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THE REGULATORY ROLE OF THE TRANSCRIPTION FACTOR ZNF224 IN CHRONIC MYELOGENOUS LEUKEMIA

TUTOR
Prof.ssa Paola Costanzo

CANDIDATE
Gaetano Sodaro

COORDINATOR **VITTORIO ENRICO AVVEDIMENTO**

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INDEX

ABSTRACT

The transcription factor ZNF224 was recently identified as a proapoptotic factor in chronic myelogenous leukemia (CML), mediating ara-C drug-induced apoptosis. Most recently, we demonstrated that the fusion protein Bcr-Abl negatively regulates ZNF224 expression in CML, while Bcr-Abl inhibition by the tyrosine kinase inhibitor (TKI) Imatinib, used as first-line treatment in CML, triggers ZNF224 up-regulation in CML cells.

On the other hand, Bcr-Abl via Jak2 activation increases the expression of c-Myc gene, which is required for Bcr-Abl oncogenic transformation in CML. Coherently, Bcr-Abl inhibition by Imatinib results in c-Myc reduction, which represents a key step for Imatinib and new generation TKIs responsiveness in CML.

In this work, we demonstrated that ZNF224 is a novel transcriptional repressor of c-Myc oncogene in CML and coherently, hampers c-Myc proliferative network, reducing CML cells proliferation and DNA synthesis. Intriguingly, our data describe a crucial role for ZNF224 induction in Imatinib repression on c-Myc in CML and subsequently demonstrate that alterations in ZNF224 binding sites on c-Myc promoter significantly affect Imatinib transcriptional repression of c-Myc gene in CML cells, thus suggesting a potential implication of this region in Imatinib responsiveness. In addition, we further reveal that AG490, a potent Jak2 inhibitor, negatively regulate c-Myc, at least in part via ZNF224 up-regulation.

A crucial problem linked to TKIs treatment in CML is the possibility to develop resistance towards these drugs. Here we demonstrate that ZNF224 expression is lower in Imatinib-resistant CML cells and that its induction by Imatinib is impaired, but can be restored by AG490. Consistently, AG490

treatment or ZNF224 induction in Imatinib-resistant CML cells results in c-Myc reduction and apoptosis induction.

Moreover, enforcing ZNF224 implications in Imatinib responsiveness, we further demonstrate that ZNF224 is a transcriptional repressor of AXL tyrosine kinase in CML, which plays a crucial role in Imatinib resistance in CML.

Taken together, our results identify new mechanisms by which ZNF224 transcription factor exerts an anti-oncogenic effect in CML and highlight the crucial role of ZNF224 in Imatinib responsiveness, thus providing convincing evidence that ZNF224 induction could play a role in overcoming Imatinib resistance in CML cells.

SOMMARIO

Il fattore trascrizionale ZNF224, è stato recentemente descritto come un fattore proapoptotico in leucemia mieloide cronica (LMC), importante nel mediare l'apoptosi indotta dal farmaco ara-C. Più recentemente, abbiamo dimostrato che la proteina di fusione Bcr-Abl, regola negativamente l'espressione di ZNF224 in LMC, mentre l'inibizione di Bcr-Abl dall'inibitore tirosin-chinasico (TKI) Imatinib, usato come farmaco di prima linea in LMC, aumenta l'espressione di ZNF224.

D'altra parte, Bcr-Abl, attraverso l'attivazione di Jak2, aumenta l'espressione del gene c-Myc, che è necessario per la trasformazione oncogenica indotta da Bcr-Abl in LMC. Coerentemente, l'inibizione di Bcr-Abl da Imatinib porta ad una riduzione di c-Myc. Tale riduzione è fondamentale per la risposta ad Imatinib ed ai TKIs di nuova generazione.

In questo lavoro, abbiamo dimostrato che ZNF224 è un nuovo repressore trascrizionale dell'oncogene c-Myc in LMC e coerentemente, riduce la proliferazione e la sintesi del DNA in LMC.

In modo interessante, i nostri dati rivelano un ruolo cruciale per l'induzione di ZNF224 nel mediare la repressione dell'Imatinib su c-Myc in LMC. In aggiunta, dimostriamo che alterazioni nel sito di legame di ZNF224 sul promotore di c-Myc, significativamente riducono la repressione trascrizionale dell'Imatinib su c-Myc in cellule LMC, suggerendo un importante ruolo per questa regione per la risposta ad Imatinib.

Successivamente, abbiamo inoltre riportato che l'AG490, un potente inibitore di Jak2, reprime c-Myc, almeno in parte, attraverso l'induzione di ZNF224.

Un problema cruciale del trattamento con TKIs in LMC è legato alla possibilità di sviluppare resistenza a questi. Qui dimostriamo che

l’espressione di ZNF224 è più bassa in linee cellulari di LMC resistenti ad Imatinib e che la sua induzione dall’Imatinib è bloccata, ma può essere revertita dall’AG490. Consistentemente, il trattamento con AG490 o l’induzione di ZNF224 nelle cellule di LMC resistenti ad Imatinib, riduce l’espressione di c-Myc ed induce apoptosi.

In aggiunta, dimostrate le implicazioni di ZNF224 nella risposta ad Imatinib, abbiamo ulteriormente dimostrato che ZNF224 è un repressore trascrizionale del recettore tirosin-chinasico AXL in LMC, che ha un ruolo fondamentale nella resistenza ad Imatinib in LMC.

In conclusione, i nostri dati descrivono nuovi meccanismi anti-tumorali del fattore trascrizionale ZNF224 in LMC e suggeriscono un suo ruolo cruciale nella resistenza ad Imatinib, aprendo dunque la strada allo sviluppo di nuovi approcci molecolari per indurre l’espressione di ZNF224 nelle cellule di LMC.

1. BACKGROUND

1.1 Chronic Myelogenous Leukemia

Chronic Myelogenous Leukemia is a hematopoietic stem cell disorder characterized by an uncontrolled expansion of clonal bone marrow myeloid precursor cells, which are deregulated in their normal differentiation towards mature granulocytes (neutrophils, eosinophils and basophils). Finally, the deregulated differentiation and growth of myeloid precursor cells in the bone marrow is followed by the accumulation of these cells in the blood.

CML is classified into three phases on the basis of clinical characteristics. CML begins in the chronic phase and can progress to an accelerated phase and finally to a blast crisis phase. In fact, blast crisis behaves like acute leukemia and represents the terminal phase of CML. The main causes of the progression from chronic phase to accelerated and blast crisis phases are mainly represented by the accumulation of new chromosomal abnormalities.[1]

A crucial role in chronic myelogenous leukemia (CML) pathogenesis and progression is represented by the reciprocal chromosomal translocation between the long arms of chromosome 9 and chromosome 22 t(9;22)(q34;q11) which causes the formation of Bcr-Abl fusion oncoprotein. Bcr-Abl fusion oncoprotein is characterized by a constitutive tyrosine kinase activity which activates proliferative and survival pathways and transcription factors, such as c-Myc, driving and sustaining the oncogenic transformation. [2,3,4,5,6]

Although the bone marrow transplantation represents the only curative treatment for CML, chemotherapy approaches using alkylating agents, interferon alfa 2b, steroids and antimetabolites such as cytarabine (ara-C), have been widely exploited and used for leukemia treatment, even though their mechanisms of action remain still largely unexplored and not extremely specific towards tumoral cells.

Nevertheless, the wide exploration of the molecular mechanisms involved in CML pathogenesis and progression improved the therapeutic approaches and led to new first line treatments for CML, using Tyrosine-Kinase inhibitors (TKIs) targeting specifically the Bcr-Abl fusion oncoprotein. [7]

Imatinib (Gleevec) was the first signal transduction inhibitor approved by FDA in 2001 for the treatment of CML and gives patients a normal life expectancy. Indeed Imatinib, by specifically targeting and blocking Bcr-Abl tyrosine kinase activity, inhibits the CML progression in the 65–75% of patients, allowing the re-growth of the normal proportions of maturing white blood cells. However, a proportion of leukemic cells persist in almost all patients and the treatment must be continued indefinitely. [8]

Among the tyrosine kinase inhibitors targeting Bcr-Abl, other new generation TKIs, such as Nilotinib and the dual Bcr-Abl and Src kinase inhibitors such as Dasatinib and PD166326, have been developed and are now used for CML treatment.

However, a crucial problem linked to TKIs treatment is that, possibly in all the CML phases, the residual leukemic cells of many CML patients accumulate new mutations in Bcr-Abl fusion protein or in other downstream signaling or effector molecules, thus resulting in a refractory response and resistance to TKIs. [9] In this context, several molecular studies contributed to reveal new molecular effectors strictly linked to TKIs resistance in CML, including LYN, FYN, and AXL. [10,11,12] Moreover, several findings showed

that increased expression of the c-Myc transcription factor, found in CML blast crisis, is strictly correlated with poor response to Imatinib. [13]

Therefore, the identification of new molecular mechanisms implicated in CML pathogenesis, could lead to new diagnostic approaches and could be exploited as the basis for the development of new therapeutic approach in Imatinib-resistant CML.

1.2 c-Myc role in CML

Bcr-Abl possesses a constitutive tyrosine kinase activity which drives the activation of a multitude of signaling pathways, including Jak2 [14], PI3K/Akt [15,16], Ras [17] and NF-kB [18], which can lead to the induction of several proto-oncogenic transcription factors, playing a pivotal role in sustaining the oncogenic transformation in CML.

c-Myc represents one of the crucial oncogenic transcription factors induced by Bcr-Abl pathways in CML. In fact, c-Myc plays a central role in the regulation of apoptosis, proliferation, differentiation, and tumorigenesis of hematopoietic cells and consistently, it is necessary for Bcr-Abl oncogenic transformation in CML.[19] More in details, Bcr-Abl induction of the c-Myc oncogene occurs mainly via Jak2 activation, which positively regulates c-Myc protein stability and transcription, although the transcriptional factors involved in this regulation remain still largely unexplored. [20,21,22]

Consistently, the inhibition of Bcr-Abl tyrosine kinase activity by Imatinib or new generation TKIs, strongly reduces c-Myc expression and hematopoietic tumoral features of CML cells. Moreover, the reduction of c-Myc is a key step for Imatinib induced differentiation and apoptosis in CML; in accord, during CML blast crisis phase, elevated c-Myc levels are found and correlate with a refractory response to Imatinib [13,23,24].

A central role for c-Myc oncogene in CML oncogenic transformation is mainly linked to its function in increasing CML cell proliferation rate. Among others, c-Myc proliferative network in CML is strictly correlated to cyclin D1 and p21 regulation. In fact, c-Myc exerts a positive regulation on cyclin D1 expression, [27] while it decreases p21 expression in CML cells [28,29,30,31,32,33].

Cyclin D1 and p21 genes are cell cycle regulators in CML. More in details, Cyclin D1 promotes the progression to G1/S phase of the cell cycle [34], while p21 blocks cyclin/CDK complexes and the proliferating cell nuclear antigen (PCNA), which is strictly correlated to S-phase, thus causing G1 and G2 cell cycle arrest [35,36,37].

Consistently, multiple options have been and are still considered to counteract CML progression targeting c-Myc. These options range from the direct inhibition of c-Myc protein or its regulators, to more genetic targeting approaches based on c-Myc promoter repression.

In accord with the pivotal role of the Jak2 pathway in Bcr-Abl positive regulation of c-Myc oncogene in CML, different Jak2 kinase inhibitors which have been developed, such as AG490, rely most of their anti-oncogenic effect on c-Myc downmodulation[20,22,38]. For these reasons, Jak2 inhibitor molecules could provide a means to overcome Imatinib resistance in CML. However, further studies are needed to consider the pre-clinical/clinical suitability of AG490 compound, eventually combined with first-line treatments [39].

An AG490-related compound, WP-1066, has been already developed. WP-1066 possesses biological activity at significantly lower concentrations compared to AG490 and blocks the Jak pathway in Acute Myeloid Leukemia (AML) cell lines, suppressing proliferation and inducing cell cycle arrest and apoptosis.

In several c-Myc-driven tumors, including CML, c-Myc overexpression is due to its increased transcription; hence, blocking or hampering c-Myc promoter activation could represent a pertinent option to contrast c-Myc oncogenic transformation.

c-Myc promoter is composed of two tandem principal promoters, termed P1 and P2. The downstream promoter P2 represents the highest regulated c-Myc promoter region[41]. Upstream P1 and P2 promoters there is a nuclease

hypersensitive element (NHE) III1, a G-rich sequence, which has a regulatory role on c-Myc transcription and which is rich in binding sites for zinc-finger protein, such as ZF87 and MAZ. This region can modulate the activation or repression of c-Myc transcription. Indeed, the double helix form of this region allows the binding of RNA polymerases and other transcription factors; by contrast, the G-quadruplex form prevents the transcription factors interactions with the promoter and leads to the down-regulation of c-Myc transcription.

Since G-quadruplexes play a crucial role in the repression of c-Myc oncogene, small-molecules that can specifically induce or stabilize the G-quadruplex formation has been developed as promising anti-cancer drugs. [42,43]. However, only CX-3543 (Quarfloxin) small-molecule compound, which selectively stabilizes the c-Myc G-quadruplexes, has entered in phase II clinical trials for neuro-endocrine carcinomas so far.

1.3 AXL emerging role in response to Imatinib in CML

As mentioned before, a critical problem linked to TKIs treatment in CML is the possibility to develop resistance to the treatment.

In this context, many studies have been focused on the exploration of the molecular mechanisms correlated to TKIs resistance in CML in order to specifically target resistant cells and, hopefully, induce re-sensitization.

As already mentioned, LYN, FYN and AXL molecules have been recently reported as strictly linked to drug resistance in cancer [10]. Interestingly, it has been demonstrated that AXL overexpression is a feature of several TKI-resistant CML cell lines. Consistently, AXL knockdown re-sensitized TKI-resistant CML cells to Imatinib and, by contrast, overexpression of AXL confers Imatinib resistance to Imatinib-sensitive CML cells. [12]

Furthermore, a very recent study has reported that AXL is overexpressed in primary cells of CML patients compared to healthy individuals and its expression is further increased in patients with resistance to Bcr-Abl TKIs, compared to the sensitive counterpart.

