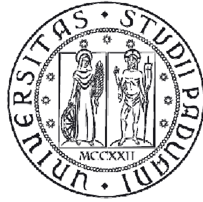

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A NEW FEATURE OF CK2 FUNCTION: ITS ROLE IN GRANULOCYTIC DIFFERENTIATION INDUCED BY RETINOIC ACID

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ABBREVIATIONS

Ab antibody

B-act β-actin

AML acute myeloid leukemia

APC allophycocyanin

[-P^{33}]ATP ATP labelled with radioactive phosphorus

ATRA all-trans retinoic acid

AV annexin V

BSA bovine serum albumin

CBFs core binding factors

CD cluster OF differentiation

CDK cyclin-dependent kinase

CDKi inhibitor of cyclin dependent kinase

CDP CCAAT displacement protein

C/EBPε CCAAT/ enhancer binding protein

CK2 protein kinase CKII

cpm counts per minute

CRABP retinoic acid binding protein

Ct threshold cycle

C-terminal carboxy terminal

DAPI 4, 6 diamidino-2phenilindole

DMSO Dimethyl sulfoxide

Dvl dishevelled

EDTA Ethylene diamine tetraacetic acid

EGTA ethylene glycol tetraacetic acid

ERK extracellular signal regulated kinase

FAB	French-American-British
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
HAT	histone acetyl-transferase
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDACs	Histone deacetylase
Hh	hedgehog
Hrp	horseradish peroxidise
HSC	hematopoietic stem cell
HSP90	heat shock protein 90
Ig	immunoglobulin
K17	4,5,6,7-tetrabromobenzimidazole
K27	4,5,6,7-tetrabromo-2-amino-benzimidazole
LSC	leukemic stem cells
M-CSF	monocyte colony stimulating factor
MEK	MAP ERK kinase
MAPK	mitogen activated protein kinase
NF-κB	nuclear factor κB
NLS	nuclear localization signal
N-terminal	amino terminal
PARP	Poly (ADP-Ribose) Polymerase
PBS	phosphate-buffered-saline
PCR	polymerase chain reaction
PerCP	peridin chlorophill protein
PI	propidium iodide
PIP_3	phosphatidylinositol 3,4,5-trisphosphate

PI3-K	Phosphatidylinositol 3-kinases
PLZF	promyelocytic leukemic zinc finger
PMA	12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
RA	retinoic acid
RARα	retinoic acid receptor α
RAREs	retinoic acid response elements
RTK	Receptor tyrosine kinases
RXR	retinoic receptor x
SDS/PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
siRNA	small interfering RNA
shRNA	short hairpin RNA
TBB	4,5,6,7-tetrabromobenzotriazole
TBS	tris buffer solution
Tm	melting temperature
TNFα	tumor necrosis factor α
TNFR	tumor necrosis factor receptor
UDG	uracil DNA glycosylase
WB	Western Blot

AMINO ACID ABBREVIATIONS

A	**Ala**	**Alanine**
C	**Cys**	**Cysteine**
D	**Asp**	**Aspartic acid**
E	**Glu**	**Glutamic acid**
F	**Phe**	**Phenylalanine**
G	**Gly**	**Glycine**
H	**His**	**Histidine**
I	**Ile**	**Isoleucine**
K	**Lys**	**Lysine**
L	**Leu**	**Leucine**
M	**Met**	**Methionine**
N	**Asn**	**Asparagine**
P	**Pro**	**Proline**
Q	**Gln**	**Glutamine**
R	**Arg**	**Arginine**
S	**Ser**	**Serine**
T	**Thr**	**Threonine**
V	**Val**	**Valine**
W	**Trp**	**Tryptophan**
Y	**Tyr**	**Tyrosine**
X	**generic amino acid**	

ABSTRACT

The Ser/Thr kinase CK2 promotes cell survival and proliferation [1]; it also modulates a number of molecules involved in cell development [2, 3, 4, 5, 6]. In this study, we aimed to study a potential novel role for CK2 in myeloid cell differentiation. We took advantage of the well-established model of acute promyelocytic leukemia cells (APL) maturation induced by retinoic acid (RA).
Both CK2 expression and activity were high in APL cell lines. RA caused a modulation of CK2 subunits expression. CK2 inhibition, with chemicals or RNA interference 1) prevented RA-induced cell cycle arrest in the G1 phase 2) blocked the phenotypical, morphological and functional differentiation of APL cells 3) impaired the transcription of RARα target genes p21 and CEBPε. Interestingly, CK2 blockade determined delocalization of RARα from the nucleus to the cytoplasm even in presence of RA. Upon CK2 inhibition, lower-molecular weight RARα specific bands appeared, which likely correspond to dephosphorylated forms of the receptor. Moreover, CK2 blockade resulted to slow down the decrease of RARα expression due to degradation triggered by RA. Experiments performed after protein synthesis inhibition showed that RARα turnover is fast and blockade of proteasome with MG132 partially restores RARα expression. Thus, other mechanisms of degradation could operate on RARα protein. Remarkably, the combined treatment with CK2 inhibitors and MG132 favoured the shift from higher forms to lower forms of RARα. CK2 blockade in the presence of RA and MG132, did not increase significantly the huge accumulation of the lighter forms of RARα already produced by retinoic acid treatment.
Taken together our results indicate that CK2 is essential in granulocytic differentiation upon RA treatment and could operate at several levels: (1) modulation of RARα transcriptional activity; (2) retention of RARα in the

nucleus; (3) phosphorylation of RARα and consequent RARα localization, stability, and control of its turnover during differentiation. Further investigation is needed to clarify the mechanism of CK2-RARα interactions; the kinase could perform its activity in a direct or most likely in an indirect way through the modulation of other kinases or phosphateses. Moreover,. it would be interesting to elucidate the possibility that CK2 could regulate proteins involved in RARα shuttling from nucleus to cytoplasm compartments, influencing also in this way its transcriptional activity.

RIASSUNTO

CK2 è una protein-chinasi ampiamente studiata e nota per la sua capacità nel promuovere la sopravvivenza e proliferazione cellulare [1]. Sulla base della dimostrata modulazione CK2-mediata di molecole coinvolte nel processo differenziativo mieloide [2, 3, 4, 6, 5, 7], l'obiettivo di questo studio sperimentale è stato quello di dimostrare un possibile ruolo, ad oggi mai esplorato, nel differenziamento granulocitico e di conseguenza anche nel blocco differenziativo che interviene nella patogenesi delle leucemie mieloidi acute. A questo scopo abbiamo scelto il modello di differenziamento granulocitico indotto da acido retinoico nelle cellule di leucemia promielocitica acuta (LPA).

Attraverso studi di espressione dell' mRNA e della proteina abbiamo dimostrato alti livelli di CK2 rispetto ai controlli normali e al contempo una spiccata attività chinasica. In presenza di stimolo differenziativo si è osservata una diversa modulazione di espressione della subunità catalitica alpha rispetto alla controparte regolatoria beta, con una riduzione precoce dei livelli di quest'ultima. L'attività chinasica si è mantenuta elevata nel corso delle 72 ore, nonostante la diminuzione della sub unità beta. Questo ci lascia presupporre che in corso di differenziamento la subunità alpha sia la maggior responsabile dell'azione chinasica.

Lo studio approfondito del differenziamento granulocitico ci ha permesso di dimostrare come l'attività di CK2 sia fondamentale per garantire la maturazione indotta dall'acido retinoico sia sul piano fenotipico, morfologico che funzionale. Tale risultato è stato ottenuto impiegando sia inibitori chimici della chinasi sia la metodica dell'RNA *interference* andando a colpire l'mRNA della subunità α.

Abbiamo escluso la possibilità che la riduzione di cellule differenzianti, a seguito del blocco della chinasi, fosse dovuto ad una maggiore propensione

delle stesse all'apoptosi; successivamente ci siamo concentrati sui possibili meccanismi adottati dalla chinasi sul differenziamento. CK2. non è risultata essere coinvolta nel processo di ricostituzione dei *PML-nuclear bodies* promossa dall'acido retinoico [8], tuttavia dall'analisi condotta sul ciclo cellulare si è potuto constatare che CK2 è determinante per garantire il blocco in fase G0/G1 mediata dai retinoidi, propedeutica al differenziamento successivo. Abbiamo inoltre dimostrato che CK2 modula l'attività trascrizionale di RARα, infatti l'inibizione della chinasi ha portato ad una riduzione significativa dell'espressione di p21 e c/EBPε, geni target di tale recettore, essenziali per il differenziamento granulocitico terminale [9,10]. CK2 influenza anche la distribuzione intracellulare di RARα, permettendo al recettore di mantenere la localizzazione nucleare. È stato interessante notare come il blocco della chinasi porti all'accumulo di forme più leggere di RARα: verosimilmente corrispondono a forme meno fosforilate del recettore che tendono ad accumularsi a livello citoplasmatico. Infine CK2 agisce sul turnover di RARα, favorendo la rapida degradazione indotta dall'acido retinoico sia della forma *wild-type* del recettore che della proteina di fusione attraverso proteasoma.

I dati ottenuti nel presente lavoro sperimentale ci consentono di concludere che CK2 svolge un ruolo essenziale e mai provato sino ad oggi nel differenziamento granulocitico indotto dall'acido retinoico agendo su più livelli, anche se l'esatto meccanismo d'azione molecolare deve ancora essere chiarito

1.INTRODUCTION

1.1 Acute myeloid leukemia.

Acute myeloid leukemia (AML) represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and overproliferation in the stem cell compartment result in accumulation of non-functional cells termed myeloblasts[11]. AML represents 30% of all acute leukemias affecting preferentially adults from 18 to 60 years; its incidence increases with age with a peak at 50 years. The symptoms of AML are caused by replacement of normal bone marrow with leukemic cells: the early signs of AML include fever, weakness and fatigue, reduction of weight and appetite, and pains in the bone or joints. Other signs include red spots in the skin, easy bleeding due to platelet loss, decreased resistance to infection linked to neutrophil loss and anemia.

AML progress rapidly and is typically fatal within weeks or months if left untreated. AML generally involve the bone marrow and the diagnosis of AML need the presence at least of 20% of blasts in this compartment; in some cases, AML cells may spread to peripheral blood and other organs, such as the liver and spleen. In the 1970s, a group of French, American, and British leukemia experts divided acute myeloid leukemias into subtypes, M0 through M7, on the basis of morphology, special stains, cytogenetics, and cell surface markers; this method was called FAB classification [12] (Table 1).

Table 1. FAB classification of AML

FAB subtype	Name	% of adult AML patients	Prognosis compared to average for AML
M0	Undifferentiated acute myeloblastic leukemia	5%	Worse
M1	Acute myeloblastic leukemia with minimal maturation	15%	Average
M2	Acute myeloblastic leukemia with maturation	25%	Better
M3	Acute promyelocytic leukemia (APL)	10%	Best
M4	Acute myelomonocytic leukemia	20%	Average
M4 eos	Acute myelomonocytic leukemia with eosinophilia	5%	Better
M5	Acute monocytic leukemia	10%	Average
M6	Acute erythroid leukemia	5%	Worse
M7	Acute megakaryoblastic leukemia	5%	Worse

The FAB classification system is useful and is still commonly used but it does not take into account many of the factors that are known to impact prognosis such as cytogenetic aberration and other genetic defects. The World Health Organization (WHO) has proposed a newer system that includes some of these factors to try to help better classify cases of AML [13]

The WHO classification system divides AML into several broad groups:

1) AML with certain genetic abnormalities

- AML with a translocation between chromosomes 8 and 21
- AML with a translocation or inversion in chromosome 16
- AML with changes in chromosome 11
- APL (M3), which usually has translocation between chromosomes 15 and 17

2) AML with multilineage dysplasia (more than one abnormal myeloid cell type is involved)

3) AML related to previous chemotherapy or radiation

4) AML not otherwise specified (includes cases of AML that do not fall into one of the above groups; similar to the FAB classification)

- Undifferentiated AML (M0)
- AML with minimal maturation (M1)
- AML with maturation (M2)
- Acute myelomonocytic leukemia (M4)
- Acute monocytic leukemia (M5)
- Acute erythroid leukemia (M6)
- Acute megakaryoblastic leukemia (M7)
- Acute basophilic leukemia
- Acute panmyelosis with fibrosis
- Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)

5) Undifferentiated or biphenotypic acute leukemias (leukemias that have both lymphocytic and myeloid features). Sometimes called ALL with myeloid markers, AML with lymphoid markers, or mixed lineage leukemias.

The evolution of the classification system in AML from morphology to cytogenetic/genetic-based reflects the recognition of the importance of subtype-specific biology. The two major prognostic factors in newly diagnosed AML, patient age and chromosome status, form the basis of important treatment decisions.

The therapeutic approach to adults aged 18-60 years classically involves separate treatment phases. The first one consists of induction chemotherapy in which the goal of myelosuppression is to empty the bone marrow of all hematopoietic elements (both benign and malignant) and to allow the repopulation of the marrow with normal cells, thereby yelding remission (<5% marrow blasts).

Cytarabine (AraC) is the cornerstone of induction therapy and consolidation therapy for AML. A standard form of induction therapy consists of AraC (100–200 mg/m^2), administered by a continuous infusion for 7 days, combined with an anthracycline, administered intravenously for 3 days. Consolidation therapy comprises treatment with additional courses of intensive chemotherapy after the patient has achieved a complete remission (CR), usually with higher doses of the same drugs that were used during the induction period. High-dose AraC (2–3 g/m^2) is now a standard consolidation therapy for patients aged <60 years. Despite substantial progress in the treatment of newly diagnosed AML, 20% to 40% of patients do not achieve remission with the standard induction chemotherapy, and 50% to 70% of first CR patients are expected to relapse within 3 years. The optimum strategy at the time of relapse, or for patients with the resistant disease, remains uncertain. Allogenic stem cell transplantation has been established as the most effective form of anti-leukemic therapy in patients with AML in first or subsequent remission[14]. However, this approach carries a high degree of initial mortality and a significant degree of long-term morbidity in the form of chronic graft-versus-host disease (GVHD). Chemotherapy-based

approaches with or without autologous stem cell rescue can be performed relatively safely, but there remains a high chance for disease recurrence [11].
There is a problem linked to the common use of agents directed toward the inhibition of proliferation: the rapid response to chemotherapy of acute leukemias indicates that these cancers are made up of mostly cells that are actively proliferating. However the malignant genetic change is also present in the "resting or G_0" stage stem cells that are not proliferating when the drug is given. This implies that the leukemia arises in the reserve hematopoietic stem cells, which can only be eliminated by total bone marrow ablation. Differentiation therapy is an alternative approach to leukemia treatment, which does not directly kill the proliferating cells but induces them to mature and die[15]. New therapies have been approved for clinical trials that include agents promoting differentiation such as azacitidine, a DNA hypomethylating agent, or using histone deacetylase inhibitors valproic acid (VPA) [16], and all trans retinoic acid. Other new therapies include small molecules that inhibit signal transduction (i.e thymidine kinase inhibition) and antibody-directed cytotoxicity (anti-CD-33 immunotoxin).

Genetic alterations in AML.

Human leukemia, like all cancers, results from multiple mutations that lead to abnormalities in the expression or function of gene products that affect the delicate balance among proliferation, differentiation, and apoptosis. Leukemias are characterized by acquisition of recurring genetic aberrations and chromosomal translocations. These last ones include loss of function mutations in transcription factors that are required for normal hematopoietic development. However they are not sufficient to cause leukemia and appear to be one hit in the multistep pathway. Recent studies indicate that activating mutations, that confer prolipherative and survival signals to the progenitors

cells, could represent "the second hit", thus cooperating with loss of function mutations in the transcription factors towards uncontrolled proliferation and impaired differentiation.

- Chromosomal translocations. They are the most common aberration in AML and in the majority of cases are balanced rearrangements. To date more than 700 recurrent non-random translocations have been identified: different studies have provided evidence that gene rearrangements correlate with specific tumor phenotypes, but at the meantime they uncover the presence of a shared molecular plateau that translocations use to transform cells. Indeed these translocations lead to the expression of fusion proteins that show a common structural and functional theme: they are usually composed of a transcriptional factor, that retains the DNA-binding motifs of the wild-type protein, and of a non correlated protein, that is able to interact with a corepressor complex, thus altering the expression of target genes necessary for myeloid development [17]
- The most prevalent fusion protein are:
  - t(15;17) PML-RARα found in 95% of acute promyelocytic leukemia, that will be discussed in more detail in the next paragraph;
  - t(6;21) AML1-ETO where AML1 is a transcription factor crucial for hematopoietic differentiation and ETO is a protein with transcriptional repressor activity;
  - Inv(16) CBFβ-MYH11 where the first aminoacid of core binding factor β are fused to the c-terminal region of a smooth muscle myosin heavy chain;
  - MLL rearrangements. MLL is implicated in leukemias of various types: acute linfoblastic leukemia (ALL), in AMLs, biphenotypic ALs, and infant leukemias. In general the prognosis is poor. MLL protein can fuse to one of more 50 partners, resulting in a MLL-fusion protein that acts as a potent oncogene [18].

- Mutations in transcription factors involved in myelopoiesis and granulopoiesis such as PU.1, GATA-1, c-EBPα [19].

Other genetic defects are activating mutations that confer proliferative and survival signals:

- Receptor tyrosine kinases which become constitutively active, such as c-KIT

  and FLT3. This last one is found in 15-42% of patients with AML and is associated with a poor prognosis. Two types of mutations results in constitutive activation of the receptor: internal tandem duplications and missense point mutations.
- Other signalling molecules:

  -RAS proteins involved in the transduction through receptor tyrosine kinases: the mechanism of activation is constituted by point mutations in the GTP binding site and occur at a frequency of 25% in AML;

  -STAT proteins in particular STAT3 and STAT5;

  -over-expression of anti-apoptotic genes of the BCL-2 family;

  - alterations in the genes that control cell cycle: loss of Rb, overexpression of cyclin D and cyclin E, increased activity of cdk kinases [20].

1.2 Acute promyelocytic leukemia (APL).

Acute promyelocytic leukemia (APL) is a form of acute myelogenous leukemia (AML), designated as AML-M3 by the French-American (FAB) classification [12]. This unique form of AML accounts for approximately 10% of all acute myelogenous leukemia cases [21]. The defining features of APL include a maturation arrest at the promyelocytic stage of myeloid development and a reciprocal chromosomal translocation involving the retinoic acid receptor gene

(RARα) on chromosome 17 with the promyelocytic leukemia gene (PML) on chromosome 15, resulting in the expression of the aberrant PML- RARα fusion protein. Although PML- RARα is the predominant chromosomal translocation present in APL cells, rare alternative chromosomal translocations are also seen, in which the PML gene is replaced by the promyelocytic zinc finger (PLZF), by the nucleophosmin (NPM) gene, the nuclear mitotic apparatus (NUMA) and by the signal transducer and activator of transcription 5b (STAT5b) gene located on chromosome 11, 5, 11, or 17, respectively [22].

Distinct translocations are associated with distinct responses to treatment. The majority of APL respond indeed to treatment with all-trans retinoic acid (ATRA), by contrast t(11;17)/PLZF-RARα and possibly t(17;17)/STAT5b- RARα APL show little o no response to treatment with ATRA and poor response also to chemotherapy [22].

Using such an exclusive ATRA-based treatment approach, cure rates of 70 –80% appear to be attainable in APL patients. Unfortunately, relapses are still seen in approximately 20–30% of such patients, underscoring the need for the development of other treatments for such patients. An alternative to ATRA is represented by arsenic trioxide (As_2O_3) that has been introduced in the treatment of relapsed or refractory APL [23]. Moreover recent studies in animal models demonstrated that association of ATRA and As_2O_3 leads to a clear synergy in inducing APL cells maturation [24].

Two groups of retinoic acid receptors have been identified to date; retinoid receptors (RARs types α, β, γ) which are activated by all *trans*-retinoic acid and 9-*cis*-retinoic acid and rexinoid receptors (RXRs types α, β, γ) which are activated by 9-*cis*-retinoic acid only [21]. Upon ligand-binding, these receptor subtypes form either heterodimers (RAR/RXR) or homodimers (RXR/RXR), and such complexes bind to retinoic acid response elements (RARE), to initiate the transcription process. RXRs are also capable of forming heterodimers with other nuclear receptors, namely thyroid hormone receptors, the vitamin D3 receptor,

and the peroxisome proliferator-activated receptor (PPAR) [21]. RARs and RXRs are expressed in a tissue specific manner during development reflecting their pleiotropic roles [25]. Gene knockout studies demonstrate that no single RAR is essential for myeloid development, showing a redundancy and overlapping in RARs function, but simultaneous disruption of both RARα and RARγ blocks granulocytic differentiation. Nevertheless, individual RARs have distinct roles in hematopoiesis [26].

Although retinoic acid (RA) generally functions by binding to RARs, RA can also signal by binding to non canonical retinoid receptors. RA was reported to be a ligand for the PPARβ/δ orphan receptor and promotes survival in cells that express high levels of fatty acid-binding protein 5 [27]. Another orphan receptor is RORβ: it is evolutionary close to RARs and is thought to be involved in the regulation of genes that control the integration of sensory input as well as circadian rhythm [27].

In the absence of ligand-binding, the RAR/RXR heterodimer acts as a transcriptional-repressor by recruiting histone deacetylases (HDACs), nuclear co-repressors (N-Cor or SMRT), and Sin 3A or Sin 3B, resulting in histone deacetylation and chromatin condensation, events that silence gene transcription.

Structural studies have established that physiological concentrations of RA result in allosteric changes in the ligand-binding domain (LBD) of the receptor. Such events lead to dissociation of the corepressor complex, recruitment of histone acetyl transferase (HAT), co-activators and chromatin de-condensation, ultimately resulting in transcriptional activation [21]. Co-activator molecules recruited in the retinoic acid receptor complexes exhibit important regulatory effects on retinoid-mediated gene transcription, as they possess the capacity to induce *trans*-activation of the AF-2 domain of the retinoid receptors [21]. Some of the identified nuclear receptor co-activators include NcoA-1/SRC-1, CBP/p300, p/CIP, and ACTR, all of which possess intrinsic histone acetyltransferase

activity [9,10]. Induction of histone acetyltransferase activities in the transcriptional complex results in histone acetylation with subsequent relaxation of the condensed chromatin that allows DNA accessibility to the basal transcription machinery in the promoters of RA-dependent genes.

In the presence of chromosomal translocations, the fusion proteins X-RARα maintain the DNA binding domains of RARα, keep the ability to heterodimerize with RXRs and to interact with ligand ATRA. However there is evidence that PML-RARα binds more tightly to the corepressor complex, most likely because PML-RARα is present as homodimers which have two strong binding sites for these corepressors. The PML-RARα bound transcriptional repressors complex requires a higher concentration of retinoic acid (RA) to be released and be replaced by the activator complex[24].

The t(15;17) chromosomal translocation results not only in the generation of the PML/RARα product but also of the RARα-PML fusion protein. The PML/RARα detectable in essentially all of the patients suffering from acute promyelocytic leukemia, while the RARα/PML transcript is detected in many, but not all, cases [28]. In vivo analysis demonstrated that X-RARα proteins are necessary but not sufficient for leukemogenesis, since full-blown leukemia is always preceded by a long latency varying from 6 months to 1 year[29]. Thus, additional genetic events are likely accumulating in order to trigger full-blown leukemia in X-RARα transgenic mice (TM). In human APL blasts, one of such events could be represented by the RARα-X fusion protein. RARα/PML TM do not develop leukemia nor alteration of the myeloid cellular compartment, however in PML-RARα/RARα-PML double TM, the RARα-PML transgene increases penetrance of leukemia, thus acting as a classical tumor modifier: APL in double TM can be seen as the outcome of two concomitant aberrant activities affecting distinct molecular pathways [22]. It has been proposed that RARα-PML confers to the system further survival/proliferative advantage; alternatively it could affect genomic stability perhaps increasing the rate of additional genetic hits and

worsening the feature of the disease [22]. Another implication of this analysis is that, if RARα-X molecules are not present, as it happens in 30% of APL patients, their function could be vicariated by additional genetic events: an example could be represented by FLT3 mutations [21].

As mentioned above, extensive studies have established the leukemogenic capacity of PML-RARα, but the mechanisms by which such effects occur remain to be fully defined; nevertheless it appears that one mechanism, by which PML-RARα transforms cells, involves dominant-negative function: indeed PML-RARα can simultaneously interfere with both PML and RARα pathways. In addition to the disruption of the functions of the normal RARα by the abnormal PML-RARα protein, the normal functions of the wild-type promyelocytic leukemia (PML) protein are also disturbed in acute promyelocytic leukemia. The PML gene exhibits tumorsuppressor characteristics and the PML protein product mediates growth inhibitory and pro-apoptotic effects. PML has a characteristic cellular localization in the, so called, PML nuclear bodies, where is complexed with other proteins, such as SUMO-1, Sp 100, Sp 140, CBP, DAXX, p53, and Rb [30] After ATRA-induced remission, the normal appearance of PML-nuclear bodies is restored [30].

PML exhibits antitumor effects, partly via regulation of Fas- and caspase – dependent apoptosis and its function is essential for the induction of apoptosis in response to tumor necrosis factor alpha (TNFα), ceramide, and interferons [31].

Expression of the PML-RARα fusion protein in hematopoietic progenitor cells renders them resistant to Fas-,TNF-, and IFN-dependent apoptosis, via the exhibition of dominant-negative effects on these PML-activities.

This raises the possibility that one step in the pathogenesis of APL may be the lack of PML-mediated apoptosis, due to the expression of the abnormal PML-RARα fusion protein [31].

Another potential mechanism by which PML-RARα may mediate leukemogenesis via inhibition of a normal function of PML, relates to the

recently described interactions of PML with components of the Jak-Stat pathway. It has been shown that the normal PML interacts with the Stat3 protein, via its B-box and carboxyl terminus region, and exhibits negative regulatory effects on its activation by inhibiting its ligand-dependent DNA binding capacity [36]. In cells expressing PML-RARα, there is a dissociation of Stat3 from PML, despite the fact that PML-RARα does not interact directly with Stat3. Such dissociation leads to enhancement of Stat3 transcriptional activity and promotes Stat3-mediated anti-apoptotic responses [32].

PML-RARα fusion protein shows another function, it influences the action of RAR-RXR heterodimers. Indeed PML-RARα keeps the ability to interact and so to sequester RXR from other heterodimers, such as RAR-RXR, ultimately resulting in a dominant negative silencing of RARE-dependent gene transcription [33].

How ATRA or arsenic trioxide treatments work in promoting cell differentiation and APL remission?

It is well established that these two specific APL therapies have a common property: they both induce PML-RARα catabolism. Induction of the catabolism by RA was demonstrated long after the identification of the therapeutic effect of RA on the disease. Degradation of the fusion protein is clearly responsible for RA-induced re-localization of PML, which could then promote apoptosis induction. Some evidence suggests that degradation of the fusion protein occurs on DNA and that NB-associates proteins are secondarily released to reform NBs [8].

A first mechanistical analysis demonstrated that caspase activation was responsible for cleavage of the fusion protein upon RA exposure [24]**.** The specific cleavage site was mapped to the c-terminus of PML. Since several caspases become activated during hematopoietic cell differentiation independently of cell death induction, this cleavage reflects an ongoing differentiation and is not observed in RA-resistant cell lines. Therefore caspase cleavage of the fusion is

predicted to occur only in a subset of APL and could account for the different behaviours proteins, either in clinical outcome or RA response [24].

A second pathway of PML-RARα degradation was through ubiquitin/proteasome system upon RA activation. The DNA-bound fusion and wild type heterodimer RAR/RXR appears to be degraded in an AF-2 dependent manner, where AF-2 is the c-terminal helix of RAR receptor that controls the interaction with coregulators.

The exact role of proteolitic activity of the proteasome in RARα mediated transcription is not clear. A first hypothesys considers degradation as a way to limit gene activation, as a classical negative feedback. Alternatively protein catabolism could be required for transcriptional activation. This is in accord to the new concept that dynamic exchanges and coregulators are required for transcription to proceed, proteolysis might serve to clear out corepressors and/or activators so that other coregulators can subsequently bind [34].

Arsenic trioxide treatment modulates the microspeckled pattern of PML distribution within the nucleus of APL cells and induces rapid formation of PML NBs. As RA arsenic trioxide targets PML- RARα for degradation, however in contrast to RA, which targets the RARα AF-2 domain of the fusion protein, arsenic targets its PML moiety [35]. Arsenic induces covalent modification of the PML protein by the ubiquitin-related peptides SUMO. Sumoilation is required for the targeting of NB-associated protein onto NBs. Moreover sumolation on a specific lysine residue is required for both PML and PML/ RARα degradation [36].

Since unliganded RARα is a repressor and PML/ RARα is a more potent repressor than RARα, degradation of PML/ RARα fusion protein is most likely to have major effects on transcription and differentiation. Thus treatment of APL cells exerts two effects: derepression through degradation triggered both by RA and arsenic, and direct transcriptional activation linked exclusively to RA exposure. [24]

1.3. Retinoic acid receptors structure and regulation.

Nuclear retinoic acid receptors (RARs) consists of three subtypes: α, β, γ encoded by separate genes. For each subtype, there are at least two isoforms, which are generated by differential promoter usage and alternative splicing and differ only in their N-terminal regions [37].

As with most nuclear receptors, such as oestrogen (ER) and androgen receptors (AR), progesterone (PR) and glucocorticoid receptor (GR), RARs exhibit a modular structure composed of six regions of homology (called A to F regions, from the N-terminal to the C-terminal end) each one showing specific functions (Fig.1).

The DNA binding domain (DBD) and ligand binding domain (LBD) are the most conserved and important domains and control the classical model of RARs transcriptional activity.

- The DBD domain.

