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The hematopoietic tumor suppressor interferon regulatory factor 8 (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

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ABSTRACT

The transcription factor interferon regulatory factor-8 (IRF8) is highly expressed in myeloid progenitors, while most myeloid leukemias show low or absent expression. Loss of IRF8 in mice leads to a myeloproliferative disorder, indicating a tumor-suppressive role of IRF8. The Wilms tumor gene 1 (WT1) protein represses the IRF8-promoter. The zinc finger protein ZNF224 can act as a transcriptional co-factor of WT1 and potentiate the cytotoxic response to the cytostatic drug cytarabine. We hypothesized that cytarabine upregulates IRF8 and that transcriptional control of IRF8 involves WT1 and ZNF224. Treatment of leukemic K562 cells with cytarabine upregulated IRF8 protein and mRNA, which was correlated to increased expression of ZNF224. Knock down of ZNF224 with shRNA suppressed both basal and cytarabine-induced IRF8 expression. While ZNF224 alone did not affect IRF8 promoter activity, ZNF224 partially reversed the suppressive effect of WT1 on the IRF8 promoter, as judged by luciferase reporter experiments. Coprecipitation revealed nuclear binding of WT1 and ZNF224, and by chromatin immunoprecipitation (ChIP) experiments it was demonstrated that WT1 recruits ZNF224 to the IRF8 promoter. We conclude that cytarabine-induced upregulation of the IRF8 in leukemic cells involves increased levels of ZNF224, which can counteract the repressive activity of WT1 on the IRF8-promoter.

Key-words:

leukemia, IRF8, WT1, ZNF224, cytarabine, transcription

1. INTRODUCTION

Interferon regulatory factor-8 (IRF8) (also called interferon consensus sequence binding protein, ICSBP) is an interferon γ -inducible transcription factor, expressed during hematopoiesis in cells of the myeloid, as well as of the B-lymphocyte lineage [1,2]. In normal hematopoiesis, IRF8 expression is high in myeloid, lymphoid and dendritic progenitors [3]. On the other hand, levels of IRF8 are low or absent in leukemic blasts from a majority of chronic myeloid leukemia (CML) or acute myeloid leukemia (AML) patients [4], often due to promoter methylation [5,6], suggesting an anti-leukemic role for IRF8. Several mechanisms downstream of IRF8 that are relevant for an anti-oncogenic function have been reported, including repression of *BCL2L1* (Bcl-X_L) [7], *MYC* [8], *BCL2* [9], *PTPN13* [10], *CTNNB1* (β -catenin) [11,12], and enhanced expression of *CASP3* [7], and *NF1* [13]. Loss of IRF8 in mice leads to deregulated myeloid differentiation with an accumulation of neutrophil-like cells, resembling human CML [14]. CML is driven by the oncogenic fusion protein BCR-ABL with constitutive tyrosine kinase activity [15]. Forced expression of IRF8 antagonizes the BCR-ABL-induced leukemic phenotype *in vitro* and *in vivo* [8,9]. Moreover, treatment of leukemic cells with the BCR-ABL-inhibitor imatinib or with retinoic acid, both clinically used in the treatment of leukemia, increases expression of IRF8 [16,17], further emphasizing the tumor suppressor function of IRF8 in myeloid malignancies.

Transcriptional control of IRF8 in leukemic cells is incompletely understood. The Wilm's tumor gene 1 (WT1) protein is a zinc-finger transcription factor normally expressed in a small subset of hematopoietic progenitor cells [18,19,20,21,22], suggesting a role in early hematopoiesis. WT1 is commonly overexpressed in myeloid leukemias [21,23,24,25,26] and WT1 cooperates with the leukemia fusion protein RUNX1 (AML1-ETO) to rapidly induce leukemia in mice, demonstrating a leukemogenic role for WT1 [27]. Recently, we showed that WT1 targets the *IRF8*-promoter, resulting in transcriptional repression [16]. Moreover, expression levels of *WT1* and *IRF8* in primary acute myeloid leukemias are highly anticorrelated [16]. Thus, WT1-mediated repression of IRF8 provides one explanation for the generally low expression of IRF8 in CML and AML. Interestingly, the constitutive tyrosine kinase activity of BCR-ABL causes increased expression of WT1, indicating a BCR-ABL-WT1-IRF8 pathway in CML [16,28].

The zinc-finger protein ZNF224 was first identified as a repressor of the human aldolase A gene [29]. In contrast to the restricted expression of WT1 in adult tissues, ZNF224 is ubiquitously expressed. A functional interaction between ZNF224 and WT1 was shown as ZNF224 functions as a coactivator of WT1 on the promoter of the vitamin D-receptor (VDR), demonstrating that ZNF224 can also activate transcription [30]. The role of ZNF224 in WT1-mediated transcriptional regulation was recently extended to a number of WT1-target genes involved in the regulation of apoptosis. On the promoter of these genes, ZNF224 acts as a co-activator of proapoptotic genes, while repressing expression of antiapoptotic WT1 target genes [31]. In this way, ZNF224 shifts the balance of antiapoptotic and proapoptotic signals in favor of the latter. Consistent with a proapoptotic role, overexpression of ZNF224 in leukemic cells potentiated the cytotoxic response to the cytostatic drug and antimetabolite cytarabine [31], while WT1 can confer resistance to this treatment [32].

Cytarabine is one of the most commonly used drugs used for treatment of AML [33]. Within the cell, the pyrimidine nucleoside analog cytarabine is activated into ara-CTP which is incorporated into DNA of proliferating cells in place of deoxycytidine triphosphate (dCTP), thus blocking DNA synthesis, resulting in proliferation arrest and cell death [34]. Cytarabine may also be incorporated into DNA repair patches of quiescent cells, leading to inhibition of DNA-repair [35,36].

The aim of this work was to elucidate whether expression of the tumor suppressor IRF8 is affected by cytarabine, and whether ZNF224 cooperates with WT1 in transcriptional regulation of the *IRF8*-promoter.