Most interestingly, AXL blocking with the new small inhibitor BGB324 showed intriguing additive therapeutic effects in combination with Imatinib in CML cell lines and, importantly, decreases the growth and induces apoptosis of Bcr-Abl TKI-resistant cells, mutated in Bcr-Abl [44]. Altogether, the entry in clinical trials of the first AXL-branded inhibitor in 2013, followed by the developing of this new promising AXL inhibitor BGB324, enforce the role of AXL as an anticancer target, especially in CML.

AXL is a Receptor Tyrosine Kinase (RTK), belonging to the TAM family (TYRO3, AXL, and MER), discovered in 1991 and reported as an unidentified transforming gene in CML. [45]

TAM proteins comprise a β-stranded N-terminal lobe at the extracellular region, composed of two immunoglobulin like (Ig-like) domains and two fibronectin type III repeats (FTIII). A transmembrane region connects the extracellular region to a helical C-terminal lobe, containing an intracellular kinase domain. Once AXL has been activated, the intracellular region is phosphorylated and trans-activates, by cross-phosphorylation, downstream signaling effectors. [46]

A variety of mechanisms can activate AXL, but the most common is the ligand-dependent activation exerted by GAS6, which leads to the formation of a dimer consisting of two AXL molecules, bound to two GAS6 molecules. [47]

The phopsphorylated AXL protein activates different signaling pathways, mainly involved in cell survival. More in details, AXL activates RAS and PI3K/Akt pathways, thus resulting in an activation of multiple cell survival proteins such as: IKK, MDM2 and mTOR. By contrast, there is an inactivation of pro-apoptotic proteins, such as the Bcl-2 family member BAD. [48]

Intriguingly, AXL also crosstalks with other RTKs, such as VEGFR [49], EGFR [50], and MET [51], influencing their signaling as well. This further enforces the complex regulatory role of AXL in different mechanisms of drug resistance and even metastasis.

Apart from ligand-dependent activation of AXL, ligand-independent activation can also occur, when AXL is greatly overexpressed; this is consistent with the strict correlation between AXL overexpression and its enhanced function in tumor cells. [47]

Regarding AXL regulation, it has been described that AXL gene expression is modulated by CpG methylation of its promoter and its transcription is mediated by the transcription factors SP1 and SP3 [52]. Moreover, it has been reported a post-transcriptional down-regulation of AXL mRNA by two

specific microRNAs, miR-34a and miR 199a/b, which are suppressed by promoter methylation in several solid tumors [53].

Nevertheless, although AXL overexpression has been found in different cancer types and correlates with poor prognosis, AXL regulation needs to be still largely investigated.

These studies could pave the way to develop new molecular tools to counteract tumor progression and, most importantly, TKIs resistance in CML.

1.4 Role of ZNF224 transcription factor in CML

ZNF224 is a Kruppel-like zinc-finger protein, which consists of 707 aminoacids. It comprises a C-terminal region, containing 19 tandemly repeated C2H2 zinc finger domains and an N-terminal region, containing the KRAB-A repressor domain of 45 aminoacids [54].

Through its KRAB repressor domain, ZNF224 recruits the KAP-1 co-repressor complex and the arginine methyltransferase type II, PRMT5, to repress gene transcription [55,56,57].

Initially identified as the transcriptional repressor of Aldolase A, ZNF224 has been further reported as a transcriptional repressor of the mitochondrial carrier citrate gene as well, thus highlighting its role in controlling cell metabolism [58].

Complex new roles have been recently described for ZNF224 in regulating positively or negatively cancer progression, acting respectively as an oncogene or oncosuppressor, depending on different tumoral contexts and the interaction with specific sets of interactors [59].

The first oncogenic role for ZNF224 was reported in bladder carcinogenesis, in which ZNF224 exerts its tumoral effect by interacting with DEPDC1 protein [60]. Moreover, it has been recently showed that ZNF224 acts as an oncogene in breast cancer cells, increasing the transcription of miR-663, which plays crucial roles in the initiation and progression of the malignancy [61].

Importantly, we have recently highlighted a role for ZNF224 as an oncogene in hematological malignancies. Indeed, we demonstrated a significative correlation between ZNF224 and cyclin D3 expression in Chronic Lymphocytic Leukemia (CLL) patients, further reporting that ZNF224

transcriptionally activates cyclin D3 expression in CLL, thus resulting in an increased CLL cell growth. Consistently, we further demonstrated the involvement of ZNF224 in fludarabine-resistance mechanisms of CLL, showing that fludarabine-induced apoptosis is inhibited by ZNF224 [62].

The oncosuppressor function for ZNF224 instead, has been clearly established in CML. In this context, it has been found that ZNF224 modulates the transcription in a DNA-binding-independent mode, by acting as a transcriptional cofactor of the Wilms Tumor protein 1 (WT1), a pro-survival transcription factor highly expressed in CML. Interestingly, ZNF224 affects the balance between the antiapoptotic and proapoptotic WT1 target genes in favor of the latter in CML. More in details, ZNF224, acts as a co-activator of WT1 on VDR, Bax and Bak pro-apoptotic genes, while it counteracts WT1 positive regulation of the anti-apoptotic A1/Bfl1 and Bag3 genes. Moreover, we have recently identified a new mechanism by which ZNF224 acts in the fine tuning of WT1-dependent gene expression, leading to a positive regulation on the Interferon regulatory factor-8 (IRF8) gene expression in CML cells. IRF8 is a tumor suppressor in some leukemias and its expression is repressed by WT1 [63]. We demonstrated that WT1 repressive activity on IRF8 promoter is counteracted by ZNF224 and that ZNF224 induction by cytarabine (ara-C) is able to restore IRF8 expression in CML cells [64]. Consistently, we also demonstrated that ZNF224 induction is necessary for ara-C-induced apoptosis [65].

CML is a myeloproliferative disorder in which the oncogenic progression is driven by Bcr-Abl fusion protein, which promotes uncontrolled proliferation and anti-apoptotic processes [66,67]. However, these processes remain to be largely characterized at a molecular level.

Starting from these findings, revealing the significant role of ZNF224 in ara-C mediated apoptosis in CML, we further clarified whether ZNF224 was a Bcr-Abl downstream target.

As first, we reported a ZNF224 down-modulation in both Bcr-Abl positive cell lines and in primary CML samples, compared to Bcr-Abl negative counterparts and, subsequently, we found that the inhibition of Bcr-Abl tyrosine-kinase activity by Imatinib or new generation TKIs triggers ZNF224 transcriptional up-regulation. Moreover, we reported a good correlation between ZNF224 expression at diagnosis and Imatinib responsiveness in CML patients, further suggesting a potential role for ZNF224 in Imatinib responsiveness. Furthermore, exploring Bcr-Abl negative regulation of ZNF224 expression in CML, we indentified WT1 as a direct transcriptional repressor of ZNF224, downstream Bcr-Abl/phosphatidylinositol-3 kinase (PI3K)-Akt pathway [68].

Since we previously observed that ara-C reduces WT1 expression in CML cells [65], the identification of WT1 as a transcriptional repressor of ZNF224 gene in CML well correlates with ZNF224 induction by ara-C treatment. Altogether, our findings are consistent with the hypothesis that different signaling pathways induced by ara-C and Imatinib could converge on ZNF224 induction, via downmodulation of WT1.

In addition, these molecular results also suggest the existence of a fine-regulatory loop in CML, by which WT1 transcriptional repression on ZNF224 could prevent ZNF224/WT1 complex formation and function, further enhancing CML cells survival. On the contrary, the induction of ZNF224 expression allows the formation of ZNF224/WT1 complex, which positively modulates the expression of proapoptotic genes.

In the present study, starting from a good anti-correlation between ZNF224 and c-Myc expression observed in CML cells treated with Imatinib, we demonstrate that ZNF224 is a novel transcriptional repressor of c-Myc and is responsible for Imatinib-mediated downregulation of c-Myc in CML.

Moreover, exploring ZNF224 role in Imatinib resistance we further showed that ZNF224 is also involved in negative regulation of AXL that, as mentioned before, represents a molecular effector strictly correlated to TKIs resistance in CML.

Altogether, our data revealed appealing basis to consider ZNF224 induction for the development of new therapeutic approach in CML, hopefully, overcoming drug resistance.

2. AIM OF THE WORK

Recently, we demonstrated a crucial pro-apoptotic role for ZNF224 transcription factor in CML. In fact, ZNF224 acts as a transcriptional cofactor of WT1 and positively regulates the expression of different WT1 pro-apoptotic target genes, such as VDR, bax, bak and IRF8, while it suppresses A1/Bfl1 and bag3 anti-apoptotic genes in CML.

Most interestingly, we recently found that ZNF224 is negatively regulated by Bcr-Abl fusion oncoprotein and, consistently, it is positively regulated by Imatinib and next-generation TKIs in CML cells.

These data provided the basis for the present study, aimed to better characterize the regulatory role of ZNF224 in CML.

Prompted by a good anti-correlation between ZNF224 and c-Myc expression in CML cells, the first aim of the present study was to investigate ZNF224 as a potential novel transcriptional repressor of c-Myc oncogene, downstream Bcr-Abl in CML.

In addition, our recent findings showing that ZNF224 is significantly down-regulated in CML patients with poor response or resistance to Imatinib led us to further investigate the molecular mechanisms underlying ZNF224 involvement in Imatinib responsiveness in CML.

The identification of novel targets of ZNF224 and a better characterization of the pathways downstream Bcr-Abl involved in the down-regulation of ZNF224 expression could pave the way to develop new prognostic and therapeutic approaches in CML based on ZNF224 induction, hopefully useful to overcome drug resistance.

3. MATERIALS AND METHODS

Cell lines and Reagents

HEK293T human cell line was cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, 100 µg/ml streptomycin-penicillin mix (Sigma-Aldrich) at 37°C in 5% CO2.
K562 and Jurl-MK1 human cell lines were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal calf serum and 100 µg/ml penicillin-streptomycin mix (Sigma-Aldrich) at 37°C in 5% CO2.
K562 cells were treated with 1 µM Imatinib (Novartis Pharma), 100 µM or 50 µM AG490 (Sigma-Aldrich), 500ng/ml Puromycin (Sigma-Aldrich).
K562 Imatinib were estabilished as described in [10], in wich it is described that the TKIs resistance is indipendent by mutations in Bcr-Abl.
K562 Nilotinib resistant clones were estabilished as described in [69] Jurl-MK1 Imatinib resistant clones were estabilished as described in [80].

RNA isolation, reverse transcription and real-time qPCR

Total RNA was isolated using the Quick-RNA™ MiniPrep Plus (Zymo research) according to the manufacturer's protocol. 1 µg of RNA was reverse transcribed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad), as recommended by the manufacturer. Real-time PCR was carried out in a Real-Time CFX 69 System (Bio-Rad, Berkeley, CA, USA) using the Master Mix SYBR Green (Bio-Rad).

The sequences of the primers are reported in the table below:

ZNF224	F 5′-GGGCTGTCTTGGCACAATTC-3′ R 5′-TTGCCTCCTTGAACGTGGTC-3′
c-Myc	F 5’-ACTCTGAGGAGGAACAAGAA-3’ R 5’- TGGAGACGTGGCACCTCTT-3’
AXL	F 5’-GGTGGCTGTGAAGACGATGA-3’ R 5’- CTCAGATACTCCATGCCACT-3’
Abl	F 5’-GATGTAGTTGCTTGGGACCCA-3’ R 5’- TGGAGATAACACTCTAAGCATACT-3’
β2 microglobulin	F 5’- CCGTGGCCTTAGCTGTGCT-3’ R 5’- TCGGATGGATGAAACCCAGA-3’

The relative quantification in gene expression was determined using the ΔΔCT method.

Cell lysates and western blot assays

Total cell lysates were prepared by homogenization in modified RIPA buffer as previously described [81]. The membranes were incubated with the following antibodies: anti-ZNF224 (rabbit polyclonal, T3) diluted 1:300 in Super-Block Blocking Buffer (Thermo Scientific, Waltham, MA, USA), anti-c-Myc, anti-Cyclin D1, anti-p21 and anti-PCNA (Santa Cruz Biotechnology, CA, USA) diluted 1:500, anti-G3PDH (Santa Cruz Biotechnology, CA, USA) diluted 1:1000, anti-phospho-Jak2 (Santa Cruz Biotechnology, CA, USA) diluted 1:200, anti-β-actin (Sigma) diluted 1:1000, anti-Flag and anti-ß-Tubulin (Upstate, Lake Placid, NY) diluted 1:1000, anti-AXL (Santa Cruz Biotechnology, CA, USA) diluted 1:300. Signals were detected with ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

Transient and stable transfection

HEK293 cells were transiently transfected using Metafectene (Biontex) with either 0.5 μg or 1 μg or 2 μg of ZNF224-Flag expression plasmid. As control, 1.5 μg of 3X-Flag empty vector were transfected.

K562 cell lines were transiently transfected using Lipofectamine 2000 (Thermo Fisher) with either 1.5 μg of ZNF224-Flag expression plasmid or two different shRNA specific for ZNF224 (shC3 and shE7). As control, 1.5 μg of 3X-Flag empty vector or shRNA specific for GFP were transfected.

To obtain stable ZNF224 knock-down, K562 transfected cells, were cultured in RPMI supplemented with 10% FBS and 500 ng/mL Puromycin (Sigma-Aldrich). To obtain stable ZNF224 over-expressing clones, K562 transfected cells with ZNF224-Flag(Neo) or 3X-Flag(Neo) expression plasmid, were cultured in RPMI supplemented with 10% FBS and 500 μg/mL Neomycin (Sigma-Aldrich).

