germany). At least 100 cells per slide were analyzed.

2.11 RNA extraction, Retrotranscription and qPCR

Transfected K562 cells were collected and total cellular RNA was extracted using the RNeasy minikit (Ambion) according to the manufacturer's instructions. The purity and amount of RNA were analyzed measuring their assorbance at 260-280nm through Nanodrop. 2 μg of total RNA were reverse transcribed using 0.5μg of Oligo(dT) 15 Primer and sterile H_2O was added to the solution up to a final volume of 15μl. The mix was incubated for 5 minutes at 70^{o} C to eliminate all the secondary structures of RNA. Next, Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), RNAse and dNTPs (Promega) were added following the manufacturer's instructions in order to obtain a 25μl solution. Gene expression of PLCβ1a, PLCβ1b, PKCα and Cyclin B1 was determined by using the 7300 real-time PCR system. Briefly, the reaction was performed in MicroAmp Optical 96-well plates at a final volume of 25μl. Every solution was composed using 12.5μl of TaqMan® Gene Expression Master Mix (Applied Biosystems), 1μl of cDNA, 1.25μl of the right probe (specific for the gene of interest) and 10,25 μl of H_2O. Every sample was analyzed in triplicate and the results were normalized to the level of the ubiquitously expressed RNA 18S ribosomal 1 gene (RN18S, Hs03928990_g1) and were expressed as $2^{-\Delta\Delta Ct}$. The reaction conditions were

as follows: 2 minutes at 50° C, 10 minutes at 95° C followed by 40 cycles at 95° C of 15 seconds and 1 minute at 60° C. For PKCα the Hs00925193_m1, for PLCβ1a the Hs01008373, for PLCβ1b the Hs01001939, for Cyclin B1 the Hs00820463_g1 probes were used (Applied Biosystems).

2.12 Statistical analysis

Statistical analyses were performed by Student *t*-test, using GraphPad Prism (GraphPad Software Inc. version.6) (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$).

2.13 Cell counting

To determine cell growth, cells were synchronized using Nocodazole or starvation in RPMI without FBS. 24 hours later, the block was removed seeding them in complete RPMI 10% FBS at a cell density of 1 x 10^5/ml in 6-well plates. Growth curves were determined by direct counting of cells harvested for 24, 48 and 72 h after seeding. Viable cells were handly counted by a hemocytometer using 0.2% Trypan Blue.

2.14 Quantification of DAG

K562 cells were cultured and synchronized as previously described. Nuclei or intact cells were labeled with [^{3}H]-glycerol (10μCi/ml/1 x 10^6 cells) for 90 minutes. Next, nuclei and total cells were precipitated with 10% TCA and, then, 10 volumes of chloroform/methanol/concentrated HCl (300:300:1.5) were added and lipids extracted for 20 hours at 4°C. After centrifugation, supernatants were preserved and the pellets were re-extracted twice with 10 volumes of chloroform/methanol/concentrated HCl (400:200/1.5). The combined supernatants were dried under steam of nitrogen and lipids were dissolved in 100μl of chloroform and washed three times in 4 volumes of chloroform/methanol/water (3:48:47). Finally the samples were dried under a steam of nitrogen and [^{3}H]-labelled lipids were analyzed by TLC on silica gel 60 plates developed with ether/exane/NH_4 (50:50:0.25). TLC plates were sprayed with Enhancer (Du Pont, NEN) and fluorographed at -80° C. Spots corresponding to lipids were scraped off, extracted with 1.5ml of 0.6N HCl-Methanol (60:40 by volume) for 48 hours with gentle stirring and counted with a liquid scintillation counter using 9ml of Packard Pico-Fluor 40 scintillation cocktail .

2.15 PLC activity assay

The activity of nuclear PLCβ1 was assessed using a specific protocol set by Martelli et al. PLCβ1 was silenced and, 48h later, nuclei and cytoplasms were separated. A scramble siRNA was used as control. 60μg of nuclear proteins were treated with 3nmol [^{3}H]-PIP_2 (specific activity 30000 d.p.m./nmol), 100mM MES, 150nM NaCl, 0.06% taurodeoxycholate and reaction was performed for 30 minutes at 37^o C. Hydrolysis of PIP_2 was stopped adding HCL-chloroform-methanol and the aqueous phase was separated from the organic one through centrifugation at 2000g for 5 minutes. PIs recovered from the aqueous phase were analyzed by HPLC. The amount of radioactivity corresponding to IP_3 was analyzed (in counts per second, c.p.s.) [47].

Results

3.1 INVOLVEMENT OF PLCβ1 AND PKCα IN THE MODULATION OF CYCLIN D3 EXPRESSION AND CELL PROLIFERATION OF K562 CELL LINE

3.1.1 Human erythroleukemia cells, K562, overexpressing PLCβ1 show increased levels of Cyclin D3

In order to understand whether PLCβ1 could regulate expression of Cyclin D3 also in human erythroleukemia cells as well as in murine models [102], we used a model of human cell line, K562, transiently transfected with two vectors encoding the two splicing variants of PLCβ1, PLCβ1a and PLCβ1b. An empty vector was used as control for the experiments. Cells, seeded at a cell density of 5 x 10^5/ml were transfected to overexpress these enzymes and, 24 hours later, were collected and lysed to extract their protein content. Total lysates were analyzed via western blotting in order to study the possible modulation of cell cycle related proteins due to the increase of PLCβ1 in the cells. Interestingly, we found that the overexpression of both the isoforms of PLCβ1 (PLCβ1a OV and PLCβ1b OV) could lead to an important increase of Cyclin D3 levels if compared with the controls (Empty vector). We screened also some others cyclins and Cdks, such as Cyclin A, Cyclin E, Cdk6 and Cdk4, which did not show any change in their expression. These findings indicated that the connection between PLCβ1a and PLCβ1b with Cyclin D3 was positive and specific. Indeed, no other molecules were targeted by PLCβ1 increase (Figure 12).

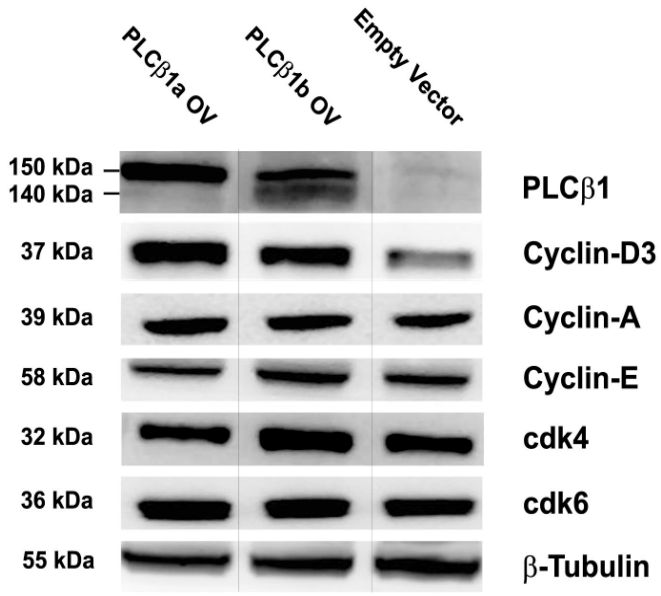

Figure 12 : PLCβ1 overexpression positively affects only Cyclin D3 levels. *K562 cells were transfected in order to overexpress both the splicing variants of PLCβ1, PLCβ1a and PLCβ1b (PLCβ1a OV/ PLCβ1b OV). An empty vector was used as control (Empty Vector). Lysates were analyzed via Western Blotting and the expression of several proteins involved in cell cycle progression was studied.*

3.1.2 PLCβ1 leads to a delay in cell proliferation prolonging the S phase of the cell cycle in proliferating K562 cells

In order to understand if the positive effects of overexpression of the splicing variants PLCβ1a and PLCβ1b on Cyclin D3 could influence the behavior of the cells in terms of proliferation and cell cycle progression, we decided to create stably transfected clones overexpressing PLCβ1a, PLCβ1b and the empty vector (PLCβ1a OV, PLCβ1b OV and Empty vector). K562 cells were seeded at a density of 5 x 10^5/ml in complete RPMI 1640 10% FBS and transfected using a Lipofectamine 2000 protocol, following manufacturer's instructions. After the transfections, we kept the cells growing and recovering for 72 hours and, then, we added G418 to the medium to start the selection of stable clones. A week later, the specificity and the quality of PLCβ1 isoforms overexpression in the stable clones was analyzed via qPCR using specific probes for each splicing variant (Figure 13a). Once we obtained stably transfected cells, we started to focus our attention on cell cycle and cell proliferation analyses. As Cyclin D3 is involved is G1 phase [13-15], we synchronized K562 clones at G0/G1 phase. Briefly,

cells were grown for two days and blocked at G0/G1 by 24h of starvation in RPMI 1640 without FBS. After this treatment, cells were split in two aliquots: one was collected immediately (24h starved cells) and one was centrifuged, washed in sterile PBS 1X and seeded back in complete RPMI 1640 with FBS for another 24h. The day after, also this second aliquot (proliferating cells) was collected. Analyses via western blotting and FACS were performed in order to study the modulation of Cyclin D3 in stable clones overexpressing PLCβ1a and PLCβ1b and the subsequent effects on cell cycle progression. As shown in Figure 13b and c, starved cells were characterized by accumulation at G0/G1 with no modulation of Cyclin D3 expression in all the clones. On the contrary, proliferating clones overexpressing PLCβ1 showed an increased Cyclin D3 expression and a prolonged S phase if compared with the control. In addition, in order to better understand the meaning and the effects of these differences in the S phase among clones, we decided to study which was their behavior in terms of cell proliferation. Cells were then grown for three days, seeded in 6-wells at the same number and, then, counted after 24-48-72 hours. As shown in Fig. 12D, clones overexpressing both the isoforms of PLCβ1 were characterized by a decrease in cell proliferation compared with the controls. These data confirmed a role of PLCβ1 in G1/S transition: targeting Cyclin D3 in proliferating cells, overexpression of both the isoforms of PLCβ1 prolonged the S phase of the cell cycle leading to a severe slow-down of cell proliferation in K562 cells.

