The RNA binding proteins RBM38 and DND1 are repressed in AML and have a novel function in APL differentiation

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ABSTRACT

The RNA binding proteins RBM binding motif protein 38 (RBM38) and DEAD END 1 (DND1) selectively stabilize mRNAs by attenuating RNAse activity or protecting them from micro(mi)RNA-mediated cleavage. Furthermore, both proteins can efficiently stabilize the mRNA of the cell cycle inhibitor $p21^{CIP1}$. Since acute myeloid leukemia (AML) differentiation requires cell cycle arrest and RBM38 as well as DND1 have antiproliferative functions, we hypothesized that decreased RBM38 and DND1 expression may contribute to the differentiation block seen in this disease. We first quantified RBM38 and DND1 mRNA expression in clinical AML patient samples and $CD34^+$ progenitor cells and mature granulocytes from healthy donors. We found significantly lower RBM38 and DND1 mRNA levels in AML blasts and $CD34^+$ progenitor cells as compared to mature neutrophils from healthy donors. Furthermore, the lowest expression of both RBM38 and DND1 mRNA correlated with t(8;21). In addition, neutrophil differentiation of $CD34^+$ cells *in vitro* with G-CSF (granulocyte colony stimulating factor) resulted in a significant increase of RBM38 and DND1 mRNA levels. Similarly, neutrophil differentiation of NB4 acute promyelocytic leukemia (APL) cells was associated with a significant induction of RBM38 and DND1 expression. To address the function of RBM38 and DND1 in neutrophil differentiation, we generated two independent NB4_RBM38 as well as DND1 knockdown cell lines. Inhibition of both RBM38 and DND1 mRNA significantly attenuated NB4 differentiation and resulted in decreased $p21^{CIP1}$ mRNA expression. Our results clearly indicate that expression of the RNA binding proteins RBM38 and DND1 is repressed in primary AML patients, that neutrophil differentiation is dependent on increased expression of both proteins, and that these proteins have a critical role in regulating $p21^{CIP1}$ expression during APL differentiation.

Highlights

- RBM38 and DND1 expression is attenuated in primary AML patients
- Normal and leukemic neutrophil differentiation induces RBM38 and DND1 expression
- New function for the RNA binding proteins RBM38 and DND1 in APL differentiation

Keywords: RBM38, DND1, acute myeloid leukemia, acute promyelocytic leukemia, neutrophil differentiation

Abbreviations

ABL1 - ABL proto-oncogene 1

AML - Acute myeloid leukemia

APL - Acute promyelocytic leukemia

ATRA - all-trans retinoic acid

CDKN1A - cyclin-dependent kinase inhibitor 1A

DND1 - DEAD END 1

FAB - French American British classification

FBS - Fetal bovine serum

G-CSF – granulocyte colony stimulating factor

G-CSF-R – granulocyte colony stimulating factor receptor

HMBS - hydroxymethylbilane synthase

HOVON/SAKK - Dutch-Belgian Hematology-Oncology/Swiss Group for Clinical Cancer Research Cooperative Group

MDM2 - MDM2 proto-oncogene

mRNA – messenger RNA

miRNA – micro RNA

MWU – Mann-Whytney-U-test

LATS2 - large tumor suppressor kinase 2

Onco-miR – oncogenic micro RNA

PML-RARa – onco-fusionprotein of promyelocytic leukemia gene and retinoic acid receptor-alpha (*RARA*) gene