Luciferase reporter assays

HEK293 cells were transiently transfected using Metafectene (Biontex) with either a luciferase reporter plasmids containing the c-Myc promoter (0.2 μg of c-Myc Del-2/Del-3/Del-6 plasmid) or AXL promoter (0.5 μg of AXL p6 plasmid) and pRL-CMV plasmid coding for the renilla luciferase to normalize (1:10 the ratio between renilla and c-Myc constructs; 1:20 the ratio between renilla and AXL construct). c-Myc Del-2 and Del-3 plasmids were a gift from Bert Vogelstein (Addgene plasmid # 16603) [82]. c-Myc Del-6 plasmid was a gift from Joan Massague (Addgene plasmid # 14969) [83]. AXL p6 plasmid was a gift from Heike Allgayer [52]. HEK293 cells were co-transfected with 0.2 or 0.4 or 0.6 μg or 0.8 μg of ZNF224-Flag expression plasmid, or with the 3X-Flag empty vector, as negative control.

K562 cell lines were transiently transfected using Lipofectamine 2000 (Thermo Fisher) with either a luciferase reporter plasmids containing the c-

Myc promoter (0.2 µg of c-Myc Del-2/Del-3/Del-6 plasmid) or AXL promoter (0.5 µg of AXL p6 plasmid) and pRL-CMV plasmid coding for the renilla luciferase to normalize (1:10 the ratio between renilla and c-Myc constructs; 1:5 the ratio between renilla and AXL construct). K562 cells were co-transfected with 1 µg of ZNF224-Flag expression plasmid, or with the 3X-Flag empty vector, as negative control.

After 48h, luciferase activity was measured in HEK293 or K562 cells, using the Dual-Luciferase Reporter Assay System (Promega Corporation, WI, USA), according to the manufacturer's instructions.

Chromatin Immunoprecipitation Assay

Cross-linked chromatin was prepared from HEK293 or K562. Briefly, 6 milions cells were cross-linked with 1.4% of fixing solution (11% HCHO, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes, pH 8.0). Next, cells were harvested in 1 ml of cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, protease inhibitors). Collected nuclei were resuspended in 300 µl of nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.8% SDS, and protease inhibitors). DNA was sheared by sonication. Sonicated DNA (500-1000bp) was diluted in dilution buffer (1:3) (10 mM Tris-HCl, pH 8.0, 0.5 mM EGTA, 1% Triton X-100, 140 mM NaCl, protease inhibitors) and pre-cleared with protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 1 h. The precleared nuclear extract was divided in two: one was used for negative immune-precipitation control, incubated with Anti – IgG (Sigma-Aldrich) antibody and the other was incubated with Anti-ZNF224 antibody (G16 – Santa Cruz Biotechnology, Santa Cruz, CA). After antibody incubation, A/G plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the samples and incubated for 1 h at 4°C , then collected by centrifugation and washed four times with LSB buffer (10mM Hepes pH 7.8, 250mM

NaCl, 5mM EDTA, 0.1% NP40, 1 mM sodium orthovanadate, 50mM NaF) and once with TE buffer (1 mM Na-EDTA, 10 mM Tris-HCl, pH 8.0). After washing, the beads were resuspended in 100 µl of TE buffer, and incubated at 65 °C overnight to reverse the cross-link. The day after, 0.5% SDS and 0.5 mg/ml proteinase K were adjusted in the samples. After, the samples were incubated for 3 h at 50 °C. Finally, by phenol-chloroform extraction, DNA was purified. ChIP samples were then analyzed by RT-qPCR using specific primers for: c-Myc promoter (P2 region) (Fw: 5'-TCGGGGCTTTATCTAACTCG-3', Rev: 5'-GCTGCTATGGGCAAAGTTTC-3') and Unrelated c-Myc region (5'-GAAGCGGAAATTGCAGTGAG-3', Rev:5'-AGGGATAGGGTCTTGCTACG-3').

The ΔΔCt related to the total input chromatin (% of input) of immunoprecipitated ZNF224 P2 Myc region and c-Myc unrelated region relative were compared.

Cell counting and Annexin V assay

For cell number determination of K562 cells stably silenced for ZNF224, cells were plated at 1 x 10^4/mL density in a 24 well plate and viable cells were counted from 1 to 4 days by trypan blue exclusion every 24 hours. For cell doubling time determination, 4 x 10^4/mL cells were plated in a 12 well plate and viable cells were counted every 24 hours from plating and re-plated at initial density. Cell doubling time was calculated by Log_2 (n° counted cells/n° plated cells).

For annexin V staining, 1 x 10^5 cells were washed twice in cold PBS, then resuspended in cold annexin V-binding buffer (5 M NaCl, 1 M CaCl2, 1M HEPES buffer/NaOH pH 7.4) and stained with Annexin V-APC (550474, BD Pharmingen). After incubation in the dark on a shaker for 15 min at 4°C,

cells were analyzed on FACS flow cytometer (BD Biosciences Accuri C6 Flow Cytometer).

Site-directed mutagenesis of c-Myc promoter reporter construct

Mutant constructs were generated using the QuikChange® site-directed mutagenesis kit (Stratagene) using c-Myc del-6 (−109 to +334) as the DNA template. Mutagenesis was performed in according to the manufacturer's instructions. Complimentary oligonucleotide for mutation of the ZNF224 binding sites to convert GCAGAGCTG to GCGCGGCTG, on c-Myc promoter, was synthesized (5' - GTGGAAGAGCCGGGCGAGCGCGGCTGCGCTGCGGGCGTCC -3' N bp=40; %GC=80; %MM=7.5; Tm=89.925°C). Successful incorporation of the mutations was confirmed by DNA sequencing.

BrdU incorporation assay

Cell proliferation measured with 5-bromo-20-deoxy-uridine labelling was performed with a detection kit (Detection Kit II, Roche Diagnostics Corporation, Indianapolis, IN, USA) following the manufacturer's instructions. Briefly, K562 cells stable silenced for ZNF224 were plated 3x 10^5 per 1.5mL into 6-well plates. While, 4x10^5 K562 cells were transiently transfected for 24 hours with 7µg of 3X-Flag or ZNF224-Flag expression vectors, and then 2x10^5 of these cells per 1.5mL were plated into 6-well plates. Then, 10 mM BrdU was added to the cultures, and, after 3 hours or 5 hours, respectively stable silenced cells or transiently transfected cells were collected, fixed with ethanol, and incubated with anti-BrdU monoclonal antibody. The percentage of BrdU incorporation was measured on FACS flow cytometer (BD Biosciences Accuri C6 Flow Cytometer).

Caspase Activity Measurement

After treatment, K562 cells were lysed for 30 min at 4°C in lysis buffer [84], and lysates were cleared at 10,000 *g* for 15 min at 4°C. Each assay was done with 25 µg of protein. Cellular extracts were incubated in a 96-well plate with Ac-DEVD-AMC (7-amino-4-methylcoumarin) or Ac-LEHD-AMC (0.2 mmol/L) for various times at 37°C as described earlier. [84]

Samples from CML patients and healthy donors

Human Peripheral blood mononuclear cells (PBMC) were obtained from 3 healthy donors and 6 CML patients with informed consent, according to the Declaration of Helsinki and recommendations of an independent scientific review board.

Statistical analysis

All data are presented as mean±SD. The Student's t test was used to evaluate the statistical signicance of differences using the non-parametric Mann-Whitney test, with a p value < 0.05 or < 0.005 indicating a signicant difference.

4. RESULTS

4.1 ZNF224 exerts a transcriptional repression on c-Myc expression

We previously showed that Bcr-Abl fusion protein negatively regulates the expression of the proapoptotic transcription factor ZNF224 in CML. Consistently, Bcr-Abl inhibition by Imatinib and new generation tyrosine kinase inhibitors (TKIs), such as Dasatinib and Nilotinib, increased ZNF224 expression and was accompanied by apoptosis induction in CML cells. [68]

The c-Myc oncogene is a target of Bcr-Abl transforming activity, indeed Bcr-Abl inhibition by Imatinib strongly reduces c-Myc expression.

Coherently, we observed that ZNF224 induction by Imatinib well correlated with c-Myc oncogene reduction and cell death induction in K562 CML cells (fig.1A).

Moreover, *in silico* analysis revealed three putative ZNF224 binding sites on a c-Myc promoter region extending from -1237 to +334, including the two transcription start sites (TSS) P1 and P2 (fig.1B, upper panel), thus suggesting that c-Myc gene could be a target of ZNF224 transcriptional repression in CML.

To explore whether c-Myc promoter activity was repressed by ZNF224 and to identify the potentially involved regions, three luciferase reporter plasmids containing progressive deletions of the c-Myc promoter (Fig.1B lower panel) were introduced into HEK293 cells and luciferase activity was analyzed in the presence of increasing amounts of the ZNF224 expression vector. The result of this experiment showed that the transcriptional activity of c-Myc promoter was progressively decreased by the overexpression of ZNF224 in all three deletion mutants (Fig.1C), thus indicating that ZNF224 repression on c-Myc gene occurs through ZNF224 binding at the high regulatory P2 region, included in the DEL-6 construct.

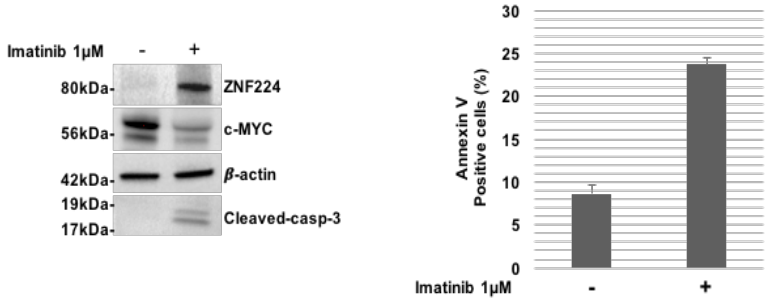
Coherently, ZNF224 overexpression in HEK293 cells was associated with a dose-dependent reduction of c-Myc protein levels and with a decrease in the protein levels of cyclin D1, a positive c-Myc target gene (Fig.1D).

Chromatin immunoprecipitation assays (ChIP) in HEK293 cells were performed to validate ZNF224 binding on the P2 region of the c-Myc promoter. Chromatin was immune-precipitated with a ZNF224 antibody and analyzed by RT-qPCR, using oligonucleotides flanking the P2 region. As shown in figure 1E, the results confirmed ZNF224 occupancy on the P2 region of c-Myc promoter (P2 region).

Moreover, in agreement with the lower expression of ZNF224 in K562 CML cells compared to HEK293 cells, we found that the basal transcriptional activity of DEL-6 construct was higher in K562 CML cells compared to HEK293 (Fig. 1F).

These data demonstrate that ZNF224 is able to bind c-Myc promoter to repress its transcription.

1A



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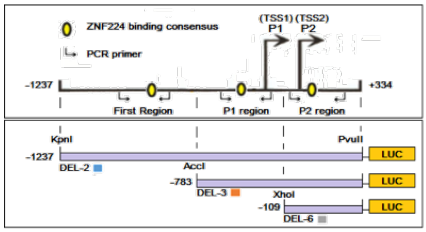

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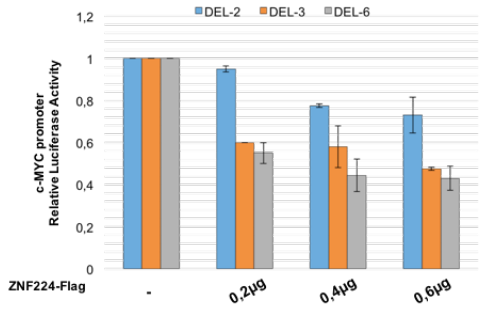

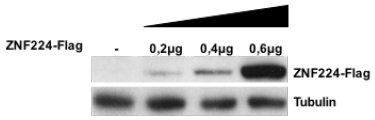

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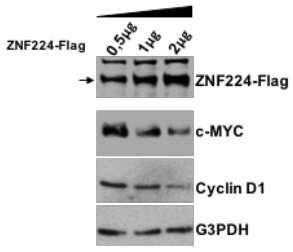

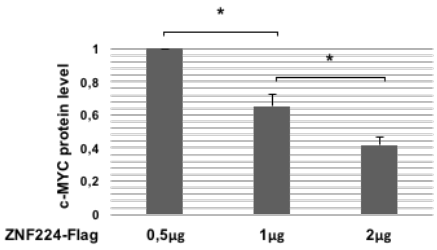

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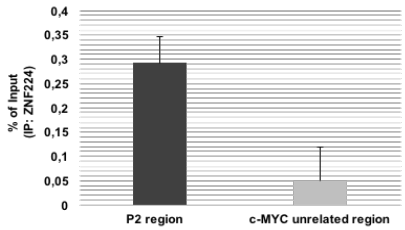

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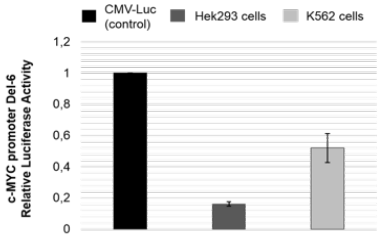


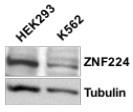


Figure 1: ZNF224 reduces c-Myc expression via a transcriptional mechanism.
(A) K562 cells were treated with 1 µM Imatinib for 24h or vehicle only (DMSO) as control. ZNF224, c-Myc and cleaved caspase-3 protein levels were analyzed by Western blot. ß-actin was used as loading control. One representative blot out of two performed is shown. Cell death was evaluated by annexin V staining followed by flow cytometry. Results represent the means +/- SD of two independent experiments. (B) Schematic representation of c-Myc promoter region and position of the three putative ZNF224 binding sites (First region, P1 region and P2 region) (upper panel). Schematic representation of DEL-2, DEL-3 and DEL-6 deletion constructs of the c-Myc promoter (lower panel). (C) Luciferase reporter assay showing c-Myc promoter activity. 0.2 µg of c-Myc deletion constructs (DEL-2, DEL-3 and DEL-6) were transfected into HEK293 cells together with 0.2µg, 0.4µg, 0.6µg of 3X-Flag ZNF224 or 3X-Flag expression plasmid as control (-). 24h hours after transfection, promoter activity was determined by normalizing Firefly to Renilla luciferase activity. Error bars represent standard deviations of two independent experiments. ZNF224 overexpression was controlled by Western Blot analysis. Tubulin was used as loading control. One representative blot out of three performed is presented. (D) Western blot analysis of ZNF224-Flag, c-Myc and cyclin D1 protein levels in HEK293 cells transfected with 0.5µg, 1µg and 2µg of 3X-Flag ZNF224. G3PDH was used as loading control (left panel). Densitometric analysis of c-Myc protein levels. Results were normalized by arbitrarily setting the densitometry of cells transfected with 0.5µg of 3X-Flag ZNF224 to 1. Error bars represent standard deviations of three independent experiments; *= p-value <0.05. (E) ChIP assay performed in HEK293 cells with an anti-ZNF224 antibody. Immunoprecipitation with non-specific IgG was used as negative control. Quantitative real-time PCR analysis was performed using specific c-Myc primers, covering ZNF224 putative binding element on the c-Myc promoter (P2 region: -30/+150 to TSS P2). A region downstream c-Myc locus was used as negative control (c-Myc unrelated region). Shown is the amount of immunoprecipitated P2 region or c-Myc unrelated region relative to that present in total input chromatin (% of input). Error bars indicate the mean value +/- SD of two independent experiments. (F) DEL-6 transcriptional activity in HEK293 cells and K562 CML cells. 0.2µg of the DEL-6 construct were transfected into HEK293 cells and K562 cell. 24h after transfection, promoter activity was determined by normalizing firefly to Renilla luciferase activity. DEL-6 activity was compared to CMV Luciferase activity obtained in each cell line. Error bars represent standard deviations of two independent experiments. ZNF224 protein expression was measured by Western Blot analysis. Tubulin was used as loading control. One representative blot out of two performed is shown.