2. MATERIAL AND METHODS:

2.1 Cell culture

The human kidney cancer cell line HEK293T/17 was cultured in Dulbecco's modified Eagle's medium, (Hyclone Laboratories Inc, Utah, U.S.A), supplemented with 10% fetal calf serum and 100 µg/ml streptomycin-penicillin mix (Bio-Whittaker Inc, MD, USA) at 37°C in 5% CO₂. The leukemic cell line K562 (DSMZ, Braunschweig, Germany) was cultured in RPMI 1640 (Gibco Life Technologies, NY, USA) supplemented with 10% fetal calf serum at 37°C in 5% CO₂. K562 cells were treated 1 µM cytarabine (Pfizer AB, Sollentuna, Sweden) for 72 h. Concentration of cytarabine was carefully titrated in initial experiments to generate maximal proliferation arrest.

2.2 Immunoblotting

Harvested cells for immunoblotting were resuspended in Laemmli buffer (#161-0737, Bio-Rad Laboratories, Hercules, CA, USA) containing 0.2M β-mercaptoethanol (Sigma-Aldrich). Proteins were separated by SDS-PAGE (12% TGX gel, #456-1043, Bio-Rad) and transferred to a Hybond ECL membrane (GE Healthcare, Uppsala, Sweden). Primary antibodies used were: rabbit anti-IRF8 antibody (MBS224027, MyBioSource.com, CA, USA), rabbit anti-ZNF224 (T3) antibody (29), rabbit anti-WT1 (C-19, Santa Cruz, CA, USA), and mouse anti-GAPDH (7-B, Santa Cruz, CA, USA). The EZ-ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel) was used for analysis of protein bands with a ChemiDocTMXRS⁺ system (BioRad).

2.3 shRNA-mediated knockdown of ZNF224

Pools of K562 cells expressing ZNF224 shRNA, were obtained as previously described [31]. Briefly, K562 cells were transfected with 1.5 µg of short-interfering RNA plasmid SH2351C3 (Open Biosystem, Huntsville, AL, USA) using the HiPerFect Reagent (Qiagen, Venlo, Netherlands), according to the manufacturer's protocol; transfection of a non-silencing shRNA (scrambled shRNA) (Open Biosystem) was used as negative control. Transfected cells were selected by culture in the presence of puromycin (500 µg/ml) (Promega Corporation, Wisconsin, USA) for 4 weeks. Suppression of ZNF224 protein expression in response to shRNA expression was previously characterized by immunoblotting [31].

2.4 RNA isolation, reverse transcription and quantitative PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, after which RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA) with random hexamer primers according to the manufacturer's instructions. Quantitative PCR (qPCR) was carried out using TaqMan probe-based chemistry (Applied Biosystems); the probe for ZNF224 (Hs00273760_m1), IRF8 (Hs00175238_m1), WT1 (Hs00240913_m1), and the endogenous controls beta-actin (Hs99999903_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were purchased as Assay-on-Demand (Applied Biosystems). The amplification reactions were all performed in triplicates in a StepOnePlus (Applied Biosystems). Data were collected and analyzed using the Applied Biosystems StepOne™ Real-Time PCR Software v2.0. The relative quantification in gene expression was determined using the $\Delta\Delta C_t$ method [37]. Efficacy of the PCR amplification of controls and test was identical; parallelism of standard curves of the control and test was confirmed.

2.5 Transient transfection and luciferase-reporter assays

HEK293T/17 cells were transiently transfected using Lipofectamine reagent (Life Technologies, CA, USA) in 12-well plates with 200 ng of a luciferase reporter plasmid containing the proximal IRF8 promoter including a WT1 response element [16], and the 3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(+/-) expression plasmids at indicated combinations and concentrations. To normalize the luciferase assay a pRL-CMV plasmid (20 ng) encoding the renilla luciferase was used. Dual-Luciferase Reporter Assay System (Promega Corporation, WI, USA) was performed 48h after the transfection, according to the manufacturer's instructions.

2.6 Chromatin Immunoprecipitation (ChIP) assay

ChIP experiments were performed using Chromatin Immunoprecipitation assay kit (Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Briefly, cross-linked chromatin was prepared from HEK293T/17 cells transfected with 3XFLAG-CMV-7.1-ZNF224 or with pcDNA3WT1(+/-), or co-transfected with

3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(+/-). The antibodies anti-ZNF224 (G-16), anti-WT1 (C19), and anti-HA were from Santa Cruz Biotechnology, TX, USA. ChIP samples were analyzed by qPCR as described (Montano 2013), using 2 µl of immunoprecipitated material and 1 µl of input control diluted 1:30 as templates. Specific primers for a WT1-binding region at -52 to -38 on IRF8 promoter were 5'-ttctcggaagcagagcacttc-3' (forward), and 5'-gccttaaaaagggtcgtggg-3' (reverse) (16); for a WT1-binding region at the aldolase A promoter 5'-ccctctgttcactgggcaagt-3' (forward) and 5'-ccattccagttcccaggcctgggtg-3' (reverse) (31); and for the GAPDH promoter (used as negative control) 5'-ggtcgtattgggcgcctggtcacca-3' (forward) and 5'-cacacccatgacgaacatgggggc-3' [31].

2.7 Co-immunoprecipitation assay

HEK293 cells were transfected with the plasmids p3XFLAG-ZNF224 and pcDNA3-WT1(+/-). Total protein extract, nuclear and cytoplasmic extracts were obtained as previously described [30]. Immunoprecipitation using an anti-WT1 antibody (F6 Santa Cruz Biotechnology) or the IgG control antibody (Santa Cruz Biotechnology) was performed as described [30].

2.8 Statistical analysis:

Statistical analysis was performed using the paired one-tailed t-test. Stars represent conventional significance levels; single star indicates, $p < 0.05$, double stars $p < 0.01$, and triple stars $p < 0.001$.

3. RESULTS

3.1 Induction of IRF8 by cytarabine is correlated to upregulation of ZNF224.

Clinical intermediate doses of cytarabine produce plasma levels of 7 μM or higher [38]. We have previously shown that treatment of leukemic K562 cells with 1 μM cytarabine for 72 hours induces some degree of apoptosis, correlated to induction of ZNF224 [31]. Given the anti-oncogenic function of IRF8, we now asked whether this cytostatic drug also affects the levels of IRF8. As shown in Fig 1, treatment of K562 cells with cytarabine indeed upregulated the amount of IRF8 protein. The increase of IRF8 protein was correlated to strongly raised levels of IRF8 mRNA in K562 cells (Fig 2A), indicating transcriptional effects. Confirming previous data [31], cytarabine induced an upregulation also of ZNF224 (Fig 2B). Cytarabine caused a prompt proliferation arrest (Fig 2C) and significant cell death (Fig 2D). We conclude that cytarabine-induced IRF8 expression is correlated to upregulation of ZNF224.