qPCR - quantitative real-time polymerase chain reaction

RBM38 - RBM binding motif protein 38

RBP - RNA binding proteins

RRM - RNA recognition motif

RT-PCR - real-time polymerase chain reaction

shRNA - Small hairpin RNA

UTR - untranslated region

WIG1 - wild-type p53-induced gene 1

ZMAT3 - zinc finger, matrin-type 3

1. Introduction

Post-transcriptional gene expression is regulated by a variety of mechanisms including polyadenylation, RNA splicing, transport, stability and translation. RNA binding proteins (RBP) containing one or more RNA-binding motifs are involved in all of these processes.[1] For instance, the RNA recognition motif (RRM) is the most important binding motif in eukaryotic cells.[2] Given the important function of RBPs in gene expression, it is not surprising that deregulated or mutated RBPs contribute to cancer progression.[3],[4],[5] Dysfunctional or mutated RBPs can cause increased expression of oncogenes or decreased expression of tumor suppressor genes such as the p53 family members.[6],[7] Recent studies demonstrated that RBPs can protect mRNAs by preventing micro(mi)RNA access to the 3'-UTR and thus protecting them from degradation.[8],[9] miRNAs are small non-coding single-stranded RNA molecules that regulate gene expression at a post-transcriptional level by mRNA degradation, destabilization, or translational inhibition.[10] miRNAs control a variety of cellular and metabolic pathways including hematopoietic differentiation, e.g. miR-15a/144/451 regulate erythropoiesis and miR-223 is key to granulocytic differentiation.[11],[12],[13]

AML is a heterogeneous leukemic disease affecting the myeloid lineage and characterized by a block of differentiation at different stages of myelopoeisis.[14] According to the French-American-British (FAB) classification AML can be classified into eight subtypes based on their blast cell morphology. Acute promyelocytic leukemia (APL or FAB AML-M3 subtype), which is characterized by a chromosomal translocation involving the promyelocytic leukemia gene (PML) on chromosome 15 and the retinoic acid receptor alpha (RARA) on chromosome 17, resulting in the expression of the oncogenic fusion protein PML-RARA. Among others, PML-RARA causes a block in differentiation which can be nevertheless resolved with pharmaceutical doses of all-*trans* retinoic acid (ATRA).[15] The oncogenic fusion protein AML1-ETO is result of a translocation involving the genes AML1 on chromosome 8 and ETO on chromosome 21 present in the AML-M2 subtype. AML1-ETO enhances stem cell maintenance and inhibits myeloid differentiation (as reviewed by Nimer et al).[16] Based on the fact that several miRNAs are key to successful myeloid differentiation, a general decrease in miRNA expression is associated with AML pathology.[17] Moreover, we recently published that several members of the miRNA processing machinery, particularly DICER1, are downregulated in primary AML.[18]

Two RNA binding proteins, the RNA binding motif protein 38 (RBM38 also known as RNPC1) and the DEAD END 1 (DND1), have been linked to cellular differentiation. RBM38 is induced during late erythrocyte development where it mainly regulates alternative splicing. RBM38 knockout mice

exhibit decreased erythropoiesis and other hematopoietic defects.[19],[20] Furthermore, RBM38 and DND1 can regulate miRNA activity at the level of miRNA-mRNA interactions by blocking miRNA access to their target mRNAs.[8],[21] Several studies identified RBM38 as a downstream effector of p53 family members that stabilizes the mRNA of the cell cycle inhibitor $p21^{CIP1}$.[22] The stabilization of the $p21^{CIP1}$ mRNA by RBM38 is at least in part caused by preventing access of miR-17/106b to the $p21^{CIP1}$- 3'UTR.[8] Of interest for this study, $p21^{CIP1}$ has been linked to retinoic acid-induced differentiation of AML cells.[23] In addition to p53 its relative p73 is also stabilized by RBM38.[24] Lastly, RBM38 is not the only RBP that is regulated by p53, e.g. transcription of the zinc finger, matrin-type 3 (ZMAT3 also known as WIG1) depends on p53.[25]

Although RBM38 was originally identified as a potential oncogene, the majority of hepatocellular carcinoma and breast cancer published reports on RBM38 function point to a tumor suppressor role.[26],[27] RBM38 also contributes to the destabilization of the MDM2 mRNA, a gene promoting p53 degradation.[28] Interestingly, p73 contributes to myeloid differentiation by modulating $p21^{CIP1}$, and RBM38 may contribute to this process by regulating p73 mRNA stability.[29],[30] Although limited information is available on the role of DND1 in tumors, DND1 mutations have been implicated in germ cell loss and testicular germ cell tumors.[31] This effect has been partially explained by inhibiting miRNA mediated cleavage of mRNAs bound by DND1.[9],[32] Furthermore, DND1 can block skin oncogenesis by preventing miR-21 binding to its targets.[33] Lastly, similarly to RBM38, DND1 can also protect $p21^{CIP1}$ mRNA from degradation.[34] Thus, the published evidence supports a tumorsuppressive role for DND1 as well.