4.2 ZNF224 binds to a regulatory element in the c-Myc promoter in CML

Next, we further confirmed ZNF224 transcriptional repression on c-Myc in K562 CML cells and investigated the region of c-Myc promoter involved in this repression. To this aim, we performed a site-directed mutagenesis of three nucleotides within the ZNF224 binding consensus on the DEL-6 construct, thus obtaining the mutant c-Myc promoter construct DEL-6 MUT (Fig. 2A).

Next, we compared ZNF224 transcriptional repression on both wild-type and mutant c-Myc promoter in K562 cells. K562 cells were transfected with DEL-6 or DEL-6 MUT constructs together with the ZNF224-Flag expression vector and luciferase activity was measured. As expected, ZNF224 overexpression significantly reduced DEL-6 luciferase activity, while it was not able to repress the luciferase activity of DEL-6 MUT construct (Fig. 2B).

Furthermore, by introducing DEL-6 wild-type or DEL-6 MUT constructs in K562 cells stably knocked-down for ZNF224 with two different shRNA (shC3 and shE7) we observed an increased DEL-6 wild-type promoter activity, while no significant variations in DEL-6 MUT luciferase activity were detected (Fig. 2C).

Accordingly, a substantial reduction in c-Myc protein levels was found when ZNF224 was overexpressed in K562 CML cells (Fig.2D), while the stable knock-down of ZNF224 in shC3 and shE7 clones was associated with an increased c-Myc protein levels (Fig.2E).

c-Myc oncogene plays a role in regulating cell death and survival but is also involved in CML oncogenic transformation, mainly increasing cell proliferation rate.

Hence, to further support ZNF224 repression on c-Myc, we evaluated CML cell proliferation rate in K562 cells stably knocked-down for ZNF224 with

two different shRNA (shC3 and shE7). Coherently with c-Myc increased levels, ZNF224 knocked-down cells showed an increased cell number (Fig. 3A) and doubling time (Fig. 3B). Moreover, BrdU incorporation assays demonstrated a significant increase in DNA synthesis of ZNF224 silenced K562 cells (Fig. 3C), while DNA synthesis was significantly decreased when ZNF224 was overexpressed in K562 cells (Fig. 3D). In addition, by western blot assay we consistently showed that c-Myc reduction after ZNF224 overexpression was accompanied by a reduction in cyclin D1 and PCNA, while p21 was increased (Fig. 3E).

Taken together, these data showed that ZNF224 exerts a transcriptional repression on c-Myc expression in CML cells and coherently inhibits cell proliferation. Furthermore, our results revealed that a binding element near to P2 transcriptional start site is necessary for ZNF224 repression on the c-Myc gene in CML cells.

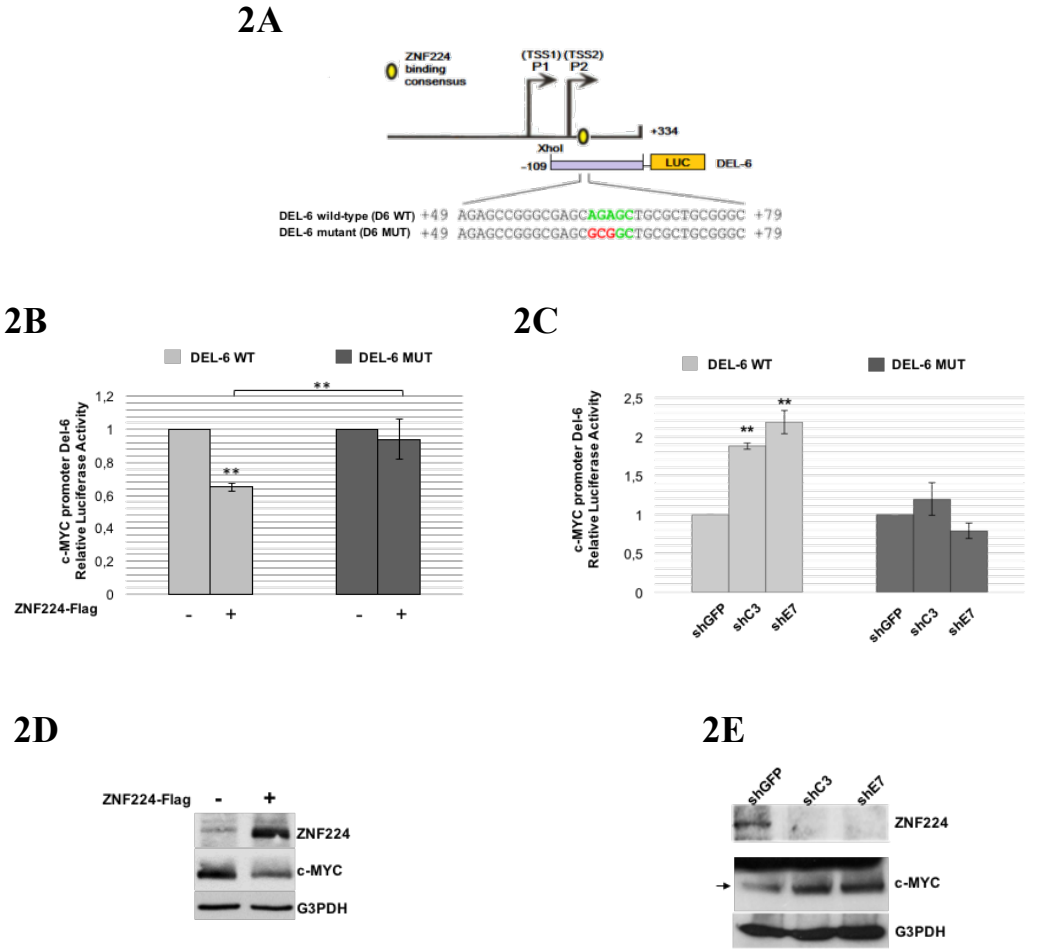


Figure 2: ZNF224 binding on P2 region is crucial for its repression activity on c-Myc promoter in CML.

(A) Schematic representation of DEL-6 MUT and DEL-6 WT constructs. (B) Luciferase reporter assay showing ZNF224 effect on the promoter activity of DEL-6 WT and DEL-6 MUT constructs in K562 cells. K562 cells were transfected with DEL-6 WT or DEL-6 MUT together with 1µg of 3X-Flag ZNF224 or 1µg 3X-Flag empty vector as control (-). 48 hours after transfection promoter activity was determined by normalizing Firefly to renilla luciferase activity. Shown is promoter activity compared to control cells (-). Error bars represent standard deviations of three independent experiments; **= p-value <0.005. (C) K562 cells stably silenced with two different shRNA versus ZNF224 (shC3 and shE7) or with a control shRNA (shGFP) were transfected with DEL-6 WT or DEL-6 MUT. 24h hours after transfection the Firefly luciferase activity was measured and normalized to Renilla luciferase activity. Error bars represent standard deviations of three independent experiments; **= p-value <0.005. (D) Western blot analysis of ZNF224-Flag and c-Myc protein levels in K562 transfected with 1µg of 3X-Flag ZNF224 or 1µg 3X-Flag empty vector as control (-). G3PDH was used as loading control. (E) Western blot analysis of ZNF224 and c-Myc protein levels in K562 cells stably silenced for ZNF224 (shC3 and shE7) and control K562 cells (shGFP). G3PDH was used as loading control. Arrow indicates the specific band

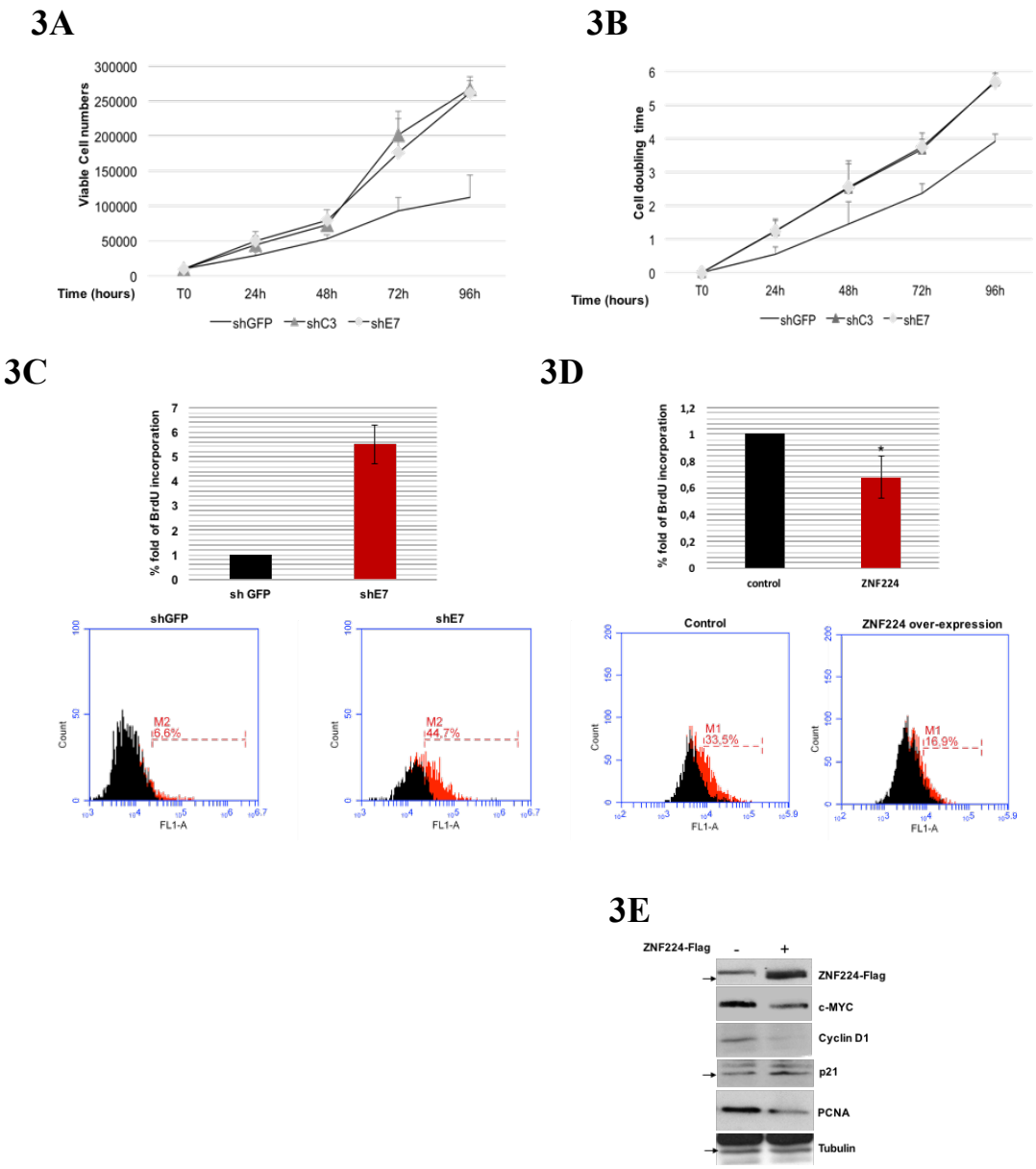


Figure 3: ZNF224 hampers CML cells proliferation

(A) Proliferation rate of K562 cells stably silenced for ZNF224 (shC3 and shE7) and control K562 cells (shGFP) evaluated by cell counting. Viable cells were counted by trypan blue exclusion every 24 hours for 4 days. Error bars represent standard deviations of three independent experiments.

(B) Every 24 hours, viable cells were counted by trypan blue exclusion and cell doubling time was calculated. Error bars represent standard deviations of two independent experiments. (C) Relative BrdU incorporation in K562 cells stably silenced for ZNF224 (shE7) compared to control K562 cells (shGFP). Error bars represent standard deviations of two independent experiments. Percentage of BrdU incorporation of one representative plot out of two is presented. (D) Relative BrdU incorporation in K562 cells transiently transfected with 1.5µg of 3X-Flag ZNF224 expression vector compared to K562 cells transfected with 3X-Flag empty vector, used as control (-). Error bars represent standard deviations of three independent experiments (*= p-value < 0.05). (E) Western blot analysis of ZNF224-Flag, c-Myc, cyclin D1, p21 and PCNA levels. Tubulin was used as loading control. One representative blot out of two performed is presented. Arrows indicate specific bands.

4.3 ZNF224 mediates the Imatinib-dependent transcriptional repression of c-Myc in CML and induces cell death in Imatinib-resistant CML cells

Prompted by these data, we decided to investigate the involvement of ZNF224 in Imatinib-dependent transcriptional repression on c-Myc oncogene in CML, which represents a key step for Imatinib responsiveness [13,23,24].