  This region includes several conserved sequences that define or contribute to the response element's specificity, to a dimerization interface within the DBDs and to contacts with the DNA backbone. RARs bind with RXRs to specific sequences or RA response elements (RAREs) located in the regulatory regions of target genes. The classical RARE is a 5bp-spaced direct repeat, called DR5. However, the heterodimers also bind to repeats separated by 1bp (DR1) or 2bp (DR2). RXR homodimers also bind to DR1.

- The LBD domain

  It is a functionally complex region as it contains:

  - the ligand binding pocket: the shape of LBP matches the volume of the ligand, maximizing the hydrophobic contacts and contributing to the selectivity of ligand binding;

- the heterodimerization surface: constitute the core of the dimer interface for RARa/RXR interaction;

-the c-terminal helix 12, named AF2: it controls the ability of RARs to interact with coregulators.

- The N-terminal AF-1 domain (NTD)

  It is composed of A and B regions and includes the activation function AF-1 and is implicated in the control of transcription of RA target genes. The A region differs between the various bubtypes and between isoforms, in contrast the B region is rather conserved and contains phosphorylation sites. In contrast to the DBD and the LBD, NTD are of naturally disordered structure, which provides the flexibility that is needed for modification by enzymes such as kinases and ubiquitin ligases. Such modification may induce changes in the structural properties of the domain with profound impacts on its interactions with coregulators and/or the dynamics of adjacent structural domains.

- The D region

  It is poorly conserved and serve as a hinge between the BDB and the LBD, allowing rotation of the DBD.

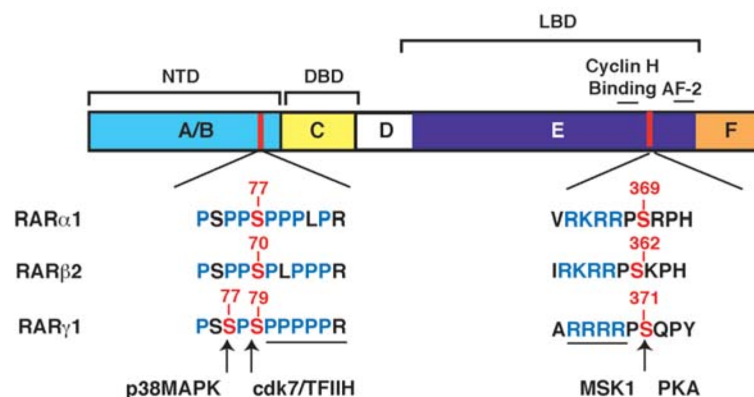

Fig.1 Schematic representation of the RAR proteins with the functional domains and the main phosphorylation sites [37].

- The F region

This region extends C-terminal to helix12 in RARs, but is absent in RXRs. It is highly variable in length and sequence among the different RARs subtypes. Interestingly is phosphorylated at multiple position that might modify the properties of RARs. According to recent studies, this region would be also capable of binding to specific mRNA motifs. In some recent studies it was demonstrated that RARα is exported to neural dendrites, where it is associated with a subset of mRNAs such as the glutamate receptor1 mRNA. This binding, which is mediated by the C-terminal F region of RARα, represses the translation of this mRNA. RA binding to RARα, reduces its association with GluR1 mRNA and relieves translational repression. Such effect has been correlated to synaptic function and plasticity [38].

Regulation of RARs receptors appears quite complex and it is the result of well orchestrated events that act in concert: heterodimerization, ligand-dependent ubiquitination, phosphorylation and other posttranslational modification such as methylation and SUMOylation could crosstalk triggering proteasome–mediated receptor degradation, thus controlling the magnitude of retinoic effect, regulating the affinity to DNA binding, coactivators recruitment, intracellular localization[39].

Phosphorylation appears to be the key event for RARs modulation. According to some studies, RARs receptors showed several sites of phosphorylation; depending on the region in which this event occurs, phosphoryation could exert a different effect (Fig.2).

In the absence of RA, RAR target genes have also been shown to interact with other kinds of repressors such as topoisomeraseIIβ, policomb group proteins (PcG) or calmoduline kinase IIγ (CaMKIIγ). PcG proteins mediate gene

silencing, while CaMKIIγ phosphorylates RARs, thereby enhancing their interaction with corepressors.

Several exogenous signals, different from the common ligand RA, such as growth factors, insulin, stress or cytokines activate cytosolic kinase cascade pathways, ending at AKT, PKC or c-Jun N-terminal kinases (JNKs), which can enter the nucleus and phosphorylate RARs at different residues. As an example PKC can phosphorylate the DBD of RARs, resulting in abrogation of RARα heterodimerization and binding to DNA or favouring nuclear export [40]. In this last case it is already known that phosphorylation on specific target sequences (nuclear localization sequences or nuclear export sequences) can favour or inhibit the interaction of proteins with the importins or exportins receptors [41]. In response to RA it was demonstrated that RARα becomes rapidly phosphorylated at two serine residues, one located in the LBD (ser369) and the other one in the NTD (ser77). Ser 369 is located in a consensus phosphorylation motif recognized by protein kinase PKA and MSK1. In contrast ser77 has been identified as target of cdk7. Both these residues are conserved between RARs. A recent work of Bruck and collegues showed that RA-induced phosphorylation of RARα results from a coordinated phosphorylation cascade starting with ser 369 and ending with ser 77 and this is essential for RARα recruitment to target promoters. Such result corroborate the importance of NTD phosphorylation in the transcriptional activity of RARα. In contrast in the case of RARγ, the promoters are already occupied by the receptor in absence of ligand: in this situation phosphorylation of the NTD might modulate transcription through controlling protein-protein interaction [42].

Lefebvre et al. demonstrated also the importance of phosphatases in regulating the phosphorylation state of RAR receptors; in particular inhibition of the Ser-Thr phosphatases PP1 and PP2A through okadaic acid or calyculin-A increased DNA binding capacity and transcriptional activity of RARs receptors [43], favouring granulocytic differentiation induced by ATRA.

Another aspect is that in response to RA, RARs as well as their coactivators, are ubiquitinated and degraded by the 26S proteasome which consists of the 20S proteolitic core capped by the 19S regulatory complex that recognizes the ubiquitinated proteins and prepares them for the entry into 20S core (Fig.3). [44]. The degradation of RARα depends on RA-induced recruitment at the AF-2 domain of the ubiquitin. In the particular case of the RARγ subtype, degradation and ubiquitination depends on the prior phosphorylation on the NTD. Srinivas and collegues demonstrated that RARα can be phosphorylated by JNK kinase, activated by stress signals, and this new phosphorylation state enhances degradation of the receptor through proteasome in a RA-independent manner [45] .

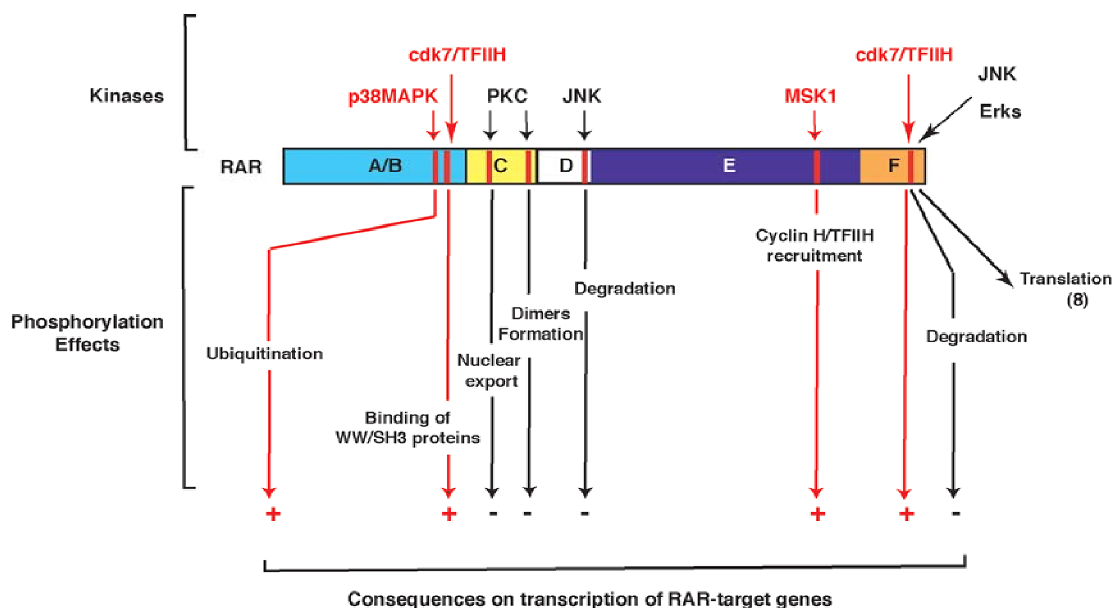

Fig.2 The different signaling pathways involved in RAR phosphorylation.

The pathways that are induced by RA are in red. The consequences (positive or negative) of RAR phosphorylation on RA target genes transcription are also indicated [37].

However there is an increasing evidence that the ubiquitin-proteasome system would also play a role in the control of RA-mediated transcription without proteolysis. Indeed SUG-1, one of the six ATPases of the 19S, interacts with

RARs and SRC-3 and is recruited to the promoters of RA target genes, contributing to their transcription. It has been proposed that the ATPase subunit of the 19S regulatory subcomplex would unfold or refold the components of the regulatory transcriptional complexes in order to facilitate their loading and/or removal at promoters [46].

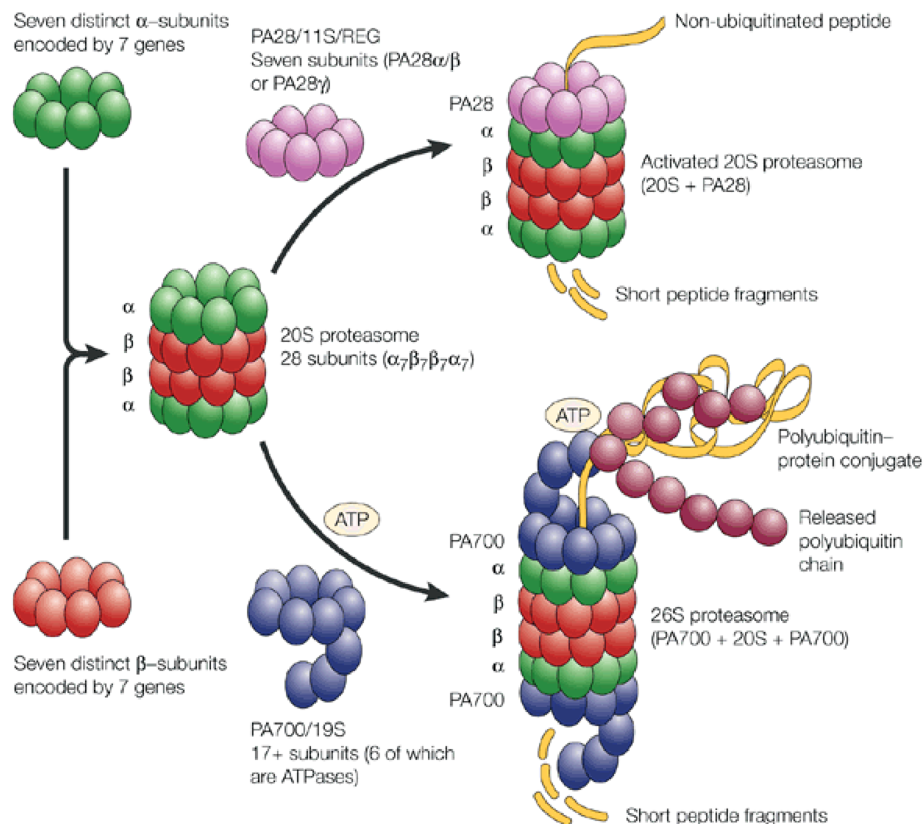

Fig.3 Proteasome structure. The 26S proteasome is a multicatalytic protease. It consists of a 28-subunit catalytic core — 20S proteasome— which is an assembly of two outer and two inner heptameric rings that form a hollow cylindrical structure in which proteolysis occurs. Each of the two inner rings of the 20S proteasome host the three different catalytic sites on the inner surface of the 20S proteasome complex, preventing the indiscriminate degradation of intracellular proteins. These proteolytically active sites mediate the hydrolysis of proteins at the carboxyl terminus of hydrophobic, basic and acidic residues, and are referred to, respectively, as the chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolytic activities. The β -subunits of 20S proteasome might be constitutively or inducibly expressed, and assembled in a selective manner to form complexes with distinct proteolytic characteristics. The outer rings comprise seven different α-subunits, none of which has catalytic activity, but which serve as an anchor for the multisubunit ATPase-containing PA700 (19S) regulator that binds to form a

complex referred to as the 26S proteasome. Linking of the 19S regulatory complex serves two functions: it opens the channel through the 20S proteasome, which is normally gated by the amino termini of the α-subunits, and it unfolds ubiquitinated proteins to allow entry to the catalytic core; both processes require ATP. The PA28 (11S) regulatory (REG) complex can also bind to the 20S proteasome and open the channel through the complex, but this process is ATP-independent, and mediates the degradation of non-ubiquitinated short peptides [47].

In order to complete the vision we have to consider also the role of the heterodimerization partners RXRs in the control of RARs susceptibility to degradation. For exemple ligand dependent RARγ2 degradation is strictly linked to its heterodimerization with RXRα and on phosphorylation of its B region: liganded RXRα may cause an allosteric transformation in its partner, which would result in an increase in RARγ2 ubiquitination. This last event is dependent on the recognition of phosphorylated ser66 and 68 by the ubiquitin machinery, thus facilitating degradation. In contrast to what was observed for RARγ2, requirements for RA-induced degradation of RARα appears to be different: phosphorylation of RARa1 in the A/B region prevents its degradation. Interestingly, heterodimerization with RXRα increased the degradation of phosphorylated RARα1 [39].
In conclusion these findings suggest a dynamic model with rapid and sequential exchanges between RARs and coregulators at the promoter. Such exchanges, carefully coordinated by phosphorylations, would act as a transcriptional time clock so that the correct proteins are present with the right activity, at the right place and at the right moment [37].

1.4. Myelopoiesis.

The coordinated production of all blood cells from a common stem cell is a highly regulated process involving subsequent stages of commitment and

differentiation. The myelopoietic system includes the hematopoietic cells derived from a common hematopoietic stem cell and includes erythroid, granulocytic, monocytic and megakaryocytic lineages (Fig.4). The development of distinct lineages is controlled by a myriad of transcription factors which regulate the expression of essential genes, including those encoding growth factors and their receptors, enzymes, adhesion molecules, and transcription factors themselves. A recent model for induction of hematopoietic differentiation hypothesizes that these factors are expressed at low levels in CD34+ stem cells, but under direction of signals such as the influence of stromal interactions or growth factor signalling, specific transcription factors are upregulated or downregulated, initiating the cascade of maturation.

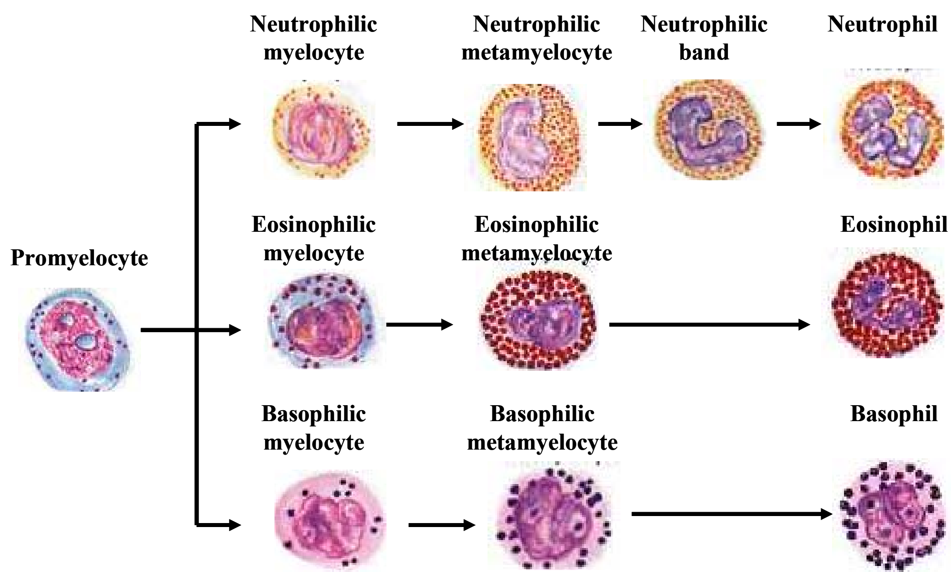

Fig.4. Schematic representation of granulopoiesis.

Various growth factors are responsible for myelopoiesis, the most important ones are granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) which exert their action through tyrosin kinase receptors,

followed by the activation of cell signalling pathways involved in cell survival and proliferation such as RAS-MAPK, PI3K/AKT, Jak/Stat pathways.

A number of transcription factors have been identified that play a role in the myeloid lineages. Their central role has been studied by gene inactivation studies, promoter analysis and ectopic expression of lineage restricted factors. Rather than being controlled by single master regulators, lineage-specific gene expression appears to depend on the combination of factors in overlapping expression domain.

- **C-Myb** is a 75-kDa transcription factor. It binds to consensus sequences and activates transcription via interaction with co-activators proteins, in particular CBP/p300, which provides a bridge between c-Myb and the transcription machinery. In addition, they show histone acetyltransferase activity, influencing in this way chromatin status at promoter level. Studies in hematopoietic cell lines demonstrated that c-Myb expression is above all confined in progenitors cells, with expression being down-regulated as differentiation goes on. Mice that lack c-Myb die embrionically with failures of erythroid and myeloid development. However, this transcription factor plays a role also in non-hematopietic cells. While c-Myb is generally not expressed in terminally differentiated cells, it can be up-regulated in mature B and T cells upon activation. Although multilineage defects can be observed in c-Myb deficient embryos, definitive hematopoietic stem cells (HSC) are generated in the absence of c-Myb. The c-Myb-/- fetal livers does contain some cells with a hematopoietic progenitor phenotype, even if they are reduced in number. Thus, these studies point out that the failure of erythroid and myeloid development in c-Myb-/- embryos is not due to a lack of HSCs, but rather the consequence of a defect in expansion and /or maturation of these cells [48]**.** While primitive erythrocytes do not express c-Myb, expression is detected at the onset of definitive erythropoiesis. Moreover,

this factor is highly expressed in primitive T cells, when they are double negative and then double positive for CD4 and CD8 markers; instead c-Myb is reduced in single positive T cells [49]. Various factors including NF-kB, WT-1 and Ets-1 have been suggested to regulate c-Myb transcription. Posttranslational modifications that generally inhibit c-Myb activity include phosphorylation, ubiquitination and sumoylation. For exemple, p42MAPK and casein kinase II can phosphorylate c-Myb [50].

- **CBFs (core binding factors)**, such as CBFα2 or AML1 are critical for the early commitment of hematopoietic stem cells (HSC) towards limphoid or myeloid progenitors. CBF (-/-) mice die in utero and lack all the components of the hematopoietic system.
- **PU.1** is a member of the Ets transcription family. It binds as a monomer to the consensus DNA site via its C-terminal domain an activates transcription via its N-terminal glutamine-rich and acid domains. PU.1 is expressed in B-lymphoid, early T-lymphoid, granulocytic and monocytic cells. Lymphoid-myeloid progenitors (LMP) express higher levels of PU.1 than megakaryocyte-erythroid progenitors (MEP). PU.1 (-/-) mice lack B cells and monocyte and have greatly reduced neutrophils. Expression of low levels of PU.1 (-/-) cells induces granulopoiesis, whereas high levels induce monopoiesis. Thus PU.1 favour monopoiesis over granulopoiesis. Moreover increased expression of PU.1 favours myeloid over lymphoid development. It binds and activates its own promoter, potentially to variable degrees in different lineages and developmental stages dependent on cooperating factors. PU.1 interacts with other transcriptional regulators such as C/EBPδ, c-Jun, RB, CBP, C/EBPα. PU.1 in myeloid cells directly binds and represses *trans*-activation of GATA-1 to downmodulate erythroid program; in turn, GATA-1 inhibits PU.1 activity in cooperation with Rb to ensure continued maturation of erythroid and megakaryocytic cells. PU.1 is

phosphorylated *in vitro* by protein kinase CK2 and JNK kinase, but not by ERK1 (MAP) kinase. Phosphorylation of PU.1 within its central PEST domain on serine 148 allows binding with interferon regulatory factor 4 (IRF4) or (IRF8) and this interaction regulates genes for monocyte lineage commitment [51]. PU.1 regulates also CSF receptor, IL-1β, the macrophage scavenger receptor, the human high affinity IgG receptor, CD 11b, the β subunit of of the IL-3, IL-5 receptors [52].

- **C/EBPs** To date at least 6 distinct members of of the C/EBP family of DNA binding proteins have been identified. These include C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε and C/EBPζ (also known as CHOP or GADD153). All members of this family contain a highly conserved carboxy terminal (bZIP) consisting of a basic region involved in DNA recognition and an adiacent helical structure, the leucine zipper, which mediates subunit dimerization. C/EBPs are often expressed in a cell type-restricted manner and have been shown to be important in regulation of differentiation. C/EBPα and C/EBPε in particular, play an important role during myeloid differentiation. C/EBPs either homodimerize or heterodimerize with other proteins, such as additional C/EBP members or activating transcription factor (ATF), and modulate the expression of various target genes. C/EBPs can interact with a number of transcription factors including RB, jun/fos zipper family and NFKB; in particular C/EBPα and C/EBPβ cooperate with NF-kB p50 to induce bcl-2 transcription and inhibit apoptosis; moreover these complexes interact to induce genes required for monocyte development. Also C/EBPε can bind NFkB p65 favouring transcriptional induction. C/EBPα is most abundantly expressed in adipose tissue, placenta, liver and is also detected in a variety of other organs such as lung, kidney, small intestine, and brain. Within hematopoiesis C/EBPα expression predominates in immature cells and is detected in HSC, CMP, GMP, but

not the common lymphoid (CLP) or MEP progenitor population. C/EBPα (-/-) neonatal mice lack neutrophils and eosinophils; although they retain monocytes in their peripheral blood [53]. C/EBPα controls M-CSF and G-CSF expression, it regulates lineage specific genes such as myeloperoxidase and neutrophil elastase, it promotes the transcription of PU.1 and C/EBPε. It also binds and and activates its own promoter, reflecting a feed-forward mechanism to fix commitment decisions. In some circumstances C/EBPβ can compensate for loss of C/EBPα during myelopoiesis. While C/EBPβ stimulates the proliferation of myeloid cells, C/EBPα potently inhibits G1 to S cell cycle progression via several mechanisms including direct binding of E2F1 or cyclin-dependent kinase cdk2/cdk4 [9]. C/EBPε is found in later-stage of maturation; it is induced by retinoic acid and is a key regulator of terminal differentiation of granulocytes [10].. CHOP is a small nuclear protein that is expressed ubiquitously in many cell types at low levels but it is markedly induced by a variety of stressors. CHOP was initially determined as a negative inhibitor of C/EBPs, indeed it interacts with them and prevents their ability to bind DNA. However, later studies showed that under certain circumstances, CHOP/C/EBPs heterodimers are capable of recognizing novel DNA target sequences and, hence, of activating gene transcription. Gery and collegues demonstrated that CHOP is upregulated after ATRA treatment: this induction of expression is specific to myeloid cells undergoing granulocytic differentiation and they showed that in this context CHOP also heterodimerizes with C/EBPε, decreasing the activity of this transcription factor. In presence of ATRA, CHOP may promote granulocytic maturation by *trans*-activation of a unique set of target genes yet to be identified [10].

- **AP-1s** are a subfamily of bZIP transcription factors. Within this family, Jun proteins heterodimerize with c-Fos, FosB, Fra1 and Fra2 to bind

DNA at specific consensus sites. C-Jun, JunB and JunD levels increase during monocytic maturation. C-Jun enhances the ability of PU.1 to *trans*-activate the M-CSF receptor promoter. Moreover c-Jun or c-Fos interacts with and prevents C/EBP DNA binding. Other transcription factors such as MafB can zipper with c-Fos; MafB seems to play an important role in the late stages of monocyte lineage development [51].

- **Retinoic acid and vitamin D receptors**. RARs are widely expressed, with RARα preferentially found in myeloid cells. Dominant inhibition of RARα arrest granulopoiesis at the promyelocyte stage. RARα regulates C/EBPε expression and also activates the CD18 promoter in maturing myeloid cells. Retinoic acid increases the formation of CFU-G at the expense of CFU-M from marrow progenitors, on the contrary vitamin D, which activates the related vitamin D receptor (VDR)-RXR cpmplex, icreases CFU-M [51].
- **Gfi-1, Egr-1/2, HoxA10.** Gfi-1 is expressed in lymphoid and granulocytic cells but not in the monocyte lineage. Gfi (-/-) mice display defects in neutrophil maturation. It represses PU.1 transcription inhibiting monopoiesis. Analysis of genes induced by high levels of PU.1 identified Egr proteins and Nab-2 as candidates for repressing granulopoiesis, while at the same time contributing to monopoiesis. Egr/Nab-2 represses the Gfi-1 promoter via an Egr binding site. The same study found that Gfi-1 also represses Egr-2 promoter. HoxA10 is preferentially expressed in immature myeloid cells within hematopoiesis and exogenous HoxA10 stimulates monopoiesis while blocking terminal granulopoiesis [51].
- **Histone deacetylase 1 and 2 (HDAC1-2).** Histone deacetylases catalyse the removal of acetyl groups from histone tails and are therefore considered as transcriptional corepressors. HDAC1 and 2 have the ability to homo and heterodimerise; they are unable to bind to DNA by themselves, but need the recruitment of other transcription factors such

as SP1/SP3 [54]. Hematopoietic transcription factors, such as GATA-1, c-Myb or PU.1 described above, operate as transcriptional activators or repressors by recruiting histone acetyltransferases (HATs) and HDACs and controlling gene expression switches crucial for cell commitment. HATs and HDACs do not restrict their action to the modification of histones, but they can also acetylate or de-acetylate transcription factors, changing their DNA binding affinity [55]. For instance, HDAC2 has a positive influence on the expression of Pax5, EBF-1 and IKAROS transcription factors involved in hematopoiesis. This last one is important in early hematopoiesis, regulates hematopoietic stem cells activity and allow differentiation towards the limphoid lineage, restricting on the other hand myeloid fates. When it is incorporated into SWI/SNF complex, which comprises also HDAC1/HDAC2, Ikaros shows a negative transcriptional regulation on target genes [56].

Model of myeloid lineage development.

Under the influence of GATA-binding protein 1 (GATA-1), the hematopoietic stem cell (HSC) differentiates into the megakaryocyte-erythroid progenitor (MEP). PU.1 directs the HSC to develop into a lymphoid-myeloid progenitor (LMP), which then further commits to the common lymphoid progenitor (CLP) branch or, directed by CCAAT/enhancer-binding protein alpha (C/EBPα), to the granulocytemonocyte progenitor (GMP) branch. Cross-inhibition between GATA-1 and PU.1 serves to maintain HSC developmental fates, and similar cross-inhibition between C/EBPα and lymphoid regulators such as Pax5 and Notch may maintain CLP developmental decisions. Increased PU.1 activity directs the GMP to further commit to the monocytic lineage. C/EBPα induction of PU.1 transcription helps elevate PU.1 activity as does PU.1 interaction with c-Jun. AP-1 and NF-kB proteins may also have a role at this stage of

hematopoiesis via direct interaction with C/EBPα, either contributing to PU.1 induction or activating a parallel pathway. C/EBPα and PU.1, at reduced levels, are also required for granulopoiesis, but another factor, perhaps a protein regulated by Id1, may be essential for commitment to the granulocyte lineage. VDR, IRF-8, MafB, Egr-1 and Egr-2 direct monocytic maturation, and RARs and C/EBPε act similarly during terminal granulocytic development. Loss of CDP- and HoxA10-repressive activities and onset of Gfi-1-mediated repression contributes granulopoiesis. Cross-inhibition between Gfi-1 and Egr-1,2/Nab2 co-repressor complexes maintains GMP developmental decisions (Fig 5) [51].

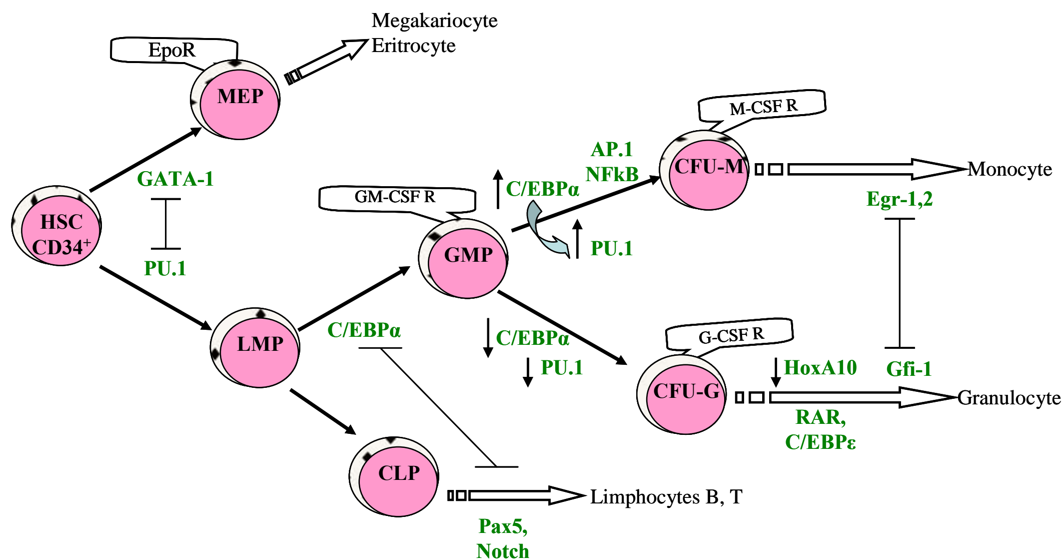

Fig.5 A model for the transcriptional regulation of granulocyte and monocyte lineage commitment and maturation. Modified image from AD Friedman, Oncogene 2007 [51].