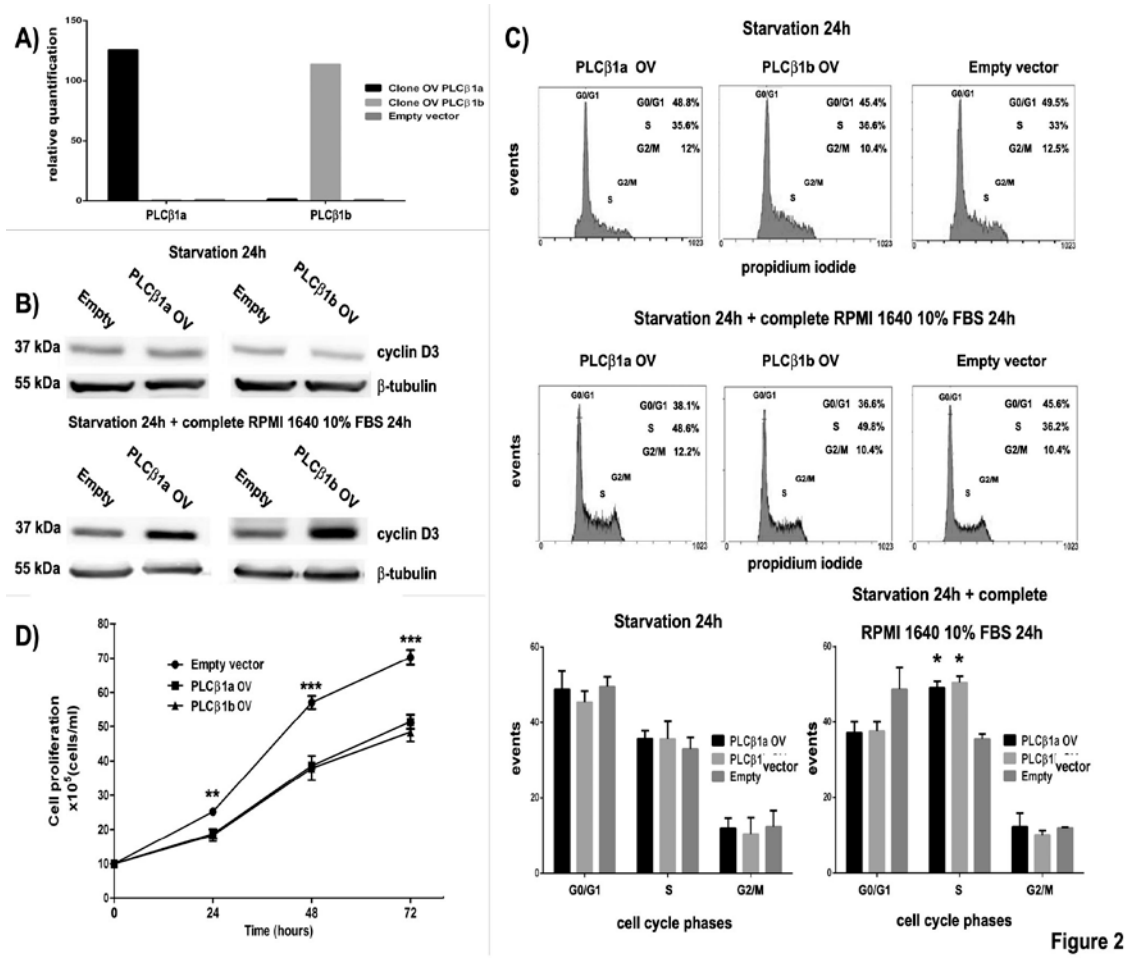

Figure 13 : PLCβ1a and PLCβ1b overexpression leads to an increase of Cyclin D3 in proliferating cells, followed by a prolongation of the S phase of cell cycle and a slow-down of cell proliferation. *a) Real-Time analyses showed the specific stable overexpression of the two PLCβ1 isoforms, PLCβ1a and PLCβ1b. b) Cells stably overexpressing PLCβ1 (PLCβ1a OV / PLCβ1b OV) and controls (Empty Vector) were starved for 24h. One aliquot was collected and lysed, one was seeded again in complete RPMI 1640 with FBS for other 24h and, then, lysed. Western Blotting analyses showed an increase of Cyclin D3 in clones overexpressing PLCβ1 only in proliferating conditions. c) Cells were treated as in b) and cell cycle analysis via FACS was performed. d) Clones overexpressing PLCβ1a or PLCβ1b and the controls were seeded in 6-well plates and counted for 24-48-72h.*

3.1.3 PLCβ1a and PLCβ1b overexpression leads to a decrease of PKCα

As PLCβ1 and Cyclin D3 were found connected to the regulation of K562 cell cycle, we decided to further investigate the pathway through which this modulation could take place. Then, we studied the class of protein known to be directly activated by PIP_2 hydrolysis, the PKC family [104-106]. Several previous findings reported the involvement of these phosphotransferases in cell cycle progression through effects on cyclins, cyclin-dependent kinases and cell cycle inhibitors [106, 133]. K562 cells resulted a very good model to study PKC signalling, because only three isoforms are expressed in this cell line: conventional PKCα and PKCβII and atypical PKCζ [143]. Thus, we screened their expression in cells stably overexpressing PLCβ1a and PLCβ1b (PLCβ1a OV, PLCβ1b OV and Empty vector as control). As previously described, cells were synchronized and lysed. Lysates were analyzed via Western Blotting and the expression of these PKC was evaluated with specific antibodies. Interestingly, overexpression of both PLCβ1 splicing variants led to a strong decrease in PKCα levels, while PKCβII and PKCζ resulted not to be affected at all (Figure 14). These data indicated a possible connection between PLCβ1 signalling and PKCα during K562 cell proliferation.

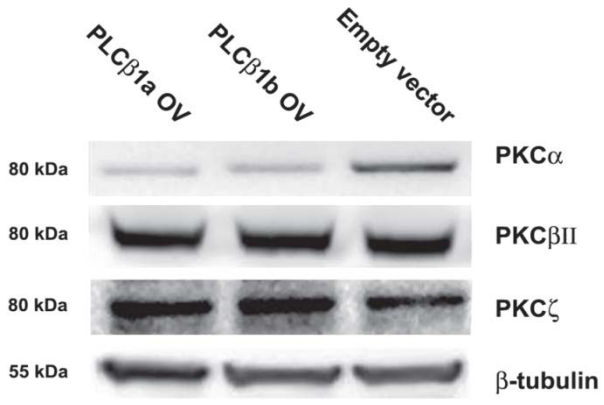

Figure 14 : PLCβ1 overexpression leads to a downregulation of the levels of PKCα. PKCβII and PKCζ are not affected. *PLCβ1a and PLCβ1b (PLCβ1a OV / PLCβ1b OV) were overexpressed in K562 cells. Empty vector was used as control (Empty Vector). Western Blotting analyses were performed to analyze the expression of the PKC isoforms expressed in K562 cells.*

3.1.4 PLCβ1 activity is necessary for PKCα decrease

In this series of experiments we decided to understand if the lipase activity of PLCβ1 was necessary in PKCα modulation. In order to achieve that, we transfected the cells with two catalytic inactive mutants of the two splicing variants of PLCβ1. As explained in materials and methods, these two vectors were characterized by two substitutions on two fundamental residues for the activity of PLCβ1; in particular His^{331} and His^{378} were substituted with two Ala [64]. Data obtained demonstrated that overexpression of a catalytic inactive mutant of PLCβ1 did not affect the expression of PKCα if compared with the wild type vectors and the controls (PLCβ1a OV, H331/H378 1a OV, PLCβ1b OV, H331/H378 1b OV and Empty vector) (Figure 15). These evidences strengthened the idea that PLCβ1 signalling could be responsible for PKCα downmodulation in this cell line.

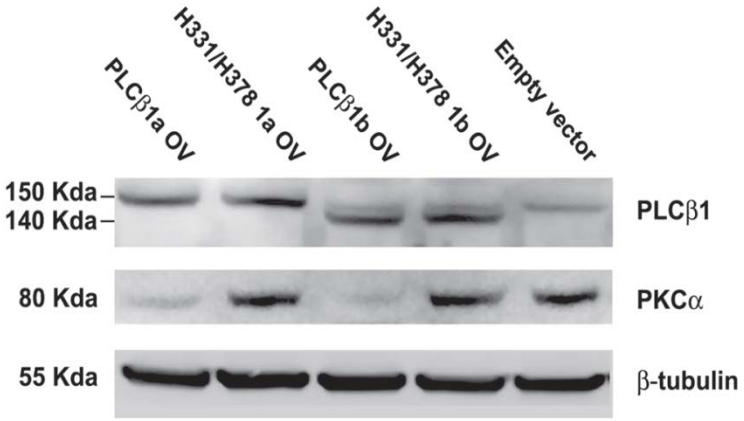

Figure 15 : The catalytic activity of PLCβ1 is necessary for PKCα downregulation. *Cells were transiently transfected to overexpress PLCβ1a or PLCβ1b (PLCβ1a OV / PLCβ1b OV) and catalytic inactive mutants of both the isoforms (H^{331}/H^{378} 1a OV / H^{331}/H^{378} 1b OV). An empty vector was used as control (Empty Vector). The expression of PKCα was analyzed via Western Blotting.*

3.1.5 PKCα knock-down mimics PLCβ1 overexpression in terms of Cyclin D3 expression and cell proliferation

As PLCβ1 mediated regulation of both PKCα and Cyclin D3 resulted clear, here we decided to investigate if PKCα modulation could be important for Cyclin D3 expression and, in turn, for cell cycle and cell proliferation. Then, mimicking the effects of PLCβ1 overexpression, we directly silenced PKCα through siRNA techniques (see materials and methods). Cells were transiently transfected with a specific siRNA for PKCα and a scrambled one as control (PKCα KD and Scrambled). 24 h later, they were collected and lysed. Western Blotting analyses showed a strong up-regulation of the levels of Cyclin D3 in PKCα knock-down conditions if compared with the control. Moreover, other proteins involved in cell cycle regulation were screened and resulted not affected by PKCα. Thanks to these evidences, we found the involvement of PKCα in Cyclin D3 regulation (Figure 16a). Moreover, we also thought to study if PKCα silencing could be important for the slow-down of K562 cell proliferation, as found in cells overexpressing PLCβ1a or PLCβ1b. Then, we transfected the cells in order to silence PKCα as already described; two hours later we moved the cells in 6-well plates at the same concentration. Finally, we counted them in triplicate after 24-48-72 hours (Figure 16b). Cells characterized by a silenced PKCα resulted slower than the controls in terms of cell proliferation. Taken together, the collected data demonstrated the similar behavior of cells overexpressing PLCβ1 or with PKCα knock-down; in particular, both the proteins resulted to be part of the same pathway which regulated Cyclin D3 expression and, in turn, cell proliferation of K562 cells.