At first, by ChIP assays we verified ZNF224 binding on the c-Myc promoter in K562 CML cells and Imatinib effect on this binding. To this aim, K562 cells were incubated in absence or presence of Imatinib 1μM for 24h, after which chromatin was immunoprecipitated with a ZNF224 antibody and analyzed by real-time qPCR with oligonucleotides covering the P2 region of the c-Myc promoter. The obtained results revealed a basal ZNF224 occupancy on P2 region of the c-Myc promoter, that was significantly increased by Imatinib treatment (Fig. 4A).

Further, we investigated the effect of Imatinib on the activity of the c-Myc promoter constructs DEL-6 WT and DEL-6 MUT. As shown in figure 4B, DEL-6 promoter activity was significantly reduced by Imatinib treatment, while a lower repression was found on DEL-6 MUT. In agreement with the observed ZNF224 induction by Imatinib (WB Fig.4A), these results indicate that ZNF224 binding on c-Myc promoter is required for Imatinib repression of the c-Myc gene.

Subsequently, we found that the reduction of c-Myc mRNA and protein levels by Imatinib was significantly impaired by ZNF224 knock-down (Fig. 4C and 4D) and, as expected, ZNF224 knock-down also impaired Imatinib induced cell death (Fig. 4E).

Collectively, these results clearly show that the induction of ZNF224 mediates Imatinib transcriptional repression on c-Myc, thus revealing an additional mechanism by which ZNF224 transcription factor contributes to Imatinib responsiveness in CML.

Prompted by these results, we explored the implication of ZNF224/c-Myc axis in Imatinib resistance. We investigated ZNF224 and c-Myc regulation by Imatinib in K562 cells resistant to Imatinib (K562 Ima-R), obtained as described in [10]. Interestingly, we observed that Imatinib inefficiency in increasing annexin V positivity and caspase activation in K562 Ima-R cells (fig.5A) was associated with the lack of ZNF224 induction and consistently of c-Myc reduction, at both mRNA and protein levels (fig.5B).

On the contrary, forced expression of ZNF224 in these cells reduced c-Myc promoter activity (fig.5C), resulting in reduced c-Myc protein levels and a significative increase in cell death (fig.5D), thus indicating that ZNF224 could contribute to overcome Imatinib resistance in CML cells.

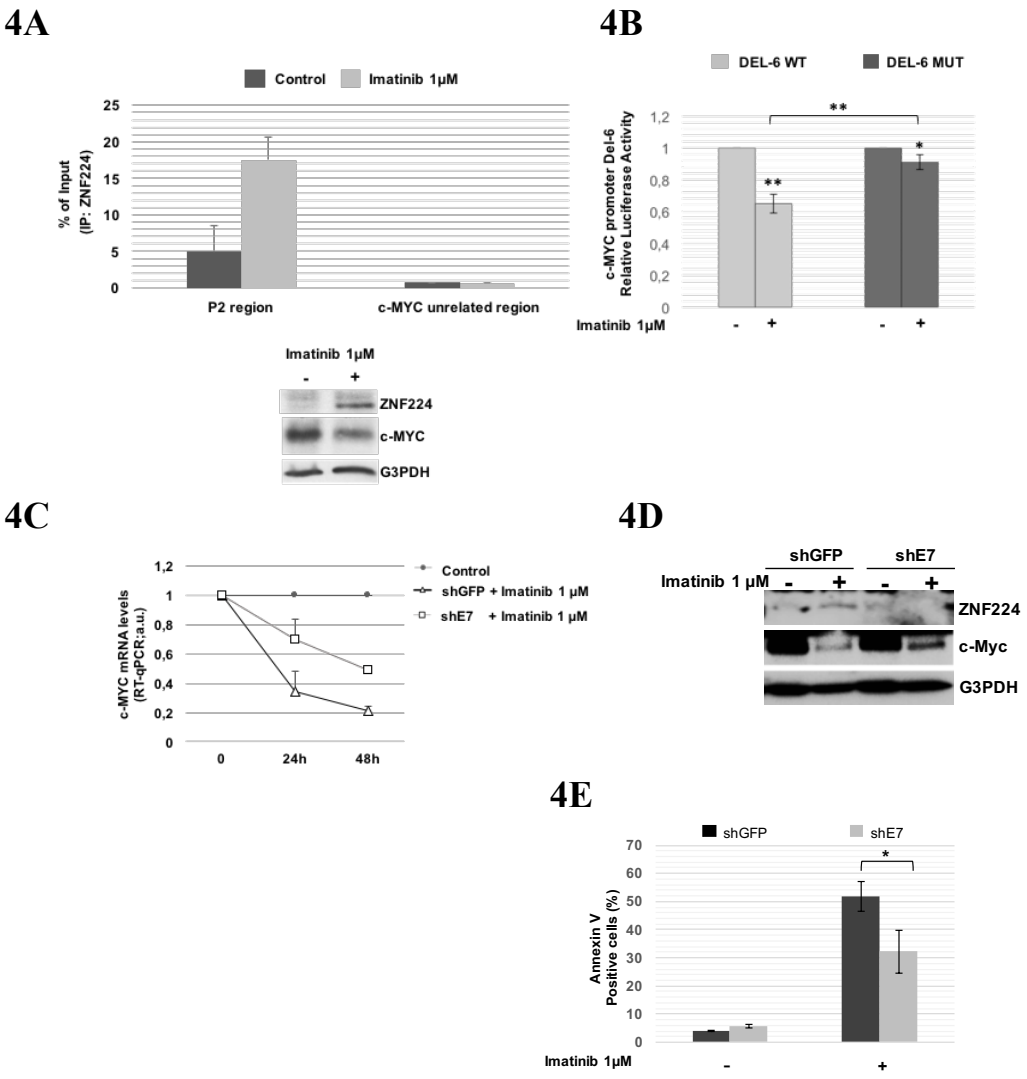


Figure 4: ZNF224 mediates Imatinib transcriptional repression on c-Myc.
(A) ChIP assay performed with an anti-ZNF224 antibody in K562 cells treated with 1 µM Imatinib or vehicle only (DMSO) as control for 24h. Quantitative real-time PCR analysis was performed using specific c-Myc primers covering the P2 region. c-Myc unrelated region was used as negative control. Shown is the amount of immunoprecipitated P2 region or c-Myc unrelated region relative to that present in total input chromatin (% of input). Error bars indicate the mean value +/- SD of two independent experiments. ZNF224 and c-Myc protein levels were analyzed by Western blot. G3PDH was used as loading control. One representative blot out of two performed is shown. (B) DEL-6 WT and DEL-6 MUT promoter activity in K562 cells after Imatinib treatment. K562 cells were transfected with 0.2 µg of DEL-6 or DEL-6-MUT constructs and after 24 hours treated with 1µM Imatinib. Luciferase activity was determined 24 hours later and promoter activity was normalized to renilla luciferase activity. Error bars represent standard deviations of two independent experiments (*= p-value < 0,05; **p-value < 0,005). (C) K562 cells stably silenced for ZNF224 (shE7) or with a control shRNA (shGFP) were exposed to 1 µM Imatinib or vehicle only (DMSO) for 48 hours. c-Myc mRNA levels were measured by RT-qPCR. Relative amounts of shGFP and shE7 treated with Imatinib were compared to shGFP and shE7 treated with DMSO (control). Error bars represent standard deviations of two independent experiments. (D) ZNF224 and c-Myc protein levels were measured by Western Blot analysis. G3PDH was used as loading control. One representative blot out of two is presented. (E) Cell death was determined by annexin V staining followed by flow cytometry. Error bars represent standard deviations of three independent experiments (*= p-value < 0,05).

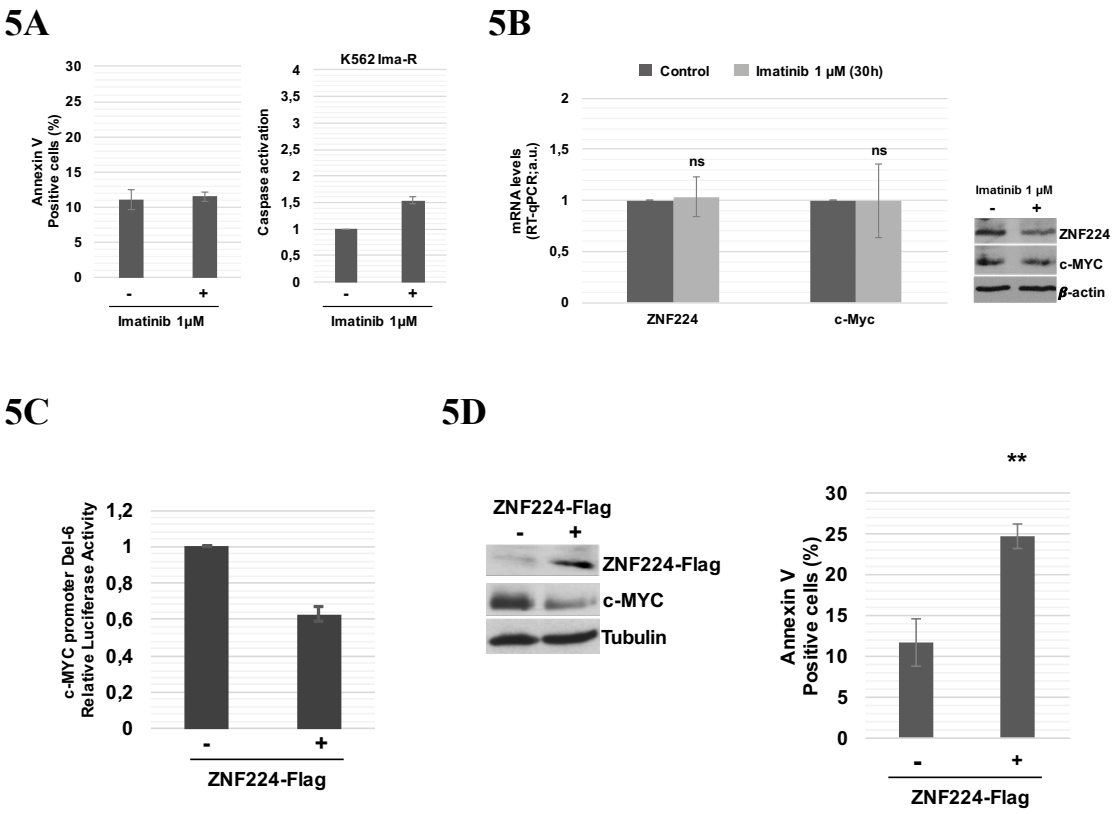


Figure 5: ZNF224 overexpression reduces c-Myc and induces cell death in K562 Ima-R cells. K562 Ima-R cells were exposed to 1 µM Imatinib or vehicle only (DMSO) as control (-) for 48 hours. (A) Caspase activity was biochemically measured. Error bars represent standard deviations of two independent experiments. (B) ZNF224 and c-Myc mRNA levels were measured by RT-qPCR. Relative amounts as compared to control are shown. Error bars represent standard deviations of three independent experiments. ZNF224 and c-Myc protein levels were measured by Western Blot analysis. ß-actin was used as loading control. One representative blot out of two is presented. (C) Luciferase reporter assay showing ZNF224 effect on the promoter activity of DEL-6 construct in K562 Ima-R cells. K562 Ima-R cells were transfected with DEL-6, together with 1.5µg of 3X-Flag ZNF224 or 3X-Flag empty vector as control (-). 48 hours after transfection promoter activity was determined by normalizing Firefly to renilla luciferase activity. Shown is promoter activity compared to control cells (-). Error bars represent standard deviations of two independent experiments. (D) K562 Ima-R cells were transfected with 1.5µg of 3X-Flag ZNF224 or 1.5µg 3X-Flag expression plasmid as control (-). c-Myc protein levels were measured by Western Blot analysis. Tubulin was used as loading control. One representative blot out of two is presented. Cell death was determined by annexin V staining followed by flow cytometry. Error bars represent standard deviations of three independent experiments (** = p-value <0.005).

4.4 Jak2 inhibitor AG490 reduces c-Myc via ZNF224 induction in CML cells

Janus Kinase 2 (Jak2) pathway plays a pivotal role in c-Myc induction by Bcr-Abl. Consistently, new inhibitor molecules targeting Jak2, downstream Bcr-Abl, have been reported as powerful tools to reduce c-Myc levels in CML and represent promising molecular tools to overcome Imatinib resistance. Therefore, since ZNF224 is implicated in c-Myc repression downstream Bcr-Abl in CML, we investigated ZNF224 involvement in c-Myc transcriptional downregulation by AG490, a specific and potent inhibitor of Jak2.

To investigate this issue, at first we treated K562 cells with AG490 and evaluated ZNF224 and c-Myc expression. As expected, AG490 induced cell death in K562 cells (fig.6A) and decreased c-Myc mRNA and protein levels (fig.6B and 6C). Interestingly, the reduction of c-Myc was associated with an increase in ZNF224 expression (fig.6C), thus suggesting the involvement of Jak2 pathway in ZNF224 suppression. Similar results were also obtained in Jurl-MK1 CML cells (fig.S1).

Subsequently, we investigated the effect of AG490 on the activity of the c-Myc promoter constructs, DEL-6 and DEL-6 MUT in K562 cells. We observed that AG490 treatment, similarly to Imatinib (fig.4B), strongly reduced DEL-6 WT promoter activity, while exerted a lower repression on DEL-6 MUT (fig.6D), thus indicating that ZNF224 binding on c-Myc promoter is important for AG490-dependent repression of the c-Myc gene.

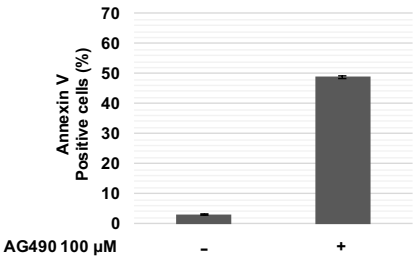
Finally, to definitively demonstrate the involvement of ZNF224 in c-Myc repression by AG490, we analyzed the effect of AG490 on c-Myc expression in K562 cells knocked-down for ZNF224 (shE7) compared to control cells (shGFP). Interestingly, as shown in figures 6E and 6F, ZNF224 silencing strongly impaired AG490-mediated downregulation of c-Myc expression at mRNA and protein levels.