1.5.Retinoic acid signalling in myelopoiesis.

Vitamin A (retinol) is a fat-soluble vitamin essential for the formation and maintenance of many body tissues, such as skin, bone, vasculature as well as the promotion of good vision and immune function. Vitamin A also plays a role in embryonic growth and development. Vitamin A is converted to more active compounds such as retinoic acid, through which it exerts its multiple effects on embryonic development and organogenesis, tissue homeostasis, differentiation

and apoptosis. Deficiency of vitamin A leads to blindness, infectious diseases and retard growth. Conversely, excess dietary vitamin A can result in toxicity to the liver, central nervous system, musculo-skeletal system, internal organs and skin, and can reduce mineral bone density [57]. **(**

Retinoid metabolism and transport

There are over 4000 natural and synthetic molecules structurally and/or functionally related to vitamin A. This vitamin can not be synthesized by any animal species and is only obtained through diet in the form of retinol, retinyl ester, or β-carotene.

Retinoids are comprised of three units: a bulky hydrophobic region, a linker unit, and a polar terminus, which is usually carboxylic acid (Fig.6).

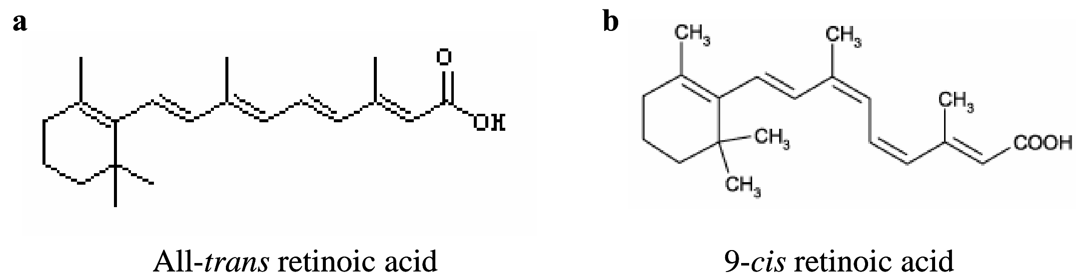

Fig.6. Two biologically-active isoforms of retinol. All-trans (a) and 9-cis retinoic acid (b), with all-trans being the predominant physiological form.

There are various types of retinoid-binding proteins, which locate in intracellular and extracellular compartments and associate with isomeric forms of retinoids. These binding proteins, along with nuclear receptors, mediate the action of retinoids, but at the same time solubilise and stabilize retinoids in aqueous spaces. For exemple retinol circulates in blood bound to serum retinol-binding protein (RBP). Inside the cell, retinol and retinal are associated with

cellular retinol binding proteins (CRBP), while all-trans RA intracellulary binds to cellular retinoic acid binding proteins (CRABP**)** [58]**.**

Retinoids absorbed from food are converted to retinol and bound to CRBP in the intestine. Then retinol is converted to retinyl esters by retinol acetyltransferase and enters into blood circulation. The liver uptakes retinyl esters, which are converted to retinol-RBP complex in the hepatocytes. The main storage site of vitamin A is the liver in particular the vitamin is accumulated in stellate cells. Retinyl esters are the intracellular storage forms of retinol. Liver is also the main site of synthesis of RBP, although other tissues, including adipose tissue, kidney, lung, heart, skeletal muscle, spleen, eye and testis, can express this protein. Secretion of RBP from the liver is regulated by the availability of retinol: in the presence of retinol, RBP associates with it, moves to the Golgi apparatus and is secreted into the blood. In the serum retinol-RBP complex is bound to transthyretin; this last one is a small protein, which in addition to associating with RBP, functions as a carrier for thyroid hormones. The constitution of this complex prevents the loss of transthyretin by filtration in the renal glomeruli. Retinol is then delivered to target tissues. The uptake of retinol by cells is mediated by a trans- membrane protein named "stimulated by retinoic acid 6" (STRA6), which is a RBP receptor. In the target cells, RA either binds to CRABP or is oxidized to retinaldehyde by retinol dehydrogenase in a reversible reaction. Then retinaldehyde can be oxidizes by retinaldehyde dehydrogenase to RA. Retinoic acid either binds to CRABP or enters the nucleus and binds to nuclear receptors. Catabolism of RA occurs principally in hepatocytes where it is oxidized by P450 enzymes (mainly CYP26) (Fig.7). Hepatocytes not only process retinoids but also are the target cells [58]**.** The intracellular concentrations of various retinoids are controlled by the activities of several metabolic enzymes and importantly the levels of these enzymes vary greatly among different types of cells and at different stages of cell maturation [27].

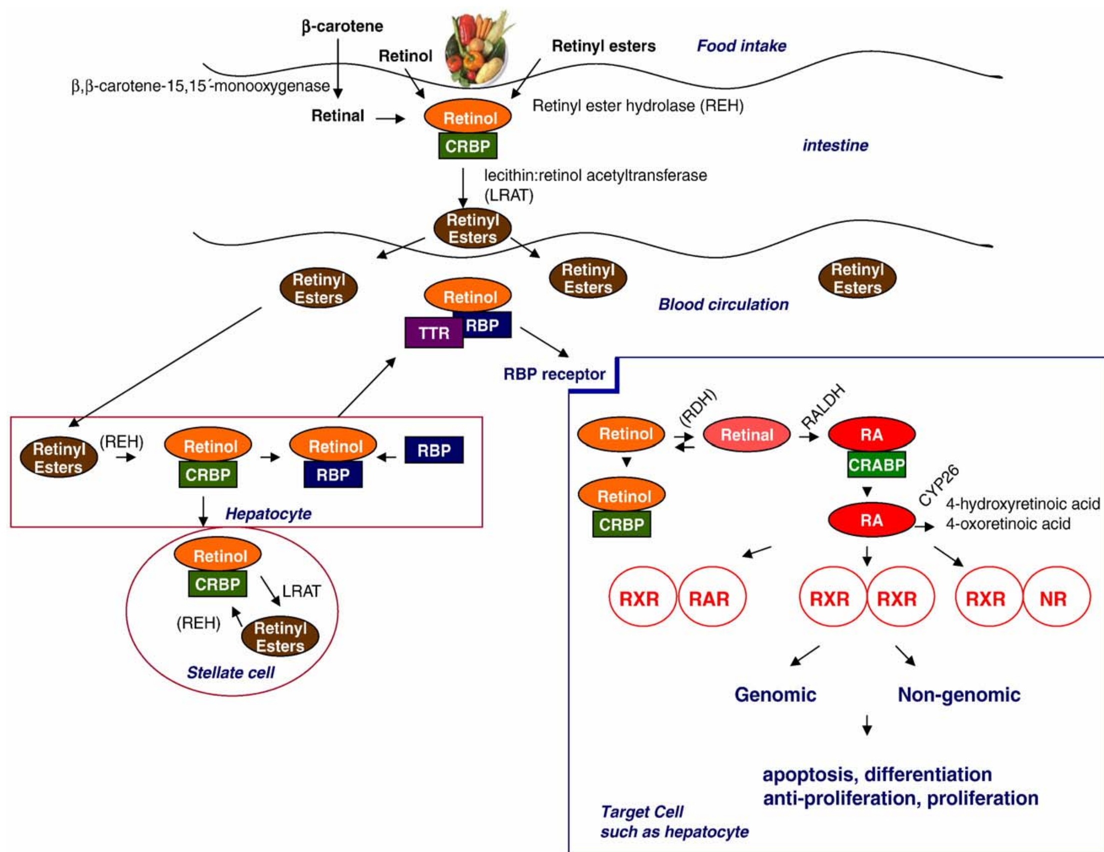

Fig.7. Retinoid pathway: from dietary uptake to metabolism in the liver and tissues.

Retinoic acid roles.

RAR receptor, as other nuclear hormone receptors, are known to act through two different types of molecular actions, classified as transcriptional dependent (genomic) and transcriptional independent (non genomic).

Genomic action involves ligand-activated receptors bound to specific DNA elements (RARE) in the promoter of their target genes. RARα is the most well studied isoform and appears to play a major role in the induction of RA effects on myelopoiesis, due to its preferential expression in cells of the myeloid lineage. The most convincing evidence on the relevance of RARα in control of normal myelopoiesis comes from experiments with target disruption of both RARα and RARγ. Bone marrows of fetuses with both mutations exhibit a maturation arrest at the myelocyte and metamyelocyte stages. In a study of Liu

and collegues [59] a total of 169 genes were found to be modulated by RA treatment in NB4 APL cell line. RA resulted in upregulation of 100 of these genes, while the transcription of 69 genes was reduced. Among the genes found to be induced were the C/EBPε transcription factor and HOXA1, the cell cycle inhibitor p21, the antiapoptotic proteins Bfl-1, DAD-1 and GADD153, and the granulocytic differentiation markers CD11b and CD18. Some of the target proteins whose gene expression is inhibited by RA are c-MYC, NFkB and GATA2, all known transcription factors that mediate cell signals that promote cell growth [59].

However, novel non genomic mechanisms have been described that involve ligand-induced modulation of signal transduction pathways in particular activation of kinase cascades (Fig.8).

- One of the activated signalling pathway involve STAT proteins. Several studies have shown that treatment of various leukemia cell lines with ATRA results in up-regulation of STAT1 and STAT2 mediated at the transcriptional level via direct effects of RAR/RXR on RAR elements in the STAT gene promoter. RA also induces STAT1 Tyr 701 and Ser 727 phosphorylation and these events are required for induction of G0/G1 arrest, apparently via regulation of c-Myc, cyclins and p27kip. Nevertheless, it does not appear that phosphorylation is the result of activation of the classic Jak-Stat pathway, as there are no evidence that ATRA activates Jak kinases in APL cells. However, it activates members of the Src-family of kinases: specifically Lyn and Fgr kinases are phosphorylated/activated during RA-induced differentiation of HL60 cells. Other studies have shown that ATRA induces tyrosine phosphorylation of the CrkL-adapter in the NB4 and HL60 APL cell lines. CrkL in an SH2 and SH3 domain containing-protein that functions as an adaptor in various cytokine signalling system [21].

- Another kinase that is activated during ATRA induced-differentiation is the phosphatidylinositol (PI) 3'kinase and its modulation is essential for ATRA- induced granulocytic maturation of APL cells. RA treatment of HL60 cells causes also an increase of Akt phosphorylation and its kinase activity [60].
- Activation of PI3K by RA appears to involve protein-protein interaction between this kinase and retinoic acid receptors. In neuroblastoma cells S.Masia and collegues demonstrated that the association of RARα with the two subunits of PI3K was differently regulated by the ligand. Cells showed stable association between RARα and p85, the regulatory subunit of PI3K, independently of the presence of RA. In contrast, ligand administration increased the association of p110, the catalytic subunit of PI3K, to this complex. RA promoted the re-localization of a small fraction of RAR to the plasma membrane, allowing the interaction with components of the signal transduction machinery leading to PI3K activation [61].
- The family of mitogen-activated protein (MAP) kinases includes three distinct groups: the extracellular signal-regulated kinases (Erk) family, the p38 Map kinase family, and the C-Jun N-terminal kinase (JNK) Map kinase family. Several studies accumulated evidence that these kinases play important roles in the induction of ATRA-responses in APL. It has been previously shown that treatment of HL60 results in phosphorylation of the Erk-2 kinase and its upstream effectors Mek1/2. Such activation is not accompanied by an increase in the levels of protein expression of these kinases [62]**.** Interestingly, Erk1 is not activated during RA-induced differentiation, suggesting that different Erk isoforms play divergent effects on the induction of RA-responses. Erk Map kinase activation is required for RA-dependent differentiation in diverse cellular backgrounds, so it is not restricted to APL cells.

- In contrast to the Erk Map kinase pathway, the JNK Map kinase cascade is inhibited during RA-tratment of cells. Such inhibitory effects are mediated by the activation of MAP kinase phosphatase-1 (MKP-1) and subsequent abrogation of Map kinase kinase4 (MKK4) activity. It is possible that ATRA downregulates JNK kinase activity in APL cells to induce an antiapoptotic state that facilitates induction of cell differentiation [63].
- Treatment of NB4 cells with ATRA results in the activation of p38 Map kinase. The induction of this kinase occurs at a relatively late time-point and appears to exhibit negative regulatory effects on the induction of differentiation and suppression of cell growth by ATRA [64].

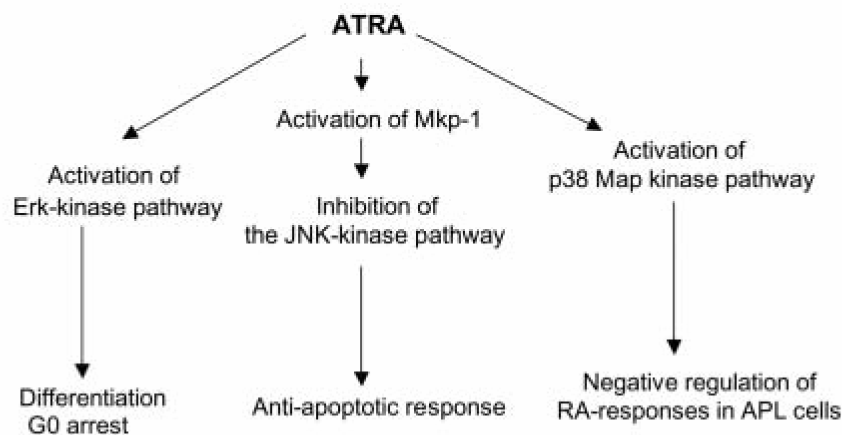

Fig.8 Effects of all-trans-retinoic acid on mitogen-activated protein (Map) kinase signalling pathways.

- Retinoic acid modulates also protein phosphatases: [65] demonstrated that RA treatment causes a decrease in PP2A activity due to a reduction in its catalytic subunit expression, while PP1 activity remains constant.

In order to understand the functions of non-genomic actions on signalling elicited by RA, E.Laserna and collegues have identified nuclear proteins, whose phosphorylation state is rapidly modified by RA treatment in neuroblastoma

cells [66]. They showed that RA exposure led to changes in the phosphorylation patterns not only of proteins related to transcriptional activation but interestingly also of proteins related to mRNA processing such as mRNA splicing. They demonstrated that RA treatment altered the regulation of splice site selection, through PI3K-dependent but ERK-independent mechanisms. Among the proteins target of RA-induced phosphorylation, they found the serine/arginine rich (SR) protein family, which are important regulators of mRNA splicing. In addition to the actions of SR proteins in splicing, several post-splicing activities have been described for a subset of shuttling SR proteins, including regulation of mRNA export and translation. RA treatment may affect the regulation of translation of specific mRNAs through specific phosphorylation of SR proteins and this stimulation could be abrogated both by inhibition of PI3K/Akt pathway as well as by inhibition of the ERK-MAPK pathway [66].

Retinoids and the cell cycle.

The cell cycle is a quite well understood process that can be divided in five steps: G_0 quiescent condition, the entry in G_1 phase, the progression in the synthetic (S) phase, G_2 phase, and finally the mitotic phase (M). Each step of cell cycle is driven by specific complexes of cyclin-dependent kinases (CDKs) and cyclins, that are quickly degradated for the entry in the later phase. CDK-cyclin complexes phosphorylate and modulate the activity of target proteins but at the same time they are under the control of cyclin-dependent kinases inhibitors (CDKi) such as p53, p21, p16INK, p19ARF, p27kip (Fig.9) [67].

Abundant evidence shows that retinoids, via varius signalling pathway, inhibit cell cycle progression by directly or indirectly modulating cyclins, CDKs and CDKi. Generally RA causes a block in G0/G1 phase and a decrease in the proportion of cells in S phase. In mammalian cells two families of cyclins are

active during the transition from Go to G1 phase and from G1 to S phase: they are respectively the D family (D1, D2, D3) ,which bind to CDK4 and CDK6, and the E family (E1 and E2), that interact with CDK2. Retinoid exposure results in an increase in ubiquitination and proteolysis of cyclin D1 and in a decrease in cyclin D1 mRNA. This reduction in cyclin D1 leads to decreased CDK activity, which subsequently reduces phosphorylation of RB oncosuppressor. Thus RB fails to release E2F transcription factor, inhibiting the expression of cyclins E1 and E2. Ultimately, cells can not enter S phase.

In addition RA causes cell cycle arrest by increasing the expression and post-translational stability of CDKi.

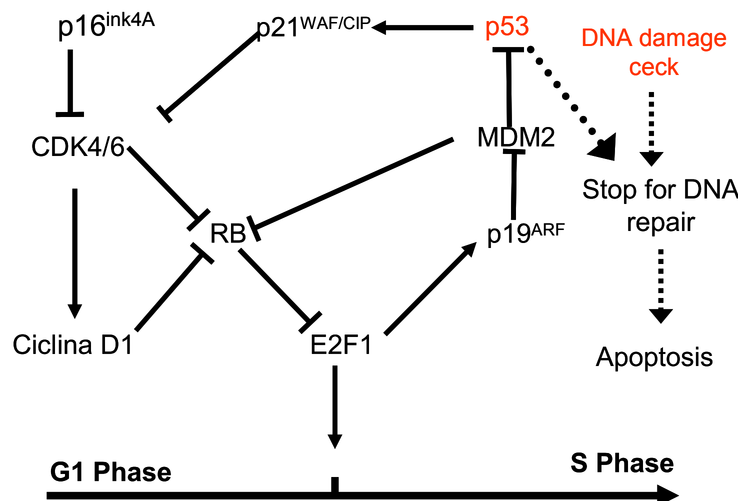

Fig.9. Scheme of the complex interaction among CDKi, cyclins and CDK in the control of cell cycle passage from G1 phase to S phase.

Among the RARs, RARβ2 is RA inducible and is the predominant receptor mediating the inhibitory effect of RA on cell proliferation. RARβ2 stimulates the induction of cell cycle inhibitors such as p21CIP and p27kip; it is not surprising that RARβ2 gene is frequently lost or epigenetically silenced in human cancers [27]. P21 inhibits the cyclin E-cdk2 kinase complex, whose activity is required for cells to go from G1 into S phase. P21 was discovered as a p53-inducible protein,

however several studies have shown a p53-independent pathway of p21 induction. M. Liu demonstrated that p21 is also a RA-responsive target gene and described a functional RA response element in this gene's promoter which is required to confer RA induction through RAR-RXR heterodimers [68].RARα plays a key role in the cell cycle blockade, stimulating, in presence of RA, the expression of p27kip1 and Myc-antagonist Mad1; however RARα does not regulate these genes in a direct way but through its target transcription factor C/EBPε, that is fundamental for terminal granulocytic differentiation [69].

<u>Retinoids and apoptosis.</u>

Considering as model APL, treatment with ATRA or 9-*cis* retinoic acid induces growth inhibition, granulocytic differentiation and apoptosis, but the relationship among these events is not well known. Retinoids are able to stimulate apoptosis also in other cancer cells, T-linphoblastic leukemia, metastatic melanoma cells, breast and lung cancer cells [27]. In HL60, a PML-RARα negative promyeloblastic cell line undergoing granulocytic maturation upon treatment with ATRA and receptor specific retinoids, retinoid-dependent differentiation and apoptosis are two independent processes. Differentiation is mainly regulated through retinoic acid receptors of the RAR type, whereas apoptosis is dependent on RXR activation. Both ATRA and 9-*cis* retinoic acid seem to be effective in inducing apoptosis but the main role is likely played by 9-*cis* RA, considering also the fact that all-*trans* retinoic acid can be converted enzymatically in 9-*cis*. Similar results were obtained also in NB4 cells which carry the PML-RARα translocation; Gianni and co-workers. demonstrated that retinoid differentiation is followed by a slow process of apoptosis which proceeds asynchronously. Indeed maximal cell maturation is observed at 4 days and from this moment on, cells show a progressive decrease in viability [70]. The beginning of apoptosis is accompanied by the downregulation of BCL-2 gene

expression as demonstrated by M.Bocchia et al [71]. Although, again PML-RARα and/or RARα activation is sufficient to trigger apoptosis, the process can be modulated by RXR activation [72]. In fact, RXR antagonistic blockade suppresses the apoptogenic response of NB4 to 9-*cis* RA. In addition stimulation of RXRs enhances the apoptogenic effect. Also the release of PML oncosuppressor after PML-RARα degradation and the reconstitution of PML nuclear bodies could have an important role in the beginning of the apoptotic program.

1.6. Protein kinase CK2.

Protein kinase CK2 (formerly known as casein kinase II) is a highly conserved and expressed serine/threonine kinase. It is now abundantly clear that it is a promiscuous enzyme as a diverse and somewhat bewildering array of more than 300 potential substrates have been identified. CK2 participates in a wide variety of cellular processes including cell proliferation, survival and differentiation [73]. There is an increasing body of evidence indicating that CK2 is involved in protein kinase networks controlling cell cycle progression and cellular responses to stress including ultraviolet light, heat shock, TNFα. Furthermore, abnormally high levels of CK2 have been observed in various types of cancer both in solid tumours (breast, prostate, lung, kidney, neck and head) and haematological malignancies (AML, multiple myeloma, burkitt lymphoma, acute myeloid leukemia in blast crisis). Based on this involvement in transformation and tumorigenesis, CK2 has recently attracted attention as a potential therapeutic target [74].

CK2 structure.

CK2 has typically been viewed as a tetrameric complex consisting of two catalytic subunits (38-42kDa in mammals) and two regulatory subunits (27kDa in mammals) (Fig.10). However the catalytic subunit can perform its activity also as monomer in the absence of the regulatory counterpart. CK2 was distinguished among other protein kinase for its ability to phosphorylate serine or threonine residues that are proximal to acidic amino acid. Pinna and colleagues defined a minimal consensus sequence for phosphorylation by CK2, however there are sites that are efficiently phosphorylated by CK2 despite of the absence of this consensus sequence. CK2 has also the ability to phosphorylate tyrosine residues, although the kinetic parameters for this phosphorylation are much less favourable than those in ser/thr residues[75].

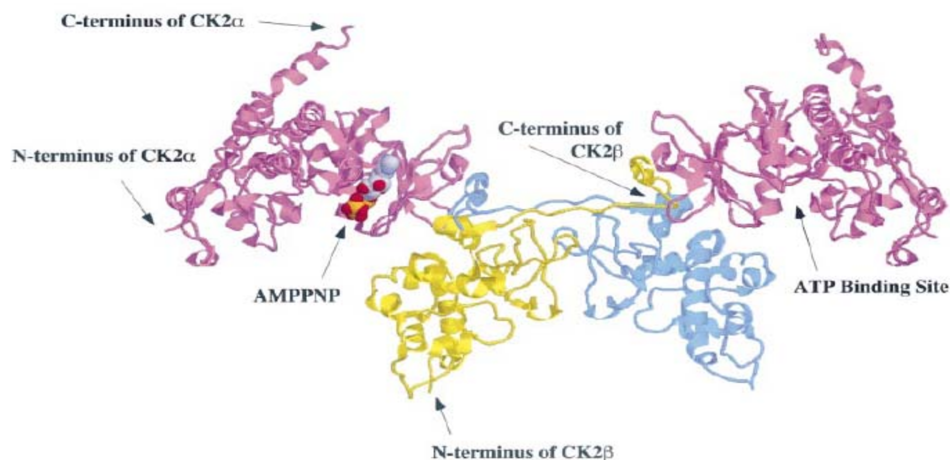

Fig.10. Ribbon diagram illustrating the high-resolution structure of tetrameric CK2

- **CK2α.** In humans, two different forms of its catalytic subunits (designated CK2α and CK2α') which are encoded by distinct genes, were initially characterized. With exception of their unrelated C-terminal domains, these two isoforms are very similar to one another exhibiting approximately 90% identity within their catalytic domain. Recently a

third isoform, CK2 α", was identified, that is almost completely identical to CK2α; the only distinguishing feature lies in the completely distinct C-terminal domain. It is known that the different CK2 isoforms are closely related and show considerable functional overlap; indeed, a knockout of the gene encoding CK2α' in mice results in variable offspring when heterozigous mice are bred to homozygosity, suggesting that CK2α has the capacity to compensate for CK2α' in the context of viability. However the male are sterile and display defect in speramatogenesis, demonstrating that the functional compensation is not absolute. There is also evidence for functional specialization of the individual CK2 isoforms in yeast, mice and mammals and there may also be differences in the subcellular localization of CK2α and CK2α' [75]

- **CK2β.** In contrast of the catalytic isoforms of CK2, only one known form of the regulatory subunit β has been identified in mammals, but multiple forms have been identified in other organisms, such as *Saccharomyces cerevisiae*. CK2β is remarkably conserved among species and x-ray crystallography studies have determined that a dimer of the CK2 β subunits forms the core of the CK2 tetramer [75].

  A large proportion of CK2β has been shown to be phosphorylated at an autophosphorylation site consisting of serine 2, 3 and 4 at its N-terminus.

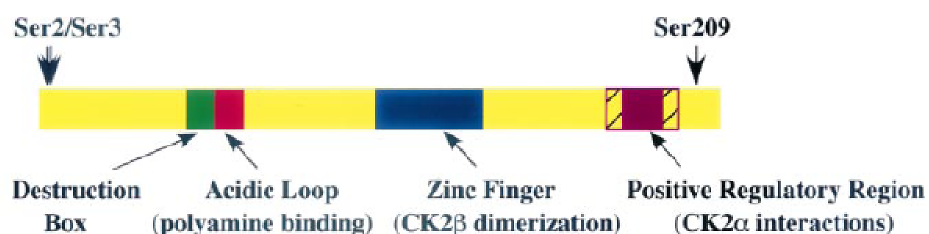

Fig.11. The regulatory CK2β subunit. Linear representation of CK2β, illustrating the main elements within its amino acid sequence.

It was hypothesized that this autophosphorylation could be mediated by an intermolucular reaction through the formation of higher order CK2 structures and it could enhance CK2β stability. CK2β is also phosphorylated at S^{209} near its C-terminus, a residue which is phosphorylated in a cell-cycle dependent manner by $p34^{cdc2}$ (Fig.11).

-It is particularly intriguing that CK2β has motifs that have been previously characterized as motifs that regulate cyclin degradation. Indeed, this sequence is similar to the amino acid motif called destruction box that plays a key role in the specific degradation of cyclin B at the end of mitosis [76].

X-ray crystallography revealed the importance of the zinc-finger region: this sequence is characterized by four cysteine residues wich mediate the interaction allowing the CK2β dimer to form the core of the CK2 holoenzyme. CK2β dimerization precedes catalytic subunit binding and it is a prerequisite for CK2 tetramer formation.

-C-terminal region is responsible for the ability of CK2β to enhance and stabilize CK2 activity.

-One additional important sequence is the acidic loop: it has been identified as the site on CK2 that binds polyamines which are known to stimulate CK2 activity *in vitro* . [75]

Challenge to the traditional view of CK2.

While consideration of CK2 as a tetrameric complex remains relevant, significance evidence has emerged to challenge the view that its individual subunits exist exclusively within these complexes. Indeed a lot of data indicate that the regulatory CK2β subunit exists and performs functions independently of CK2 tetramers. In particular:

-X-ray crystallography revealed that the CK2α and CK2β interface was relatively small and flexible; this result raises the possibility that CK2 tetramers are subject to disassembly and reassembly[76].

-Expression of CK2. Relatively little is known about how either CK2α or CK2β expression is regulated. Earlier studies had shown that CK2β protein was sythesized in excess of the catalytic subunit, underling a lack of coordinated expression. Several reports have also revealed an unbalanced expression of the two subunits in different tissues. For example the level of CK2β in testis was significantly higher in comparison to the level of CK2α.

The intriguing demonstration that aberrantly high levels of CK2β have also been observed in tumors, highlights the importance of understanding the dynamic role of CK2β both within the context of the CK2 holoenzyme and as an independent protein [76].

-Localization of CK2 subunits. Immunofluorescence studies confirmed that the catalytic and regulatory subunits of CK2 are not exclusively co-localized. While the majority of both subunits were localized to nuclear fraction, a major proportion of CK2α was tightly bound to nuclear components whereas CK2β was only loosely associated with other nuclear components. In addition, it was demonstrated in mammalian cells that all the three subunits of CK2 were localized to the smooth endoplasmic reticulum and the Golgi complex, instead only CK2α and CK2α' could be detected in the rough endoplasmic reticulum. In addition to confirming the predominantly nuclear and moderately cytoplasmic localization of both CK2α and CK2β, these studies showed that both nuclear import and export of CK2 subunits are regulated independently and can result in rapid changes of their steady-state distribution. However, when associated in stable holoenzyme complex, the two subunits are dynamically retarget in the cytoplasm. Moreover they demonstrated that the binding of fibroblast growth factor 2 (FGF-2) to the holoenzyme provokes its nuclear accumulation,

supporting the concept of a signal-mediated localization, which may result in a sophisticated regulation of the kinase {Filhol, 2003 #18**).**

CK2 beta functions.

Over the last decade a plethora of CK2β-specific interaction partners have been identified through studies performed *in vitro* and *in vivo.* Some of these proteins have undergone more extensive validation allowing for their classification as either CK2 dependent or CK2 independent partners of CK2β.

-CK2-dependent binding partners are proteins that interact with the tetrameric CK2 through binding sites of CK2β. Within CK2 complex a major role of CK2β appears to be substrate docking or recruitment where it brings the substrate protein and the catalytic subunit into close enough proximity to facilitate the phosphorylation reaction. A second function of CK2β appears to involve transmission of regulatory signals provided by other proteins in manner that could be analogous to that seen with polyamines. FGF-2 exemplifies this, as binding of FGF-2 to CK2β stimulates CK2 activity.