3.2 Cytarabine-induced upregulation of IRF8 is dependent on levels of ZNF224

Given that ZNF224 can act as a cofactor to WT1 [30,31] and that *IRF8* is a target gene of WT1 [16], the finding above led us to speculate that levels of ZNF224 can affect the expression of IRF8. To investigate this issue, we compared the response to cytarabine in K562 cells transfected with an shRNA directed against ZNF224, to the response in control cells expressing a scrambled control shRNA. These shRNA-transfected K562 cells have been previously used and the shRNA-mediated suppression of ZNF224 protein expression has been demonstrated [31] and thus is not shown here. K562 expresses high levels of WT1, giving preconditions for ZNF224 to act as a cofactor of WT1. As expected and in accordance with previous data [31], cells transfected with shZNF224 showed approximately 50% reduction of ZNF224 expression, as compared to control cells (Fig 3A). Interestingly, the reduction in ZNF224 resulted in a corresponding decrease also of IRF8 levels, suggesting that ZNF224 influences the expression of IRF8 (Fig 3B). After treatment with cytarabine, the induced increase of ZNF224, as well as the strongly induced increase of IRF8, was significantly quenched by shZNF224 (Fig 3A, B), further supporting a role of ZNF224 in IRF8 expression. While suppression of ZNF224 on its own did not affect viability, as compared to control cells, cell death caused by cytarabine was dampened by expression of shZNF224 (Fig 3C), consistent with previous data [31]. We conclude

that knockdown of ZNF224 results in increased resistance to cytarabine, correlated to quenched upregulation of IRF8.

3.3 WT1-mediated repression of the IRF8-promoter is counteracted by ZNF224

IRF8 has been defined as a target gene repressed by WT1 [16]. Our findings above, suggesting that ZNF224 positively affects IRF8-expression, therefore led us to hypothesize that ZNF224 acts as a cofactor of WT1 on the *IRF8* promoter. To investigate direct effects of ZNF224 on the transcriptional regulation of the *IRF8* promoter, we performed luciferase-reporter experiment in HEK293 cells. IRF8 expression is suppressed by WT1, in particular by the WT1(+/-) isoform, in leukemic cells [16]. The WT1(+/-) isoform represses transcription from an IRF8 promoter construct containing 970 bp upstream of the transcription start [16]. When this promoter was transfected to HEK293 cells, ZNF224 alone did not affect promoter activity, while, as expected, WT1(+/-) by itself resulted in suppression of the IRF8 promoter (Fig 4). Interestingly, increasing amounts of ZNF224 showed a dose-response alleviation of the WT1-mediated suppression, indicating that ZNF224 interferes with WT1-mediated transcriptional repression of the IRF8 promoter (Fig 4).

3.4 WT1(+/-) interacts with ZNF224 in the nucleus

The findings above raised the question whether ZNF224 interacts with WT1(+/-). We performed coprecipitation experiments to investigate that issue. Since there are no isoform-specific antibodies to WT1 available, we used HEK293 cells that lack detectable expression of endogenous WT1. The cells were transfected with ZNF224 and WT1(+/-) after which nuclear and cytosolic subcellular fractions were prepared. As shown in Fig 5A, immunoprecipitation of WT1(+/-) coprecipitated ZNF224 in the nuclear fraction, but not in the cytosol (Fig 5B). Nuclear, but not cytosolic, interaction is in agreement with previous data concerning the interaction between ZNF224 and the WT1(-/-) splice variant [30]. Analysis of c-myc and tubulin was used as markers for the nucleus and cytosol, respectively (Fig 5C and 5D). We conclude that ZNF224 binds to WT1(+/-) in the nucleus, consistent with a role for ZNF224 in modulation of WT1-mediated transcriptional control.

3.5 WT1(+/-) recruits ZNF224 to the IRF8 promoter.

To find further evidence for interaction between WT1(+/-) and ZNF224 on the *IRF8* promoter, we performed chromatin immunoprecipitation (ChIP) experiments. Again, HEK293 cells lacking endogenous WT1 expression were utilized. After transfection of cells with WT1(+/-), ZNF224, or with both, ChIP was performed with antibodies against WT1 or ZNF224. Precipitation with antibody against hemagglutinin (HA) and PCR-amplification of *GAPDH* were used as negative controls. As shown in Fig 6A, WT1 showed binding to the IRF8 promoter when transfected alone, as previously reported [16]. Also in consistence with previous data, ZNF224 alone did bind to the aldolase A promoter [29] (Fig 6B). However, ZNF224 alone failed to bind to the IRF8 promoter (Fig 6B). Interestingly, upon cotransfection with both ZNF224 and WT1(+/-), the ChIP analysis showed binding of ZNF224 to the *IRF8* promoter (Fig 6C). These results demonstrate that ZNF224 is dependent on WT1 for this particular binding and thus that WT1 recruits ZNF224 to the *IRF8* promoter. As control, Fig 6D shows immunoblot analysis of ZNF224 and WT1 protein in transfected cells from which the ChIP analyses were performed. We conclude that ZNF224 is recruited to the *IRF8* promoter in a WT1-dependent manner.

4. DISCUSSION

Preclinical and clinical data emphasize the anti-oncogenic role for the transcription factor IRF8 in hematopoietic malignancies. In consistence with a tumor suppressor function, expression of IRF8 is commonly downregulated or absent in myeloid leukemias [4,5,6]. Certain oncogenes, such as the fusion protein BCR-ABL, signal for downmodulation of IRF8-expression [39], with the transcription factors WT1 and STAT5 as downstream mediators, targeting and repressing the IRF8-promoter [16,28,40]. In this study, we show that IRF8 is transcriptionally upregulated by the cytostatic drug cytarabine involving mechanisms that include the zinc finger-protein ZNF224, which binds to and counteracts the repressive effect of WT1 on the IRF8-promoter.