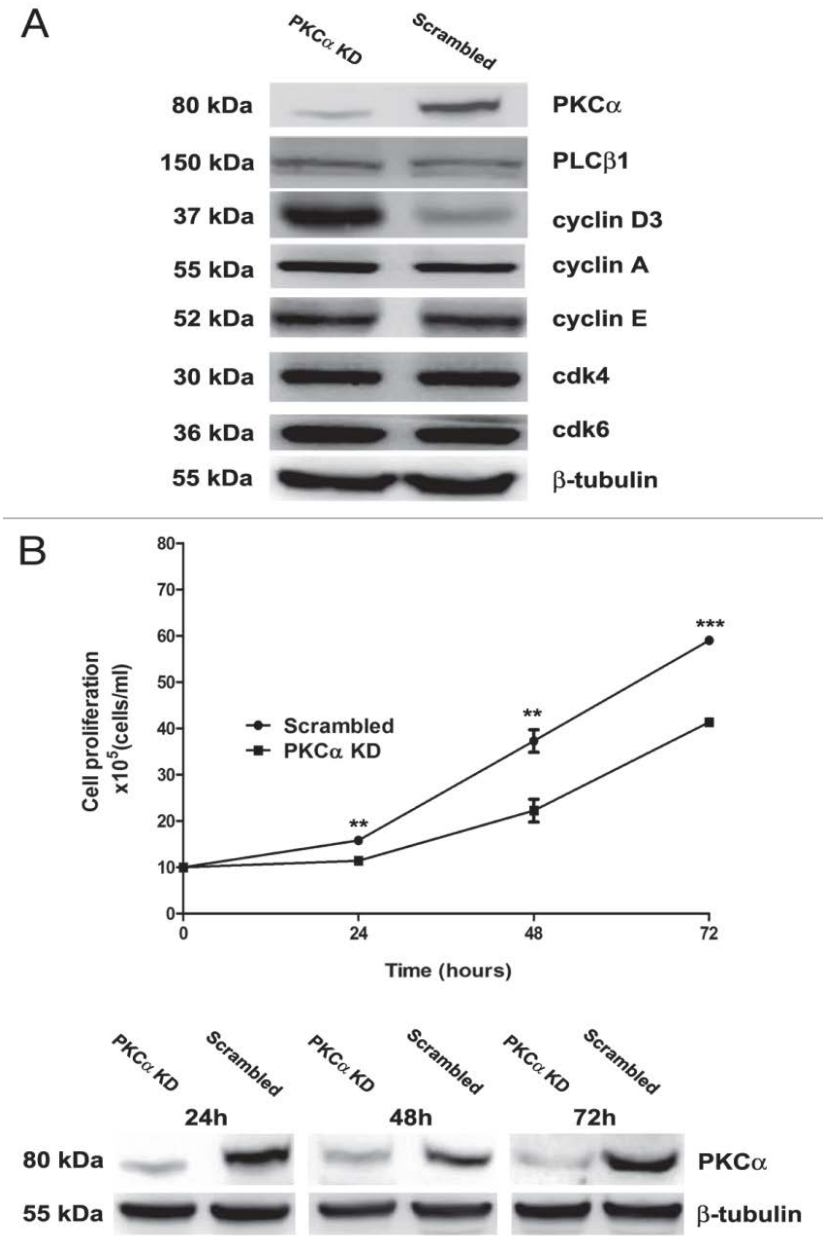

Figure 16: PKC silencing leads to an up-modulation of Cyclin D3 levels and to slow-down of cell proliferation, which results the same encountered in cells overexpressing PLCβ1. *a) PKCα was transiently silenced (PKCα KD) and, 24h later, Western Blotting analyses were performed to screen different proteins involved in cell cycle progression. A Scrambled siRNA was used as control (Scrambled). b) Cells were transfected to silence PKCα and counted for 24/48/72 hours.*

3.2 NUCLEAR DAG PRODUCTION DURING CELL CYCLE IS LINKED TO PLCβ1 ACTIVITY, WHICH RESULTS FUNDAMENTAL FOR THE REGULATION OF THE COMPLEX REPRESENTED BY PKCα AND CYCLIN B1

3.2.1 PKC decrease affects Cyclin B1 levels in cells synchronized at G2/M

As previously reported, no evidences about a possible involvement of PKC in the regulation of Cyclin B1/Cdk1 complex have been reported so far. In order to study if the two DAG dependent PKC isoforms present in K562 cells, PKCα and PKCβII, could affect Cyclin B1, we treated the cells with three different PKC inhibitors: Go6983, Go6976 and 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione anilinomonoindolylmaleimide (from here simply PKC inhibitor*), at a final concentration of 1μM for 16h. This concentration renders these compounds specific for conventional and novel isoforms. Notably, all the compounds were ATP-competitor drugs which inhibit the activity of PKC [144], but PKC inhibitor* resulted also capable to highly down-modulate the levels of these proteins, due probably to off targets effects on other molecules involved in PKC life cycle (Go6983, Go6976, PKC inhibitor* and Control). However, in all the experiments, cells were synchronized at G2/M using Nocodazole, an inhibitor of the microtubules synthesis, to avoid any cell cycle dependent oscillation of Cyclin B1 expression. We found that cells treated with PKC inhibitor* showed an important decrease of Cyclin B1 levels if compared with the controls. Surprisingly, the only inhibition of PKC activity by Go6983 and Go6976 did not affect this Cyclin isoform at all. On the contrary, we decided to increase PKC signalling using 12-O-tetradecanoylphorbol-13-acetate (TPA), also called phorbol-12-myristate-13-acetate (PMA), at a final concentration of 50nM. This phorbol-ester compound acts as a potent tumor promoter and activator of PKC, through the binding with C1B domain. As previously reported, a chronic/hyper activation of these phosphotransferases leads to their faster degradation due to changes in their structure [131, 132]. However, cells were blocked at G2/M as previously reported and the levels of Cyclin B1 were studied via Western Blotting analyses. Interestingly, PKC down-regulation due to PMA action (PMA and Control) was followed by a high down-regulation of the levels of Cyclin B1 in the cells. These findings indicated that, more

than the activity, the presence of PKC in the cells could be fundamental for the regulation of Cyclin B1 in K562 cell line (Figure 17).

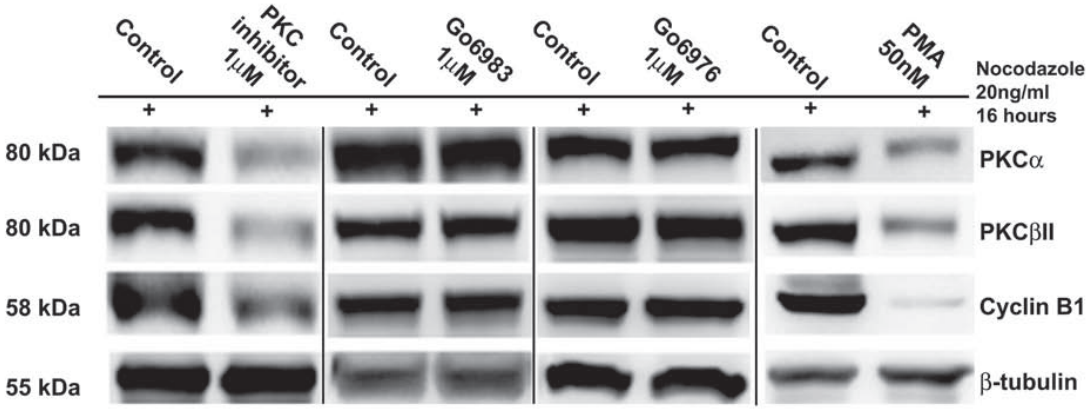

Figure 17 : PKC positively regulate the levels of Cyclin B1 in K562 cells synchronized at G2/M. *Cells were treated with three different PKC inhibitors (Go6983, Go6976 and 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione anilinomonoindolylmaleimide (PKC inhibitor*)) at a final concentration of 1μM or with PMA at 50nM for 24 hours. Then, they were synchronized by treatment with Nocodazole (16 hours at 20ng/ml) at G2/M. Western Blotting analyses of the lysates were performed to control the expression of PKCα, PKCβII and Cyclin B1.*

3.2.2 PKCα is the only conventional isoform able to modulate Cyclin B1 expression and, in turn, cell cycle progression

As it is commonly known that inhibitors can have many off target effects, in particular if used at high concentration, we decided to understand if PKC could be really involved in the mechanisms behind Cyclin B1 regulation and, in particular, which isoform between PKCα and PKCβII was responsible of that. Then, we transiently transfected K562 cells to silence PKCα or PKCβII using a Scrambled siRNA as control (PKCα KD, PKCβII KD and Scrambled). 24h later, we added Nocodazole to synchronize them at G2/M. Western Blotting analyses showed that the only PKC isoform capable to affect Cyclin B1 was PKCα. PKCβII silencing had no effects on this enzyme at all (Figure 18a). Moreover, as Cyclin B1 can be phosphorylated on a CRS region composed by 5 residues of Ser, we investigated if some of them could be modulated by PKCα silencing [29, 30]. We found that neither Ser133 nor Ser147 were affected (data not shown).