Thus, these two functions of CK2β modulates the ability of CK2 to phosphorylate specific cellular targets**.**

- CK2β independent binding partners are proteins that interact with CK2β in the absence of catalytic CK2 subunits. These proteins include A.-Raf, c-Mos, and Chk1, that are ser/thr protein kinases containing sequences reminiscent of the CK2β binding region present in the CK2 catalytic subunit. In the case of A-Raf-CK2β interaction it was demonstrated that the presence of CK2α abolishes the activation observed with CK2β, suggesting that CK2α was competing with A-Raf for binding to CK2β. Interestingly, while in the case of c-Mos, the interaction with CK2β leads to down-regulation of the latter, inducing mitotic arrest in rapidly dividing embryonic cells; in the case of A-Raf and Chk1, the kinase activity is enhanced upon interaction with CK2β [77].

Regulation of CK2 in cells.

The traditional view of CK2 looks at this protein as a constitutive active [78] and unregulated kinase, nevertheless, several studies support the idea that there are distinct mechanisms contributing to the physiological regulation of CK2:

- the first one is represented by the CK2β subunit that influences CK2 recruitment of the substrate and CK2 localization; moreover, it was demonstrated that the presence of the destruction box in CK2β, and consequently its degradation through proteasome, determines the oscillation of CK2 activity during cell cycle [76].
- Phosphorylation of CK2: several works indicate that phosphorylation is not absolutely required to activate CK2 in a manner analougous to that seen with MAP kinases. However they do not exclude the possibility that phosphorylation partecipates to some degree in aspects of CK2 regulation. Examination of CK2, isolated from mammal cells, has led to the identification of a number of physiological phosphorylation sites on both CK2α and CK2β. Indeed CK2β is phosphorylated at its autophosphorylation site and at Ser^{209}, a residue that is phosphorylated in a cell-cycle dependent manner. Autophosphorylation of CK2β could indirectly regulate CK2 activity. CK2α is phosphorylated in a cell-cycle dependent manner at four sites within its unique c-terminal domain even if these sites do not appear to effect a dramatic change in the catalytic activity of the kinase [75]. CK2 can also be phosphorylated by the Scr-family protein tyrosine kinases, by c-Abl tyrosine kinase and by the pathological counterpart Bcr-Abl fusion protein (typical of chronic myeloid leukemia). In this last context CK2 activity is inhibited by phosphorylation [79].

- Protein-protein interaction: it has been shown that CK2 interacts with proteins such as FGF-1, FGF-2, HSP90 (heat shock protein 90) and the cochaperonine cdc37 that may directly alter or stabilize its catalytic activity. CK2 also interacts with tubulin, FAF-1 and cKIP-1, that could be involved in the targeting of CK2 to specific sites or structures within cells. There are three tumor suppressors that bind and inhibit CK2 activity: p53 interacts with the β subunit affecting its function [4]; in a similar way also p21WAF1 binds toCK2 β [80]; adenomatous polyposis coli protein (APC) inhibits CK2 through the interaction with α subunit [81].
- Role of small molecules in CK2 regulation: CK2 is inhibited by negatively charged compounds such as heparin and activated by positively charged compounds, including polyamine [75].

<u>CK2 functions.</u>

CK2 always behaves as an antiapoptotic agent implying on different cellular functions, signalling pathways and biochemical reactions which ultimately cooperate to promote cell survival (Fig.12).

- CK2 is a multisite regulator of different signalling pathways that are potentiated by phosphorylation:

  -NFkB: this transcription factor is normally sequestered in the cytosol by the binding to its inhibitor IkB. CK2 acts at different levels: it phosphorylates IkB promoting its degradation through proteasome, increases the expression of IKK kinases, phosphorylates p65 subunit of NFkB increasing its transcriptional capability.

  -Wnt pathway: in the presence of wnt, the destruction complex which targets β-catenin to the proteasome is inhibited by the stabilizing protein dishevelled (Dvl). CK2 phosphorylates Dvl and β-catenin promoting

their stabilization, and TCF/LEF, facilitating its association to partner molecules [1];

-PI3K/Akt: here again CK2 operates as multisite regulator: a first level is represented by the tumor suppressor PTEN, the phosphatase which dephosphorylates PIP3 (phosphatidylinositol 3, 4, 5 triphosphate), thus maintaining the PI3/Akt signal down, under resting conditions; it has been demonstrated that the constitutive phosphorylation of PTEN by CK2, while regulating the PTEN protein stability, has an inhibitory effect on its phosphatase activity as well, with the final effect of stimulating Akt-dependent signalling. A second level of CK2 intervention on this pathway is represented by Akt itself: beside a physical interaction between the two kinases, a direct phosphorylation of Akt on Ser 129 by CK2 has been found, which promotes an hyper-activated state of Akt[82]. There is moreover an indirect effect of this CK2-mediated phosphorylation, since it contributes to maintain an high level of phospho Thr-308, by ensuring a stable association with the chaperone protein Hsp90, known to protect Thr308 from dephosphorylation.

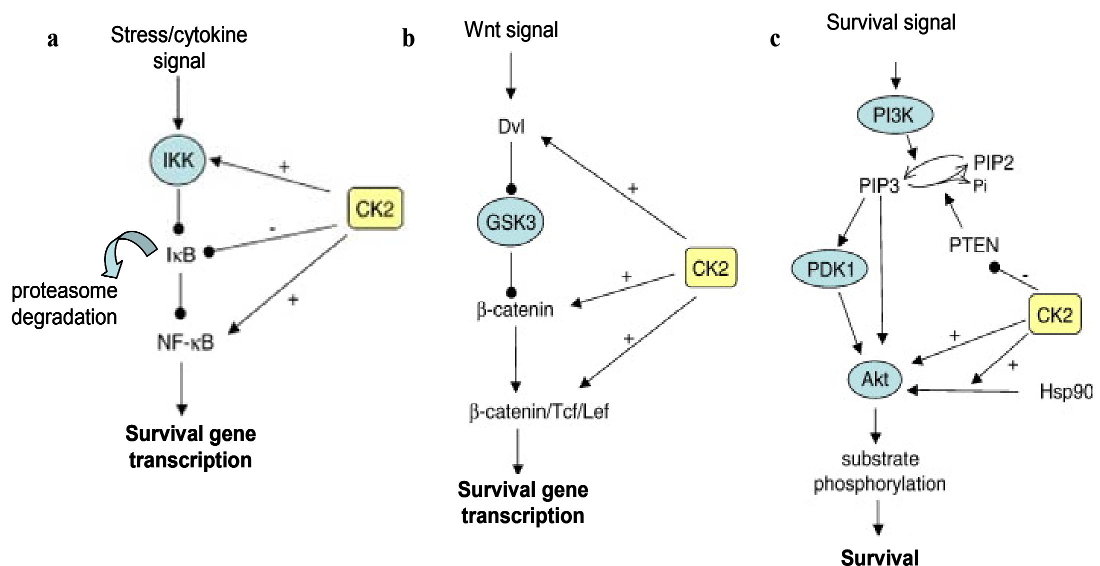

Fig.12. CK2 –dependent multisite regulation of NF-kB (a), β-catenin (b), and Akt (c) signalling.

- Jak-Stat pathway: Zheng and co-workers provided the first evidence that ser/thr kinase CK2 binds and phosphorylates Jak2 and these events are critical for the activation of Jak2-Stat signalling pathway [83].
- CK2 and apoptotic signalling: the caspase inhibitor ARC is phosphorylated and activated by CK2 while survivin, a member of the inhibitor of apoptosis protein (IAP) family, is upregulated whenever CK2 expression is increased. Other CK2 targets are Bid, Max, HS1, presenilin, connexin, whose previous phosphorylation generates caspase resistant sites. Caspase 9 itself falls in this category, since its phosphorylation by CK2 protects caspase 9 from caspase 8 cleavage [84].
- PML: CK2 regulates PML protein levels by promoting its ubiquitin-mediated degradation dependent on direct phosphorylation at Ser517. [85]
- CK2 participates in the regulation of proteins that have important functions associated with cell cycle progression: topoisomerase II, p34, cdc34, $p27^{kip}$, MDM2, p21WAF/CIP and p53 [73].
- CK2 cooperates also with proto-oncogenes such as c-Myc, c-Myb, c-Jun, Ha-Ras and A-Raf [1].
- The RNA polymerase I and RNA polymerase II complexes were among the first substrates to be discovered. Then RNA polymerase III was also shown to be target of CK2. Phosphorylation by CK2 of the TATA-binding protein (TBP), a subunit of TFIIIB (the core component of the Pol III transcriptional machinery), promotes a remarkable increase in Pol III activity, favouring the synthesis of tRNA and 5SrRNA. Thus CK2 enhances rRNA and tRNA biogenesis [73].
- Y. Miyata and colleagues demonstrated that the cochaperone cdc37 is a CK2 target. Cdc37 is involved in the folding process of several protein kinases in tight collaboration with Hsp90; however, cdc37 shows molecular chaperone activity *per se*. Phosphorylation of cdc37 by CK2 is

essential for the proper function of the chaperone, moreover, CK2 itself operates in a cdc37-dependent manner being directly associated with the latter one. Thus CK2 may control many growth-related protein kinases simultaneously via the cdc37 phosphorylation (Fig.13) [86].

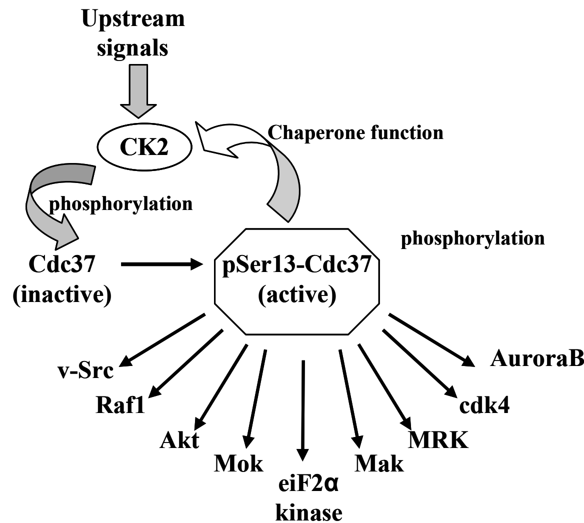

Fig.13. Cdc37 as substrate of CK2. CK2 and Cdc37 constitute a positive feedback to control a number of important kinases.

The implication of CK2 in signalling cascades is atypical with respect to the other protein kinases because it is not hierarchical. Owing to its lack of molecular mechanisms capable to readily turn on or off its activity, CK2 is exempted from the control of other kinases and it is excluded from the canonical signalling pathway "dropping vertically" into the cell from the membrane to the nucleus. It rather plays a "lateral role" impinging on many of these longitudinal pathways. CK2 can be seen as a "master regulator" of cellular functions, committed to the integration and crosswise consolidation of different pathways [1].

CK2 and cancer.

A number of genetic alteration can occur which bypass the physiological mode of activation of a kinase, given the rise to a constitutively active enzyme no more subjected to its physiological mechanism of control. The molecular alterations that interfere with protein kinase activities and are causative of cancer may be several, including gain/ loss of function, gene deletion, translocation with generation of fusion proteins. However CK2 does not conform to this general paradigm, merely because only active forms of it apparently exist. In addition, mutations of CK2 have never been reported, while its physiological concentration is one of the highest found, suggesting that CK2 is one of the more represented kinase. Remarkably, CK2 levels has been found to be invariably higher in malignant cells than it is in normal cell of the same type. There is also a correlation between the grading of the malignancy and the level of CK2: the higher is this latter, the worse the prognosis is [87]. It is felt now-a-days that such elevated CK2 is neither the cause nor the consequence of neoplastic transformation while it may well reflect the tendency of the tumour, regardless the genetic alterations causing it, to preferentially colonize those cells where CK2 is higher. Ruzzene and Pinna introduced the concept of "non oncogene addiction" in order to understand the role of CK2 in ensuring survival of a variety of cancer cells, where its elevated activity seems to be relied to epigenetic events [1]. They propose a model: assuming that stochastically such high CK2 level occurs only in a little part of the cellular population, its contribution to the overall tissue phenotype will be almost null. Any oncogenic mutation occurring in these cells will be counteracted by a number of opposing mechanism in the majority of cells, where CK2 is normally represented; in contrast it will find a more favourable environment in the few cells were CK2 is abnormally elevated and which are predisposed to evade apoptosis, to stabilize

the onco-kinome, to develop drug resistance etc..(Fig.14) These cells are destined to proportionally increase in number, conferring to the tissue a malignant phenotype which will be associated and maintained by elevated CK2. At this stage it can be said that the tumour has become "addicted" to CK2 [1]. There is the possibility that tumour cells more strictly rely on CK2 functionality for their survival pathway than normal cells do. This concept is supported by the observation that, when primary tumour cells are treated with CK2 inhibitors, their susceptibility to cell death tends to be higher than in the case of normal precursor cells. This has been found e.g. for multiple myeloma (MM) [22], for acute myeloid leukemia [88], and for T-Acute lymphoblastic leukemia (T-ALL) [89].

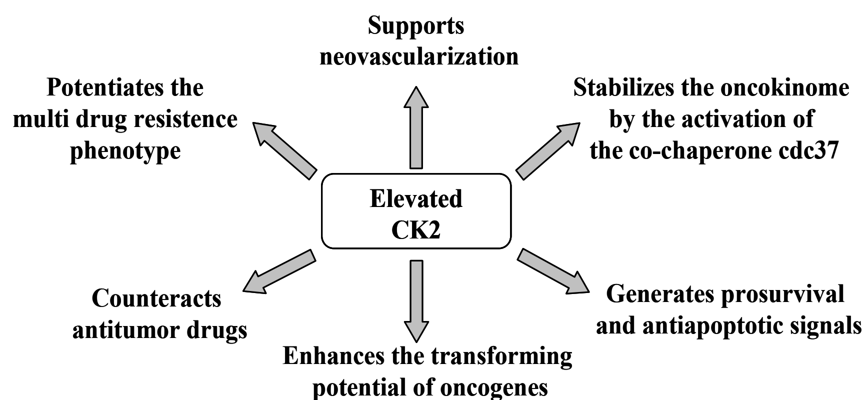

Fig.14. CK2 as a cancer driver. Effects promoted by abnormally high CK2 levels.

CK2 and hematopoiesis.

The function of CK2 in normal hematopiesis is up to now not fully known. However, some studies demonstrated that this kinase regulates several hematopietic transcription factors and molecules as well as inflences signaling pathways involved in hematopoietic stem cell biology.

Among the transcription factors targets of CK2 we annoverate:

-c-Myb: it is phosphorylated at the amino terminal serine 11 and 12 by CK2. These sites are constitutively phosphorylated and this post-translational modification is essential for high affinity specific DNA binding activity of c-Myb [2].

-PU.1 is phosphorylated by CK2 at Ser 148. Phosphorylation at this site is required to allow interaction between PU.1 and IRF-4 and to allow the trans-activation function of PU.1 in LPS-stimulated murine macrophages [3].

-Ikaros: it is a transcription factor essential for hematopoiesis and an established tumor suppressor. Studies by Georgopoulos and colleagues have identified several amino acid that are phosphorylated by CK2. Phosphorylation of Ikaros at its terminalc-region regulates its ability to control cell cycle progression from G1 to S phase [90]. Following studies identified four novel CK2 phosphorylation sites located at the N-terminal end of Ikaros: for instance phosphorylation at 13 and 294 aminoacids causes a decrease in Ikaros DNA binding affinity and influences its subcellular localization. It was demonstrated that its phosphorylation changes during T-cell differentiation: Ikaros, indeed 13 and 294 sites undergo dephosphorylation, resulting in increased Ikaros binding to TdT regulatory gene and repression of its transcription. Moreover Ikaros contains PEST sequences (associated with increased degradation of protein following phosphorylation) that show multiple phospho-sites targeted by CK2. Thus, CK2-mediate phosphorylation might regulate Ikaros stability and turnover [90, 4].

-HDAC1 and HDAC2: the first one can be phosphorylated by CK2, cAMP-dependent protein kinase, and protein kinase G, the second one results to be phosphorylated uniquely by CK2. Thus, CK2 influencing HDACs phosphorylation alter the balance of hystone deacetylase and acetyltransferase activities [6]. HDAC1 and 2 are associated with Sp1 and Sp3 transcription factors and also these proteins are targets of CK2 [40]; indeed Sp1 is phosphorylated at the c-terminus, decreasing its DNA binding activity [5, 91].

-c-Myc and Max **:** Bousset and colleagues identified these proteins as targets of CK2. C-Myc is a proto-oncoprotein that plays a pivotal role in cell growth control. It promotes cell cycle progression into S phase and inhibits differentiation. The importance of c-Myc is further supported by the this protein is essential for murine embryonic development. To perform its functions, c-Myc heterodimerizes with Max. Max can interact also with Mad proteins that negatively regulate cell growth [7]**..** CK2 phosphorylation of Ser2 and 11 in Max resulted in enhanced DNA binding kinetics of both Max/max homo- and Myc-Max heterodimers [92]**.**

CK2 modulates also signaling pathways involved both in normal and maligant hematopoiesis such as:

-Wnt pathway: as showed in the previous paragraph, CK2 displays several site of phosphorylation, dishevelled (Dvl), β-catenin, promoting their stabilization, and TCF/LEF, facilitating its association to partner molecules. Wnt proteins were found at sites of fetal hematopiesis and Wnt5a was involved in self-renewal and proliferation of HSC. Wnt3a also increases HSC self-renewal *in vivo* in adult mice. Human CD34+ lineage also expand under Wnt stimulation. β-catenin constitutive expression determined a depletion of HSC and a multilineage differentiation block, instead Wnt signaling appeared fundamental for HSC long-term repopulating activity and for the leukemia stem cells (LSC) repopulating activity of chronic myeloid leukemia (CML). Therefore these results underline the importanmce of Wnt/ β-catenin signaling in normal and in leukemic stem cells [1].

-PI3/PTEN pathway: some studies performed in mice have demonstrated that PTEN deletion in hematopoietic compartment caused the entry of HSC into the cell cycle and fast depletion of HSC pool. PTEN-/- HSC showed the lack of long-term repopulating activity, furthermore mice null for this protein developed myeloproliferative disease and ALL. Thus the presence or absence of PTEN allows the discrimination between normal HSC and leukemic LSC. As

represented in the previous Fig.12, CK2 through phosphorylation inhibits PTEN and activates Akt [82].

-The Hedgehog (Hh) pathway is transduced through a reception system at the plasma membrane that includes the receptor complex Patched (Ptc-Ihog) and the signal transducer Smoothened (Smo). Binding of Hh to Ptc-Ihog relieves the inhibition of Smo by Ptc, which allows Smo to activate the Cubitus interruptus (Ci)/Gli family of zing finger transcription factor (Fig.15) [93]. The Hh signal is essential for normal embryonic development and tissue repair. Hh has been reported to play a role in hemangioblast formation [94]; B cell, T cell, and thymocyte development [95]; erythrocyte proliferation and differentiation [96] and the HSC and progenitor cell compartment [97]. Thus, the available literature indicates that there is an important contribution of Hh signal transduction in normal hematopoietic development. Of further interest, mutations in the Hh signaling pathway lead to severe developmental abnormalities and have been associated with several types of cancers. The role of Hh signaling in hematopoiesis has been studied primarily by modulating the activity of Patched and Smoothened, but results have been conflicting. Merchant and colleagues have analyzed hematopoietic stem cell (HSC) and progenitor function in mice with a homozygous deletion of *Gli1* (*Gli1^{null}*). *Gli1^{null}* mice have more long-term HSCs that are more quiescent and show increased engraftment after transplantation. In contrast, myeloid development is adversely affected with decreased in vitro colony formation, decreased in vivo response to granulocyte colony-stimulating factor (G-CSF), and impaired leukocyte recovery after chemotherapy. Levels of the proto-oncogene *Cyclin D1* are reduced in *Gli1^{null}* mice and may explain the loss of proliferation seen in HSCs and progenitor cells [98]. CK2 is a positive regulator of the Hh signal transduction pathway. Jia and co-workers demonstrated that Smo is phosphorylated by CK2 at multiple Ser residues in the C-terminal region. These event is necessary for Hh-induced Smo accumulation. In addition, they found that CK2 regulates the pathway also

downstream of Smo; indeed, they showed that phosphorylation of Ci prevets its ubiquitination and degradation through proteasome. (Fig.15) [99]. There are also other kinases involved in the control of this pathway such as PKA, GSK3, CK1, which phosphorylate Smo and Ci [99].

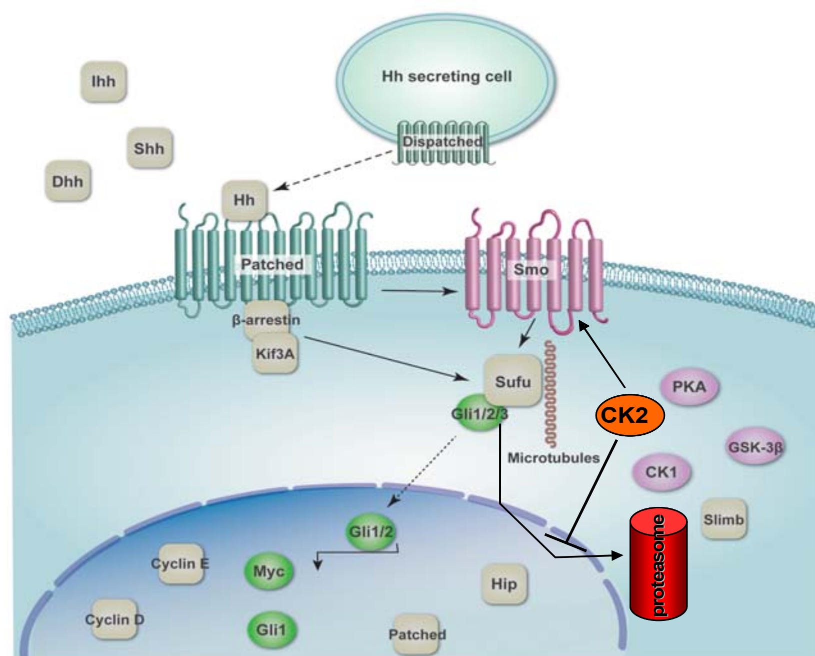

Figure15. The Sonic Hedgehog signaling pathway.

1.7. Protein kinase CK2 inhibitors.

Interest in developing small molecule inhibitors of CK2 was heightened with the identification of adenosine -5′-triphosphate (ATP)-binding sites specific chemotypes. An increasing number of reports dealing with the development and usage of CK2 inhibitors appeared in literature. However, as with many inhibitors of other kinases, questions regarding their specificity arose immediately. This cautionary note has to be considered especially for these kind of compounds that are competitors of ATP, since ATP is the substrate for all members of protein kinase family in addition to a vast array of other cellular

enzymes. Some years ago five inhibitors, all competitive with respect to ATP, the structure in complex with the catalytic subunit of maize CK2 has been solved: emodin, MNX (1,8-dihydroxy-4-nitro-xanthen-9-one), DAA (1,4-diamino5,8-dihydroxyanthraquinone), TBB (4,5,6,7-tetrabromobenzotriazole), and IQA (5-oxo-5,6-dihydroxyindolo-1,2-a-quinazolin-7-ylacetic acid)[100]. Although these inhibitors belong to different classes of chemical compounds, all are accommodated in a hydrophobic pocket which in CK2 is smaller than in the majority of protein kinases. This may well account for the selectivity of these inhibitors, which, with the partial exeption of emodin, are more effective on CK2 than they are on a panel of >30 different protein kinases. Among these inhibitors, TBB proved especially successful for in cell and *in vivo* studies (Fig.16c). While the selectivity of TBB is remarkable, its potency, though higher than that of the most other CK2 inhibitors, is not outstanding: *in vitro* it inhibits purified CK2 with IC_{50} values around 1μM, while the concentration required to induce half-maximal apoptosis of Jurkat cells is 17 μM. The relatively low potency of TBB, as well as of other CK2 inhibitors, is due to their mode of binding, which is almost exclusively based on apolar contacts with unique hydrophobic side chains, while polar interactions, which are common with potent inhibitors, are nearly absent. On the basis of these assumptions, Pagano and colleagues started to develop TBB derivates in which the tetrabromobenzene moiety, responsible for selectivity is conserved, while the triazole ring is replaced by an imidazole one derivatized with substituents that could provide interactions with polar side chains of the kinase. Some TBB analogues revealed a markedly higher potency and specificity than the previous compounds both *in vitro* and *in vivo* [101] such as K17 (4,5,6,7-tetrabromobanzinidazole; IC_{50} 0.5 μM) and K27 (4,5,6,7-tetrabromo-2-amino-benzimidazole; IC_{50} 0.25 μM) (Fig. 16a and b respectively).

Recently, a new selective CK2 inhibitor has been developed: CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthy ridine-8-carboxylic acidis) the first

orally bioavailable small molecule inhibitor of CK2 to advance into human clinical trials, thereby paving the way for an entirely new class of targeted treatment for cancer (Fig.15d). It was designated by Cylene Pharmaceuticals and entered Phase I clinical trials for advanced solid tumors and multiple myeloma. [102]

The crystal structure of human CK2α in complex with CX-4945 shows two direct protein-inhibitor hydrogen bonds with CK2α. Two well ordered water molecules mediate additional protein-inhibitor contacts between the carboxylate group of CX-4945 and CK2α**.** Collectively, this extensive combination of direct and water-mediated hydrogen bonds and van der Waals contacts observed between CX-4945 and CK2α establishes the structural basis for the high affinity binding for the small molecule inhibitor [102]**.**

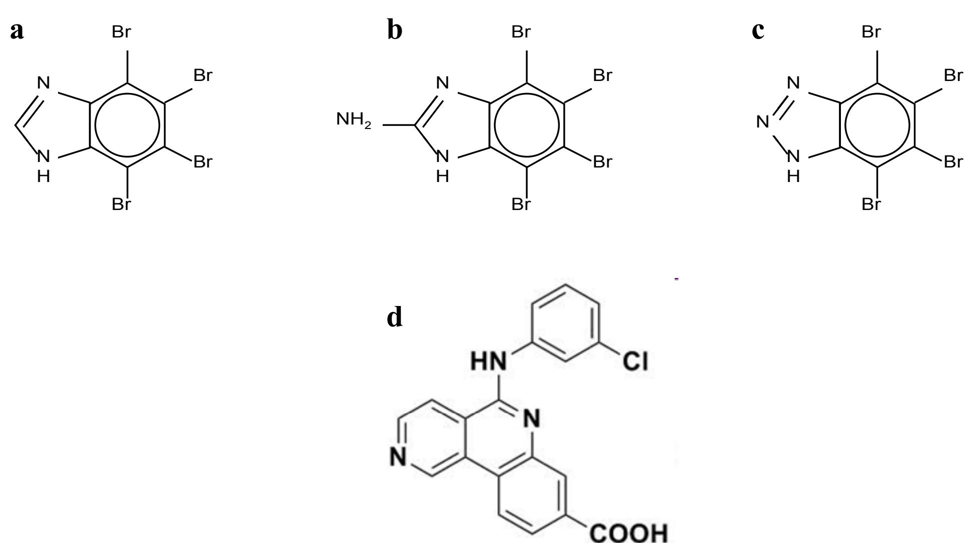

Fig.16. The most specific inhibitors of CK2. (a) K17; (b) K27; (c) TBB; (d) CX-4945

2. AIM OF THE THESIS

CK2 is a Ser/Thr kinase which is highly conserved and constitutively expressed. It is involved in several molecular processes and it can be considered a "master regulator" in the cell, playing a lateral role at different levels on signalling pathways rather than the canonical vertical action on the signal cascade that moves from the cell membrane to the nucleus [1] CK2 is essential for cell survival and proliferation. Remarkably, CK2 levels have been found to be invariably higher in malignant cells than it is in normal cell of the same type, and this over-expression promotes the neoplastic growth, evasion from apoptosis, neovascularisation and multidrug resistance. Thus, several studies support the Ser/Thr kinase CK2 as a potential target for cancer therapy. While almost undetectable in normal granulocytes, CK2 is markedly increased also in highly proliferating myeloblasts from patients with acute myeloid leukemia (AML) or with chronic myelogenous leukemia (CML) in blast crisis. In this respect, known molecular targets of CK2 pathogenetic activity would include proto-oncogenes such as c-Myc and c-Myb [74].

AML represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and over-proliferation in the stem cell compartment result in accumulation of non-functional cells termed myeloblasts. A specific AML subtype is acute promyelocytic leukemia (APL): it is characterized by the typical translocation t(15;17), which generates the PML-RARα fusion protein responsible for the differentiation blockade at the promyelocytic stage. Retinoic acid (RA) treatment allows to bypass this block and restores myeloid differentiation of APL blasts [23].

CK2 was reported to regulate also molecules involved in hematopoiesis. In particular CK2-dependent phosphorylation modulates transcriptional factors such as PU.1 [3], c-Myb [2], c-Myc and Max [92, 7], Ikaros [90], HDAC1 and HDAC2 [5, 91],

protein kinases such as AKT/PBK[82], as well as components of the Wnt and NF-kB pathways [1]. Moreover, it was demonstrated that CK2 is a phosphorylation target of Abl kinase, which is translocated and hyper-activated in CML and in some acute lymphoblastic leukemias. This phosphorylation has a negative effect on CK2, blocking its activity, and they observed that administration of imatinib, an inhibitor of Abl, restored differentiation [79]. Taking together these assumptions we could hypothesize that CK2 could be involved in haematopietic maturation and in the pathogenesis of acute myeloid leukemias. Thus, in this work we aimed to investigate a potential novel role of CK2 in myeloid cell differentiation and we took advantage of the well-established model of acute promyelocytic leukemia cells (APL) maturation induced by retinoic acid (RA).