Taken together, these results indicate that ZNF224 is a new transcription factor in the Bcr-Abl/Jak2-c-Myc transcriptional pathway in CML and demonstrate that ZNF224 induction mediates, at least in part, AG490 transcriptional repression on c-Myc oncogene in CML cells.

Most interestingly, AG490 treatment of cultured peripheral mononuclear cells (PBMC) obtained from CML patients at diagnosis and sensitive to Imatinib (Fig.S2), led to an increased ZNF224 expression which was associated with caspase-3 cleavage (fig.7A) and cell death (fig.7B). As shown in fig.S3, ZNF224 protein levels in PBMC from CML patients at diagnosis was lower compared to PBMC from healthy donors.

Altogether, these preliminary results are in agreement with our previous results, reporting lower ZNF224 mRNA levels in CML patients compared to healthy donor [68] and strengthened AG490 potential in increasing ZNF224 expression and cell death in CML patients.

6A



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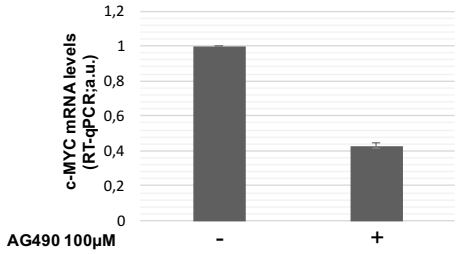

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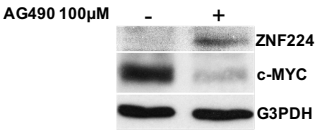

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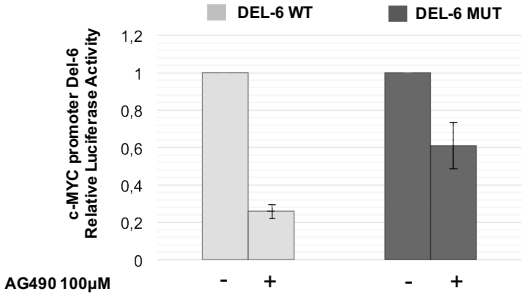

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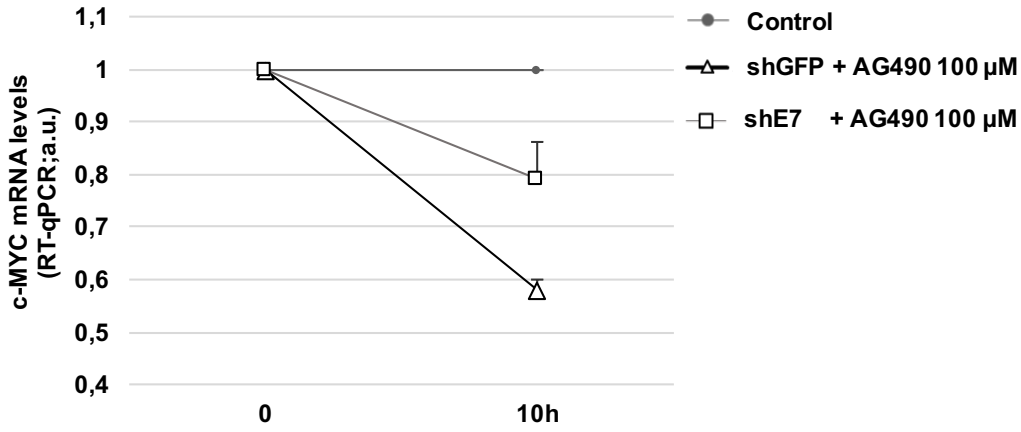


6F

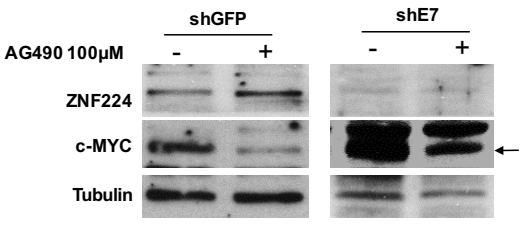


Figure 6: ZNF224 mediates AG490 transcriptional repression on c-Myc.
(A) K562 cells were treated with 100 µM AG490 for 24h or vehicle only (DMSO) as control (-). Cell death was evaluated by annexin V staining followed by flow cytometry. Results represent the means +/- SD of two independent experiments. (B) K562 cells were treated with 100 µM AG490 for 10 hours or vehicle only (DMSO) as control (-). c-Myc mRNA levels were measured by RT-qPCR. Relative amounts as compared to control are shown. Error bars represent standard deviations of two independent experiments. (C) Western blot analysis of ZNF224 and c-Myc protein levels. G3PDH was used as loading control. One representative blot out of two performed is shown. (D) K562 cells were transiently transfected with 0.2µg of DEL-6 WT or DEL-6-MUT constructs and treated with 100µM AG490; after 10 hours, luciferase activity was determined by normalizing firefly to renilla luciferase activity. Error bars represent standard deviations of two independent experiments. (E) K562 cells stably silenced for ZNF224 (sh E7) or with a control shRNA (shGFP) were treated with 100µM AG490 for 10 hours. c-Myc mRNA levels were measured by RT-qPCR. Relative amounts as compared to shGFP and shE7 treated with vehicle only (DMSO) as control are shown. Error bars represent standard deviations of two independent experiments. (F) ZNF224 and c-Myc protein levels were measured by Western Blot analysis. Tubulin was used as loading control. One representative blot out of two is shown. The arrow indicates specific band.

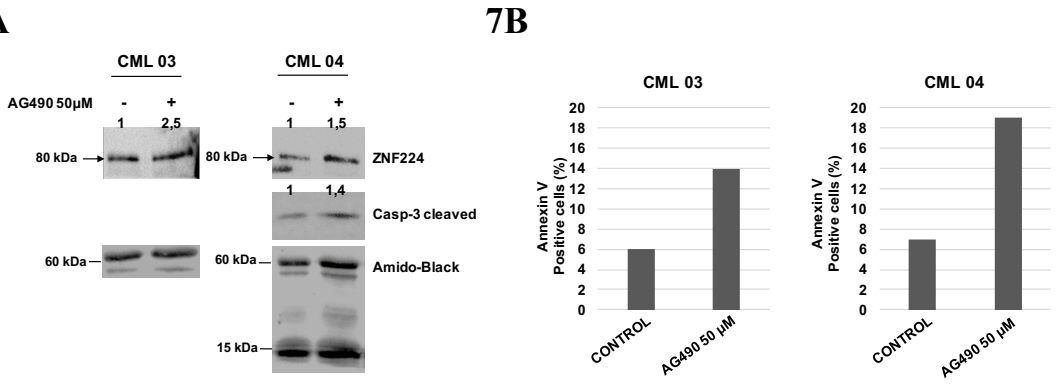


Figure 7: Increased ZNF224 expression and cell death induction in PBMC from CML patients treated with AG490.
(A) PBMC from 2 patients with CML at diagnosis (CML03, CML04) were treated with 50 µM AG490 for 24 hours, after which ZNF224 and cleaved caspase-3 protein levels were measured by Western Blot analysis. Amido-black membrane-coloration or β-actin was used as loading control. (B) Cell death was determined by annexin V staining followed by flow cytometry.

4.5 AG490 induces ZNF224 expression and cell death in Imatinib-resistant CML cells

Subsequently, we evaluated the effect of AG490 on cell death and ZNF224/c-Myc axis in Imatinib-resistant CML cells. Interestingly, we found that AG490 significantly induces cell death (fig.8A) and caspase activation (fig.8B) in K562 Ima-R and similar effects were also observed in Jurl-MK1 Imatinib-resistant cells (Jurl-MK1 Ima-R) (fig. S4), obtained as described in [80].

Most interestingly, we observed that the induction of apoptosis in AG490 treated K562 Ima-R cells was associated with Jak-2 inactivation, ZNF224 induction and c-Myc reduction (fig.8C).

Similar results were obtained in K562 Nilotinib-resistant cells (K562 Nilo-R) [69]. Indeed, Nilotinib treatment in K562 Nilo-R did not affect ZNF224 and c-Myc expression, while AG490 treatment was able to induce apoptosis, increasing ZNF224 and reducing c-Myc expression (fig. S5).

Furthermore, coherently with the role of ZNF224 in repressing c-Myc and inducing cell death in Imatinib-resistant K562 cells, we demonstrated that ZNF224 silencing, with two different siRNA (indicated as siRNA ZNF224 #1 and siRNA ZNF224 #2), increased c-Myc expression and significantly impaired AG490 induced cell death of K562 Ima-R cells (fig.8D).

Altogether, these results demonstrate that induction of ZNF224 expression by AG490 may contribute to override Imatinib resistance in CML cells.

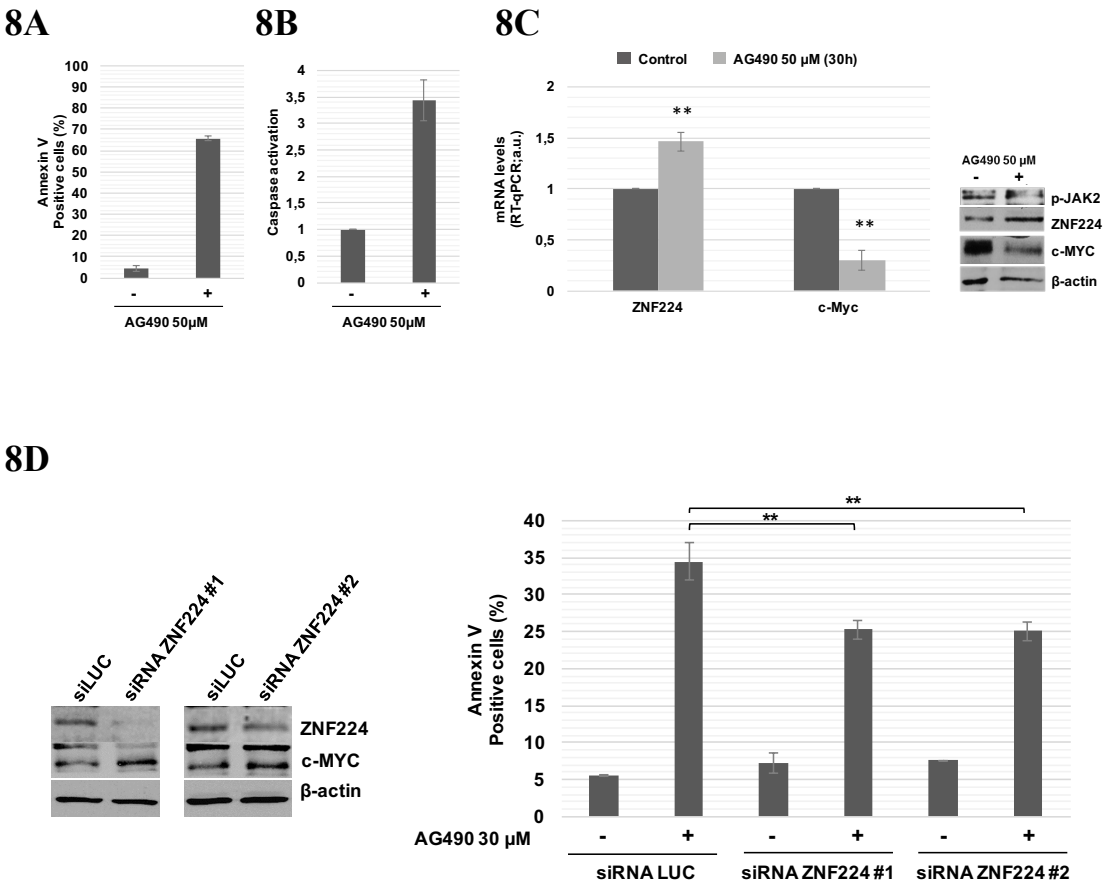


Figure 8: ZNF224 is involved in AG490 induced cell death of K562 Ima-R cells.
K562 Ima-R cells were exposed to 50µM AG490 or vehicle only (DMSO) as control (-) for 48 hours. (A) Cell death was determined by annexin V staining followed by flow cytometry. Error bars represent standard deviations of three independent experiments. (B) Caspase activity was biochemically measured. (C) ZNF224 and c-Myc mRNA levels were measured by RT-qPCR. Relative amounts as compared to control are shown. Error bars represent standard deviations of three independent experiments (** = p-value <0.005). ZNF224, c-Myc and p-JAK2 protein levels were measured by Western Blot analysis. ß-actin was used as loading control. One representative blot out of two is presented. (D) K562 Ima-R cells were silenced with two siRNA versus ZNF224 (siRNA ZNF224 pool #1 and siRNA ZNF224 pool #2) or with a control siRNA (siLuc) and were exposed to 30 µM AG490 or vehicle only (DMSO) as control (-) for 30 hours. ZNF224 and c-Myc protein levels were evaluated by Western Blot analysis. ß-actin was used as loading control. One representative blot out of two is presented. Cell death was determined by annexin V staining followed by flow cytometry. Error bars represent standard deviations of three independent experiments (** = p-value <0.005).

4.6 ZNF224 represses AXL expression in CML cells

As previously described by Auberger et al., Imatinib resistance in K562 Ima-R cells, is mostly due to the overexpression of AXL tyrosine kinase receptor [12]. Indeed, AXL knock-down restored Imatinib sensitivity of K562 Ima-R cells.

By western blot analysis, we intriguingly observed that the higher expression of AXL was associated with a lower expression of ZNF224 in K562 Ima-R as compared to K562 (Fig.9A). In addition, the anti-correlation between ZNF224 and AXL expression levels was also observed when we compared K562 resistant to Nilotinib (K562 Nilo-R) or Jurl-MK1 resistant to Imatinib (Jurl-MK1 Ima-R) to their sensitive counterparts (Fig.9A). Resistant cells were obtained as described in [12] and [69]. Consistently, by real-time PCR we also confirmed a significative increase in AXL mRNA levels in K562 Ima-R cells as compared to K562 (Fig. 9B).