Finally, we focused our attention on cell cycle profile of cells characterized by PKCα knock-down and, then, to decrease of Cyclin B1. 24h after transfection, cells were synchronized at G2/M with Nocodazole. Then, the block was released seeding them again in complete RPMI 1640 10% FBS where they were left to grow for 24h. Cell cycle analyses by FACS revealed that silencing of PKCα led to an increase of the percentage of cells in G2/M compared to the controls, probably due to their difficulty to exit this stage of the cell cycle for the lack of Cyclin B1 (Figure 18b).

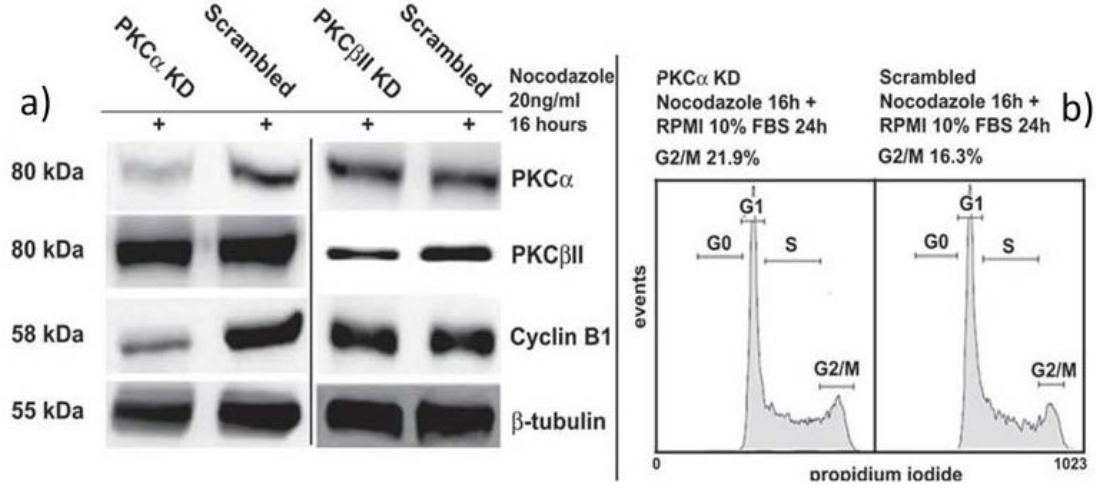

Figure 18: PKCα is the PKC isoform responsible for Cyclin B1 modulation. This regulation leads to effects on the G2/M progression of cell cycle. *a) Cells were transiently transfected to silence PKCα or PKCβII (PKCα KD / PKCβII KD). A Scrambled siRNA was used as control (Scrambled). 24h later, they were synchronized at G2/M using Nocodazole. Lysates were analyzed via Western Blotting. b) Cells were transfected and, 24h later, synchronized at G2/M with Nocodazole. Then, the G2/M block was removed seeding the cells in complete RPMI 1640 10% FBS for 24h. Cell cycle was analyzed via FACS.*

3.2.3 PKCα overexpression increases Cyclin B1 levels in K562 cells: the kinase activity of the enzyme resulted not to be necessary for this effect

In this series of experiments we decided to understand if PKCα activity was necessary for Cyclin B1 modulation. Then, using two different vectors, one encoding a wild type PKCα (WT) and one a dominant negative mutant of the protein (DN, given by a puntiform mutation in the ATP-binding site, Lys^{376} was substituted with Arg), we overexpressed the enzyme in the cells. An empty vector was used as control. 24h later, Nocodazole was added for only 6h, in order to drive the cells to Cyclin B1 increase and,

in turn, to G2/M accumulation. This partial synchronization was performed to avoid a complete block and high increase of the levels of Cyclin B1, which could render the differences among the samples impossible to be detected. Notably, we found an up-modulation of Cyclin B1 levels in cells characterized by overexpression of either PKCα WT or DN if compared to the controls (PKCα WT OV, PKCα DN OV and Empty vector). Interestingly, no differences between the WT or DN samples were encountered (Figure 19a). This was a further indication that the kinase activity of PKCα was not necessary in this mechanism. Moreover, cell cycle analyses indicated that overexpression of both the PKCα vectors led to a faster G2/M accumulation of the cells, probably connected to a major presence of Cyclin B1 in the system (Figure 19b).

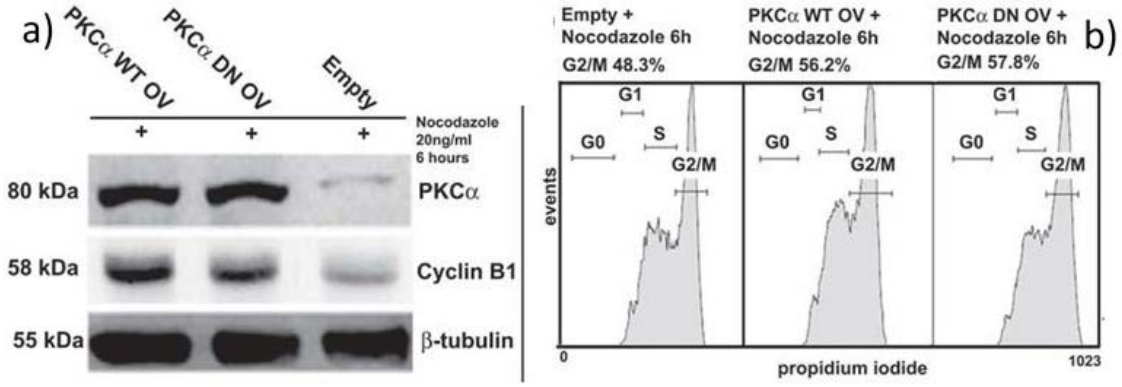

Figure 19 : Overexpression of a wild type or dominant negative PKCα leads to the same modulation of Cyclin B1 and to the same cell cycle profile in K562 cells. *Cells were transiently transfected to overexpress PKCα using a wild type vector (PKCα WT OV) and a dominant negative mutant (PKCα DN OV). Empty vector was used as control (Empty Vector). Partial synchronization was obtained adding Nocodazole for 6h, in order to stimulate G2/M and Cyclin B1 accumulations. Lysates were analyzed via Western Blotting and cell cycle profile via FACS.*

3.2.4 Absence of PKCα triggers Cyclin B1 degradation

Since we found a positive correlation between PKCα and Cyclin B1, we decided to deeply study how this modulation could work. Then, we thought to understand at which level it could take place. First, we performed a gene expression analysis via real-time PCR. We silenced or overexpressed PKCα and synchronized the cells as previously described. RNA extracted was retrotranscribed to obtain cDNA, which was used for the real-time PCR. Notably, no changes in gene expression of Cyclin B1 have been found (Figure 20a), suggesting the existence of a post-transcriptional mechanism of regulation

by PKCα. As Cyclin B1 undergoes degradation after the G2/M progression, we investigated the possible involvement of PKCα in this process. In order to study that, we transiently silenced PKCα and, 24h later, we divided the cells in two aliquots. One of them was treated with a proteasome inhibitor, MG-132, at a final concentration of 15μM for 2h, to inhibit Cyclin B1 degradation. Western Blotting analyses showed an accumulation of Cyclin B1 levels in cells treated with this compound compared with the control (Figure 20b) (PKCα KD, PKCα KD + MG-132 2h). These evidences suggested that the presence of PKCα could limit Cyclin B1 degradation in K562 cells. Moreover, as further control of this findings, we decided to screen the levels of the Cip inhibitor p21, known to be involved in this process [34]. Indeed, its levels have been found to raise during Cyclin B1 degradation. Then, we silenced PKCα and synchronized cells at G2/M. PKCα knock-down cells were characterized by a strong increase of p21/Cip1 expression concomitant with Cyclin B1 down-modulation (Figure 20c) (PKCα KD and Scrambled). All these data indicated that lack of PKCα drove to a faster degradation of Cyclin B1 in K562 cells.