The conclusion that IRF8 is transcriptionally upregulated in response to cytarabine is based on our findings that mRNA levels of IRF8 are 30 to 40-fold upregulated in response to the cytostatic drug, making posttranscriptional mechanisms unlikely. The concentration of cytarabine used (1 μ M) is lower than serum levels obtained after intermediate dosage of cytarabine in clinical use (>7 μ M), concentrations at which the conversion of cytarabine into active ara-CTP is saturated [38]. The relatively low concentration used in this study might therefore suggest that non-cytotoxic effects downstream of cytarabine may be involved in IRF8 regulation, consistent with our data showing rather weak effects on the viability of K562 cells. Indeed, previous reports show that low amounts of cytarabine (10-250 nM) can induce differentiation of K562 as shown by surface markers and hemoglobin production (41,42). In the present investigation, we cannot exclude that some differentiation of the K562 cells occurred prior to the induction of apoptosis. However, also other WT1 target genes involved in the apoptotic pathway, including *BAK*, *BAX* and *BCL2A1*, are induced in K562 cells by cytarabine (31), consistent with an apoptotic response. Although the exact mechanism by which cytarabine induces increased transcription of IRF8 remains to be defined, our finding that knock-down of ZNF224 lessens cytarabine-induced increase of IRF8 indicates the importance of ZNF224 for IRF8 transcription. ZNF224 specifically binds to DNA on the promoter of the aldolase A gene [29], but can also indirectly bind to certain promoters via interaction with the transcription factor WT1 [30], thus fine tuning the effect of WT1 on transcription [31]. Here, we show that ZNF224 is recruited to the IRF8 promoter via binding to WT1, as demonstrated by co-

precipitation and ChIP-experiments. Our finding that the WT1^{+/-} isoform interacts with ZNF224 in the nucleus is in accordance with previous data for the WT1^{-/-} isoform [30], indicating that the 17AA insert in the WT^{+/-} isoform is dispensable for binding to ZNF224. Importantly, our ChIP experiments clearly show the functional relevance of the interaction between WT1^{+/-} and ZNF224, since the latter is not able to bind to the IRF8 promoter, unless WT1^{+/-} is also present. The ChIP analysis demonstrates recruitment of ZNF224 to the IRF8 promoter in the absence of cytarabine treatment. Whether cytarabine signaling results in protein modifications of WT1 or ZNF224 that increase their interaction, or if the effect of cytarabine is merely a result of increased levels of ZNF224 (Fig 2 and 3) and decreased expression of WT1 [31], remains to be defined.

The notion that ZNF224 is dependent on WT1 to be active on the *IRF8* promoter is further supported by our results showing that ZNF224 has no effect on its own on the *IRF8*-promoter reporter construct. However, as judged by impaired inhibition of promoter-reporter activity, ZNF224 counteracts the repressive effect of WT1 on the *IRF8* promoter. Moreover, knock down of ZNF224 significantly repressed cytarabine-induced upregulation of IRF8, demonstrating the positive effect of ZNF224 on IRF8 expression. Together with the previously shown downregulation of WT1 in response to cytarabine [31], upregulation of ZNF224 therefore provides one mechanistic explanation for the strong effect on IRF8 expression by this cytostatic drug. Although we cannot exclude additional mechanisms, our results indicate that WT1 and ZNF224 are important mediators of the effect of cytarabine on IRF8 expression.

These data extend our previous findings that ZNF224 can act as a transcriptional co-factor of WT1 [30,31]. Here, we demonstrate that ZNF224 is able to counteract the repressive effect of oncogenic WT1 on the tumor suppressor IRF8, providing novel insights into the regulative mechanisms triggered by the WT1/ZNF224 complex in leukemic cells.

5. CONCLUSIONS:

This work shows that the myeloid tumor suppressor IRF8 is induced in leukemic cells by the commonly used cytostatic drug cytarabine. This effect of cytarabine is dependent on ZNF224, which modulate transcription of IRF8 by binding to WT1 on the promoter, thus counteracting the repressive effect of the oncoprotein WT1 on IRF8 expression. We conclude that WT1 and ZNF224 are regulators of the tumor suppressor IRF8 in the response to cytarabine.

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REFERENCES

1. Wang H, Morse HC 3rd. IRF8 regulates myeloid and B lymphoid lineage diversification. *Immunol Res*. 2009;43:109-17.
2. Tamura T, Kurotaki D, Koizumi S. Regulation of myelopoiesis by the transcription factor IRF8. *Int J Hematol*. 2015;101:342-51.
3. Wang H, Yan M, Sun J, Jain S, Yoshimi R, Abolfath SM, Ozato K, Coleman WG Jr, Ng AP, Metcalf D, DiRago L, Nutt SL, Morse HC 3rd. A reporter mouse reveals lineage-specific and heterogeneous expression of IRF8 during lymphoid and myeloid cell differentiation. *J Immunol*. 2014;193:1766-77.
4. Schmidt M, Nagel S, Proba J, Thiede C, Ritter M, Waring JF, Rosenbauer F, Huhn D, Wittig B, Horak I, Neubauer A. Lack of interferon consensus sequence binding protein (ICSBP) transcripts in human myeloid leukemias. *Blood*. 1998;91:22-9.
5. Hu X, Yang D, Zimmerman M, Liu F, Yang J, Kannan S, Burchert A, Szulc Z, Bielawska A, Ozato K, Bhalla K, Liu K. IRF8 regulates acid ceramidase expression to mediate apoptosis and suppresses myelogenous leukemia. *Cancer Res*. 2011;71:2882-91.
6. Otto N, Manukjan G, Göhring G, Hofmann W, Scherer R, Luna JC, Lehmann U, Ganser A, Welte K, Schlegelberger B, Steinemann D. ICSBP promoter methylation in myelodysplastic syndromes and acute myeloid leukaemia. *Leukemia*. 2011;25:1202-7.
7. Gabriele L, Phung J, Fukumoto J, Segal D, Wang IM, Giannakakou P, Giese NA, Ozato K, Morse HC 3rd. Regulation of apoptosis in myeloid cells by interferon consensus sequence-binding protein. *J Exp Med*. 1999;190:411-21.
8. Tamura T, Kong HJ, Tunyaplin C, Tsujimura H, Calame K, Ozato K. ICSBP/IRF-8 inhibits mitogenic activity of p210 Bcr/Abl in differentiating myeloid progenitor cells. *Blood*. 2003;102:4547-54.
9. Burchert A, Cai D, Hofbauer LC, Samuelsson MK, Slater EP, Duyster J, Ritter M, Hochhaus A, Müller R, Eilers M, Schmidt M, Neubauer A. Interferon consensus sequence binding protein (ICSBP; IRF-8) antagonizes BCR/ABL and down-regulates bcl-2. *Blood*. 2004;103:3480-9.
10. Huang W, Zhu C, Wang H, Horvath E, Eklund EA. The interferon consensus sequence-binding protein (ICSBP/IRF8) represses PTPN13 gene transcription in differentiating myeloid cells. *J Biol Chem*. 2008;283:7921-35.
11. Huang W, Zhou W, Saberwal G, Konieczna I, Horvath E, Katsoulidis E, Platanias LC, Eklund EA. Interferon consensus sequence binding protein (ICSBP) decreases beta-catenin activity in myeloid cells by repressing GAS2 transcription. *Mol Cell Biol*. 2010 Oct;30:4575-94.