Interestingly, ZNF224 stable knock-down in K562 cells (shC3 and shE7) led to an increased expression of AXL (Fig. 10A, 10B), which was associated with impaired response to Imatinib (Fig. 10C). On the other hand, ZNF224 overexpression reduced mRNA and protein expression levels of AXL (Fig. 11A, 11B) and was associated with an increased response to Imatinib (Fig.11C).

These data, coherently with ZNF224 pro-apoptotic role in CML suggested that the anti-apoptotic factor AXL is a new target of ZNF224 transcription factor.

In consistency with the potential role of ZNF224 as a transcriptional repressor of AXL gene, we found a putative ZNF224 binding site on AXL promoter region, extending from -614 to +7 (Fig. 12A).

In order to confirm ZNF224 transcriptional repression on AXL gene, we performed luciferase assays in HEK293 cells. A luciferase reporter plasmid

containing the AXL promoter region -614/+7 (P6-AXL) (Fig.12A) was introduced into HEK293 cells together with increasing amounts of the ZNF224 expression vector and luciferase activity was measured. We observed that AXL promoter activity was reduced in a dose-dependent manner by ZNF224 overexpression (Fig. 12B). Furthermore, we also confirmed that ZNF224 repressed AXL promoter activity in K562 cells (Fig. 12C). Interestingly, ZNF224 forced expression in K562 Ima-R cells resulted in a reduction of AXL promoter activity (Fig.13A) and protein expression levels (Fig.13B), thus suggesting a potential role for ZNF224 induction in Imatinib re-sensitization in this context.

9A **9B**

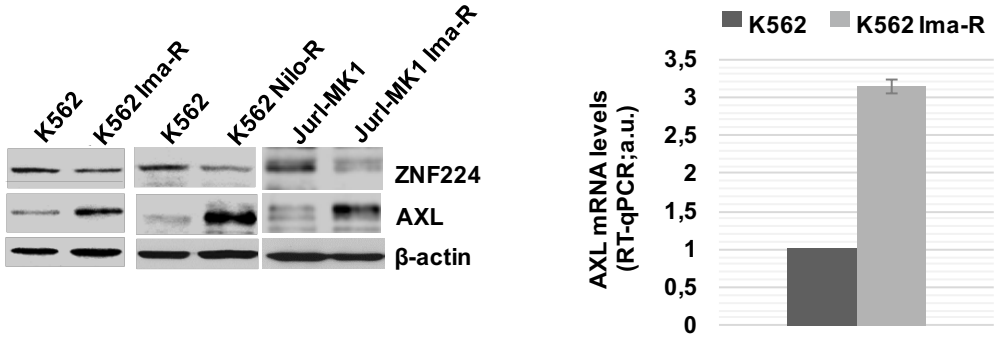
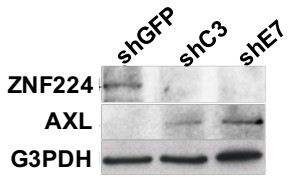
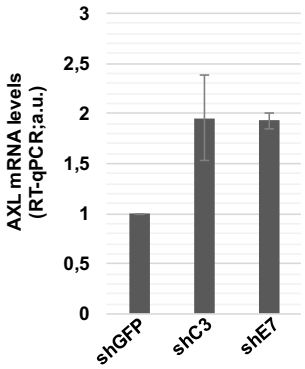


Figure 9: ZNF224 and AXL expression in TKIs sensitive and resistant CML cells
(A) ZNF224 and AXL protein levels were measured in K562, K562 Ima-R, K562 Nilo-R, Jurl-MK1 and Jurl-MK1 Ima-R CML cells by Western Blot analysis. ß-actin was used as loading control. One representative blot out of two is presented. (B) AXL mRNA levels were measured by RT-qPCR in K562 Ima-R cells. Relative amounts as compared to K562 cells are shown. Error bars represent standard deviations of two independent experiments.

10A



10B



10C

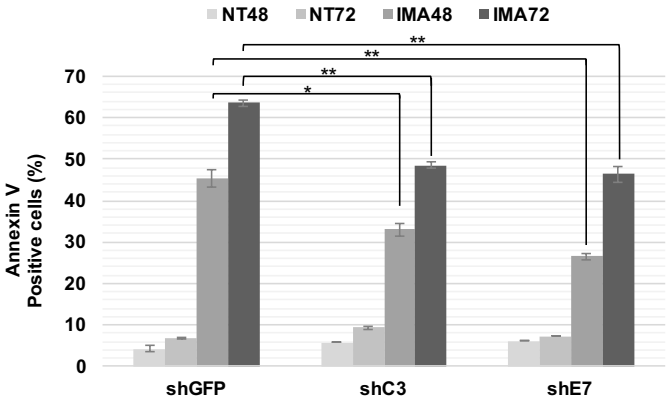
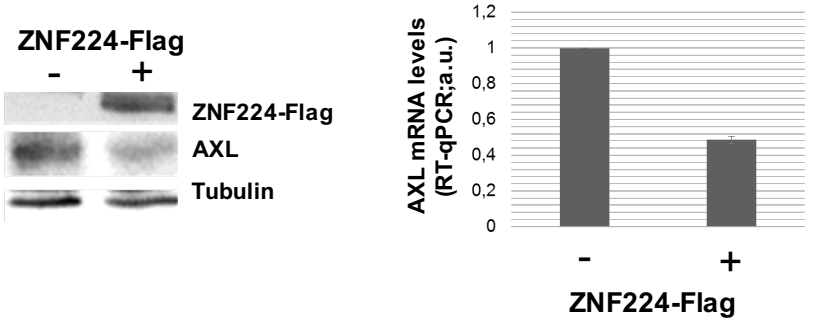


Figure 10: ZNF224 knock-down increases AXL expression and reduces Imatinib responsiveness in CML cells

(A) Western blot analysis of ZNF224 and AXL protein levels in K562 cells stably silenced for ZNF224 (shC3 and shE7) or with a control shRNA (shGFP). G3PDH was used as loading control. (B) AXL mRNA levels were measured by RT-qPCR in K562 cells stably silenced for ZNF224 (shC3 and shE7) or with a control shRNA (shGFP). (C) K562 cells stably silenced for ZNF224 (shC3 and shE7) or with a control shRNA (shGFP) were exposed to 1 µM Imatinib or vehicle only (DMSO) for 48 and 72 hours. Cell death was determined by annexin V staining followed by flow cytometry. Error bars represent standard deviations of three independent experiments (*= p-value < 0,05; **= p-value < 0,005).

11A **11B**



11C

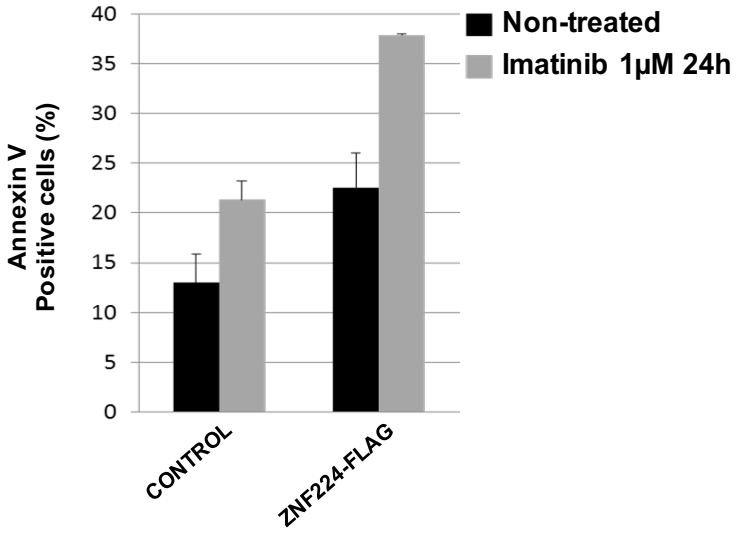
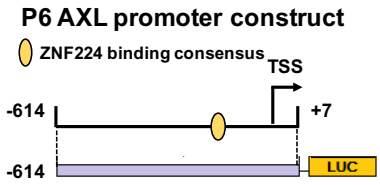


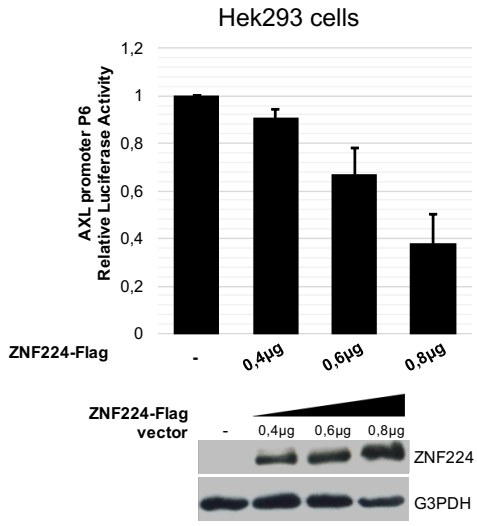
Figure 11: ZNF224 over-expression reduces AXL expression and increases Imatinib responsiveness in CML cells

K562 cells were transfected with 1,5µg of 3X-Flag ZNF224 or 1,5µg 3X-Flag empty vector as control (-). (A) Western blot analysis of ZNF224-Flag and AXL protein levels. Tubulin was used as loading control. (B) AXL mRNA levels were measured by RT-qPCR. Relative amounts as compared to control are shown. Error bars represent standard deviations of two independent experiments. (C) K562 cells stably transfected with ZNF224-Flag or 3X-Flag empty vector (control) were treated with Imatinib 1 µM or vehicle only (DMSO) for 24 hours. Cell death was determined by annexin V labeling followed by flow cytometry. (* = p-value <0.05; ** = p-value <0.005)

12A



12B



12C

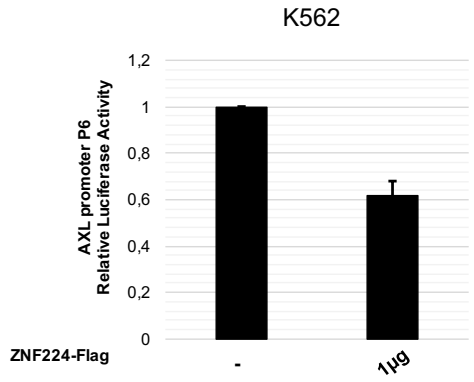


Figure 12: **ZNF224 reduces AXL expression via a transcriptional mechanism**
(A) Schematic representation of AXL promoter region and P6 constructs of the AXL promoter. (B) Luciferase reporter assay showing AXL promoter activity. 0.5 µg of AXL P6 construct were transfected into HEK293 cells together with 0.4µg, 0.6µg, 0.8µg of 3X-Flag ZNF224 or 3X-Flag empty vector as control (-). 24h hours after transfection, promoter activity was determined by normalizing Firefly to Renilla luciferase activity. Error bars represent standard deviations of two independent experiments. ZNF224 overexpression was controlled by Western Blot analysis. G3PDH was used as loading control. One representative blot out of two performed is presented. (C) Luciferase reporter assay showing ZNF224 effect on the promoter activity of P6-AXL construct in K562 cells. K562 cells were transfected with P6-AXL together with 1µg of 3X-Flag ZNF224 or 1µg 3X-Flag empty vector as control (-). 48 hours after transfection promoter activity was determined by normalizing Firefly to renilla luciferase activity. Shown is promoter activity compared to control cells (-). Error bars represent standard deviations of three independent experiments.

13A **13B**

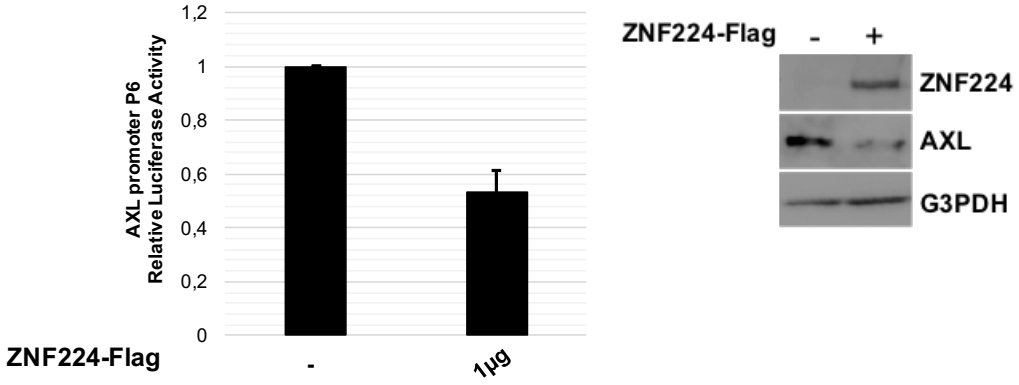
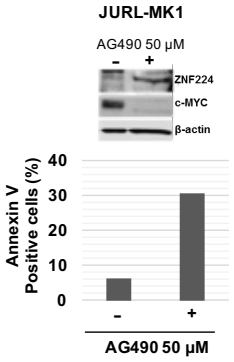


Figure 13: **ZNF224 reduces AXL expression in K562 Ima-R cells**
(A) Luciferase reporter assay showing ZNF224 effect on the promoter activity of P6-AXL construct in K562 Ima-R cells. K562 Ima-R cells were transfected with P6-AXL, together with 1µg of 3X-Flag ZNF224 or 1µg 3X-Flag empty vector as control (-). 48 hours after transfection promoter activity was determined by normalizing Firefly to renilla luciferase activity. Shown is promoter activity compared to control cells (-). Error bars represent standard deviations of two independent experiments. (B) K562 Ima-R cells were transfected with 1.5µg of 3X-Flag ZNF224 or 1.5µg 3X-Flag empty vector as control (-). ZNF224-Flag and AXL protein levels were measured by Western Blot analysis. G3PDH was used as loading control. One representative blot out of two is presented.