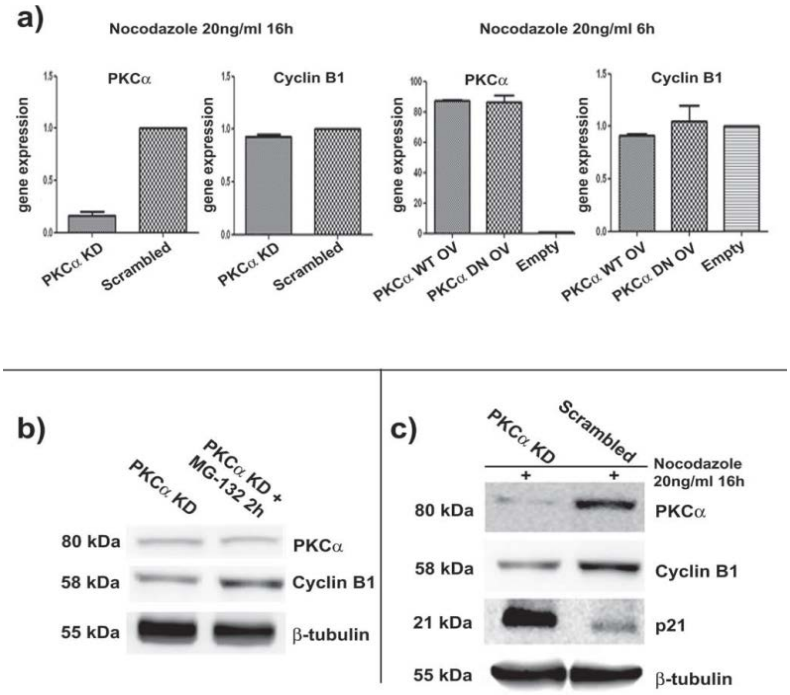

Figure 20 : PKCα is involved in mechanisms that mediate Cyclin B1 degradation. *a) Cells were transfected to silence or overexpress PKCα and gene expression of PKCα and Cyclin B1 were analyzed via real-time PCR. b) PKCα was silenced for 24h, then, cells were split in two aliquots, one of them was treated with the proteasome inhibitor MG-132 for 2h at a final concentration of 15μM. c) PKCα was silenced and cells synchronized at G2/M. Both the lysates of b) and c) were analyzed via Western Blotting.*

3.2.5 Cyclin B1 and PKCα share the same behavior during cell cycle progression and are able to interact in the cytoplasmic fraction

In order to study the localization and the expression of PKCα and Cyclin B1 along the cell cycle progression, we decided to set a protocol to specifically synchronize K562 cells in the different cell cycle phases. First, we starved them over/night with HBSS to obtain a partial synchronization at G0/G1; G1/S phase was referred to proliferating cells in complete RPMI 1640 10% FBS; finally, Nocodazole was again used for G2/M arrest (Figure 21a). Western Blotting analyses of total lysates of cells in the different cell cycle stages showed a progressive increase of Cyclin B1 and PKCα peaking at G2/M. In particular, separating nuclei from cytoplasms, we found an important G2/M related accumulation of both these molecules in the nuclear fraction (Figure 21b) (G0/G1, G1/S and G2/M). This similar behavior of the two enzymes was also confirmed by immunocytochemistry experiments (Figure 21c). Furthermore, considering their colocalization along cell cycle progression, we decided to understand if they could be part of a common complex of proteins. Then, we Co-Immunoprecipitated both the enzymes in cells synchronized at G2/M. Interestingly, we found a perfect interaction between them. As data obtained by IP techniques could be misleading, we decided to control the quality of our experiments. First, as positive control, we pulled down both Cyclin B1 and PKCα developing the following Western Blotting with specific antibodies to detect these same enzymes. Data obtained showed that the experiments were performed properly. Second, we immunoprecipitated again both the proteins with Cdk1/cdc2, the Cdk isoform known to interact with Cyclin B1 [27]. Both PKCα and Cyclin B1 resulted complexed with this enzyme, confirming our previous findings (Figure 21d/e). Finally, we performed immunoprecipitation using nuclear or cytoplasmic lysates, finding that the interaction between PKCα and Cyclin B1 took place in the cytoplasm (Figure 21f). These findings, together with the evidences indicating that the kinase activity of PKCα was not linked to Cyclin B1 modulation, supported our idea about a possible role for this phosphotransferase as a scaffold protein necessary to inhibit the degradation of Cyclin B1 along cell cycle progression.

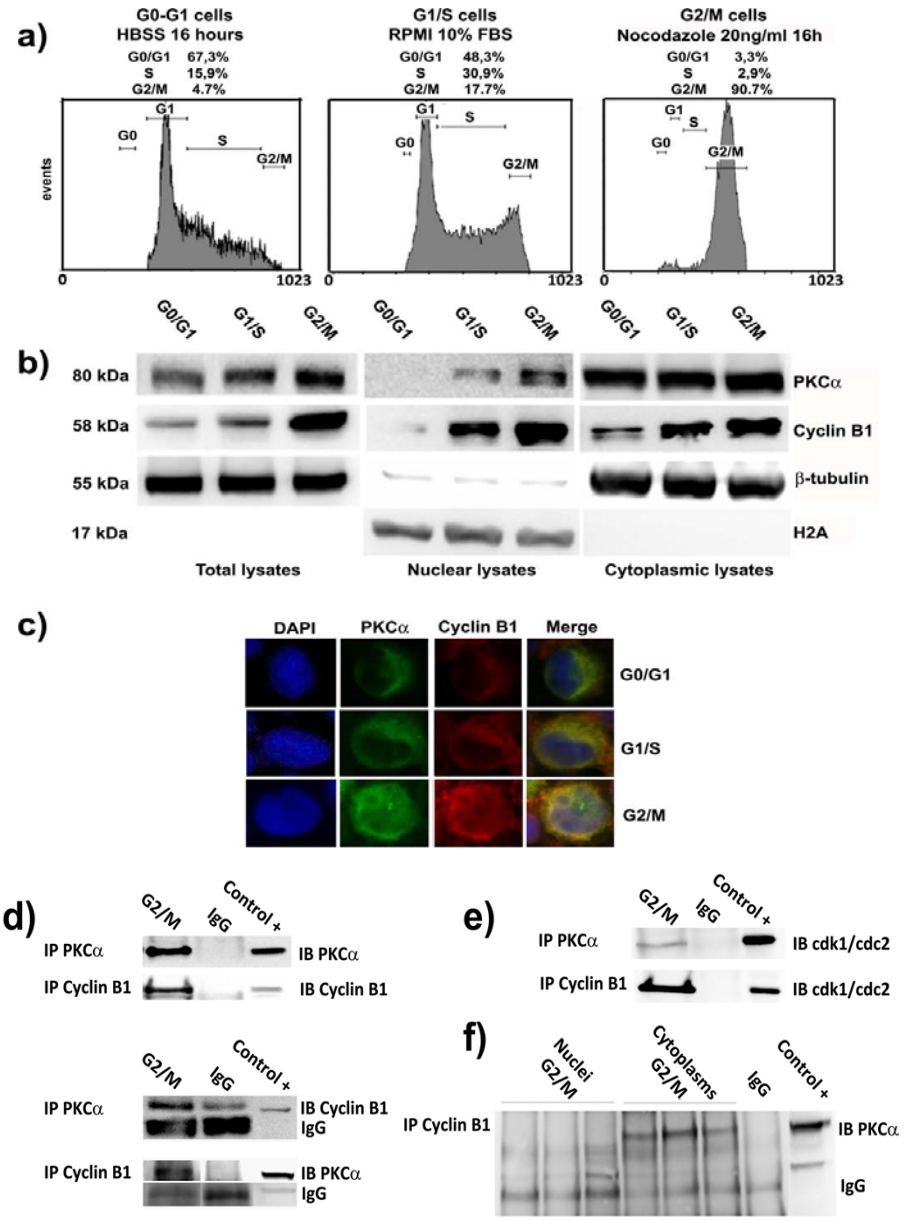

Figure 21 : PKCα and Cyclin B1 share the same behavior during cell cycle progression, highly translocating in the nucleus at G2/M. In addition, they interact at cytoplasmic level along cell cycle phases. *a) Cells were synchronized as follow: G0/G1 cells were starved with HBSS over/night, G1/S cells are cell growing in complete RPMI 1640 10% FBS, G2/M cells via Nocodazole. Cell cycle profile was analyzed via FACS. b) Cells were synchronized as a). Total, nuclear and cytoplasmic lysates were analyzed via Western Blotting to study the expression and the localization of PKCα and Cyclin B1. c) Cells were treated as a) and b) and immunocytochemistry experiments were performed. d) Co-IP of Cyclin B1/PKCα were performed in cells synchronized at G2/M. e) IP of PKCα and Cyclin B1 with Cdk1/cdc2. f) Nuclear and cytoplasmic fractions were used to immunoprecipitate Cyclin B1.*

3.2.6 Cyclin B1 nuclear levels are modulated by PKCα

Since the interaction and co-localization of PKCα and Cyclin B1 was found to take place along cell cycle and peak at G2/M, we decided to study if PKCα modulation could affect the nuclear translocation of Cyclin B1. In this series of experiments, we found that increase or decrease of PKCα levels in the cells, and its related major or minor nuclear accumulation, affected Cyclin B1 nuclear import. In order to achieve that, we silenced or overexpressed PKCα and, 24h later, we synchronized the cells using Nocodazole as previously described. Then, nuclei and cytoplasms were separated and lysed. PKCα knock-down cells were characterized by a minor accumulation of Cyclin B1 in the cytoplasms and in the nuclei if compared with the controls (Figure 22a) (PKCα KD and Scrambled). On the other hand, the samples overexpressing both WT and DN isoforms showed a major accumulation in both the compartments (PKCα WT OV, PKCα DN OV and Empty vector) (Figure 22b). Data were supported by immunocytochemistry (Figure 22c). Taken together, these findings indicated that the more PKCα translocated into the nuclei at G2/M the more Cyclin B1 could do the same.