CK2 expression levels and kinase activity were firstly evaluated in APL cells as well as in normal myeloid progenitors. Then we tested our hypothesis inducing granulocytic differentiation with RA in the presence or absence of CK2 blockade both using chemical inhibitors and RNA interference. In order to find potential molecular targets of CK2, we studied also the interaction of the kinase with transcription factors and pathways activated downstream retinoic acid signal.

3. MATERIALS AND METHODS

3.1 Cell culture.

Stabilized AML cell lines were obtained from DSMZ (Deutsche Sammlung von Mikroorganosmen und Zellculturen GmbH):

-NB4: human acute promyelocytic leukemia (FAB: M3) with t(15;17) PML-RARα fusion gene;

-HL60 human acute promyelocytic leukemia that lacks p53 gene.

They were maintained at 0,5-1x10^6 cells/ml in RPMI 1640 (Euroclone, Italy) containing Hepes and L-glutamine and supplemented with 10% fetal bovine serum, FBS, (Euroclone, Italy).

HeLa cell line, derived from human epithelial carcinoma cells from cervix, was kindly provided by Prof. E. Carafoli. Cells were harvested in Dulbecco's modified Eagle's medium, DMEM, (Euroclone, Italy) with Hepes and L-glutamine, supplemented with 10% FBS.

100U/ml penicillin and 100mg/ml streptomycin were addicted to all mediums and cells were harvested at 37°C in an atmosphere containing 5% CO_2.

APL cells from patients were obtained from peripheral or medullar blood after stratification over Ficoll (GE Healthcare) and following centrifugation.

CD34+ blood stem cells were isolated through cell sorting of apheresis obtained from patients with leukemia free medullar blood. Patients previously had undergone treatment for mobilization of stem cells.

PBMC (peripheral blood mono-nuclear cells) were obtained after density gradient centrifugation of healthy donor blood.

Samples were obtained after informed consent and were provided by the Department of Medicine, Hematology and Immunology branch. (Prof. G. Semenzato, University of Padova).

3.2. Cell treatments.

Cells were counted with tripan blu (Sigma-Aldrich, Steinheim Germany) in Neubeuer chamber, centrifuged at 800rpm for 5′ and plated. They were treated with different compounds:

-9-cis retinoic acid (Sigma-Aldrich, Steinheim, Germany)

-CK2 synthetic inhibitors:

- K17 and K27 supplied by Dr. Z. Kazimierczuk (Warsaw);
- TBB supplied by Dr. G. Fabbretti (University of Padua);
- CX-4945 (Activate Scientific GmbH);

-proteasome inhibitor Mg-132 (Sigma);

-Cycloheximide, antifungal antibiotic which inhibits protein synthesis in eukaryotes (Calbiochem).

3.3. Protein extraction.

Whole cell protein extraction.

All steps were performed at 4°C. Cells (1-5x10^6) were collected and washed in PBS. Pellets were resuspended with 30-50µl of lysis buffer composed of:

-Triton	0,5%
-Okadaic acid	1µM (Sigma)
-phosphatase inhibitors cocktail(100X)	1X (Thermo scientific)
-DTT	1mM (Sigma)
-PMSF	1mM (Sigma)
-Proteases inhibitor (100X)	1X (Sigma)

-Buffer made up of TRIS (pH7.5) 20mM, NaCl 150mM, EDTA 2mM, EGTA 2mM to final volume.

Samples were incubated for 30', vortexed every 5' and centrifuged for 10' at 13000 rpm. Supernatants were collected and stored at -20°C.

Cytoplasmic and nuclear protein extraction.

A large amount of cells are required for this procedure (4-5.10^6 cells each condition). After wash in PBS the pellet was lysed in 50µl buffer A (Hepes 10mM, KCL 10mM, EDTA 0,2mM, DTT 1mM, PMSF 1mM, Okadaic acid 1uM, Phosphatase inhibitor cocktail 1X, protease inhibitors 1X). Samples were incubated for 20' resuspending gently every 5 minutes. Then 5µl of NP40 (4%) were added and samples were vortexed for 15''. Cell lysates were centrifuged at 2000rpm for 3' and supernatants, which contain cytosolic proteins, were collected and stored at -20°C. The left pellets were resuspended in buffer B (Hepes 20mM, NaCl 0,4M, EDTA 0,2M, DTT 1mM, PMSF 1mM, Okadaic acid 1uM, Phosphatase inhibitor cocktail 1X, protease inhibitors 1X and glicerol 10% v/v), vortexed vigorously for 20' and centrifuged at 13000 rpm for 10'. Supernatants, containing nuclear proteins, were stored at -80°C.

3.4. Protein quantification.

To measure the concentration of protein in solution we used the Bradford (Sigma) protein assay . It is based on an absorbance shift of the dye Comassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed. The bound form of the dye has an absorption spectrum maximum at 595 nm. The binding of the dye to the protein stabilizes the blue anionic form. The increase of

absorbance at 595nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample.

Bradford was diluted 1:2 in distilled water and 1-2μl of cell lysate was added; the solution was mixed and incubated 3' in the dark, and absorbance was performed using a spectrophotometer (Ultrospec 1100pro; Amersham).

Concentration values were obtained applying Lambert-Beer formula:

$$A = \varepsilon x\ c \qquad \varepsilon = \text{molar extinction coefficient}$$

Molar extinction coefficient was calculated from a calibration curve, obtained using rising concentrations of BSA.

3.5. Electrophoresis SDS-PAGE.

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a method that allows to separate proteins according to their size, and no other physical feature.

SDS is a detergent that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it. SDS can disrupt hydrophobic areas and coat proteins with many negative charges which overwhelm any positive charges the protein had. The resulting protein is denatured by SDS (reduced to its primary structure) and as a result is linearized. Moreover all proteins, having a large negative charge, will all migrate towards the positve pole when placed in an electric field.

When polyacrylamide, which is a polymer of acrylamide monomers, undergoes the process of polymerization, it turns into a gel and we will use electricity to pull the proteins through it. The acrylamide concentration of the gel can be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight proteins, while much higher

percentages are needed to resolve smaller proteins. Polyacrylamide gel is not solid but is made of a laberynth of tunnels through a meshwork of fibers. Small molecules can move through the polyacrylamide mesh faster than big molecules.

Sample preparation:

20-50μg of protein lysate are mixed with sample buffer (1:4 v/v) composed by SDS 20%p/v, Tris (pH6.8) 1,5M, bromophenol blu 0,05% v/v, DTT 6% v/v, and β-mercaptoethanol 1:20 v/v. The samples are heated at 100°C for 3' to favour denaturation.

Preparing acrylamide gel:

This gel is composed of two different phases: the upper phase called stacking gel (pH6.8) and lower phase called separating gel. The first one allows the protein to compact and makes them enter the separating gel simultaneously. The last one allows the real separation of proteins according to their size. In this work we used both fixed concentration of acrylamide (10%v/v for sepatating gel; 5% for stacking gel) or precast gradient gels with a concentration of acrylamide varing from 4% to 20% (Thermo scientific) for the separating gel. Gradient sds page is best suited for showing high and low molecular weight proteins in the same gel; it still helps resolution in a couple of ways: first, by getting more and more restrictive as the protein moves down the gel, it helps maintain stacking. Sharper bands will not overlap as much. Second, by engineering the gradient properly one can enhance the separation of closely moving bands.

Protein samples and a standard sample, as molecular weight reference (Seeblue Plus2 Prestained Standard 1X-Invitrogen), are loaded into the gel and the electric field applied was 25mA. We used Amersham electrophoretic chambers and a specific saline running buffer (pH 8.3) (25 mM Tris, 192 mM glycine, 0.1% SDS).

3.6. Western blot.

Following electrophoresis, the protein must be transferred from the electrophoresis gel to a membrane. The transfer method that is most commonly used is electrophoretic transfer: this method involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose or other suitable, protein-binding support and "sandwiching" this between two electrodes submerged in a conducting solution. The sandwich is composed into a grid in the following manner: sponge, watman paper, nitrocellulose, gel, paper, sponge. When an electric field is applied, the proteins move out of the polyacrylamide gel onto the surface of the membrane, where the proteins become tightly attached. The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel. The transfer was done in specific saline buffer containing (Tris 250mM, glycine 1.92M, deionized water and methanol 20%v/v).

After the transfer it is important to block the remaining surface of the membrane to prevent unspecific binding of the detection antibodies during subsequent steps. Saturation is performed for 1 hour in a solution composed of non fatty milk 5% v/v (Ristora) and TBS1X (tris saline buffer) supplemented with tween 0,05%.

Saturation is followed by washing steps in TBS plus Tween-20 0,05% v/v (Sigma) in order to remove unbound reagents and reduce background.

The membrane is incubated for 1 hour with a primary antibody that recognizes a specific protein or epitope on a group of proteins. The primary antibody is not directly detectable. Therefore, tagged secondary antibodies are used as the means of ultimately detecting the target antigen (indirect detection). Our secondary antibodies were enzymatically labelled with Horseradish peroxidase (HRP), which is conjugated to the antibodies. After a final series of washes,

antibodies on the membranes are ready to be detected. An appropriate chemiluminescent substrate, which produces light as a byproduct, is then added to the membrane. The light output can be captured using film, (Kodak). The intensity of the signal should correlate with the abundance of the antigen on the membrane. We used different chemiluminescent substrates:

-Pierce ECL western blotting substrate (Thermo Scientific);

-Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific);

-LiteAblot EXTEND Long Lasting Chemiluminescent Substrate (Euroclone).

In order to detect more antibodies with the same specificity and similar molecular weight it is necessary to strip the membrane. Stripping buffer reagent (Thermo scientific) allows the cleaning and the efficient removal of primary and secondary antibodies from immunoblots without removing or damaging the immobilized antigen. This allows blots to be re-probed with new antibodies. Membranes are covered with this buffer and incubated for 10'-15' at room temperature; some washes in TBS are performed and finally the membrane can be saturated again with milk.

3.7. Antibodies.

We used antibodies directed against the following human proteins:

Western blot antibodies:

Primary antibodies: anti-rabbit CK2α provided by Dr. S. Sarno, University of Padova, Italy; anti-mouse CK2β (BD Biosciences, USA); anti-mouse PML (Santa Cruz Biotechnology, INC, California,USA); anti-rabbit RARα (Santa Cruz Biotechnology, INC, California,USA); anti-mouse p21 (BD Pharmingen, USA).

Secondary antibodies: anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology); HRP labeled goat anti-mouse IgG (KPL, Gaithersburg, MD, USA).

Flow cytometry antibodies: anti-CD11b (Becton Dickinson, S.Jose, California, USA); CD 11c (Becton Dickinson, S.Jose, California, USA) labeled with PE; CD11c-PC5 (Beckman Coulter, Marseille, France).

Immunofluorescence antibodies:

Alexa Fluor 488 goat anti-mouse IgG; Alexa Fluor 594 goat anti-mouse IgG; Alexa Fluor 488 goat anti-rabbit IgG; Alexa Fluor 594 goat anti-rabbit IgG. (Invitrogen, Molecular Probes, Oregon, USA).

3.8. RNA purification.

RNA was purified using RNeasy mini kit (Quiagen). This procedure represents a well-established technology that combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100ug RNA longer that 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. RNA is then eluted in water. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides are excluded.

Protocol:

Cells are collected and wash, removing the medium; then the appropriate volume of RLT lysis buffer, that contains guanidine-thiocyanate, is added (350 μl for $<5x10^6$ cells, 600 μl for $5\text{-}9x10^6$ cells). RLT is supplemented with β-mercaptoethanol 1:100 v/v, which inhibits RNases further. Samples are

homogenized by vortexing and then 70% ethanol is added. After pipetting, lysed samples are transferred to RNeasy spin columns and centrifuged at 11000rpm for 1′, discarding the flow-through. RNA bound to the silica membrane is washed with buffer RW1 and centrifuged at 1000rpm for 1′; a mix of DNase and buffer RDD (10µl and 70 µl respectively) are added directly on the membrane and keep in incubation for 15′-30′, in order to remove contaminant DNA. Afterwards a series of washes are performed, first of all with buffer RW1 (700µl) and then with buffer RPE (500µl) (containing ethanol). Samples are centrifuged at12800rpm for 2′ paying attention that the membrane is dry from ethanol residues. At the end RNA is eluted using 30µl of RNase free water.

RNA was quantified by means of Nanodrop 1000 (Thermo Scientific).

3.9. RNA reverse transcription.

Reverse transcription is a reaction that exploits a RNA-dependent polymerase that is capable to synthesize a complementary strand of DNA, called cDNA, using a RNA strand as template.

RNA was reverse transcribed to cDNA by means of Promega system (USA). AMV, namely *Avian myeloblastoma virus,* is the reverse transcriptase enzyme used. AMV sythesizes single stranded cDNA from total or poly(A) isolated RNA; it shows polymerase activity from 5′ to 3′ versus, and RnaseH activity from 3′ to 5′, degrading the RNA strand when the hybrid cDNA/RNA is formed. The reaction takes place in 20µl of final volume:

-$MgCl_2$ (25mM)	4µl
-reverse transcription 10X buffer	2µl

-dNTPs mix (10mM) 2μl

-Oligo dT primer (0,5mg/ml) 1μl

-rRNasin RNase inhibitor 0,5μl

-AMV Reverse Transcriptase 0,75μl

-RNase free $H_2 0$ to final volume of 20μl

Then samples undergo the following thermal protocol:

42°C for 15′

95°C for 5′

4°C maintenance

3.10. Real-time PCR.

The real-time PCR is a method to quantify nucleic acid characterized by an high sensibility and specificity. This is called "real-time PCR" because it allows the scientist to actually view the increase in the amount of DNA as it is amplified. This is possible because it detects and quantify fluorescent molecules: these compounds bind the amplified DNA and emit a signal that increases in a proportional way with the rise of the amplified products. We obtain an amplification curve where the cycle numbers are found in abscissa and the fluorescence normalized on internal fluorophore is in ordinate. At the beginning of the reaction there are only little changes in fluorescence and this is called baseline region; the increasing of fluorescence above this threshold underline amplified product formation. From this point the reaction performs an exponential course that degenerate in plateau at the end of the reaction.

In the midway cycles the curve has a linear course: it represents the most important phase since in this stage the amount of amplified DNA is correlated with the amount of cDNA expressed at the beginning in the sample. In this

linear region a threshold of fluorescence is chosen: from this value it is possible to obtain the Ct (threshold cycles), namely the cycle that are necessary, for the sample, to reach that threshold of emission (Fig.17). If the amount of cDNA present at the beginning in the sample is high, the curve will rise earlier and Ct values will be smaller.

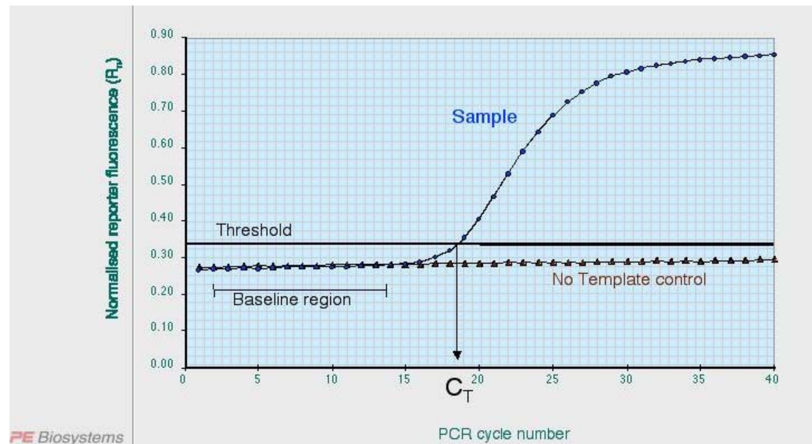

Fig.17. Amplification curve. Cycle numbers are found in abscissa, the fluorescence normalized on internal fluorophore is found in ordinate.

As detector we used SYBR GREEN: its molecules emit low levels of fluorescence if present in solution, on the contrary the signal becomes stronger if the dye binds to double strand DNA. However SYBR GREEN is not a selective dye and binds to all DNA, even to primer dimers. For this reason it is recommended the introduction of a further step after amplification, that is the dissociation protocol: temperature rises gradually until all the DNA double strands are denaturated; this method allows the identification of contaminants or unspecific amplification products since they shows different melting temperatures. There is also a second dye called ROX; it works as an internal reference used by the instrument to normalize the SYBR GREEN fluorescence.

For the evaluation of gene expression we chose a relative quantification method, using the ΔΔ Ct formula [103]:

1) ΔCt= Ct (target gene)- Ct (reference gene)
2) $\Delta\Delta$ Ct= ΔCt (of treated sample)- ΔCt (of untreated sample, the internal calibrator)
3) $2^{\wedge-\Delta\Delta C}$

The "2"value in the last formula represents the higher efficiency for reaction that means a doubling of the product at every cycle of amplification.

The thermalcycler used is the Sequence Detection System 7000 (Applied Biosystem) and the software is ABI PRISM 7000.
The reagents of the following reaction mix are provided by Invitrogen:

-Platinum SYBR GREEN supermix	6,25μl
-ROX	0,25μl
-Forward primer (10pmol/ μl)	1μl
-Reverse primer (10pmol/ μl)	1μl
-H_2O	4,7μl
-$MgCl_2$	0,3μl
cDNA	1,5μl
Final volume	20μl

Platinum Sybr Green supermix contains:
-SYBR GREEN fluorochrome;
-the Uracil DNA Glycosylase enzyme (UDG), that allows the removal of contaminant DNA, called carryover, coming from previous amplifications. Indeed UDG recognizes deossiuridine residues, that commonly substitute timidine residues in dNTPs mix, and degrade this carryover DNA, preserving cDNA. UDG is activated at 50°C and this step precede the amplification protocol; then it is inactivated at 95°C.
-Platinum Taq DNA Polymerase is an hot start polymerase.

Amplification protocol

-UDG activation	50°C 2′	
-Polymerase activation	95°C 10′	
-Denaturation	95°C 30′′	for 40 cycles
-Annealing and amplification	60°C 60′′	

-Dissociation protocol: increasing temperature from 60°C to 95°C.

The sequences of primers, used in real-time PCR, were found using Primer Express program (Applied Biosystem) (Table 2).

Table 2. Real-time primer sequences.

HUMAN GENE	FORWARD PRIMER (5′-3′)	REVERSE PRIMER (5′-3′)
βactin	TGC CGA CAG GAT GCA GAA G	CTC AGG AGG AGC AAT GAT CTT
GAPDH	GCA AAT TCC ATG GCA CCG T	AGC CTT CTC CAT GGT GGT GAA
P21	GCA GAC CAG CAT GAC AGA TTT C	AAG GCA GAA GAT GTA GAG CGG
C-EBPε	TGT GGC GGT GAA GGA GGA	TAC ACA AGG GCA AGA AGG CAG
CK2α	TCT GAA GGC CCT GGA TTA TTG T	TCG AGA GTG TCT GCC CAA GAT AT

3.11. NBT test.

The nitroblue-tetrazolium (NBT) test is the original and most widely-known test for chronic granulomatous disease (CGD). CGT is a diverse group of hereditary diseases in which certain cells of the immune system have difficulty forming the reactive oxygen compounds (most importantly, the superoxide radical) used to kill certain ingested pathogens. Phagocytes (i.e., neutrophils,

monocytes, and macrophages) require an enzyme to produce reactive oxygen species to destroy bacteria after their phagocytosis., This event is known as the respiratory burst. This enzyme is termed "phagocyte NADPH oxidase" (*PHOX*). The initial step in this process involves the one-electron reduction of molecular oxygen to produce superoxide anion, a free radical. Superoxide then undergoes a further series of reactions to produce products such as hydrogen peroxide (through the action of superoxide dismutase), hydroxyl radical and hypochlorite (bleach - through the action of myeloperoxidase on hydrogen peroxide). NBT test depends upon the direct reduction of NBT by superoxide free radical to form an insoluble formazan.. The higher the blue score, the better the cell is at producing reactive oxygen species.

In this work the NBT test was used to evaluate the functional maturation of myeloid cell lines and cells from patients after exposure to retinoic acid. NBT (Sigma) was dissolved in PBS 1X 1mg/ml; PMA was added to the solution at 3.10^{7} M as final concentration. 5.10^{5} cells were suspended in 500 μl of this solution and incubated 25′ at 37°C. To verify the formation of blu precipitates within cells, samples were spotted and fixed on slides through citospin and then coloured with May-Grunwald Giemsa method: 3′ in pure May-Grunwald; 3′in May-Grunwald diluted 1:2 v/v in water; 20′ in Giemsa diluted 20% v/v in water; final wash in water. At the end samples were analysed by means of Olympus CX 41 microscope.

3.12. Luciferase assay.

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Applications include the study of receptor activity, transcription factors, intracellular signaling, mRNA processing and protein folding. The term “dual reporter” refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Typically, the “experimental” reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected “control” reporter provides an internal control that serves as the baseline response. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by differences in cell viability or transfection efficiency. The control reporter is the TK-RL plasmid (Promega, USA), which contains the Renilla luciferase gene (Renilla reniformis, laso known as sea pansy); the experimental reporter is the PGL3- Luc plasmid (Promega, USA) where the firefly (Photinus pyralis) luciferase gene is integrated. The PGL3 vector was modified through the insertion of a promoter tht contains TATA box and retinoic acid response elements (RARE) such as DR1 and DR5 regions (Fig.18).

Lipofectamine 2000 (1mg/ml; Invitrogen) was used for transfection. Hela cells were seeded ($5X10^5$ cells/well) in 24 well plates. They were harvested in DMEM without antibiotic. When they reached 80% confluence, Hela were transfected with:

-50µl OPTIMEM medium+0,8µg DNA (TK-RL+PGL3);

-50µl OPTIMEM medium+2µl lipofectamine as negative control.

Lipofectamine was combined with DNA in ratio 1:3.

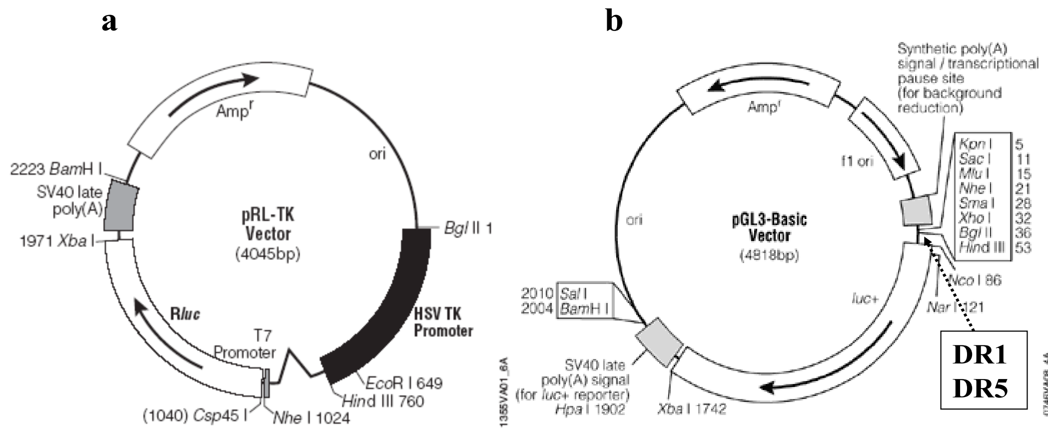

Fig.18. Structure of the plasmid vectors for the luciferase assay (a) p-RL-TK control vector; (b) pGL3 experimental vector modified with the introduction of DR1 and DR3 RARE regions.

We chose relatively small quantities of the control reporter vector to provide low-level, constitutive expression of that luciferase control activity. We used ratio of 10:1 for experimental vector/co-reporter vector combinations in order to suppress the occurrence of *trans* effects between promoter elements. After 6 hours the medium was removed and new fresh DMEM plus antibodies was added to cells. We waited 48 hours and then Hela were treated with retinoic acid and CK2 inhibitors for 6 hours. Cells were lysed directly in the plate with 100μl of passive lysis buffer. 20μl of lysate were then transferred in the luminometer tube. Gene expression study was performed by means of the Dual-Luciferase Reporter (DLR) Assay System (Promega) which provides an efficient means of performing dual reporter assays. In the DLR assay, the activity of firefly and *Renilla luciferase* are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LARII) to generate a "glow-type" luminescent signal. After quantifying the firefly luminescence, this reaction is quenced, and the *Renilla luciferase* reaction is initiated by simultaneously adding Stop and Glo Reagent to the same tube. This last reagent also produces a "glow-type" signal from the

Renilla Luciferase, which decays slowly over the course of the measurement. The luciferase signals were detected by TD-20/20 luminometer (Turner Designs).

3.13. RNA interference.

RNA interference (RNAi) is a process within living cells that moderates the activity of their genes. Two types of small ribonucleic acid (RNA) molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can bind to other specific messenger RNA (mRNA) molecules and either increase or decrease their activity, for example by preventing an mRNA from producing a protein. RNA interference has an important role in defending cells against parasitic genes – viruses and transposons – but also in directing development as well as gene expression in general.

In this work we used RNAi to down-modulate CK2 expression. In particular we chose siRNA oligos provided by Dharmacon,Thermo Scientific,: it consists of a pool of oligos directed against different regions of CK2 mRNA (CK2α or CK2β) (Table 3), a control oligo no target, and a siGLO GREEN oligo labeled with 6-FAM, used to verify transfection efficiency. Since NB4 is not an adherent cell line, transfection with lipofectamine is not efficient.

Therefore we performed electroporation using the Amaxa NUCLEOFACTOR system (Lonza).

$2X10^6$ cells were centrifuged at 90g for 6′. Medium was removed and pellet was resuspended in 100μl nucleofector solution V. 200pmol siRNA oligos, namely control oligos and oligos directed against CK2α or CK2β mRNA were added to the cellular suspension. To determine transfection efficiency cells were also transfected with siglo green oligo. Cells were electropotated using the program X-001 and then transferred in pre-warmed medium. Transfection efficiency was monitored 24 hour later through flow cytometry.

The down modulation of these genes CK2α or CK2β was verified at 72 hours through western blot and real-time PCR.

Table 3. siRNA sequences directed against CK2α or CK2β mRNA

Target gene	ON-TARGETplus SMART pool siRNA oligo sequences
CK2α	GCAUUUAGGUGGAGACUUC
	GGAAGUGUGUCUUAGUUAC
	GCUGGUCGCUUACAUCACU
	AACAUUGUCUGUACAGGUU
CK2β	CAACCAGAGUGACCUGAUU
	GCAAGGAGACUUUGGUUAC
	GCAAUGAAUUCUUCUGUGA
	CCAAGUGCAUGGAUGUGUA

Adherent Hela cells were transfected by means of Lipofectamine 2000 (1mg/ml; Invitrogen) with plasmid vector expressing shRNA (short hairpin RNA) directed against CK2α mRNA or control plasmid. We chose Origene retroviral silencing plasmids (pRS) that contain murine retroviral long terminal repeats (LTR), the puromycine resisistance gene, and U6 small nuclear RNA gene promoter. The gene specific shRNA expression plasmids were constructed using synthetic oligonucleotides cloned into Bam/Hind III cloning sites. Each of the shRNA expression plasmids has 29 base pair gene specific sequence insert immediately downstream of the U6 promoter (Fig.19). Origene provided a control plasmid vector without the shRNA cassette (TR 20003) and a pool of four shRNA plasmids against CK2α. We tested the different plasmids and finally we chose the combination of two vectors, TI378-488 and TI378-490, that gave the better down-regulation of CK2α (Table 4). To verify transfection efficiency Hela were cotransfected with pharnesilated GFP (phGFP).

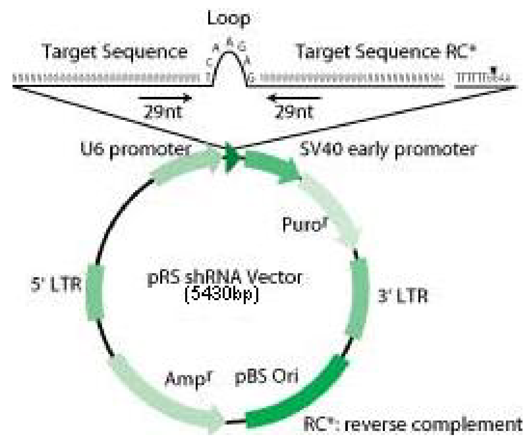

Fig.19. pRS vector map

Cells were seeded in six well plates at $4X10^5$ density in DMEM medium without antibiotic. When cells reached 50% of confluence they were transfected with plasmids-lipofectamine complexes prepared as follows:

-3µg DNA (2µg shRNA+1µg phGFP)+250µl OPTIMEM;

-7,5µl Lipofectamine (1:2,5 ratio DNA/Lipo)+250µl OPTIMEM

-the diluted plasmids were combined with the diluted Lipofectamine and incubated for 20′ at room temperature ;

-the oligomer-Lipofectamine complexes were added to each well containing cells.

-medium was changed after 6 hours and substituted with DMEM plus antibiotic.

Table 4. shRNA vectors used against *CK2α* mRNA.

Identification number	29 base pair gene specific sequences
TI378-488	AAGGCTTGTCAAGCAGTGTGCTCATCACA
TI378-490	TCTACACTGTTGTGAAGGACCAGGCTCGA

3.14. Immunofluorescence.

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on biological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. Immunofluorescence is a widely used example of immuno-staining and is a specific example of immunohistochemistry that makes use of fluorophores to display the location of the antibodies. Immunofluoresence can be used in combination with other, non-antibody methods of fluorescent staining, for example, use of DAPI to label DNA. Several microscope designs can be used for the analysis of immunofluorescence samples; the simplest are the epifluorescence microscope, and the confocal microscope.