12. Scheller M, Schönheit J, Zimmermann K, Leser U, Rosenbauer F, Leutz A. Cross talk between Wnt/ β -catenin and Irf8 in leukemia progression and drug resistance. *J Exp Med*. 2013;210:2239-56.
13. Huang W, Horvath E, Eklund EA. PU.1, interferon regulatory factor (IRF) 2, and the interferon consensus sequence-binding protein (ICSBP/IRF8) cooperate to activate NF1 transcription in differentiating myeloid cells. *J Biol Chem*. 2007;282:6629-43.
14. Holtschke T, Löhler J, Kanno Y, Fehr T, Giese N, Rosenbauer F, Lou J, Knobloch KP, Gabriele L, Waring JF, Bachmann MF, Zinkernagel RM, Morse HC 3rd, Ozato K, Horak I. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell*. 1996;87:307-17.
15. Quintás-Cardama A, Cortes J. Molecular biology of bcr-abl1-positive chronic myeloid leukemia. *Blood*. 2009;113:1619-30.
16. Vidovic K, Svensson E, Nilsson B, Thuresson B, Olofsson T, Lennartsson A, Gullberg U. Wilms' tumor gene 1 protein represses the expression of the tumor suppressor interferon regulatory factor 8 in human hematopoietic progenitors and in leukemic cells. *Leukemia*. 2010;24:992-1000.
17. Dimberg A, Kårehed K, Nilsson K, Oberg F. Inhibition of monocytic differentiation by phosphorylation-deficient Stat1 is associated with impaired expression of Stat2, ICSBP/IRF8 and C/EBPepsilon. *Scand J Immunol*. 2006;64:271-9.
18. Fraizer GC, Patmasiriwat P, Zhang X, Saunders GF. Expression of the tumor suppressor gene WT1 in both human and mouse bone marrow. *Blood*. 1995;86:4704-6.
19. Maurer U, Brieger J, Weidmann E, Mitrou PS, Hoelzer D, Bergmann L. The Wilms' tumor gene is expressed in a subset of CD34+ progenitors and downregulated early in the course of differentiation in vitro. *Exp Hematol*. 1997;25:945-50.
20. Baird PN, Simmons PJ. Expression of the Wilms' tumor gene (WT1) in normal hemopoiesis. *Exp Hematol*. 1997;25:312-20.
21. Yang L, Han Y, Suarez Saiz F, Minden MD. A tumor suppressor and oncogene: the WT1 story. *Leukemia*. 2007;21:868-76.
22. Toska E, Roberts SG. Mechanisms of transcriptional regulation by WT1 (Wilms' tumour 1). *Biochem J*. 2014;461:15-32.
23. Miwa H, Beran M, Saunders GF. Expression of the Wilms' tumor gene (WT1) in human leukemias. *Leukemia*. 1992;6:405-9.
24. Miyagi T, Ahuja H, Kubota T, Kubonishi I, Koeffler HP, Miyoshi I. Expression of the candidate Wilm's tumor gene, WT1, in human leukemia cells. *Leukemia*. 1993;7:970-7.
25. Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T, Nasu K, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood*. 1994;84:3071-9.
26. Menssen HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S, Reinhardt R, Thiel E. Presence of Wilms' tumor gene (wt1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. *Leukemia*. 1995;9:1060-7.