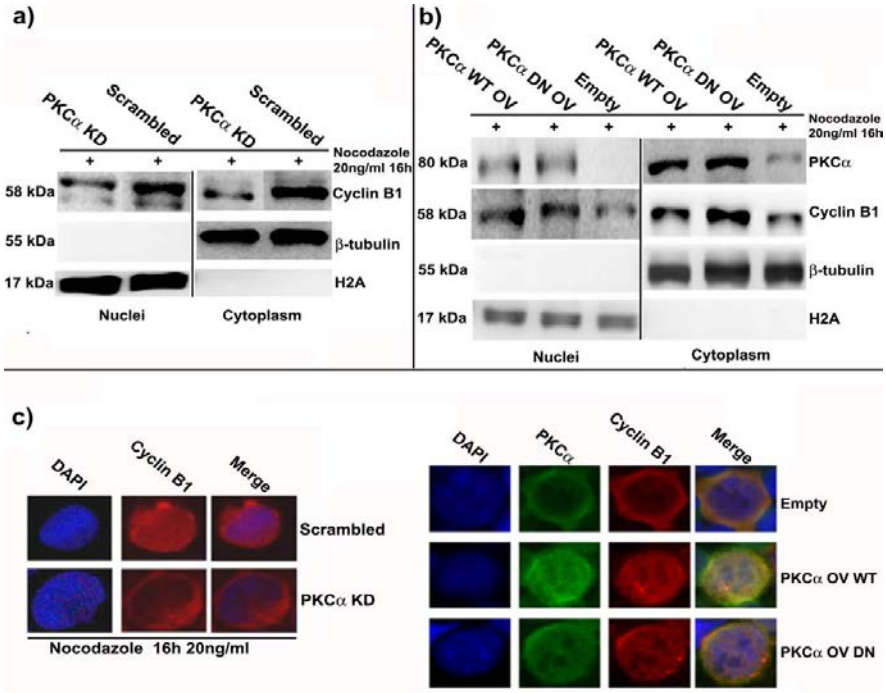

Figure 22: PKCα modulation regulates Cyclin B1 nuclear translocation. *a) PKCα was transiently silenced and cells synchronized at G2/M via Nocodazole. Nuclei and cytoplasms were separated and lysates analyzed via Western Blotting. b) PKCα was transiently overexpressed and cells partially synchronized at G2/M via Nocodazole. Nuclei and cytoplasms were separated and lysates analyzed via Western Blotting. c) Cells were treated as a) or b) and immunocytochemistry analysis was performed.*

3.2.7 Nuclear DAG oscillations peak at G2/M and trigger the accumulation of Cyclin B1 and PKCα into the nucleus

As the accumulation of Cyclin B1 and PKCα into the nucleus, in particular at G2/M, was found, we decided to shed light on the mechanisms through which this event could work. Considering that both the proteins are characterized by the lack of a common NLS, fundamental for nuclear import of many proteins, we focused our attention on the possible factors that could trigger their translocation [31]. Studies performed on different cell lines indicated nuclear DAG amount to peak at G2/M and, in turn, act as a bait to attract PKC. Then, we focused on DAG production in K562 cells along cell cycle progression. Cells were synchronized at G2/M and proliferating cells, G1/S, were used as control. Total and nuclear samples were labeled with [^{3}H]-glycerol and lipids were extracted. The percentage of total radioactivity corresponding to DAG was analyzed, indicating its slight increase in total G2/M extracts compared to the G1/S controls. On the other hand, we measured a very important accumulation of this compound in the nuclear samples synchronized at G2/M compared to the controls (Figure 23a). This peak of nuclear DAG levels, very similar to the findings reported in other studies [52, 53], led us to think it could the factor responsible for PKCα and Cyclin B1 nuclear import. Then, we treated K562 cells in two different ways: first, we used PMA (PMA 100 nM for 30 minutes) in order to mimic an increase of DAG; second, we treated the cells with U73122 (U73122 10μM for 16h), able to inhibit PIP_2 hydrolysis mediated by PLC. In both the cases we synchronized the cells at G2/M. Western Blotting analyses showed that accumulation of PKCα and Cyclin B1 was highly connected with the treatments. Indeed, major nuclear import was found in samples treated with PMA, while, on the contrary, cells treated with U73122 were characterized by minor levels of the two enzymes in the nucleus (Figure 23b). These findings indicated DAG accumulation at G2/M checkpoint as the main factor for the localization of PKCα and, in turn, Cyclin B1 in the nuclei of K562 cells.

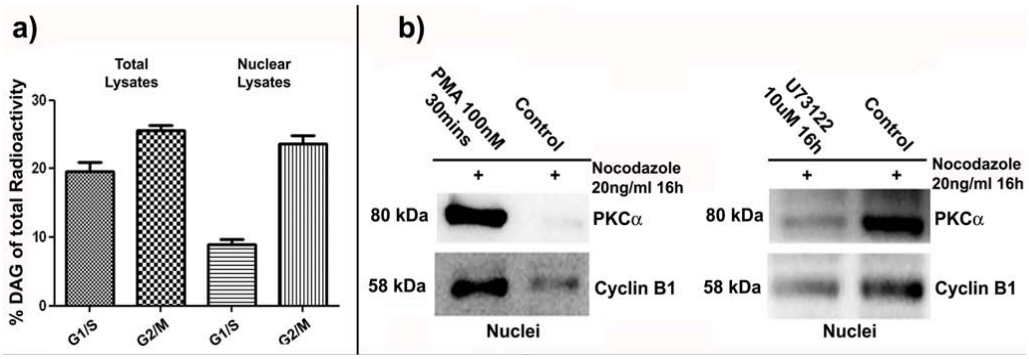

Figure 23 : DAG is responsible for nuclear accumulation of PKCα and Cyclin B1. *a) Cells were marked with [^{3}H]-glycerol and DAG extracted from total or nuclear lysates. Evaluation of the % of radioactivity representing DAG was performed. b) Cells were treated with PMA (30 minutes at 100nM) or U73122 (16 hours at 10μM) and synchronized at G2/M. Nuclear lysates were analyzed via Western Blotting.*

3.2.8 Nuclear PLCβ1 activity is fundamental for DAG production along cell cycle progression: this effect leads to modulation in PKCα and Cyclin B1 nuclear import at G2/M

Through the years, several studies indicated the existence of PIP_2 related nuclear signalling, which leads to production of DAG in the nuclear compartment. Our findings showed that nuclear production of DAG raised along cell cycle progression, in particular at G2/M. As PIP_2 hydrolysis is mediated by PLC isozymes [57], we decided to investigate which isoform could be responsible of this mechanism in K562 cells. Cells were synchronized in the different cell cycle phases, as previously described, and nuclei were separated from cytoplasms (G0/G1, G1/S and G2/M). Next, a screening of the localization of different PLC isoforms via Western Blotting was performed, indicating PLCβ1 as the only one mainly present in the nuclear compartment (Figure 24a). These data were confirmed via immunocytochemistry (Figure 24b). Then, we transiently silenced this PLC isozyme and we synchronized the cells at G2/M. Nuclear and cytoplasmic lysates were analyzed via Western Blotting showing minor PKCα and Cyclin B1 accumulation in cells characterized by PLCβ1 knock-down compared to the controls transfected with a Scrambled siRNA (PLCβ1 KD and Scrambled) (Figure 24c). Finally, in order to further confirm that PLCβ1 was really responsible of this effect on PKCα and Cyclin B1, we controlled if its silencing could lead to inhibition of DAG and

IP_3 production. Then, using a PLC assay previously described [47], we analyzed the production of IP_3 in nuclear extracts of cells synchronized at G2/M. Accordingly with our previous findings, we found a strong decrease of IP_3 production in nuclei of PLCβ1 knock-down samples compared with the controls (Figure 24d). All together, these data confirmed the importance of nuclear DAG production by PLCβ1 for PKCα and Cyclin B1 import into the nucleus at G2/M.

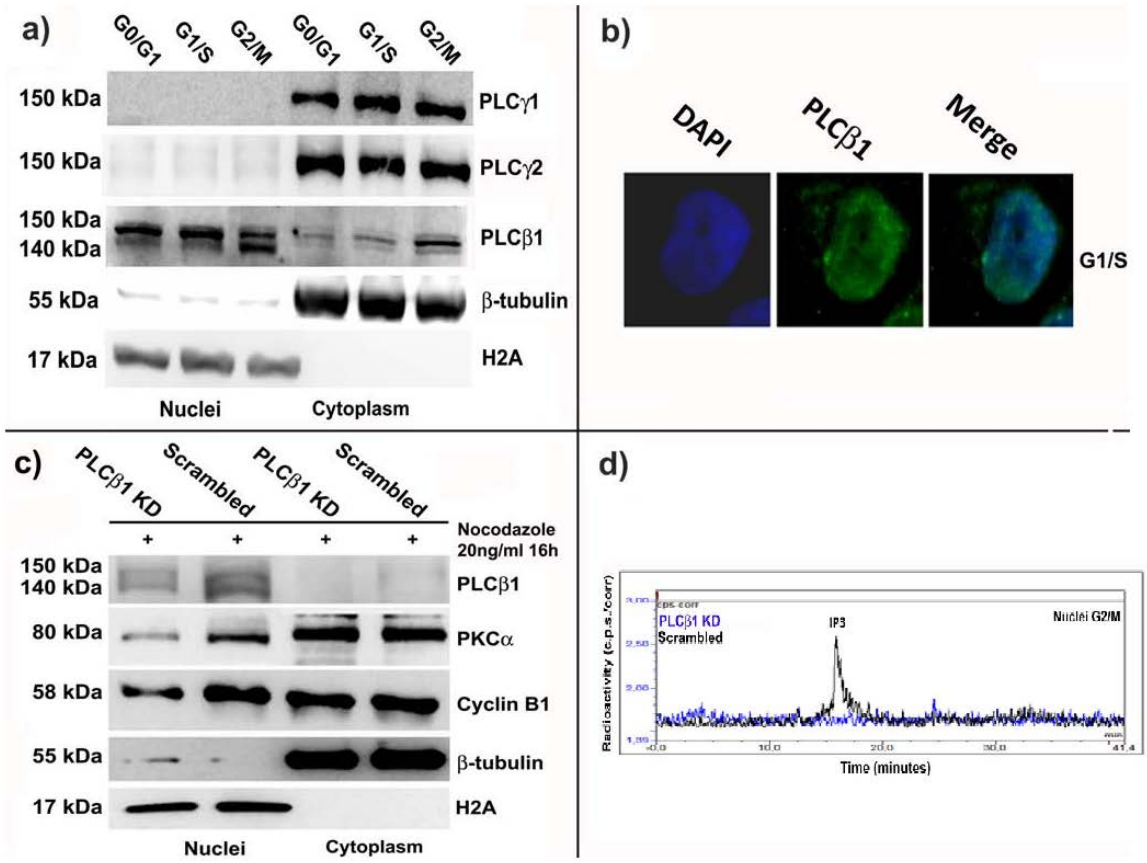

Figure 24 : PLCβ1 is the PLC isoform involved in nuclear DAG production and, then, in nuclear import of PKCα and Cyclin B1. *a) Nuclear and cytoplasmic lysates were analyzed via Western Blotting to screen the localization of different PLC isoforms. b) Immunocytochemistry was performed on G1/S cells with a PLCβ1 specific antibody. c) Cells were transfected to silence PLCβ1 and, then, synchronized at G2/M via Nocodazole. Nuclear and cytoplasmic lysates were analyzed via Western Blotting. d) PLC assay was performed in order to study the production of IP_3 mediated by PLCβ1.*