Confocal imaging aims to overcome some limitations of traditional wide-field fluorescence microscopes. In a conventional fluorescence microscope, the entire specimen is flooded evenly in light from a light source. All parts of the specimen in the optical path are excited at the same time and the resulting fluorescence is detected by the microscope's photodetector or camera including a large unfocused background part. In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal (the name "confocal" stems from this configuration). As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes.

As only one point in the sample is illuminated at a time, 2D or 3D imaging requires scanning over a regular raster (i.e. a rectangular pattern of parallel

scanning lines) in the specimen. The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical aperture of the objective lens, but also by the optical properties of the specimen. The thin optical sectioning possible makes these types of microscopes particularly good at 3D imaging and surface profiling of samples.

Procedure:

Glass slides were washed in ethanol 70% v/v and dry.Polylisine was diluted in water 1:10 ratio and 50µl of the solution were pipetted on the glass slide and incubated for 10′. Polilysine should create a film which is necessary for cell adhesion. 5.10^4 cells are put on the glass and incubated for 15′ at 37°C. In this stage cells adhere to the glass. For Hela cells polylisin step was not required, because adherent cells can be grown directly on glass coverlips. Cells were washed in PSB1X and fixed with paraformaldehyde for 15′; they were subsequently washed and permeabilized with 0,1% Triton X-100 for 10′.Then cells were washed and blocked in PBS supplemented with 5% BSA (bovine serum albumin) for 30′. This was followed by incubation with primary antibody for 1 hour. Three washes were carried out in PBS plus BSA before incubation with secondary antibodies conjugated with a fluorophore for 1 hour. Cell were finally washed with PBS, mounted with Vectashield (Vector laboratories) mounting medium containg DAPI, sealed with nail varnish, and left to dry before examination. Images were collected with Ultraview Nikon TE 200 (Perkin Elmer) confocal microscope or Leica TCS-SP2 laser scanning confocal apparatus coupled to a Leica microscope, using a 60X oil immersion objective.

3.15. Flow citometry.

Cell cycle analysis.

The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye, such as propidium iodide, that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content. Flow cytometry also enables the identification of the cell distribution during the various phases of the cell cycle. Four distinct phases could be recognized in a proliferating cell population: the G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). Diverse software containing mathematical models that fit the DNA histogram of a singlet have been developed in order to calculate the percentages of cells occupying the different phases of the cell cycle (Fig. 20).

In this work we used BD FACS Calibur flow cytometer. 1.10^6 cells were collected, centrifuged at 1600 rpm 5′ and washed with PBS 1X. Then they were fixed in 2ml of ethanol 70%v/v for 2′; then they were washed in PBS 1X to remove ethanol and the pellet was resuspended in 500μl of solution containing:

-RNase (0,2 mg/ml)

-Propidium iodide (5μg/ml)

-PBS1X

Cells were incubated for 30′ at room temperature and then citometer analysis could be performed.

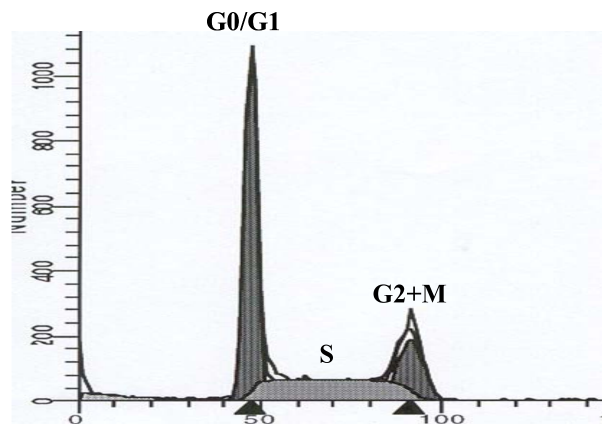

Fig.20. Different stages of cell cycle.

Apoptosis analysis.

After treatment of AML cells with CK2 inhibitors and retinoic acid, apoptosis was evaluated using the Apoptosis Detection Kit (Immunostep, Italy).
AnnexinV is a member of a highly conserved protein family that binds acidic phospholipids in a calcium-dependent manner. The protein has been shown to possess a high affinity for phosphatidylserine. Depending on the nature of the stimuli, phosphatidylserine is translocated from the inner side of the plasma membrane to the outer layer. When cells undergo death by apoptosis or cell necrosis, exposed phosphatidylserine serves as one of several signals by which cell, that are undergoing apoptosis, are recognized by phagocytes. If AnnexinV binds to the cell surface, this indicates that cell death is imminent. In order to differentiate apoptosis from necrosis, a dye exclusion test with propidium iodide is performed to establish whether membrane integrity has been conserved or whether membranes have become leaky. A combination test measuring Annexin-5 binding and dye exclusion thus allows discrimination between intact cells, apoptotic cells, and necrotic cells.

$2X10^5$ cells were washed in PBS to remove medium and resuspended in 100µl of binding buffer; 5µl of AnnexinV-Fitc were added and cells were incubated for 10′ at room temperature in the dark. 100µl of binding buffer were further added to the cell suspension and DNA was stained with 10µl of propidium iodide, added immediately before proceeding with flow cytometry analysis.

Cell maturation analysis.

To study promyelocytic cell maturation after exposure to retinoic acid and CK2 inhibitors, we evaluated terminal granulocytic differentiation markers expression, such as CD11b and CD11c by flow cytometry. $2X10^5$ cells were washed in PBS to remove medium; the cells were stained with 4-5µl of CD11b or CD11c antibodies conjugated with a fluorocrome and incubated at room temperatuire in the dark for 15′.
Cell pellets were washed in PBS to remove not bound antibody, resuspended in 200 µl of PBS and cytofluorimeter analysis was performed.

3.16. CK2 activity assay.

Treated samples were lysed with the buffer described previously in paragraph (3.3). 1 µg of protein extract was incubated for 10′ at 30°C in presence of tris-HCl 50mM(pH 7.5), $MgCl_2$ 12mM, ATP 10µM, [γ-^{P}]ATP 10µM (about 3000 cpm/pmol), NaCl 0.1M and the specific synthetic substrate (1mM) in a final volume of 20µl. The synthetic peptide $R_3AD_2SD_5$ was produced and provided by Dr. O. Marin (Padova). The reaction is stopped through absorption on phosphocellulose papers; a series of three washes are performed by means of phosphoric acid 0,5% (v/v), in order to remove the [γ-^{P}]ATP in excess that CK2 did not use for phosphorylation. The radioactivity level was evaluated by liquid

scintillation. Phosphocellulose papers were put into scintillation solution that converts the kinetic energy of nuclear emission in UV light beam energy.

3.17. Statistical analysis.

Experiments were repeated at least three times. We used t-test to analyze data as appropriate. We considered p values of 0,05 or 0,01 as significant. Analyses were performed by means of Excel (Microsoft Office) or Origene 7.0 software.

4. RESULTS

4.1 CK2 is up-regulated and its activity is high in AML cell lines.

CK2 kinase activity and CK2α subunit expression were evaluated in APL cell lines HL60 and NB4. Normal PBMC and CD34+ hematopoietic stem cells (which represent the healthy counterpart of acute myeloid leukemia blasts) were used as control. mRNA levels were determined by means of Real Time PCR: CK2α expression was significantly increased in APL cell lines compared to PBMC and CD34+ samples (Fig. 21A).

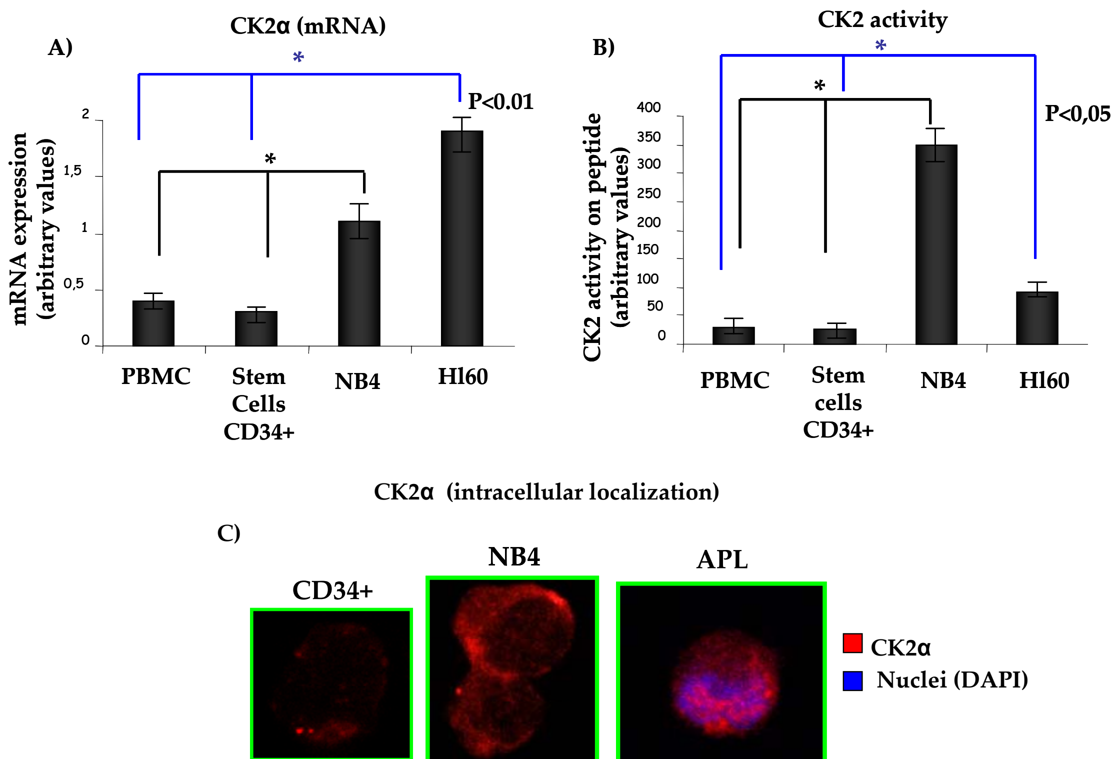

Figure 21. CK2 expression and activity in APL cells. CK2α mRNA expression (A) and activity (B) in NB4 and HL60 cells; PBMC and CD34+ were used as healthy controls; ($p< 0.01$ between NB4 and control sample mRNA; between HL60 and control sample mRNA) ($p<0.05$ between NB4 and control sample activity; between HL60 and control sample activity). C) Imunofluorescence analysis of CK2α intracellular localization. Results are representative at least of three independent experiments.

In vitro kinase assays were performed on whole cell lysates using a synthetic peptide specific for CK2: NB4 cells showed much higher levels of CK2 activity compared to the other control groups. Kinase activity was elevated also in HL60 cell line but at a lower extent compared to NB4 cells (Fig. 21B).
Immunofluorescence experiments were conducted using an antibody direct against the α subunit to study its intracellular localization. We determined a higher expression of CK2α both in NB4 and in APL cells from patient than in CD34+ sample; moreover, the catalytic subunit exhibited a diffuse localization both in the nucleus and cytoplasm but resulted more abundant in this last compartment (Fig. 21C).

4.2 CK2 expression is modulated in M3 NB4 cells induced to differentiate by retinoic acid treatment.

Since NB4 cells are confined in the promyelocytic stage, due to the t(15;17) chromosomal translocation, and this blockade can be overcome by retinoic acid (RA) exposure, we chose NB4 as a model of granulocytic differentiation. In this context we asked whether CK2 expression (of both catalytic and regulatory subunit) and activity could be influenced by treatment with retinoids.
NB4 were exposed to RA (1μM) for 24, 48, 72 hours and then CK2 expression was evaluated by Real Time PCR and Western Blot analysis: the α catalytic subunit expression remained elevated for the first 24 hours and then in the following 48 hours began to decrease compared to untreated samples (Fig. 22A; B); on the contrary the β regulatory subunit expression was already reduced at 24 hours upon RA treatment, showing a different modulation (Fig. 22B). Surprisingly, the kinase activity did not change until 48 hours despite the relevant decrease of the β subunit (Fig. 22C).

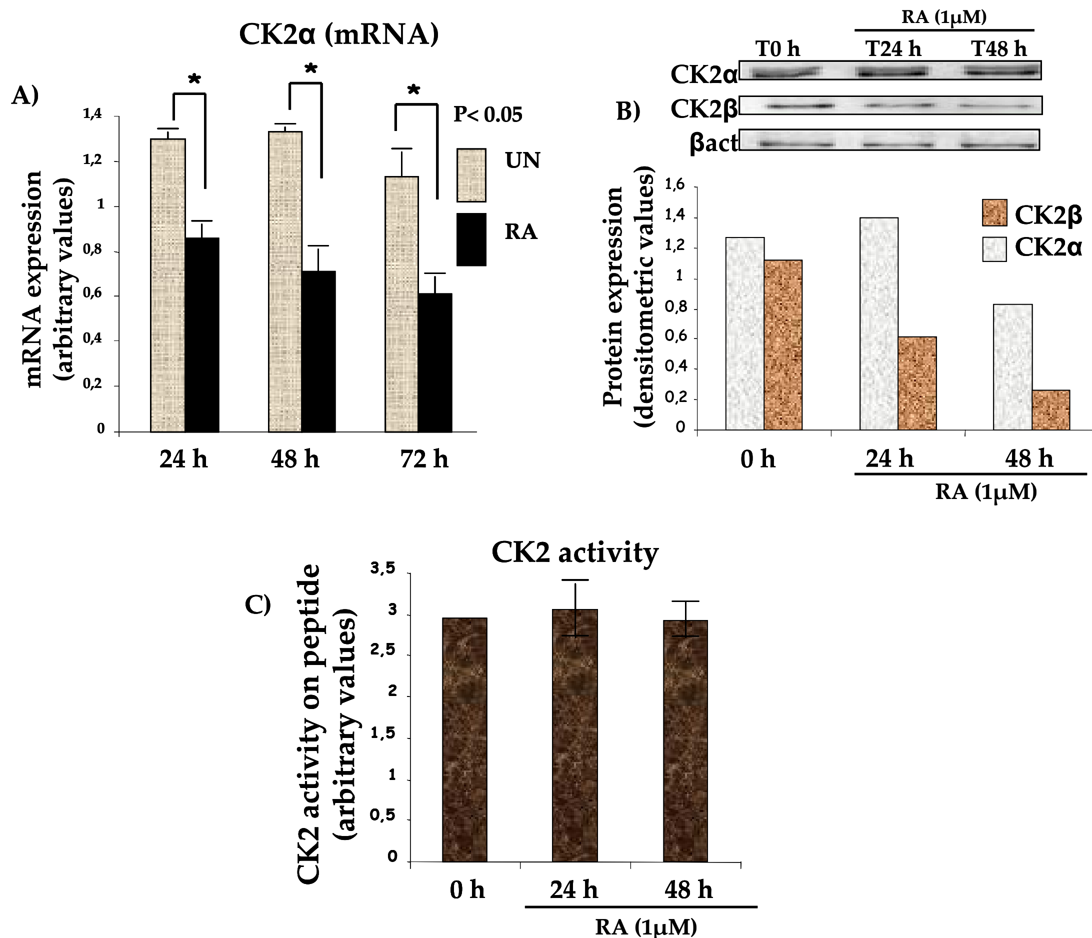

Figure 22. CK2 expression and activity in NB4 cells induced to differentiation by RA. (A) CK2α mRNA expression after addition of RA for 24, 48 and 72 hours; untreated cells (UN), used as control, were plated in RPMI medium and FBS (10%) without the stimulus and collected at the same time points ($p<0.05$ between RA-treated samples and untreated samples). (B) Western Blot and densitometrc analyses of CK2α and CK2β expression on whole cell lysate after treatment with RA for 24 and 48 hours; data were normalized on βactin (βact). (C) CK2 kinase activity upon exposure of NB4 cells to RA for 24 and 48 hours.

4.3 CK2 blockade inhibits RA-induced differentiation of APL cells.

To elucidate a possible involvement of CK2 in the differentiation process induced by retinoic acid, APL cells (NB4, HL60 cell lines and blasts from patients) were treated with RA in presence of CK2 inhibition. CK2 specific

inhibition in these cells was verified in previous experiments: kinase assays were performed on whole cell lysate of NB4 cells, treated with rising concentration of CK2 inhibitor K27. CK2 activity showed a dose-dependent reduction upon exposure to K27 (data not shown). Moreover, after K27 treatment for 48 and 72 hours cells viability was evaluated by annexinV/IP staining in order to identify the optimal dose of inhibitor, which could block the kinase without compromising heavily the cell viability: the best subapoptotic concentration of K27 resulted to be 3µM (data not shown). After these preliminary experiments, APL cells from patients and cell lines, were treated for 24 or 48 hours with RA (1µM) alone or in combination with K27 (3µM), or treated with the vehicle used to dissolve the stimulus (DMSO 0,1%) as control. Cells exposed to RA alone revealed at flow cytometry analysis a significant increase of the surface granulocytic differentiation markers CD11b and CD11c compared to untreated sample (UN). On the contrary, the combined exposure to RA and K27 caused a marked reduction of both CD11b and CD11c expression (Fig.23 A, B, C,). Any significant change in their expression was not detected after treatment with K27 alone. To exclude the possibility that the differentiation impairment after CK2 blockade was due to an unspecific effect of K27, we decided to use other approaches: 1) testing a new inhibitor of CK2, CX-4945, the most recent and specific inhibitor of this kinase already used in phase I clinical trials for multiple myeloma [102]; 2) using RNA interference against the alpha catalytic subunit of CK2. Treatment of NB4 cells with CX-4945 (5µM) and RA for 24 hours determined the reduction of the CD11c surface marker exposure (Fig. 23D), corroborating the results previously obtained with K27.

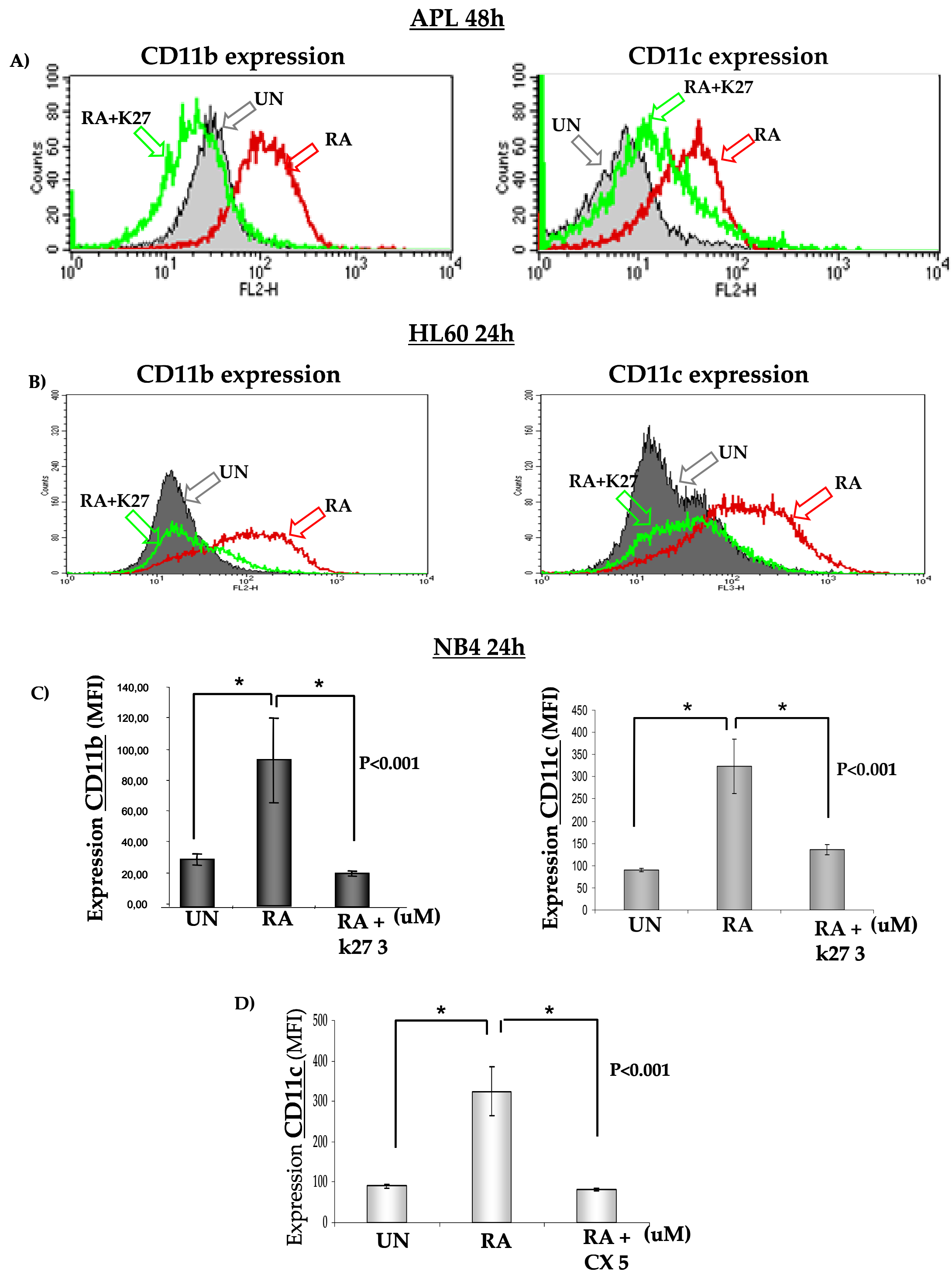

Figure 23. Flow cytometry analysis of the RA induced granulocytic differentiation markers expression in the absence or presence of CK2 inhibitors. CD11b and CD11c expression in APL blasts from patient (A) treated with RA (1μM) and K27 (3μM) for 48 hours, in HL60 (B) and NB4 (C) treated with the same stimuli for 24 hours (p<0.001). (D) CD11c expression in NB4 cells treated for 24 hours with RA in combination with the more specific CK2 inhibitor CX-4945 (5 μM) (p<0.001). Data in (C) and (D) are represented as medium fluorescence intensity (MFI).

To perform RNA interference, NB4 cells were electroporated using a mix of non targeting oligos (scr) or specific oligos against CK2α (200 pmol). After 48 hours the cells were treated with RA for 24 hours. Cells were also nucleofected with siglo green oligos (200 pmol) to asses the transfection efficiency that resulted of about 80-90% after 24 hours (data not shown). CK2 knock-down was confirmed by western blot analysis (Fig. 24A) and Real-Time PCR (0,49 mean value± SD 0,14; p< 0,001 compared to scr sample; Fig. 24B).

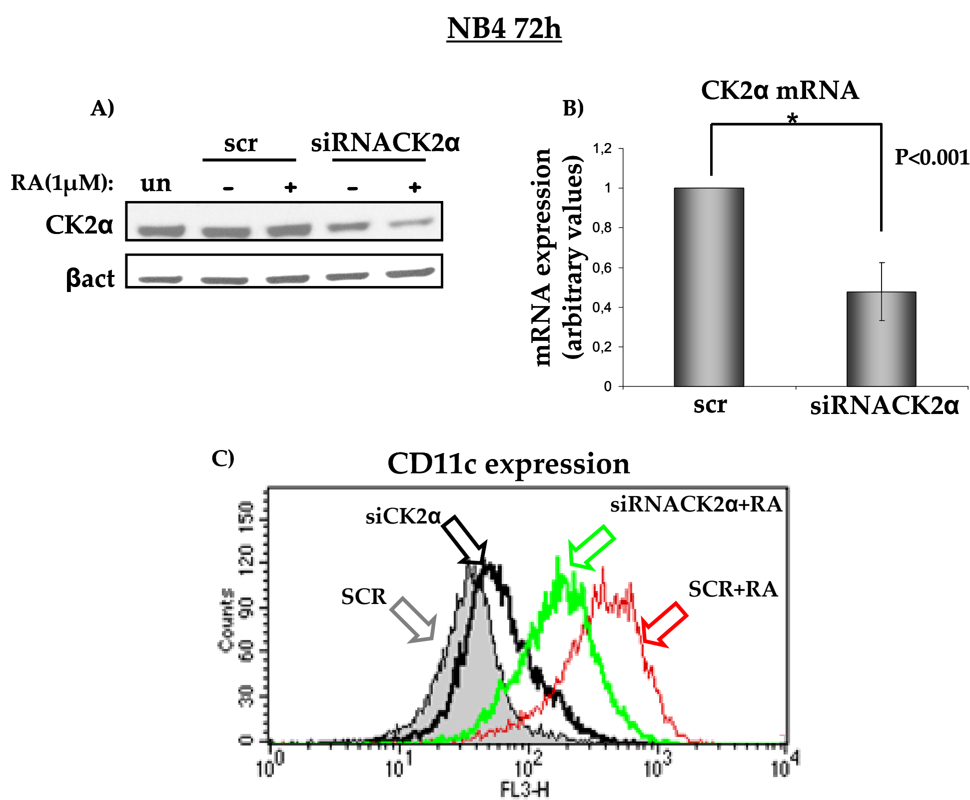

Figure 24. RNA interference against CK2α in NB4 cells. (A) Western blot analysis after 72 hours of CK2 silencing on whole cell lysate of untransfected sample (UN), sample transfected with no target oligos (scr), and sample transfected with CK2α oligos (siRNACK2α). CK2α expression was normalized on β actin (βact). (B) Real-time PCR of scr and siRNACK2α samples; expression is represented as relative quantification: siRNACK2α expression values were normalized over scr ($p<0.001$), βactin was used as housekeeping gene control; (C) Cytometer analysis of CD 11c granulocytic maker expression.

Next CD11c expression was analyzed: cells transfected with CK2α-specific siRNA displayed a significant reduction in the CD11c expression induced by RA as compared to samples transfected with scr oligos and treated with RA (Fig. 24C). Therefore siRNA experiments supported the results obtained with CK2 chemical inhibitors.

The differentiation blockade was also confirmed at a morphologic and functional level by means of May-Grunwald Giemsa staining and NBT test respectively.

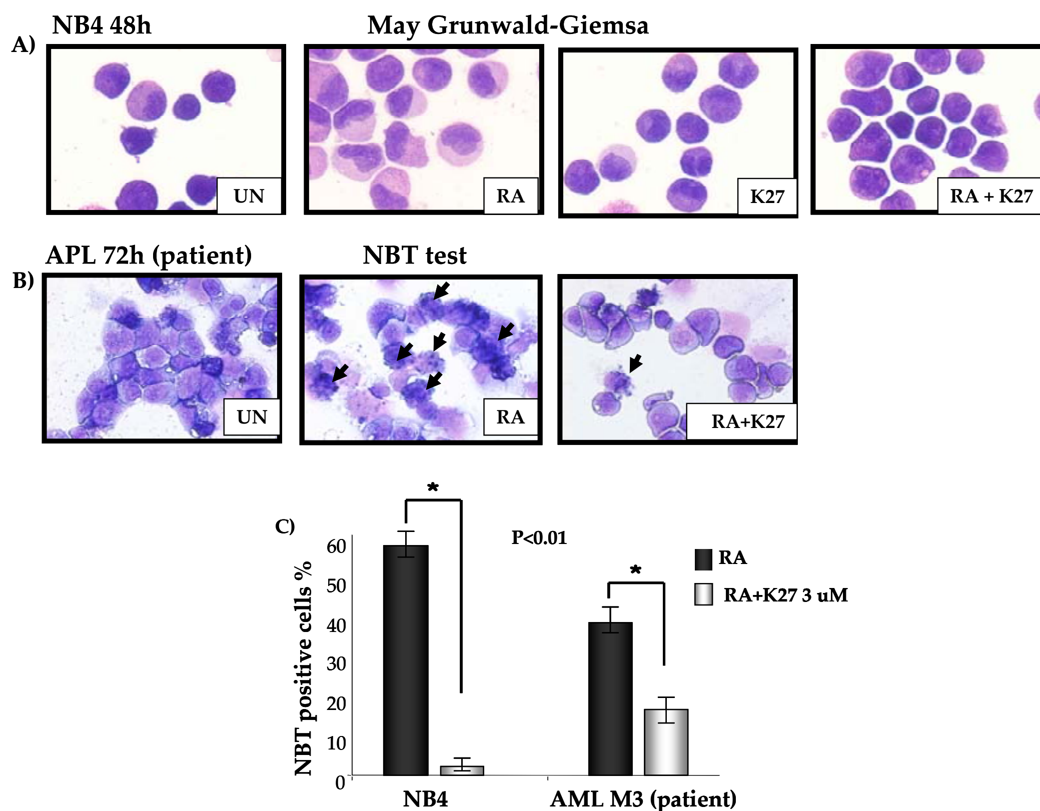

Figure 25. Morphologic and functional analysis of APL cells induced to differentiate with RA and CK2 blockade. (A) May Grunwald Giemsa staining of untreated (UN) NB4 cells, treated with RA alone, K27 or the combination of RA plus K27. (B) NBT test of APL cells from patient treated with the same compounds for 72 hours; the black arrows point out the intracellular precipitates, results of nitro blu of tetrazolium reduction. (C) NBT positive APL cells from patient and from NB4 cell lines; analysis was performed with optic microscope (p<0,01).

NB4 cell lines and APL cells from patient were treated for 48 or 72 hours with retinoic acid alone, K27 or with the combination of the two compounds. Cells were then collected, spotted onto a glass slide through cytospin and coloured.
After exposure to retinoic acid cells changed their morphology, showing a reduced nucleus/cytoplasm ratio, in favor of this last compartment, and a decreased basofilia (Fig. 25A). Moreover, they acquired the ability to produce superoxide anion: its synthesis can be evaluated indirectly through the reduction of the nitroblu of tetrazolium (NBT) which forms blu precipitates inside cells; thus, in presence of RA we could observe an increase on NBT positive cells (Fig. 25B). CK2 blockade in presence of the differentiation stimulus impaired these morphological changes and reduced significantly the number of NBT positive cells (Fig. 25B, C).