27. Nishida S, Hosen N, Shirakata T, Kanato K, Yanagihara M, Nakatsuka S, Hoshida Y, Nakazawa T, Harada Y, Tatsumi N, Tsuboi A, Kawakami M, Oka Y, Oji Y, Aozasa K, Kawase I, Sugiyama H. AML1-ETO rapidly induces acute myeloblastic leukemia in cooperation with the Wilms tumor gene, WT1. *Blood*. 2006;107:3303-12.
28. Svensson E, Vidovic K, Lassen C, Richter J, Olofsson T, Fioretos T, Gullberg U. Deregulation of the Wilms' tumour gene 1 protein (WT1) by BCR/ABL1 mediates resistance to imatinib in human leukaemia cells. *Leukemia*. 2007;21:2485-94.
29. Medugno L, Costanzo P, Lupo A, Monti M, Florio F, Pucci P, Izzo P. A novel zinc finger transcriptional repressor, ZNF224, interacts with the negative regulatory element (AldA-NRE) and inhibits gene expression. *FEBS Lett*. 2003;534:93-100.
30. Florio F, Cesaro E, Montano G, Izzo P, Miles C, Costanzo P. Biochemical and functional interaction between ZNF224 and ZNF255, two members of the Kruppel-like zinc-finger protein family and WT1 protein isoforms. *Hum Mol Genet*. 2010;19:3544-56.
31. Montano G, Cesaro E, Fattore L, Vidovic K, Palladino C, Crescitelli R, Izzo P, Turco MC, Costanzo P. Role of WT1-ZNF224 interaction in the expression of apoptosis-regulating genes. *Hum Mol Genet*. 2013;22:1771-82.
32. Simpson LA, Burwell EA, Thompson KA, Shahnaz S, Chen AR, Loeb DM. The antiapoptotic gene A1/BFL1 is a WT1 target gene that mediates granulocytic differentiation and resistance to chemotherapy. *Blood*. 2006;107:4695-702.
33. Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. *Blood*. 2005;106:1154-63.
34. Ewald B, Sampath D, Plunkett W. Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene*. 2008;27:6522-37.
35. Kufe DW, Weichselbaum R, Egan EM, Dahlberg W, Fram RJ. Lethal effects of 1-beta-D-arabinofuranosylcytosine incorporation into deoxyribonucleic acid during ultraviolet repair. *Mol Pharmacol*. 1984;25:322-6.
36. Snyder RD, van Houten B, Regan JD. The inhibition of ultraviolet radiation-induced DNA repair in human diploid fibroblasts by arabinofuranosyl nucleosides. *Chem Biol Interact*. 1984;50:1-14.
37. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol*. 2002;30:503-12.
38. Damon LE, Plunkett W, Linker CA. Plasma and cerebrospinal fluid pharmacokinetics of 1-beta-D-arabinofuranosylcytosine and 1-beta-D-arabinofuranosyluracil following the repeated intravenous administration of high- and intermediate-dose 1-beta-D-arabinofuranosylcytosine. *Cancer Res*. 1991;51:4141-5.
39. Hao SX, Ren R. Expression of interferon consensus sequence binding protein (ICSBP) is downregulated in Bcr-Abl-induced murine chronic myelogenous leukemia-like disease, and forced coexpression of ICSBP inhibits Bcr-Abl-induced myeloproliferative disorder. *Mol Cell Biol*. 2000;20:1149-61.
40. Waight JD, Banik D, Griffiths EA, Nemeth MJ, Abrams SI. Regulation of the interferon regulatory factor-8 (IRF-8) tumor suppressor gene by the signal transducer and activator of transcription 5 (STAT5) transcription factor in chronic myeloid leukemia. *J Biol Chem*. 2014;289:15642-52.

41. Fang G, Kim CN, Perkins CL, Ramadevi N, Winton E, Wittmann S, Bhalla KN. CGP57148B (STI-571) induces differentiation and apoptosis and sensitizes Bcr-Abl-positive human leukemia cells to apoptosis due to antileukemic drugs. *Blood*. 2000;96:2246-53.
42. Sztiller-Sikorska M, Jakubowska J, Wozniak M, Stasiak M, Czyz M. A non-apoptotic function of caspase-3 in pharmacologically-induced differentiation of K562 cells. *Br J Pharmacol*. 2009;157:1451-62.

FIGURE LEGENDS

Fig 1. Cytarabine increases the protein levels of IRF8 in K562 cells.

K562 cells were treated with 1 μ M cytarabine for 72 hours, after which IRF8 was analyzed by immunoblotting using an anti-IRF8 antibody (MBS224027) (MyBioSource.com, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as equal loading control. One representative blot out of three is shown.

Fig 2. Cytarabine induced expression of IRF8 is correlated to increased expression of ZNF224.

K562 cells were treated with 1 μ M cytarabine for 72 hours, after which mRNA levels of IRF8 (A) and ZNF224 (B) were determined by qPCR. Shown are relative levels, normalized to those of control. Total number of cells (C) and percentage of viable cells (D) were determined by cell counting and trypan blue exclusion, respectively. Mean values, bars \pm S.E.M. n=5. Stars indicate statistical significance (*: p<0.05; **: p<0.01; ***: p<0.001).

Fig 3. Knockdown of ZNF224 counteracts basal and cytarabine-induced expression of IRF8.

Pools of K562 cells transfected with scrambled shRNA (control) or with shRNA directed against ZNF224 were treated with 1 μ M cytarabine for 72 hours, after which mRNA levels of ZNF224 (A) and IRF8 (B) were determined by qPCR. Shown are relative levels, normalized to those of control. Viability (C) was determined by trypan blue exclusion. Mean values, bars \pm S.E.M. n=3. Stars indicate statistical significance (*: p<0.05; **: p<0.01; ***: p<0.001).

Fig 4. ZNF224 counteracts the repressive effect of WT1 on the IRF8 promoter.

A luciferase reporter plasmid with the IRF8 promoter containing a WT1 responsive element was alone transfected to 293HEK cells (promoter only), or cotransfected with ZNF224, with WT1+/-, or with WT+/- combined with increasing amounts of ZNF224, as indicated. After 48 hours, luciferase luminescence was determined and normalized to that of the internal renilla control, as described in Methods. Shown are normalized

values, relative to that obtained with promoter only. Mean values, bars \pm S.E.M. n=3. Stars indicate statistical significance (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Fig 5. Nuclear binding of ZNF224 to WT1+/-.

FLAG-tagged ZNF224 and WT1+/- were transfected to 293HEK cells. After 48 hours nuclear and cytosol extracts were prepared from which immunoprecipitation (IP) was performed with antibody against WT1 (F6) (Santa Cruz), or with non-specific IgG as negative control. Immunoprecipitated proteins were analyzed by immunoblotting (WB) using an anti-FLAG antibody against FLAG-tagged ZNF224, Immunoblotting of c-myc or tubulin served as controls of subcellular fractionation.

Fig 6. WT1+/- recruits ZNF224 to the IRF8 promoter.

WT1(+/-) (A), or ZNF224 (B), or both ZNF224 and WT1 (C), were transfected to 293HEK cells after which chromatin immunoprecipitation (ChIP) using an antibody against ZNF224 (G16, Santa Cruz) or against WT1 (C19, Santa Cruz) was performed. ChIP with anti-HA antibody was used as negative control. Following ChIP, PCR with primers specific for the IRF8-promoter with a WT1 response element was performed as described in Methods. Primers specific for the aldolaseA and GAPDH promoter were used as positive and negative controls, respectively. As control of transfected proteins, ZNF224 and WT1 protein in cell lysates were analyzed by immunoblotting (D).