Discussion

In these three years as a PhD student I have studied several cellular responses mediated by lipid signalling, particularly focusing on the signal transduction processes related to PIP_2 hydrolysis in the nucleus. As widely reported, different pathways use lipids as second messengers, such as Phosphatidylinositol (PI) and its derived [36-38]. Indeed, the metabolism of this molecule has been found to influence many processes including cell proliferation, cell cycle progression, differentiation, apoptosis and many others. PI metabolism consists in a series of coordinated events, which lead to changes in the structure of this molecule: several kinases and phosphatases are involved in this process, adding or removing phosphate groups to/from different positions on the inositol ring. One of the main products of this network is Phosphatidylinositol (4,5)-Biphosphate, $PI(4,5)P_2$, which derives by the action of PI kinases that add two phosphates at the position 4 and 5 of the inositol ring [36-38]. $PI(4,5)P_2$ can be, then, hydrolyzed by a family of lipase, the Phospholipases C (PLC), creating two important second messenger for signal transduction: Diacylglycerol (DAG) and Inositol-(1,4,5)-Triphosphate (IP_3), which in turn triggers Ca^{2+} release from the Endoplasmic Reticulum (ER) [56] [57]. As this series of events have been recognized to take place in the cytoplasm of the cells, in the last thirty years it became clear that within the nuclei of eucaryotic cells are all the enzymes and substrates to form an inositol signalling completely independent by the cytoplasmic one [39, 41, 42]. Indeed, production of DAG has been described in several cell models as connected to the action of nuclear isoforms of PLC [47-50]. In particular, among different PLC isozymes, PLCβ1 has been widely reported as mainly located in the nuclei of different cell lines [57]. This protein is known to be expressed in eucaryotic cells in two splicing variants, PLCβ1a and PLCβ1b, which present differences in the C-terminus [74]. If PLCβ1b lacks some residues where the NES should be located and, then, is localized almost entirely in the nuclei, PLCβ1a is present both at nuclear and cytoplasmic levels [86, 87]. However, our previous studies indicated PLCβ1 as a fundamental protagonist of many cellular responses, such as cell cycle progression and cell differentiation [100, 101] [102] [103]. Analyses performed on MEL cells treated with DMSO indicated that a diminished amount of nuclear PIP_2 hydrolysis was necessary for the cells to undergo differentiation [39, 46, 93, 99]. Then, experiments of overexpression of nuclear PLCβ1 inhibited the differentiative process through an increase in the production of DAG and IP_3 into the nucleus of the cells [100, 101]. Moreover, nuclear PLCβ1 was described very important both during G1/S transition and G2/M progression of the cell cycle. Evidences collected in MEL and

Swiss 3t3 cells indicated PLCβ1 as responsible of a positive regulation of a D type Cyclin, precisely Cyclin D3, known to play a fundamental role in the G1/S checkpoint [102, 146]. In particular, only up-regulation of the nuclear enzyme was necessary for Cyclin D3 modulation and, in turn, for the strong prolongation of the S phase of the cell cycle. On the other hand, several reports indicated an increase of nuclear PLC activity at G2/M, seen as a major production of DAG in the nuclear compartment, which, in turn, could attract some conventional PKC isoforms, such as PKCα and PKCβII [103] [135]. These enzymes are Ser/Thr phosphotransferases highly dependent by the PLC signalling; indeed, production of DAG and IP_3, with the subsequent Ca^{2+} release from ER, are indispensable for their maturation and activation [104-106]. PKC have been widely indicated as cell cycle modulators and their role resulted to be highly connected to different cell models and signalling environments [104-106]. In particular, PKC are known to be able to translocate into the nucleus at G2/M where they contribute to phosphorylate Lamins, triggering the disassembly of nuclear envelope during mitosis [103] [135] [141].

Since most of all the studies on PLC/PKC signalling and cell cycle were performed on murine models, my purpose was to investigate its possible involvement in human cell lines, in terms of cell proliferation and cell cycle modulation. In order to achieve that, we used a human erythroleukemia cell line, K562, as a homologue of MEL cells. The utilization of this particular cell line took some advantages in our research. Indeed, K562 cells resulted a very good model for our purpose for some characteristics they possess: a very fast doubling time (about 24h), presence of high quantity of PLCβ1 in the nucleus and expression of only two conventional PKC isoforms, PKCα and PKCβII [143]. The first part of my work was focused on G1/S transition, while the second was concentrated on G2/M progression.

As PLCβ1 was found connected to Cyclin D3 in MEL cells, even if the mechanism was not perfectly elucidated, we firstly studied if this link was still present in our model. Then, we transiently overexpressed PLCβ1a and PLCβ1b in K562 cells, finding that both the isoforms were able to lead to an up-modulation of Cyclin D3 levels. These effects appeared to be specific because other proteins involved in cell cycle machinery were not affected at all. Next, we decided to create clones stably overexpressing PLCβ1a or PLCβ1b. Working on cells stably transfected presents strengths and

weaknesses: the most important advantage can be synthesized in having an entire population of cells transfected (about 100%) and in avoiding the transfection process, usually quite harmful for the cells; on the other hand, a continuous overexpression of a protein like PLCβ1 can influence many different pathways in time. In order to overcome this problem, clones were kept in proliferation for up two weeks. Since both the clones overexpressing the two isoforms of PLCβ1 presented an upregulation of Cyclin D3, we studied the profile of their cell cycle progression. After synchronization, performed by a 24h starvation, proliferating cells with PLCβ1 overexpressed showed a prolonged S phase of the cell cycle, probably due to the upregulation of Cyclin D3. Indeed, D type cyclins concur in the G1/S transition, through the activity of the Cdk isoforms which they complex, Cdk4/6, capable to phosphorylate pRB leading to the release of the E2F and, in turn, to G1/S transition [19]. Moreover, as indicated by experiments of cell counting, this led to a decrease of the proliferation of the clones, probably due to the prolonged S phase. Next, we decided to better elucidate this mechanism and we focused on the direct down-stream of PLC signalling, conventional PKC isoforms, represented in K562 cells by PKCα and PKCβII. Our findings indicated that PKCα was downmodulated by PLCβ1 overexpression, while PKCβII was not affected at all. This peculiar effect was probably amenable to one of the most important characteristics of PKC: indeed, if they are chronically or highly activated, these protein undergo modifications on their structure and become more easily degradable [131, 132]. In order to understand if PLCβ1 activity was really involved in the system, we overexpressed two catalytic inactive isoforms of PLCβ1 characterized by two puntiform mutations on the residues His^{331} and His^{378}, which inhibited their activity [64]. This mutagenesis process was performed and analyzed accordingly with previous studies of the catalytic domain of PLCδ1 [62, 63]. However, the data confirmed the idea that PLCβ1 overexpression was linked to PKCα decrease. Indeed, the two mutants did not affect levels of PKCα at all. Finally, we decided to act directly on PKCα. In a few words, we silenced it, in order to mimic the effects of PLCβ1 overexpression on its expression, and we found that cells characterized by PKC knock-down showed the same up-regulation of Cyclin D3 and the same slow-down in cell proliferation encountered in the clones. All together our data demonstrated the existence of a PLCβ1/PKCα mediated pathway which could affect cell proliferation through Cyclin D3 modulation.

In the second part of my three years PhD course, I focused my attention on the G2/M checkpoint of the cell cycle and the role of proteins connected with PIP_2 in this phase. In particular, I studied the possible involvement of PKC in the regulation of Cyclin B1/Cdk1, never found before. As widely reported in literature, the modulation of cell proliferation by these phosphotransferases has been showed as highly context dependent both in terms of cell models and signalling environment. Many studies were performed on different cell lines describing growth stimulatory or inhibitory effects for PKC. Through the years, regulation of Cyclins, Cdks, cell cycle inhibitors and Lamins has been showed directly connected with the activity, the localization and the expression of many PKC isoforms [55, 106, 133]. Previous reports by our laboratory indicated PKC involved in the G2/M progression through the phosphorylation of Lamins [103] [135] [141]. Indeed, nuclear translocation of PKCα in MEL cells was found to help nuclear envelope disassembly at mitosis. These findings confirmed other studies performed on AML cell lines, such as HL60 [135] [141]. Since nothing was ever found about the possible connection between PKC and Cyclin B1/Cdk1 complex, we decided to understand if in our model, K562 cells, some relationship could exist affecting cell cycle progression. First, we treated the cells with three different PKC inhibitors, specific for conventional and novel isoforms: Go6983, Go6976 and PKC inhibitor*. These compounds shared the capacity to inhibit PKC activity, acting as ATP-competitors on the ATP-binding site [144]. In particular, interesting was the ability of PKC inhibitor* to lead the enzymes to a strong decrease in terms of expression, effect not encountered with Go6983 and Go6976. As this modulation was found not to be connected with a regulation of the gene expression of PKC, it was probably due to off target effects of the compound which could interfere with PKC life cycle. Particularly, for every experiment, we decided to synchronize the cells at G2/M using Nocodazole, in order to avoid any oscillation of Cyclin B1 expression related to different cell cycle phases. Then, we found an important decrease of Cyclin B1 levels only in samples treated with PKC inhibitor*, while Go6983 and Go6976 had no effects on this enzyme. On the contrary, we hyper-stimulated PKC signalling using PMA, a phorbol-ester known as a potent activator of these enzymes [131, 132]. Cells blocked at G2/M showed a strong decrease of PKC levels, due to their hyperactivation (see PKC life cycle chapter), and a subsequent downmodulation of Cyclin B1 expression. These effects were very similar to the ones encountered inhibiting PKC with PKC inhibitor*, indicating that only the modulation of the activity of these proteins was not sufficient for Cyclin B1 regulation;