4.4 CK2 blockade does not determine the apoptosis of terminal differentiated cells.

Since CK2 blockade, over a certain dose of inhibitors (3μM), determines apoptosis, we asked whether the reduction of terminal differentiated cells after exposure to RA and CK2 inhibitors, was due to a process of cell death.
To test this hypothesis NB4 cells were treated for 24 hours with RA (1μM) alone or in combination with K27 (3μM); cells were stained with CD11b and AnnexinV and flow citometry analysis was performed. The percentage of $CD11b^+/AnnV^+$ cells after the combined treatment with RA and K27 did not increase (1% ± 1,5%) compared to exposure to RA alone (1,6% ± 2%) (Fig. 26). Therefore, these results allowed us to exclude the possibility that differentiated cells could undergo a preferential process of apoptosis.

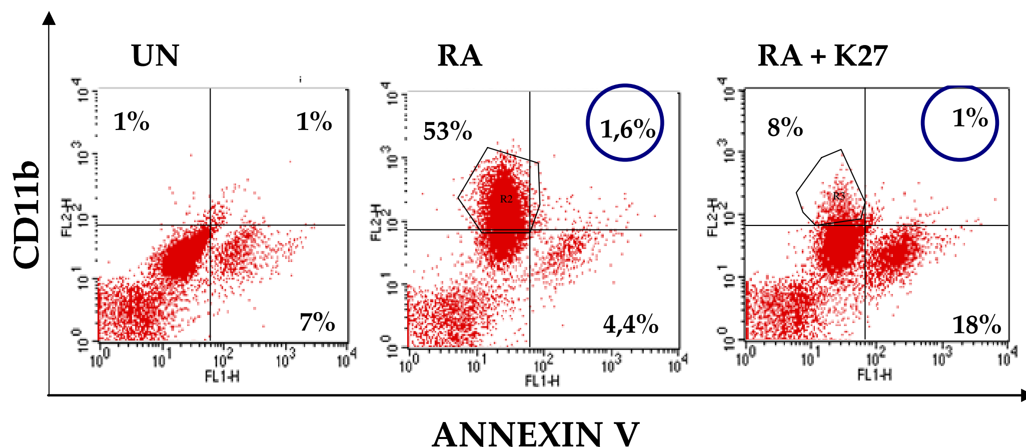

Figure 26. Flow cytometry analysis of CD11b expression and Annexin V staining in APL cells after induction to differentiation and CK2 blockade. In the X axis Annexin V staining and in the y axis Cd11b expression.The percentage of cells double positive for Annexin V and CD11b is circled. Results are representative at least of three independent experiments.

4.5 The inhibition of CK2 does not influence the reconstitution of PML nuclear bodies.

PML has a characteristic cellular localization in the, so called, PML nuclear bodies, where is complexed with other proteins [8]. It is now well established that the normal structure of the PML nuclear bodies is disrupted in APL: the fusion protein PML-RARα, which is firmly bound to DNA induces the delocalization also of the wild type PML through PML/PML/RARα heterodimers and the recruitment of the PML partners onto the cromatin [30]. ATRA treatment induces PML-RARα degradation and this event allows the reconstitution of PML-nuclear bodies. Since it is already known that CK2 interacts with PML [85], we tried to understand whether CK2 is involved in the reconstitution of PML nuclear bodies triggered by retinoic acid. Immunofluorescence analysis, using an antibody against PML, was performed in NB4 cells: in non treated cells PML showed a diffuse pattern; as expected, after exposure to RA for 24 hours the

integrity of normal PML-nuclear bodies was restored, which appeared in a macro-speckled pattern. K27 alone did not cause any change in PML distribution as compared to control samples. CK2 inhibition during differentiation did not seem to alter the reorganization of these RA-triggered nuclear structures. Thus, we could exclude the involvement of CK2 in this molecular mechanism.

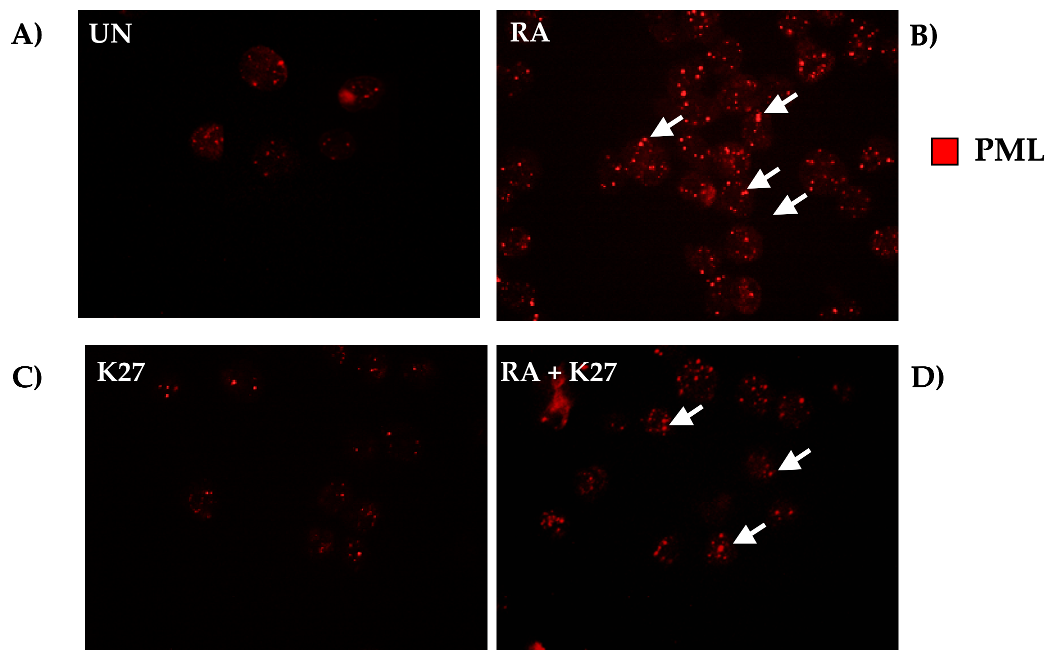

Figure 27. Immunofluorescence analysis of PML distribution in NB4 cells after treatment with retinoic acid and CK2 inhibition. RA (1μM) and K27 (3μM) NB4 treated cells were stained with an antibody directed against PML protein and analysed through fluorescent microscope. A) Control cells; B) Cells exposed to RA; C) Cells after treatment with K27 alone; D) Cells exposed to RA and CK2 inhibitor. The white arrows point out the reconstituted PML nuclear bodies that show a macro-speckled appearance.

4.6 CK2 inhibition prevents the ability of RA to induce APL cells blockade in G0/G1 phase.

In order to find a mechanistic explanation for the CK2 role in APL differentiation induced by retinoic acid, we focused on cell cycle. It is well supported in literature that exposure to RA is accompanied by a modification in cell cycle with an accumulation of cells in G0/G1 phase, an essential event for the subsequent differentiation [69]. Thus, we investigated the effects of CK2 inhibition on this process. NB4 cells were treated for 72 hours with RA (1μM), K27 (3μM), or the combination of both compounds. Similar experiments were carried out using also the more specific CK2 inhibitor, CX-4945 (5μM), for 24 hours. Cells were then fixed with ethanol and stained with propidium iodide (PI), which allows the determination of the DNA content and the identification of the cell amount present in each phase of cell cycle. Flow cytometry analysis (Fig 28A, B), as expected, showed an accumulation of cells in G0/G1 phase after exposure to retinoic acid (66% ± 5,4%; compared to control sample 43%mean ± SD 4%; $p<0,05$; Fig.28A, after 72 hours) (56% mean ± SD 1,3% compared to control sample 44,8% mean ± SD 0,6%; $p<0,05$; Fig. 28B, after 24 hours).

On the contrary, when RA was combined with K27, as well as with CX-4945, the differentiation stimulus was unable to induce the blockade in G0/G1 phase (44 mean ± SD 5%; $p<0,05$; Fig 28A, after 72 hours) (38% mean ± SD 3%; $p<0,05$; Fig. 28B, after 24 hours). The treatment with K27 or CX-4945 alone did not cause any significant change compared to untreated samples.

These results were strengthened by Real-Time PCR (Fig. 29A) and western blot (Fig.29B, C) analysis of p21 expression. P21 is important for cell cycle blockade mediated by RA and it was already demonstrated that its promoter contains RARE elements recognised by RARs receptors. [68]. NB4 cells and APL cells from

patients were collected after 6, 24 and 48 hours with RA and K27. Retinoic acid caused a significant increase of *p21* mRNA levels (2,2 mean ± SD 0,3 compared to untreated sample; p<0,01; Fig. 29A), that were not observed with K27 and RA combined treatment (0,8 mean ± SD 0,3; p<0,01; Fig 29A). A similar trend could be observed also for p21 protein expression (Fig.29B, C).

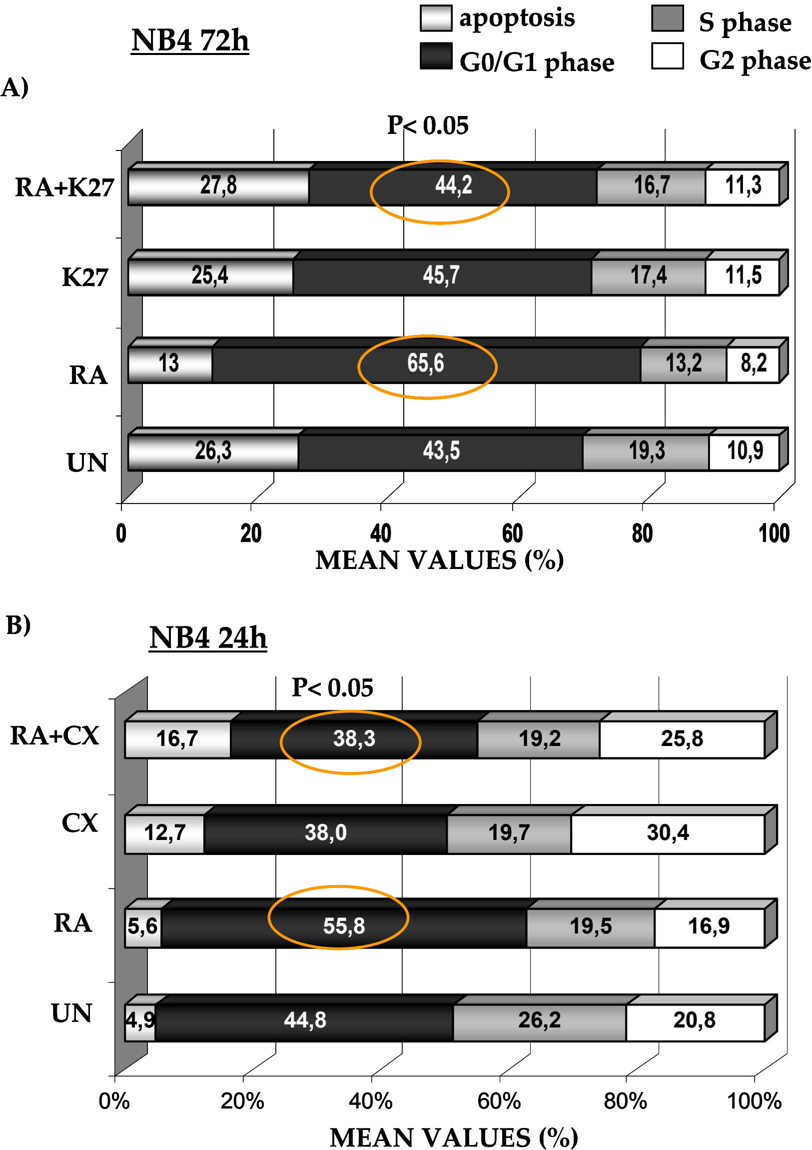

Figure 28. Cell cycle analysis during the retinoic acid induced differentiation in NB4 cells. The percentage of cells in each phase is represented in histograms in the x axis, treatments are indicated in the y axis; each phase is shown with a different color. Cell cycle analysis after treatment with RA±K27 for 72 hours (p<0,05 between RA alone and RA+K27) (A) and RA±CX-4945 for 24 hours (p<0,05 between RA alone and RA+CX4945) (B) .Results are representative of at least three independent experiments.

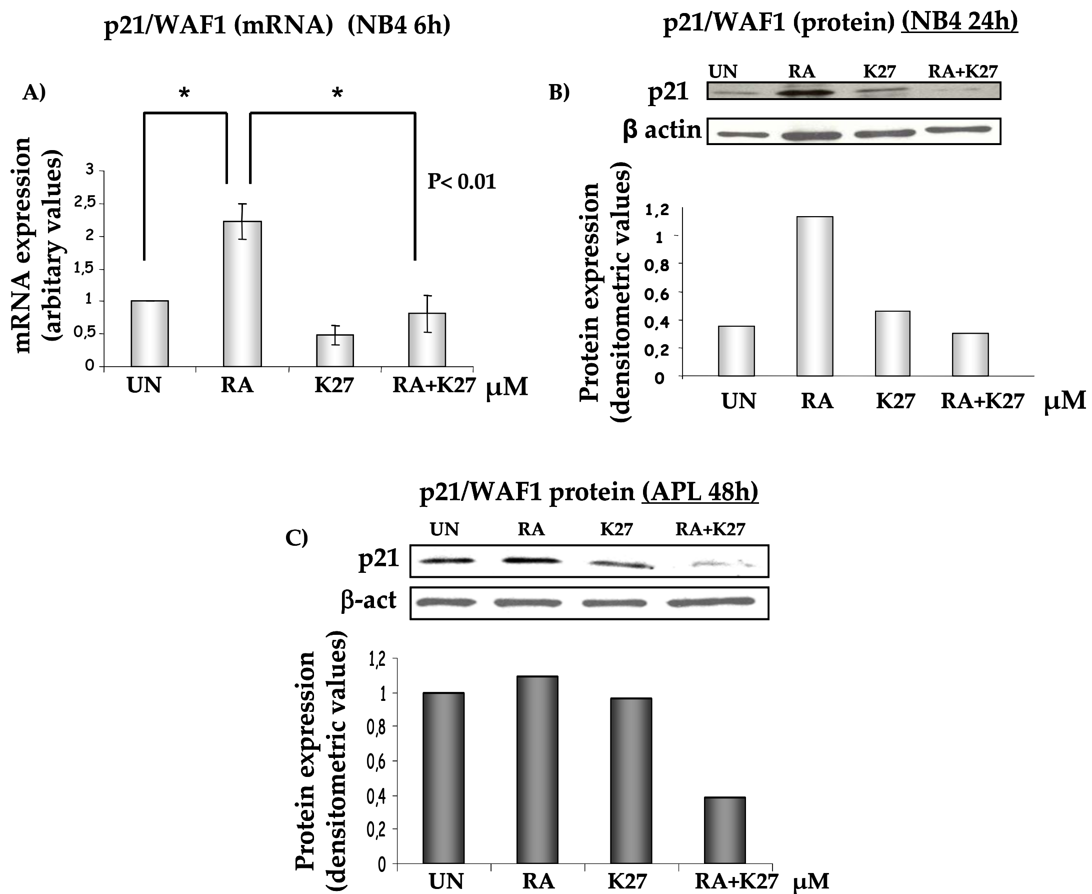

Figure 29. P21 expression in NB4 and APL cells from patient treated with RA and K27. A) p21 mRNA levels determined by Real Time PCR upon 6 hours of stimulation with RA±K27 (p<0,01 between RA and untreated samples; between RA+K27 and RA alone treated samples). βactin was used as housekeeping gene for normalization. Western blot and densitometric analysis of p21 expression on the blot above in whole cell lysate of NB4 untreated cells or treated for 24 hours with RA(1μM), K27 (3μM), or the combination of the two compounds (B) and APL cells treated for 48 hours (C); data were normalized on β actin expression

4.7 CK2 modulates RARα transcriptional activity induced by RA.

Next we asked whether CK2 could regulate RARα transcriptional activity. We tested this hypothesis by performing luciferase assays. Cervical carcinoma

HeLa cells were transfected with a plasmid vector, containing the luciferase gene under the control of RARα responsive sequences (RAR Elements), and with a control plasmid expressing luciferin, which allows an internal normalization of luminescence. Cells were treated for 6 hours with RA (1μM) alone or in combination with rising concentrations of K27 (Fig. 30A). RA determined an increase of luciferase expression (3,7 mean ± SD 1; $p<0,01$ between RA treated and control samples). On the contrary, the exposure to RA and K27 caused a dose-dependent inhibition of the luciferase activity, which resulted significant at the higher doses of K27 (3-5μM) compared to RA alone treated samples ($p<0,01$ and $<0,001$).

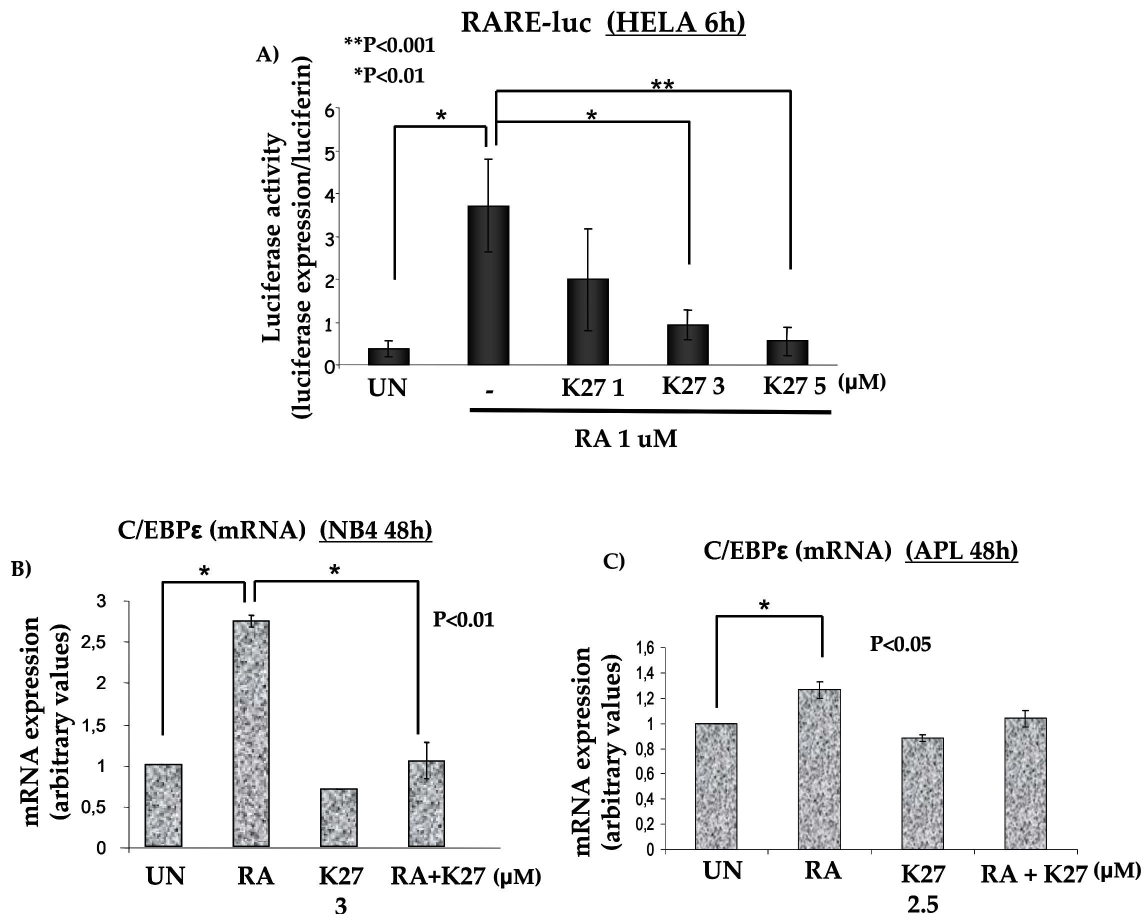

Figure 30. Transcriptional activity modulation in cells treated with RA and CK2 inhibitors. A) Luciferase assay on Hela cells transfected with a plasmid vector expressing Luciferase gene under the control of a promoter which contains RARE regions. Luciferase expression values were normalized on luciferin expression, which represents an internal control. B-C) mRNA levels of *C/EBPε* analysed by Real Time PCR in NB4 cells and APL cells.

To confirm RARα transcriptional inhibition we evaluated *C/EBPε* mRNA levels by Real Time PCR both in NB4 and APL cells from patient. This is a transcription factor, important for the expression of genes involved in terminal granulocytic maturation; moreover, it is well known that *C/EBPε* is a RARα target gene [104].

After 48h treatment with RA we observed a significant rise of *C/EBPε* compared to untreated samples both in NB4 (2,8 mean ± SD 0,1; p<0,01; Fig.30 B) and APL cells (1,29 mean ± SD 0,06; p<0,05; Fig. 30C); this increase was abrogated when K27 was added to RA (1,2 mean ± SD0,3; p<0,01 between RA+K27 and RA alone treated samples; Fig. 30B) (1 ± 0,06; Fig. 30C). In cells treated with K27 alone we did not observe any change in *C/EBPε* levels compared to control samples.

4.8 CK2 inhibition influences RARα intracellular localization.

We focused on RARα intracellular distribution to further investigate how CK2 could influence RARα transcriptional activity,.

In NB4 cells, as well as in APL blasts from patients carrying the t(15;17) translocation, wild type RARα has a nuclear localization, whereas the fusion protein PML-RARα is present both in the nucleus and the cytoplasm[105]. Immunofluorescence analysis was performed using an antibody recognizing both wild type RARα and and PML-RARα. NB4 and APL blasts in basal condition showed a pattern of RARα distribution as described above (Fig. 31A, B, left panels). Samples treated for 24 h with RA exhibited an increase of RARα nuclear levels with a macro-speckled appearance and at the meantime there was an accumulation in the *peri*-nuclear area likely corresponding to the endoplasmic reticulum (Fig. 31 A, B middle panels). After exposure to RA plus K27, RARα became diffuse in the nucleus and seemed to shuttle to the

cytoplasm (Fig. 31 A, B right panels). Next, we asked whether CK2 blockade without differentiation stimulus could determine any change in the receptor localization. We used HL60 as model: in non treated sample RARα has a nuclear distribution, since this cell line lacks PML-RARα fusion protein. After exposure to K27 for 24 hours cells displayed RARα translocation from the nucleus to the cytoplasm (Fig. 31C); thus, CK2 inhibitor used alone was sufficient to cause the intracellular redistribution of the receptor.

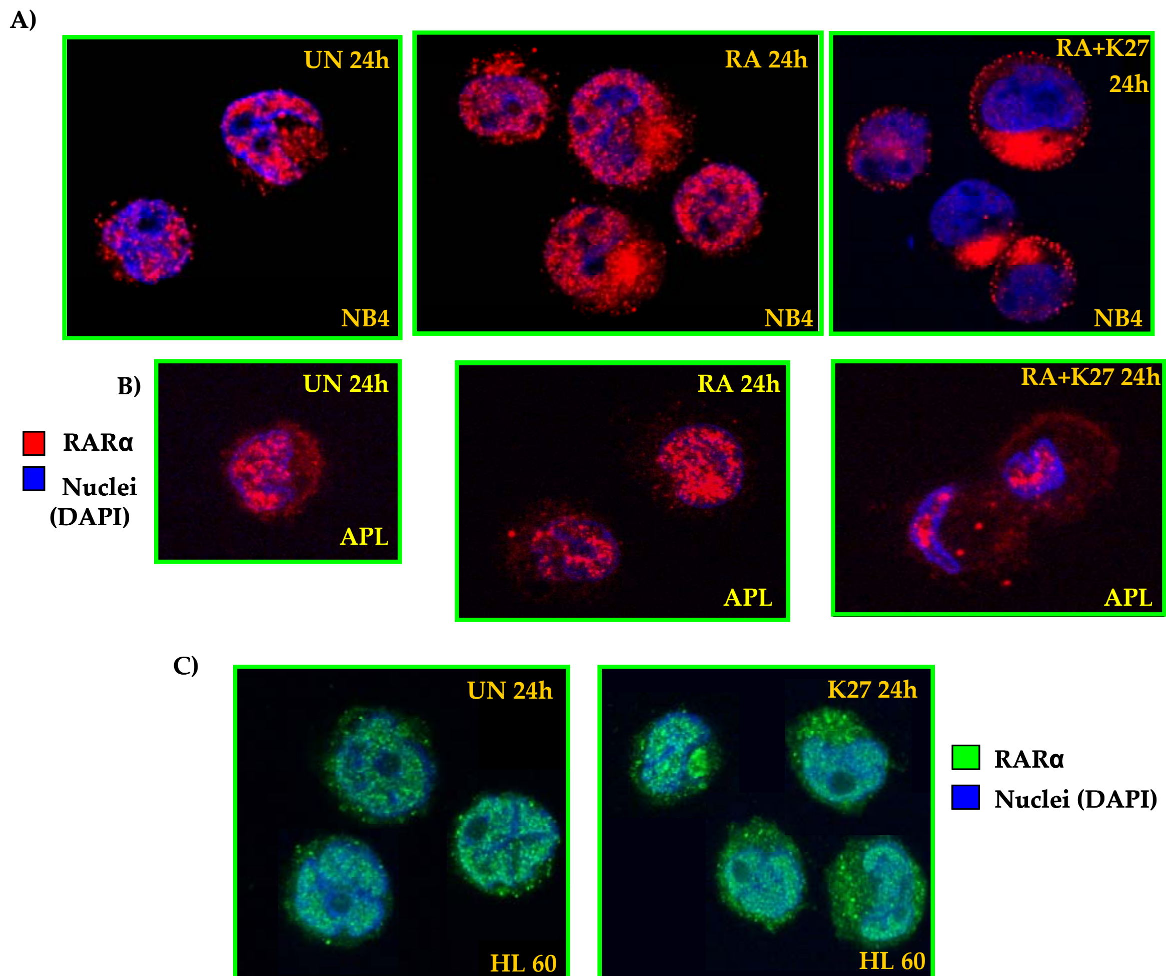

Figure 31. Immunofluorescence of RARα intracellular localization in APL cells after treatment with retinoic acid and K27.. NB4 cells (A) and APL cells from patient (B) exposed for 24 hours to RA (1μM) ± K27 (3μM); C) HL60 cell line plated in presence of K27 (3μM) for 24 hours. Cells were stained with an antibody that recognized both the wild type RARα and the fusion protein. DAPI stained the nuclei.

To confirm RARα re-localization, upon CK2 blockade, western blot analysis of nuclear and cytoplasm cellular sub-fractions was performed. NB4 cells underwent a time-course treatment with two different concentrations of K27 (3μM-5μM) and were then collected at 2, 4, 6, and 18 hours. As expected there was a dose and time-dependent reduction of RARα levels in the nucleus and a corresponding accumulation in the cytoplasm upon CK2 inhibition (Fig. 32A, B). Interestingly, we noticed that the RARα-associated bands in the cytoplasm had a lower molecular weight compared to the bands in the nucleus.

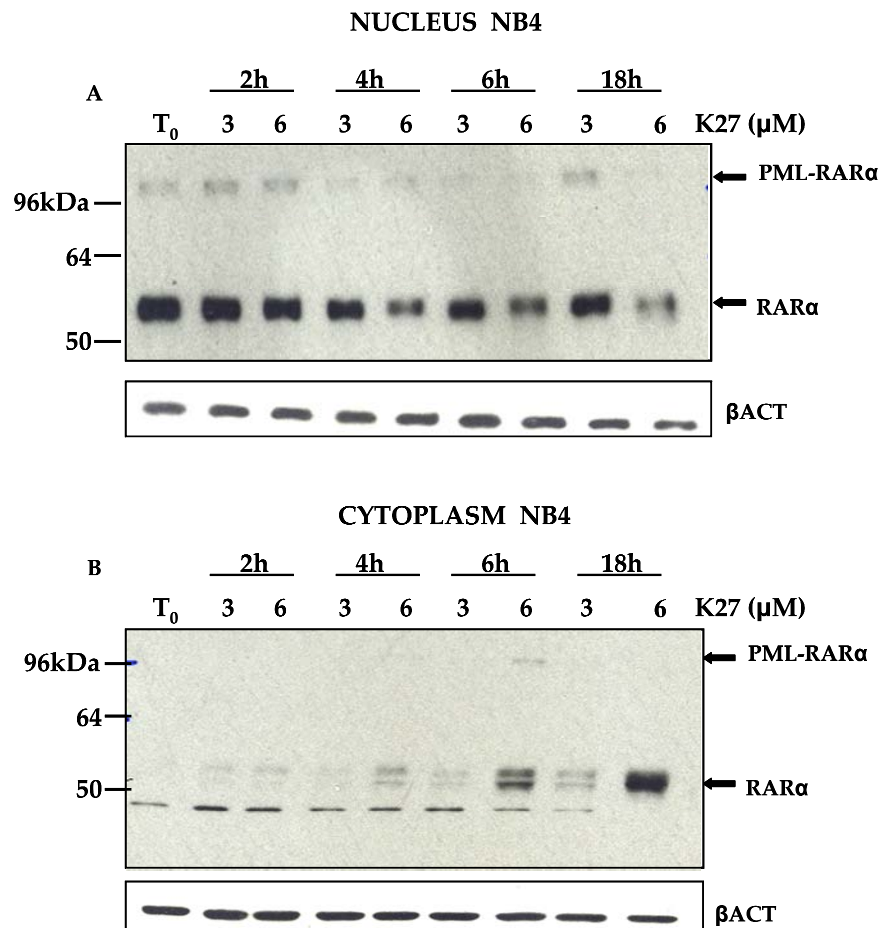

Figure 32. Western blot analysis of RARα distribution in nuclear and cytoplasm cellular subfractions. Time course treatment with K27 performed in NB4 cells collected at 2, 4, 6 and 18 hours upon exposure to K27 (3-6μM).