Fig 1

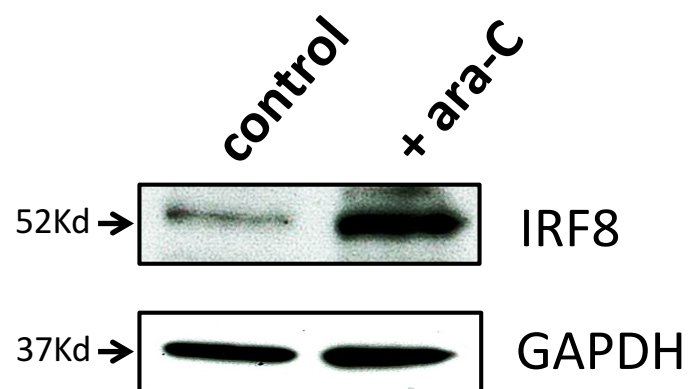


Fig 2

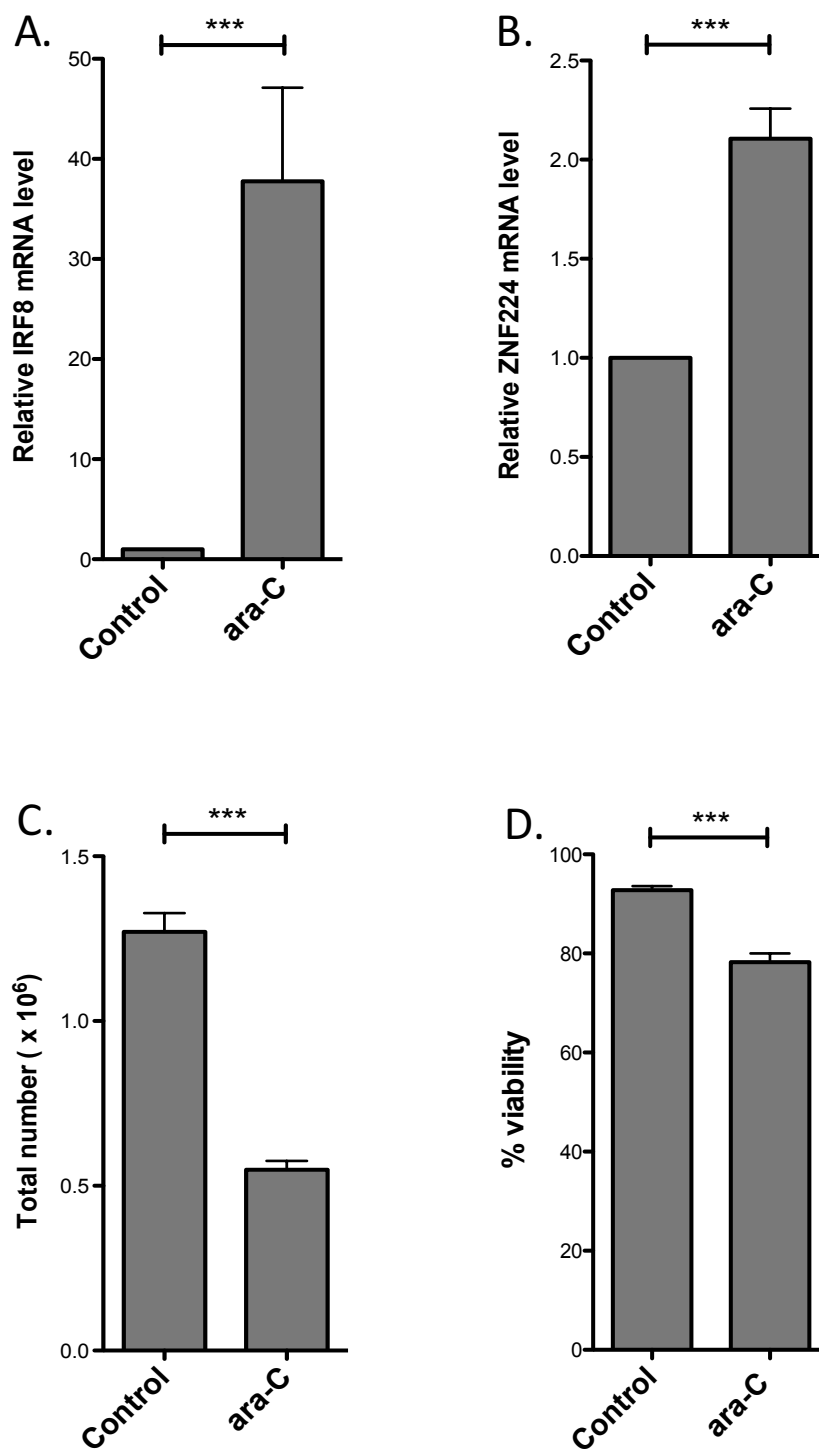


Fig 3

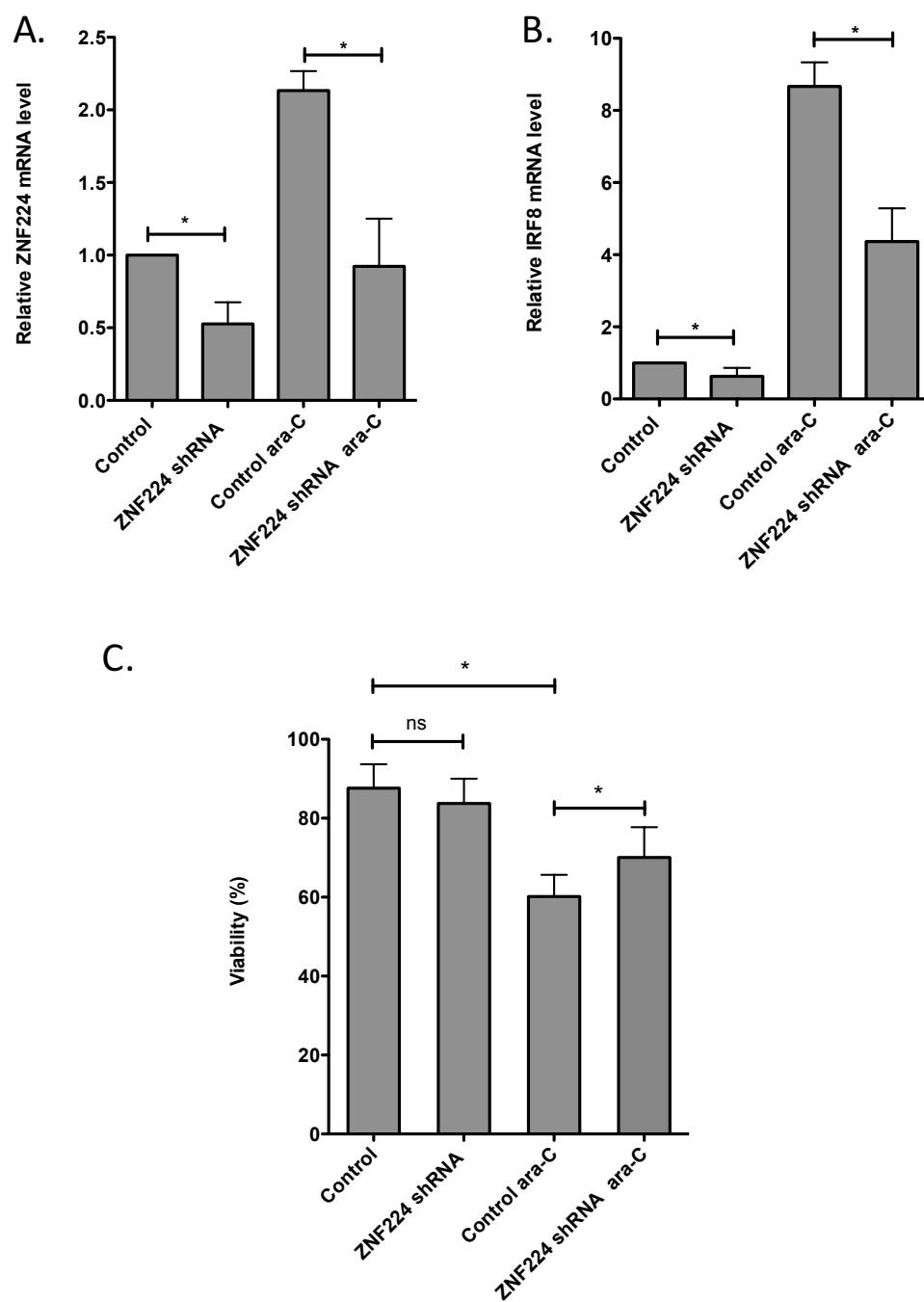