on the contrary, their physical presence in the system seemed to be necessary. However, since many inhibitors are known to have many side effects, we focused our attention on understanding if PKC were really involved in this mechanism and, in particular, which conventional isoform between PKCα and PKCβII. Experiments of silencing of these two isozymes indicated PKCα as the main responsible for Cyclin B1 modulation. Notably, analyses via FACS of the cell cycle profile of cells characterized by PKCα knock-down showed their accumulation at G2/M, probably due to difficulties in the exit from this phase for the lack of Cyclin B1, fundamental for the mitotic machinery [27]. Next, we decided to understand if the catalytic activity of PKCα was really necessary in this process. In order to achieve that, we used the same approach described for the study of the lipase activity of PLCβ1: we transfected the cells with two different vectors encoding a Wild Type and a Dominant Negative (catalytic inactive) form of PKCα finding the same up-regulation of Cyclin B1 and the same G2/M accumulation under Nocodazole stimulation. These findings further indicated that the physical presence of PKCα was more important than its activity for the process. Moreover, we studied at which level this regulation could take place. Particularly, we analyzed the gene expression of Cyclin B1 via real-time PCR after modulation of PKCα. No changes were found, indicating a possible post-transcriptional mechanism. As Cyclin B1 levels oscillate along the different phases of cell cycle degrading after G2/M exit [27, 147], we thought PKCα could be involved in this process. Then, we silenced PKCα and we treated our cells with a proteasome inhibitor, MG-132, in order to inhibit Cyclin B1 degradation. The cells were characterized by an important accumulation of this enzyme, probably due to the block of its degradation connected with the lack of PKCα. As further demonstration of that, we found an increase of the expression of p21/Cip1 in cells with PKCα knock-down. This enzyme is commonly described as an inhibitor of cell cycle progression, acting both at G1/S and G2/M checkpoints and targeting different cyclins. In particular, recent studies reported its strong accumulation and involvement in Cyclin B1 degradation after DNA damage. Here, we found that silencing PKCα led to an important decrease in Cyclin B1 and a concomitant accumulation of p21/Cip1 in the cells as well as it was described in literature [34]. All together, our findings indicated an involvement of PKCα in Cyclin B1 degradation. Next, in order to better understand the connection between these proteins, we decided to study their behavior along cell cycle progression. We set a

protocol to stimulate synchronization of K562 cells in the different cell cycle phases (see materials and methods) and we found that both the enzymes increased along cell cycle progression, peaking at G2/M. In particular, we found their high accumulation into the nucleus during mitosis. Moreover, experiments of Co-Immunoprecipitation showed PKCα and Cyclin B1 capable to interact along cell cycle in the cytoplasmic fraction. These findings supported the idea of PKCα acting as a scaffold protein for Cyclin B1 in order to avoid its degradation along cell cycle progression. Finally, we decided to shed light on the possible mechanism through which these enzymes could translocate in the nucleus at G2/M. As previously reported, both of them are characterized by the lack of a common NLS, which is usually necessary for the import of proteins through the karyopherin/importin system [31]. Then, we focused on the possible factor that could trigger this process. Through the years, many studies indicated DAG production in the nuclear compartment as the possible explanation for PKC import [47-50]. In order to study the role of DAG in K562 cells, we analyzed its production in total or nuclear lysates, finding it highly accumulated in the nucleus at G2/M checkpoint. Thus, we mimicked DAG modulations, in terms of increase or decrease, through the treatment of the cells with either PMA or U73122 (PLC inhibitor) respectively. As PMA action has already been described in this thesis, U73122 effects led to inhibition of PIP_2 hydrolysis mediated by PLC and, then, to a minor production of DAG. The evidences emerging by these experiments showed that PKCα and Cyclin B1 nuclear levels were highly connected to the treatments and, in turn, to DAG amount in the nucleus at G2/M checkpoint. Several studies in the 90's were performed to investigate different pathways involved in the production of DAG into the nucleus [47-50]. Although reports on thrombin-stimulated cells indicated DAG could derive from a phosphatidylcholine mediated pathway, others showed nuclear inositides as the main resource for this process [148]. Accordingly to this, we focused on the study of different PLC isoforms in order to identify which one was responsible for PIP_2 hydrolysis in the nucleus of K562 cells. Our findings reported that PLCβ1 was the only isoform mainly localized in the nucleus of K562 cells. In particular, the silencing of this isozyme led to minor accumulation of PKCα and Cyclin B1 in the nucleus of cells synchronized at G2/M by Nocodazole. Finally, to confirm that PLCβ1 silencing could inhibit PIP_2 hydrolysis in the nucleus, we analyzed the production of IP_3 through a PLC assay described by Martelli et al [47]. As expected, IP_3 levels in the nucleus of cells characterized by PLCβ1 knock-down were highly reduced if compared with the

controls, indicating this isoform as very important for DAG production along cell cycle and, in turn, for nuclear translocation of PKCα and Cyclin B1 at G2/M checkpoint.

All together, the findings described in this thesis indicate new roles for PLCβ1 and PKCα in the regulation of cell cycle, both at G1/S and G2/M levels. In particular, DAG production by PLCβ1 seems to be fundamental in the control of activity and location of PKCα, which, in turn, was found connected with these two cell cycle phases through the modulation of Cyclin D3 and Cyclin B1. The theorized mechanisms of cell cycle control are schematically reported in Figure 25.

The future perspectives derived by our data are many. First, the role of PKC isozymes in Cyclin B1 modulation must be further elucidated. Indeed, how this mechanism works and where/when is still not really clear. Second, the involvement of another class of proteins highly connected with nuclear DAG production, the Diacylglycerol Kinase family (DGK), could be very important for the regulation of cell cycle. These are enzymes able to catalyze the conversion of DAG in Phosphatidic Acid (PA) through the utilization of ATP as a source of phosphates [149]. Several findings reported their possible involvement in the exit from G2/M, since their activity seems to raise at the end of the mitotic process, probably to modulate nuclear DAG amount and allow the cells to divide [150]. Finally, spreading our findings on other cell models would be of high interest. As Cyclin B1 degradation has been deeply described on cervical cancer cells (Hela), it will be very important to find out if the connection between PKC and Cyclin B1 exists also in this model and, then, if it could be useful to elucidate the mechanisms of nuclear import of this protein [27, 147]. The nuclear lipid pool changes along cell cycle and can be seen as one of the main reason for the shuttle inside and outside the nuclei of many proteins characterized by absence of a common nuclear localization signal, including Cyclin B1.

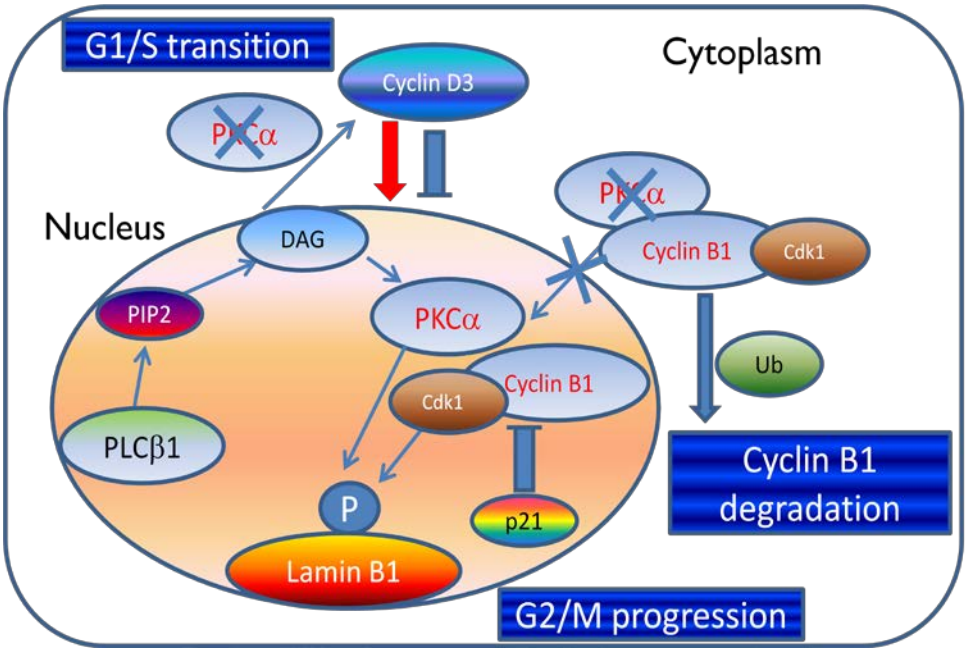

Figure 25. Schematic representation of cell cycle modulation by DAG production mediated by PLCβ1 in K562 cell line. *PLCβ1 activity is fundamental in the production of DAG in the nuclei of K562. A chronic overexpression of this enzyme leads to an increased activity of PKCα, which, in turn, undergoes faster degradation. This affects G1/S transition through the raise of Cyclin D3 expression. On the other hand, nuclear DAG production levels raise at G2/M thanks to PLCβ1 activity, modulating PKCα and Cyclin B1 nuclear accumulation. In addition, PKCα interacts with Cyclin B1 at a cytoplasmic level avoiding its fast degradation and helping its nuclear import.*

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