To validate the experiments performed with the CK2 inhibitors, we blocked CK2α using RNA interference methods. NB4 cells were nucleofected with non-target oligos (scr) or oligos against CK2α mRNA (siRNACK2α). Upon 48 hours RA was added at the medium and incubated for 24 hours. Western blot analysis of RARα was performed on nuclear and cytoplasm cellular subfractions. In untransfected sample (UN) and sample containing scr oligos, RARα and PML-RARα were highly expressed in the nucleus. Otherwise, down-modulation of CK2α determined the accumulation of a small amount of these proteins in the cytoplasm, thus supporting the results previously obtained with the chemical inhibitor.

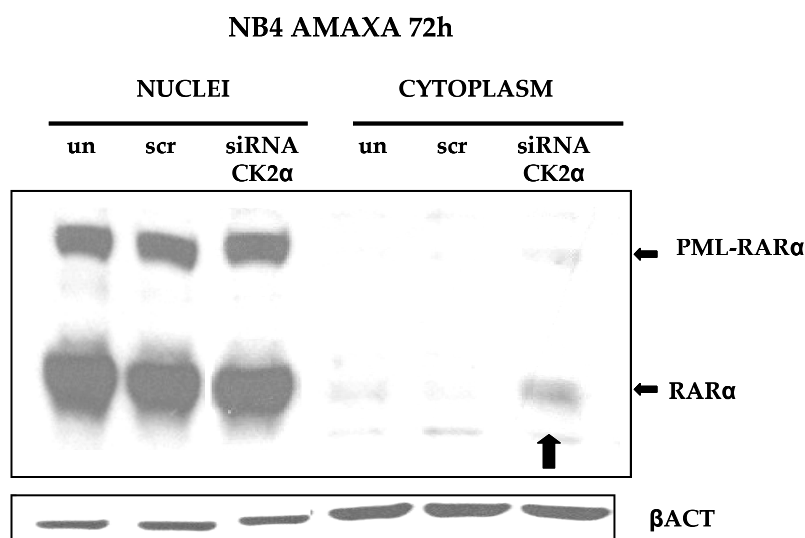

Figure 33. Western blot analysis of RARα and PML-RARα in nuclear and cytoplasmic extracts. NB4 cells were transfected with control oligos or siRNA oligos anti-CK2α mRNA. After 72 hours cells were lysed and RARα expression was analysed. The black arrow poits out the accumulation of RARα in the cytoplasmic compartment after down modulation of the alpha catalytic subunit. RARα expression was normalized on βactin (βact).

Next, we decided to strengthen our results on RARα localization using another cellular context, namely non APL cells. HeLa adherent cells were transfected with plasmids (2μg) expressing short hairpin RNA (shRNA) against CK2α

catalytic subunit mRNA or a control plasmid (scr). To verify transfection efficiency, HeLa were co-transfected with GFP plasmid (1μg) expressing a pharnesylated form of the green fluorescence protein, which localizes at the cell membrane. 48 hours after transfection, HeLa cells were treated for 24 hours with RA; they were therefore collected after a total period of 72 hours. Down-modulation of CK2α was confirmed by Real Time PCR (0,48 mean ± SD 0,15; p< 0,05 compared to scr) (shRNACK2+RA 0,45 mean ± SD 0,05 compared to scr+RA 0,9 ± 0,1, p< 0,05) and Western Blot analysis: we obtained a reduction of almost 50% of CK2α subunit expression.

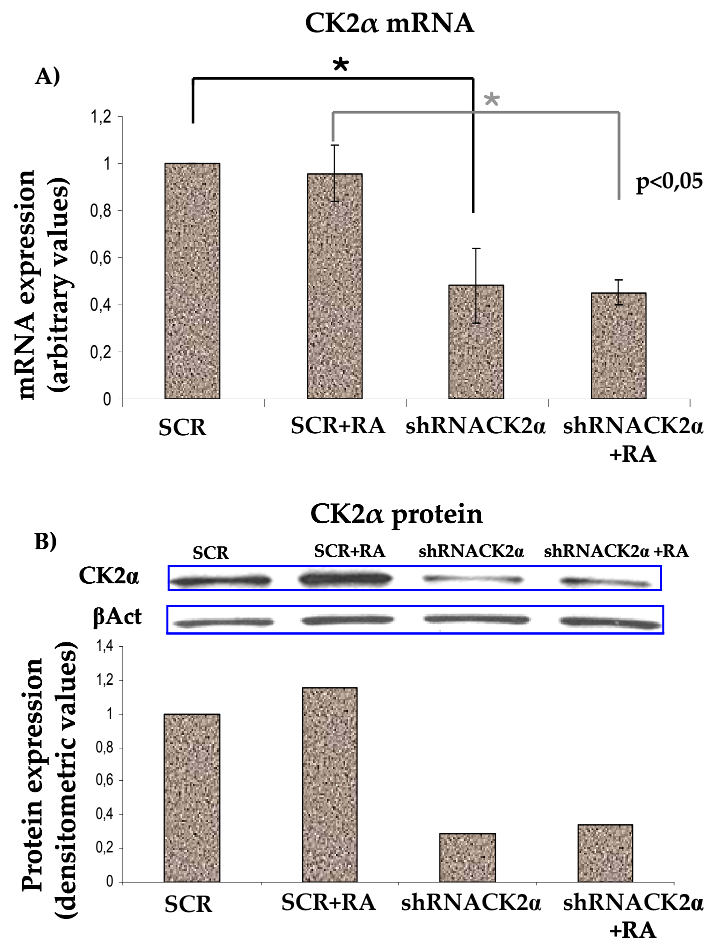

Figure 34. Analysis of CK2α expression in HeLa cells upon RNA interference of the α subunit and treatment with RA. Real Time PCR (A) and Western blot and densitometric analysis (B); βactin (βact) was used as loading control. CK2α mRNA reduction after RNA interference resulted significant (p<0,05 between shRNACK2α and scr samples; between shRNA+RA and scr+RA samples).

Immunofluorescence was carried out, staining cells with the antibody against RARα.

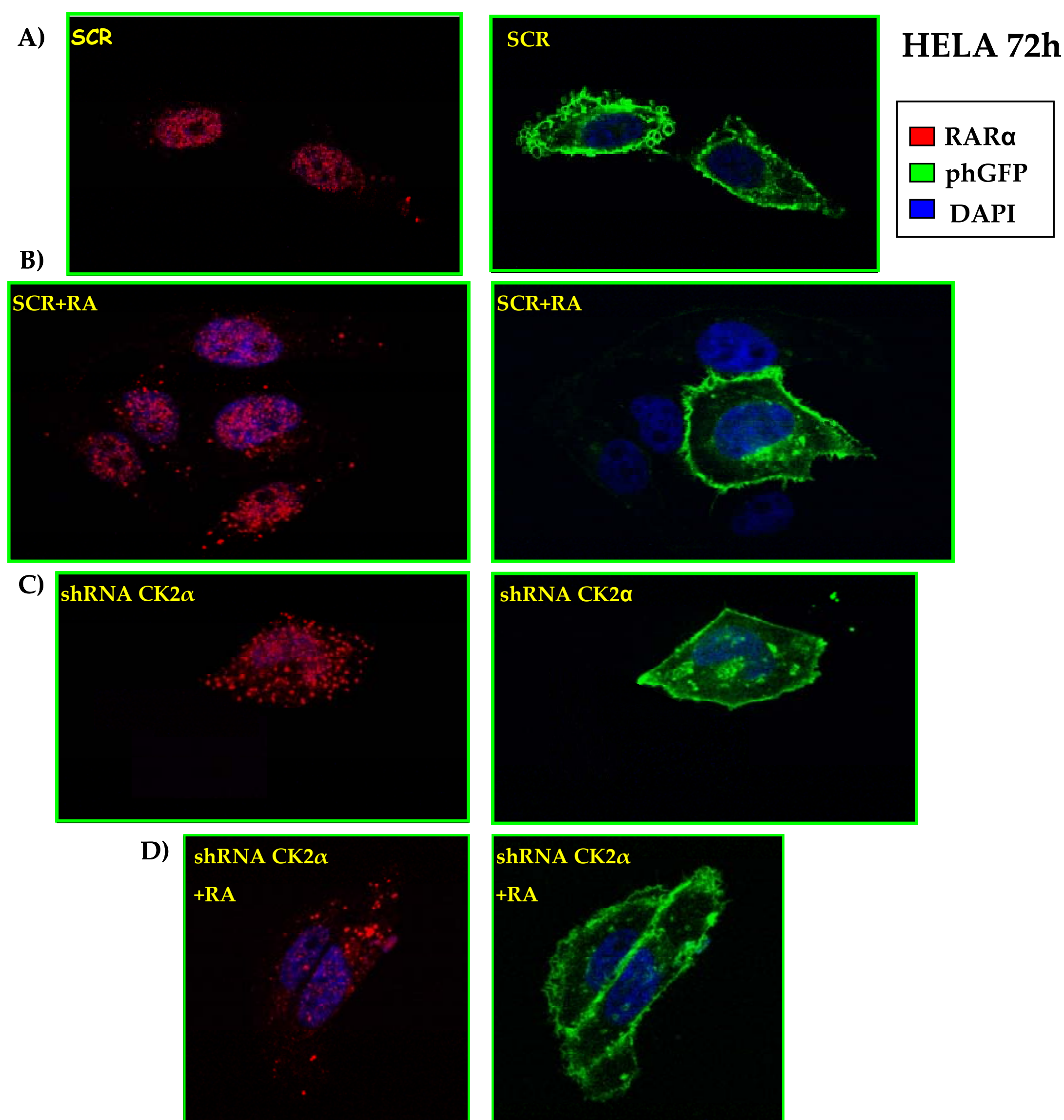

Figure 35. Immunofluorescence analysis of RARα intracellular localization in HeLa cells. HeLa were transfected with scr plasmids or plasmids expressing shRNA against CK2α in combination with a vector expressing pharnesylated GFP (phGFP). Then cells were incubated with RA for 24h and finally were stained with anti-RARα antibody. A) HeLa transfected with scr plasmid; B) Cells containing scr plasmid and treated with RA; C) HeLa expressing shRNA against CK2α mRNA (shRNACK2α); D) siRNA of CK2α followed by RA treatment (shRNACK2α+RA).

Hela transfected with control plasmid showed a nuclear distribution of RARα and this pattern did not change even after exposure to RA (Fig.35). On the contrary, after CK2α silencing, RARα migrated to the cytoplasm, despite the addiction of retinoic acid.

Previously (see Fig. 32) we demonstrated that RARα shuttled from the nucleus to the cytoplasm upon treatment with K27 in a dose and time dependent-manner (Fig.32). Similar experiments were repeated in NB4 cells adding retinoic acid to K27 and choosing a single time point for cell collection. NB4 were exposed for 6 hours to RA (1μM), K27 (3-6μM) or the combination of the two compounds. Western blot analysis was performed on nuclear and cytoplasmic protein extracts.

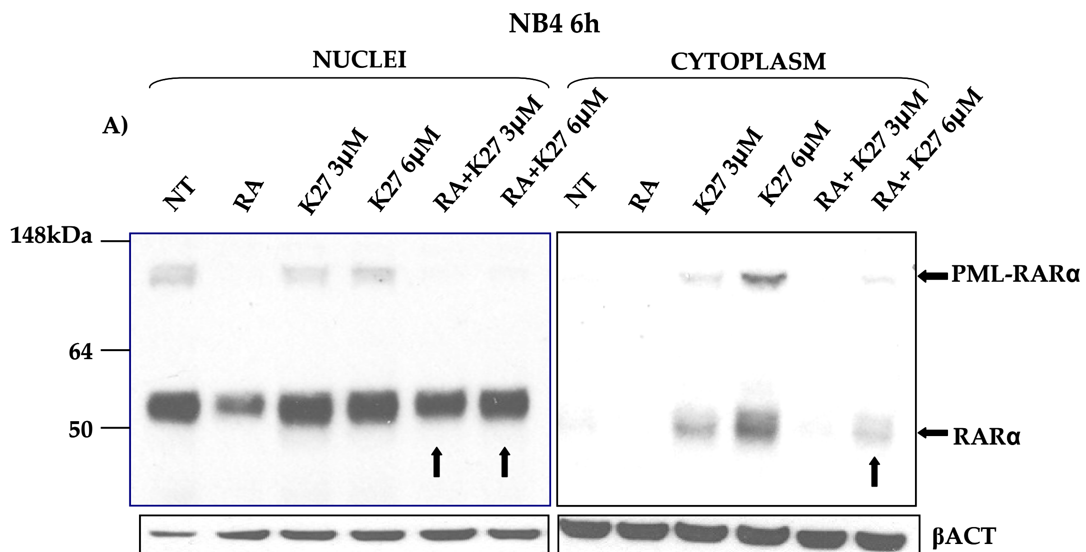

Figure 36. RARα and PML-RARα expression in nuclear and cytoplasmic protein extracts after treatment with RA and K27. NB4 cells were treated for 6 hours with RA, CK2 inhibitors or their combination. RARα expression was evaluated by Western Blot analysis; β actin was used as loading control. The black arrows highlight the accumulation of RARα in the nucleus and cytoplasm after exposure to K27: CK2 inhibition prevents RARα degradation induced by RA.

RA alone reduced nuclear RARα and PML-RARα amount because of the activation of the proteasome-dependent degradation; K27 alone determined RARα and PML-RARα translocation into cytoplasm, as previously shown; surprisingly the combined treatments with RA and K27 maintained higher levels of RARα and PML-RARα expression in the nucleus compared with RA alone; in the cytoplasm in presence of the same combined compounds there were less intense bands, which were not detectable with RA treatment alone. Otherwise, PML-RARα degradation triggered by RA seemed not to be affected by CK2 inhibition.

4.9 RARα turnover is fast and degradation begins early.

Protein degradation, especially through a proteasome–dependent pathway, represents an important mechanism to regulate RARα activity [24], therefore, we decided to focus our attention on RARα basal turnover. NB4 cells were treated with cycloheximide (an inhibitor of protein synthesis) alone, or in combination with MG132 (a proteasome inhibitor). Then cells were collected after 2, 4, 6 hours and western blot analysis was performed to evaluate RARα expression on nuclear extracts..

The blockade of RARα new synthesis reduced the receptor levels as expected; exposure to the combined treatments with cycloheximide and MG132 slowed down RARα turnover but did not block completely this process (Figure 37)

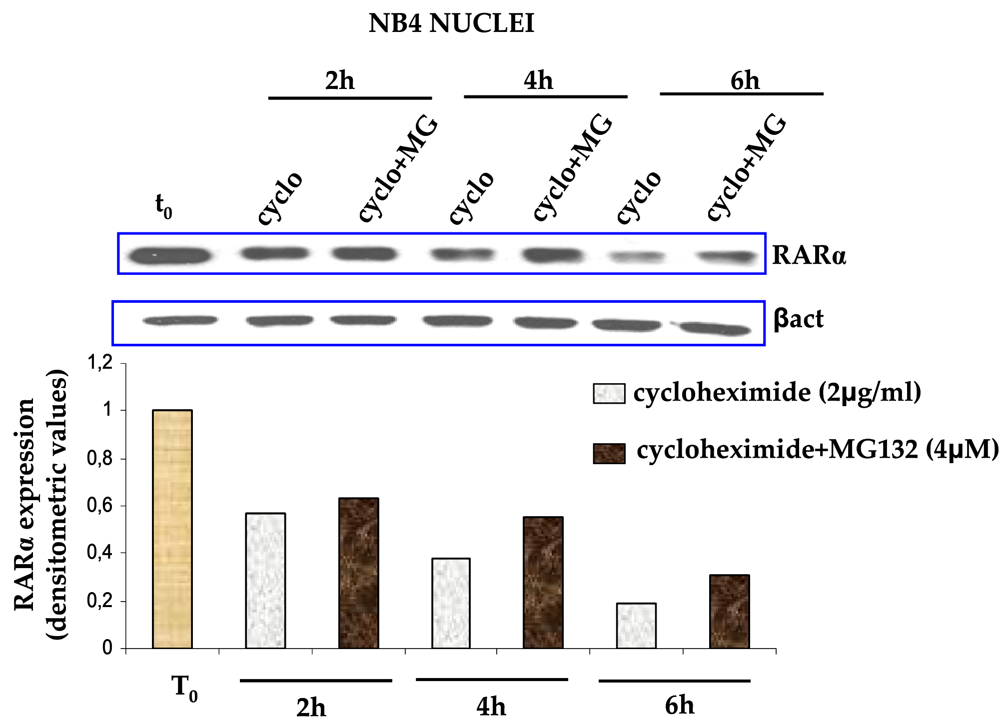

Figure 37. Western blot analysis of RARα expression in nuclear lysates. Time course treatment of NB4 cells with cycloheximide and MG132. Densitometric values of RARα expression were normalized on β actin (βact).

We previously observed (see Fig.36) that CK2 seems to influence the RA-induced degradation of RAR*α*; therfore we ckecked RAR*α* turnover in the presence of proteasome and CK2 inhibition. NB4 cells were pre-treated with Mg132 (3μM) for 30′ and subsequently treated with RA (1μM) and K27 (3 μM); 24 hour after RAR*α* expression was detected in the nuclear compartment (Fig.38).

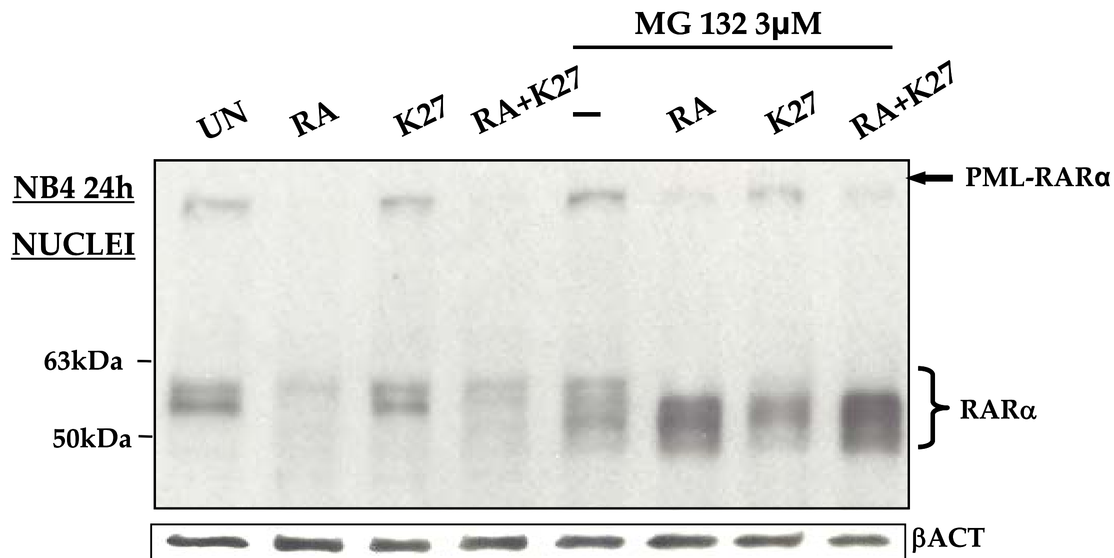

Figure 38. Western blot analysis of RAR*α* after blockade of the proteasome and treatment with RA and CK2 inhibitor. Anti-RAR*α* antibody allowed the detection of the receptor in nuclear extracts after 24 hours treatment of NB4 cells with MG132 (3μM), RA (1μM) and K27 (3μM). RAR*α* expression was normalized on βactin (βact).

When used alone, MG132 determined the appearance of heavy bands of RAR*α* (that likely correspond to highly phosphorylated state of the receptor) similar to what observed in untreated sample but also the appearance of lower forms of RAR*α* (probably corresponding to less phosphorylated forms of the protein).
A huge amount of receptor accumulated upon RA exposure and proteasome inhibition, as expected; however, it was surprising to notice that all bands showed lower molecular weight compared to untreated cells. Moreover, focusing on PML-RAR*α* fusion protein, we realized that its expression levels

increased with addiction of MG132 to RA, compared to RA alone treated samples; however, the blockade of proteasome was not sufficient to restore completely the basal amount of the fusion protein.

After proteasome inhibition, K27 treatment caused the accumulation of RARα less phosphorylated forms compared to sample exposed to MG132 alone, while the higher bands disappeared.

RA and K27 in presence of MG132 determined an accumulation of a large quantity of RARα lower bands that were little more intense compared with RA plus MG132 treated sample (Fig.38).

5. DISCUSSION

CK2 is a pleiotropic kinase involved in the integration and consolidation of different signalling pathways, behaving as a master regulator that promotes cell survival and proliferation. It is fully demonstrated that CK2 is over expressed in solid tumors and haematopoietic malignancies where it offers
some advantages to neoplastic cells: escape from apoptosis, stabilization of onco-kinome, and development of drug resistance [1]. However, CK2 controls also transcription factors such as PU.1 [3], CHOP/GADD153 [106], c-Myb [2], Ikaros [90], and signal pathways (Wnt, Sonic Hh, PI3K/Akt) that play an important role in cell maturation [82,93,97]. Starting from these assumptions we hypothesized a possible involvement of CK2 in myeloid differentiation; for this purpose we chose APL blast differentiation induced by retinoic acid as experimental model. Our results led us to the identification of a new target of CK2, namely the retinoic acid receptor alpha (RARα); by means of it the kinase influences granulocytic maturation.

We observed a high expression of CK2α catalytic subunit and elevated activity in APL blasts compared to healthy control samples represented by CD34+ and PBMC cells. This is not surprising and supports the well-established vision of CK2 as promoter of neoplastic growth.

CK2 α and β subunits showed a different modulation in expression after RA exposure; they are both down-regulated by the differentiating stimulus but CK2β expression decreased earlier at 24 hours. Despite the reduction of β subunit levels, the kinase activity remained elevated. Thus, we can hypothesize that in this context the α subunit could work alone as a monomer; this is not surprising because the beta component is not fundamental for kinase activity and it can have independent roles modulating other proteins [76].

It was already reported the importance of other Ser/Thr kinases such as Akt, Mapk, in promoting myeloid maturation; recently it was also demonstrated the involvement of the kinase GSK3 in RA-dependent differentiation where it exerts a negative modulation [107]. However, a role of CK2 in this process had never been investigated. Therefore, we inhibited CK2 using synthetic compounds, all ATP competitors, such as K27 or the more recent and highly specific CX-4945, already used in phase I clinical trials in multiple myeloma patients [108]. To further support our data, CK2 blockade was obtained also by means of RNA interference against the alpha subunit. Both chemical inhibitors and down-modulation of CK2α mRNA led to the blockade of differentiation triggered by RA: this lack of maturation could be observed at a phenotypical, morphological and functional level. siRNA effects were significant but less intense compared to those obtained with K27 or CX-4945; indeed siRNA oligos knocked down fifty per cent of CK2α mRNA and protein and likely the remaining amout was kept still active. We next excluded the possibility that the reduction of terminal differentiating cells was due to a selective sensitivity of cells to the proapoptotic effects of CK2 inhibitors.

In order to find a mechanistic explanation of how CK2 can influence differentiation of APL cells we focused on PML-nuclear bodies; it is well-established that retinoic acid favours PML-RARα degradation through proteasome and allows the correct reconstitution of these subcellular structures[109]. We observed that CK2 inhibition did not interfere with the reorganization of nuclear bodies which maintained a macrospeckled appearance in presence of RA; thus, we excluded the CK2 involvement in this process.

Since it is well known that RARα is fundamental for terminal granulocytic differentiation and controls important target genes such as *C/EBPε*, a transcriptional factor, and *p21*, involved in cell cycle control [80] [104], we investigated the effects of CK2 inhibition on cell cycle progression and RARα

transcriptional activity. We demonstrated that CK2 blockade affects the ability of RA to promote cell cycle arrest in G0/G1 phase; moreover, inhibition of the kinase down-modulated RARα activity on the transcription of p21 and C/EBPε.

Our experiments also addressed RARα intracellular distribution: we showed that CK2 is necessary to maintain RARα nuclear localization. Blockade of the kinase, both with inhibitors and RNA interference, caused the translocation of the receptor from the nucleus to the cytoplasm independently of retinoic acid exposure. It is already known that phosphorylation on nuclear receptors, in particular at the level of target regions such as nuclear localization sequences (NLS) or nuclear export sequences (NES), can enhance or prevent the interaction with importins or exportins and finally with the nuclear pore complexes. Also RARα belongs to the category of nuclear receptors and its phosphorylation can exert a different action depending on the kinase involved and the site of phosphorylation[41,110,111]. This post-translational modification can influence also interaction with other proteins that in turns could favour the retention of the receptor in one cell compartment. For example, the interaction with RXR in the heterodimers reduces RARα shuttling or the presence of the ligand, retinoic acid, limits intra-nuclear mobility, as reported by Mruvada et al. in photo-bleaching experiments[112]. Recently L. Zhu and colleagues demonstrated that the GRp58 protein (also known as ERp57 and PDIA3), an isoform of protein disulfide isomerase (PDI) [113], interacts with RARα, allows its nuclear import during RA stimulation and finally drives the receptor to endoplasmatic reticulum for degradation. Disruption of GRp58 causes the retention or the receptor into the cytoplsm [114]. Thus, CK2 might regulate RARα shuttling through a direct or indirect phosphorylation of the receptor itself or the phosphorylation of proteins involved in RARα transport, hypothesis that should be tested.

Western blot analysis, on nuclear and cytoplasmic protein extracts, confirmed the enrichment of RARα but also of PML-RARα amount in this last

compartment following CK2 blockade. Moreover, blots pointed out an interesting detail: after K27 treatement, the translocated RARα appeared as represented as more bands at lower molecular weights compared to the corresponding forms found in the nucleus. Cytoplasmic lower bands likely correspond to less phosphorylated forms of the receptor. A. Grande et al. obtained a similar pattern in NB4 cells after treatment with phosphatases: they demonstrated that these lower forms correspond to different conditions of phosphorylation and were not the result of different isoforms of RARα [115].

Retinoic acid triggers RARα and PML-RARα degradation through proteasome: this process provides an efficient way to limit RARα function and to signal the end of transcriptional process; however, at the same time proteolysis might allow the clearing of receptors, corepressors or coactivators, controlling in this way the composition of complexes at the promoter in each moment; thus, this support the vision that transcription is a dynamic process with rapid exchange of the several components [39].

We observed that RARα and PML-RARα catabolism, after retinoic acid treatment, was already prominent at 6 hours and almost complete at 24 hours. Interestingly, CK2 blockade in presence of RA appears to slow down the reduction of RARα expression due to proteasome degradation. Therefore CK2 seems to influence RARα turnover.

Our data showed that RARα basal catabolism began early; the inhibition of the proteasome with MG 132, in presence of protein synthesis blockade, delayed the decrease of RARα expression but did not block completely the turnover of the receptor. Therefore, this result let us to hypothesize the involvement of other molecular processes of RARα and PML-RARα degradation. Indeed, Isakson and colleagues demonstrated that the wild type receptor and above all the fusion protein are degraded also through the mechanism of autophagy: retinoic acid activates the formation of phagolysosomes that are more efficient

than ubiquitination to destroy macro-molecular complexes composed by the aggregation of several PML-RARα fusion proteins [116].

Upon proteasome inhibition without any other stimulation, western blot analysis of RARα expression showed higher bands already visible in the control sample but pointed out also the appearance of lower bands which likely correspond to less phosphorylated forms of the receptor. Therefore, it seems that less phosphorylated RARα is more prone to degradation.

These last findings could be strengthened by the analysis of the sample treated with retinoic acid and MG132, where it is evident the accumulation of exclusively low molecular weight bands. We can hypothesize that, as soon as RARα has carried out its transcriptional activity, retinoic acid could promote dephosphorylation of the receptor; in turn this event could favour ubiquitination and finally degradation. In presence of RA and proteasome inhibition, PML-RARα expression was not restored completely to levels observed in control sample. Thus, as said before this result confirmed the presence of alternative mechanisms of catabolism that likely correspond to authophagy process.

After proteasome inhibition, K27 treatment favoured the shift from higher forms to lower forms of RARα, indeed the heavy phosphorylated bands were no more detectable. However, these lower forms did not result to be targeted preferentially to degradation since they were similar in intensity to those observed in sample exposed only to MG132.

The combination of K27 and RA in presence of MG132, showed the accumulation of a large amount of less phosphorylated receptor, that appeared little more intense compared with RA plus MG treated sample. However, this result seemed to be the consequence of an addictive effect given by the single chemical agents rather than a synergic action, namely the lower bands produced by RA accumulated with those ones given by K27 (Fig.38).

In conclusion in this work we demonstrated the involvement of CK2 in promoting RA-induced differentiation of APL blasts. We consider the possibility that CK2 influences RARα phosphorylation state, although an indirect mechanism seems more likely: it is already known that CK2 regulates GSK3 and a recent work showed that GSK3 directly phosphorilates RARα affecting its transcriptional activity; on the contrary during differentiation GSK3 is inhibited [107]. Thus GSK3 could be the "bridge kinase" that connects CK2 to RARα, an hypothesis that we definitively will shortly test. We demonstrated that CK2 blockade prevents RARα transcriptional capability; indeed, an alteration of RARα phosphorylation state can exclude the receptor from the transcription complex, or, as we observed, can determine the de-localization of the receptor from the nucleus to the cytoplasm. Finally CK2 inhibition resulted to influence RARα turnover, slowing down its degradation through proteasome triggered by retinoic acid. We know that phosphorylation and ubiquitination are correlated events; thus, the action of CK2-dependent phosphorylation on the receptor likely enhance the degradation promoted by retinoic acid, allowing a rapid exchange of new fresh receptor at the promoter, which is essential to assure an efficient transcriptional activity. Therefore CK2 seems to operate at different levels in APL blasts differentiation, although we do not have elucidated the molecular mechanism yet.

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