Fig 4

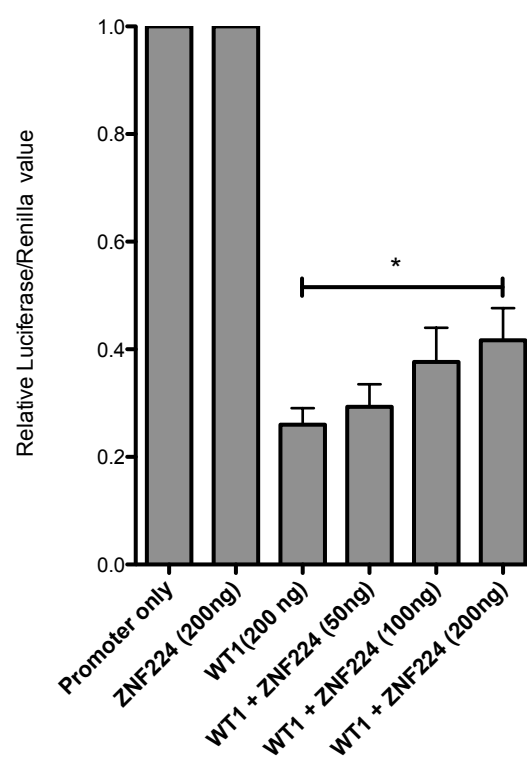


Fig. 5

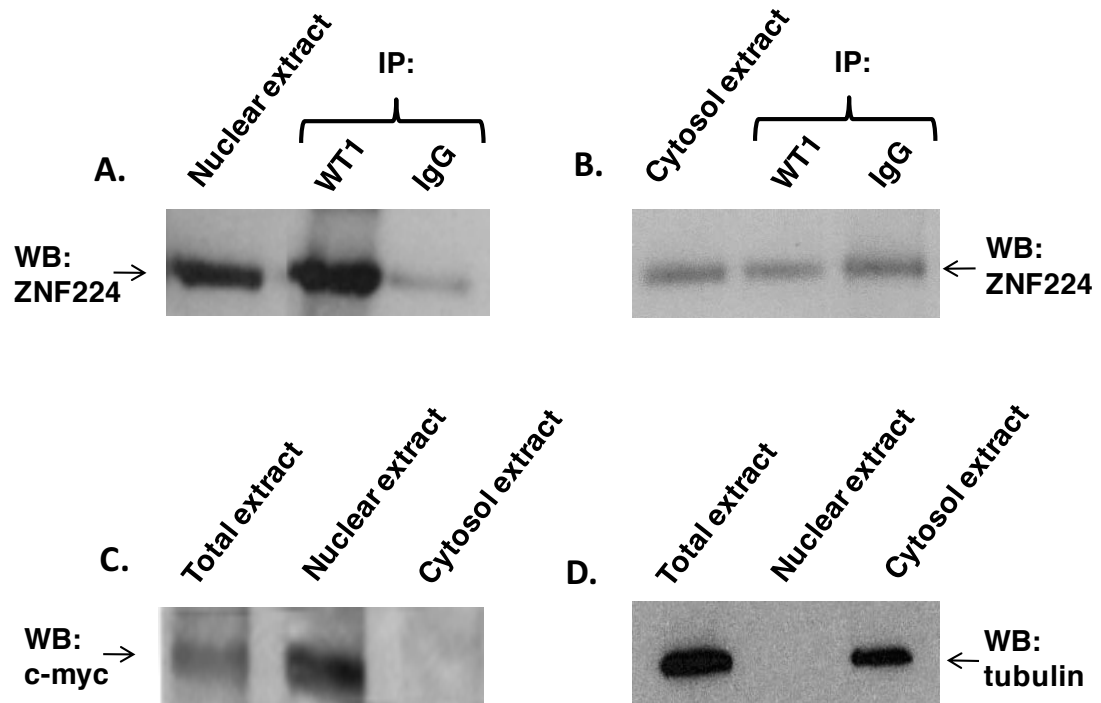


Fig. 6