4.7 Supplementary data

S1



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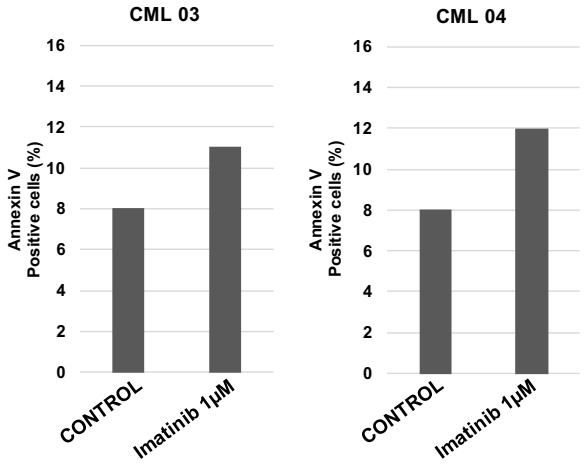

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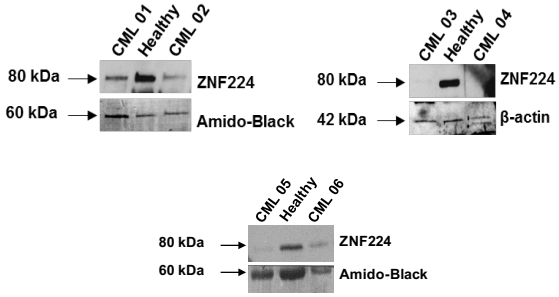

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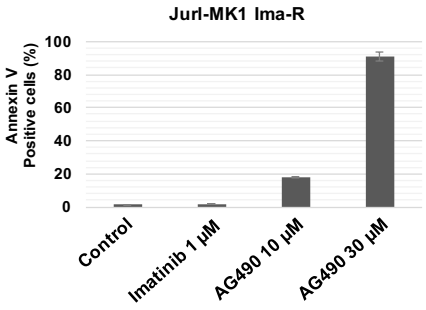

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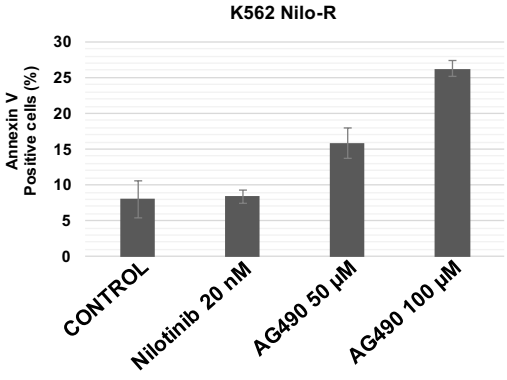

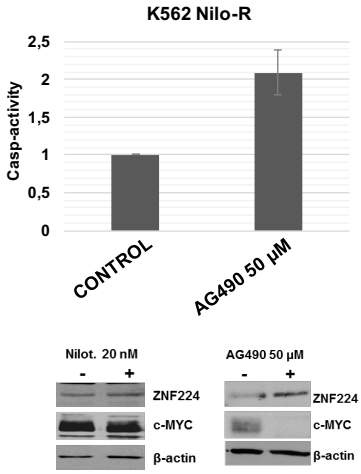

Figure S1: AG490 increases ZNF224 expression and induces cell death in Jurl-MK1 CML cells. Jurl-MK1 cells were treated with 50 µM AG490 for 48 hours or vehicle only (DMSO) as control (-). ZNF224 and c-Myc protein levels were measured by Western Blot analysis. ß-actin was used as loading control. Cell death was evaluated by annexin V staining followed by flow cytometry. Results represent the means +/- SD of two independent experiments.

Figure S2: PBMC from CML patients are sensitive to Imatinib treatment.
PBMC from 2 CML patients at diagnosis (CML03 and CML04) were treated with 1 µM Imatinib for 24 hours. Cell death was determined by annexin V staining followed by flow cytometry.

Figure S3: Evaluation of ZNF224 protein levels in PBMC from CML patients at diagnosis
ZNF224 expression level assessed by Western blot in peripheral blood of 6 patients with CML at diagnosis (CML01, CML02, CML03, CML04, CML05, CML06) and healthy donors (Healthy). Amido-black membrane-coloration or β-actin were used as loading control.

Figure S4: AG490 induces cell death in Jurl-MK1 Ima-R cells.
Jurl-MK1 Ima-R cells were exposed to 1 µM Imatinib or increasing concentrations of AG490 (10 µM and 30 µM) or vehicle only (DMSO) as control (-) for 48 hours. Cell death was determined by annexin V staining followed by flow cytometry. Error bars represent standard deviations of two independent experiments.

Figure S5: AG490 induces apoptosis and increases ZNF224 expression in K562 Nilo-R cells.
K562 Nilo-R cells were exposed to 20 nM Nilotinib or increasing concentrations of AG490 (50 µM and 100 µM) or vehicle only (DMSO) as control (-) for 48 hours. Cell death was determined by annexin V staining followed by flow cytometry. Error bars represent standard deviations of two independent experiments. Caspase activity was biochemically measured. Error bars represent standard deviations of two independent experiments. ZNF224 and c-Myc protein levels were measured by Western Blot analysis. ß-actin was used as loading control. One representative blot out of two is presented.

5. DISCUSSION

The Kruppel-like zinc-finger protein ZNF224 plays a proapoptotic role in CML by acting as a transcriptional cofactor of the Wilms' Tumor protein 1 (WT1) and leading to down-regulation of antiapoptotic genes such as bag3 and A1/Bfl1 and the up-regulation of proapoptotic genes such as VDR, Bax, Bak and IRF8. Moreover, we demonstrated that ZNF224 induction is necessary for ara-C induced apoptosis in CML [64,65,70].

Most recently, we demonstrated that Bcr-Abl oncoprotein, which drives the oncogenic transformation in CML, negatively regulates ZNF224 expression. By investigating the molecular mechanism involved in the Bcr-Abl-dependent ZNF224 dowmodulation we identified WT1, whose expression is induced by Bcr-Abl oncogenic signaling, as a transcriptional repressor of ZNF224 in CML. Accordingly, Imatinib, Nilotinib and Dasatinib, by blocking Bcr-Abl tyrosine kinase activity, are able to restore ZNF224 expression in CML cells [68]. Additional studies will be needed to identify other Bcr-Abl downstream effectors involved in ZNF224 regulation. In this work, we characterized a new mechanism by which ZNF224 exert its anti-oncogenic role in CML.

c-Myc oncogene induction by Bcr-Abl represents a necessary step for the oncogenic transformation of CML cells. In agreement, Imatinib treatment reduces c-Myc expression and hematopoietic tumoral features of CML cells. Coherently, we observed that ZNF224 induction by Imatinib well correlated with c-Myc oncogene reduction and cell death induction in K562 CML cells. Starting from these observations and from the identification of ZNF224 putative binding sites on c-Myc promoter, we demonstrated that ZNF224 is a novel transcriptional repressor of c-Myc gene

Interestingly, we demonstrated ZNF224 repression on c-Myc oncogene both in K562 CML cells and in HEK293 cells, which lacks WT1 expression, thus showing that c-Myc is a direct target of ZNF224 transcriptional repression. However, since WT1 has been reported as a transcriptional activator of c-Myc oncogene in CML [71] we cannot rule out also the involvement of ZNF224/WT1 complex in c-Myc transcriptional regulation in CML cells, as already observed for other apoptosis regulating genes [68]. Thus, we could speculate that ZNF224 downmodulates c-Myc transcription in CML cells, acting both as a transcriptional repressor that as a WT1 transcriptional cofactor.

We also showed that ZNF224 repression on c-Myc in CML coherently resulted with a decreased proliferation and c-Myc proliferative network, leading to a decreased cyclin D1 expression, a positive c-Myc target gene and an increased p21 expression, a negative c-Myc target gene.

Altogether, these data highlighted a new anti-oncogenic mechanism by which the transcription factor ZNF224 could reduce CML progression.

In agreement with the pivotal role of c-Myc oncogene in sustaining Bcr-Abl oncogenic transformation in CML, the c-Myc reduction is a key step for Imatinib induced apoptosis in CML [13,23,24]. Interestingly, we further demonstrated that ZNF224 mediates, at least in part, Imatinib repression on c-Myc. Indeed, the blocking of Bcr-Abl tyrosine-kinase activity by Imatinib, increased ZNF224 expression and its binding on c-Myc promoter in CML cells, while ZNF224 depletion significantly affected Imatinib repression on c-Myc expression. In consistence with the key role of c-Myc reduction in Imatinib responsiveness and with the already described pro-apoptotic role of ZNF224 in CML cells, ZNF224 knockdown led to an expected impairment in Imatinib-induced cell death.

Furthermore, we observed that ZNF224 forced expression in K562 Ima-R cells was accompanied by c-Myc reduction and cell death induction, thus suggesting that the induction of ZNF224 expression could contribute to overcome Imatinib-resistance in CML.

It has been previously demonstrated by Xie et al [22] that Bcr-Abl-mediated induction of c-Myc expression is dependent on activated Jak2 tyrosine kinase, that plays a key role in the stabilization of c-Myc protein and in the induction of c-Myc mRNA transcription in CML, although the molecular mechanisms of this induction have not been clarified.

Consistently, Jak2 kinase inhibitors, such as AG490, strongly reduce c-Myc expression in CML cells and induce apoptosis, thus representing promising molecular tools to override Imatinib resistance in CML.

Exploiting the newly discovered link between ZNF224 and c-Myc downstream Bcr-Abl in CML, we showed that ZNF224 expression is induced by AG490. Interestingly, ZNF224 induction mediated, at least in part, c-Myc transcriptional downmodulation by AG490. These findings suggest that ZNF224 downmodulation could represent one of the transcriptional mechanisms, still largely unexplored, by which Jak2 upregulates c-Myc expression in CML.

Most interestingly, we also demonstrated that AG490 is able to induce apoptosis in Imatinib-resistant K562 cells, at least in part, via ZNF224 induction and consequent c-Myc repression.

These new findings could be further corroborated by transcriptome analysis of K562 Ima-R cells treated with AG490 or overexpressing ZNF224, in order to find new molecular targets involved in apoptosis induction in Imatinib resistant cells.

Furthermore, in accordance with our recent results, reporting a good correlation between ZNF224 high expression at diagnosis and optimal Imatinib responsiveness in CML patients [68], we interestingly observed a lower expression of ZNF224 in different TKI-resistant cell lines, as compared to their sensitive counterparts.

In the context of TKIs resistance in CML, several studies contributed to reveal new molecular effectors strictly linked to resistance, such as LYN, FYN, and AXL [10,11,12,44]. More in details, Auberger et al. demonstrated that AXL overexpression is involved in Imatinib resistance of K562 Ima-R cells, analyzed in this work [12].

Consistently, we detected a good anti-correlation between ZNF224 and AXL expression by comparing TKIs sensitive and resistant CML cell lines.

Subsequently, we demonstrated that ZNF224 exerts a transcriptional repression on AXL gene in both K562 wild-type cells and K562 Ima-R cells. In consistence with the re-sensitization to Imatinib observed in K562 Ima-R cells silenced for AXL [12], our results indicate that ZNF224 suppression on AXL expression could be involved in this re-sensitization. Therefore, it could be of great interest to explore the epigenetic, transcriptional and post-transcriptional mechanisms potentially involved in ZNF224 regulation in CML resistant cells, in order to induce its expression. As we previously demonstrated, WT1 is a transcriptional repressor of ZNF224 downstream Bcr-Abl/PI3K in CML [68], and it is reported to contribute to Imatinib resistance [72]; therefore, we cannot exclude that the deregulation of the WT1 pathway in K562 Ima-R cells could be involved in ZNF224 downregulation.

Altogether, the present study revealed new potential ZNF224 regulatory roles in CML, paving the basis to consider ZNF224 as a new potential diagnostic tool for prognosis and drug resistance prediction in CML. Moreover, we enforced the rationale to consider ZNF224 induction as a pertinent option to hamper CML progression and hopefully overcome TKI-resistance.

6. REFERENCES

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7. LIST OF PUBLICATIONS

- Role of ZNF224 in cell growth and chemoresistance of Chronic Lymphocitic Leukemia. Busiello T, Ciano M, Romano S, **Sodaro G**, Garofalo O, Bruzzese D, Simeone L, Chiurazzi F, Romano MF, Costanzo P, Cesaro E. Human Molecular Genetics Accepted for publication. Acceptance date: 2016-12-18. DOI: 10.1093/hmg/ddw427

- The Complex Role of the ZNF224 Transcription Factor in Cancer. E. Cesaro, **G. Sodaro**, G. Montano, M. Grosso, A. Lupo, P. Costanzo. Advances in Protein Chemistry and Structural Biology December 2016. Available online 5 December 2016 DOI: 10.1016/bs.apcsb.2016.11.003

- The hematopoietic tumor suppressor interferon regulatory factor (IRF8) is upregulated by the antimetabolite cytarabine in leukemic cells involving the zinc finger protein ZNF224, acting as a cofactor of the Wilms' tumor gene 1 (WT1) protein. Montano G, Ullmark T, Jernmark-Nilsson H, **Sodaro G**, Drott K, Costanzo P, Vidovic K, Gullberg U. Leuk Res. 2016 Jan;40:60-7. doi: 10.1016/j.leukres.2015.10.014. PMID: 26563595

- WT1-mediated repression of the proapoptotic transcription factor ZNF224 is triggered by the Bcr-Abl oncogene. Montano G, Vidovic K, Palladino C, Cesaro E, **Sodaro G**, Quintarelli C, De Angelis B, Errichiello S, Pane F, Izzo P, Grosso M, Gullberg U, Costanzo P. Oncotarget. 2015 Sep 29;6(29):28223-37. doi: 10.18632/oncotarget.4950. PMID: 26320177

ONGOING MANUSCRIPT FOR PUBLICATION

ZNF224 regolatory role in Chronic Myelogenous Leukemia, downstream Bcr/Abl. **G Sodaro**, G Montano, G Blasio, F Fiorentino, E Cesaro, P Costanzo. Expected year of publication: 2017.