Based on the above-described link of RBM38 and DND1 to differentiation and their tumor suppressor functions, we investigated their role in AML pathology. We hypothesized that low RBM38 and DND1 mRNA levels and their effector proteins may contribute to the differentiation block seen in AML.

2. Material and methods

2. 1. Primary patient samples and cell culture

Primary AML patients samples from patients enrolled on HOVON/SAKK (Dutch-Belgian Hematology-Oncology/Swiss Group for Clinical Cancer Research Cooperative Group) protocols -04, -04A, -29 and -42 (available at www.hovon.nl) between 1987 and 2006 were provided by Dr. P.J.M. Valk and B. Löwenberg.[35],[36],[37] Patient data represent log2 expression levels and were normalized to the expression levels of the two house keeping genes *HMBS* and *ABL*. For better readability we multiplied the results by (-1) and excluded Ct values higher than 40 ($\Delta Ct = 40 - Ct^{GENE-1} - (\text{Mean } Ct^{HMBS} \text{ and } Ct^{ABL1}) * (-1)$).[38] The number of patient samples analyzed for *RBM38* and *DND1* mRNA expression is slightly different due to fact that in some patients the gene expression was below the detection limit of our assay (Additional file 1: Table S1).

Human mobilized $CD34^+$ cells were isolated from of healthy donors (City of Hope). The cells were then expanded for 8 days in IMDM supplemented with 10% Hyclone FBS (Thermo Fisher Scientific, Waltham, MA) 1% penicillin/streptomycin (P4333; Sigma-Aldrich), 100 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL IL-6 (Peprotech) at a density of 1x106 cells/ml. Induction of differentiation was performed in IMDM Gibco, 10% Hyclone FBS, 1% P/S Corning, 50 ng/mL hG-CSF, Peprotech, 50 ng/mL hIL-6 (Peprotech, Rocky Hill, NJ) at a density of 1x106 cells/ml for the indicated days.

The APL cell lines NB4, its ATRA-resistant subclone NB4-R2 and HT93 were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (#S0615; Biochrom AG) and 1% penicillin/streptomycin (P4333; Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2 at 37°C. For neutrophil differentiation APL cells were seeded at a concentration of 0.2 million cells/ml and treated with 1µM all-trans-retinoic acid (ATRA; Sigma-Aldrich, Switzerland) for four to six days. Successful granulocyte differentiation was evaluated by increased CD11b surface marker (#21279114; Immunotools) or granulocyte colony-stimulating factor receptor (*G-CSF-R*) mRNA expression.

2.2. Generation of RBM38 and DND1 knockdown cell lines

pLKO.1 lentiviral vectors expressing small hairpin (sh) RNAs targeting RBM38 or DND1 (shRBM38_317 NM_017495.4-317s21c1, shRBM38_1312 NM_017495.4-1312s1c1, shDND1_249 NM_194249.1-249s1c1 and shDND1_1084 NM_194249.1-1084s1c1) non-targeting shRNA control (SHC002) vector were purchased from Sigma-Aldrich (Sigma, Switzerland). Puromycin served as an antibiotic resistance marker to enable a positive selection of the infected cells. Lentivirus production and the transduction on NB4 cells were performed as previously described.[39]

2.3. TaqMan low-density arrays and real-time quantitative RT-PCR (qPCR)

RNA isolation, RT-PCR low-density arrays as well as data analysis were performed as described previously.[40] TaqMan Gene Expression Arrays bought from Applied Biosystems® for *HMBS, ABL1, RBM38 and DND1* preloaded on low-density arrays were Hs00203008_m1, Hs00377897_m1, Hs00250139_m1, and Hs00832091_s1. TaqMan gene expression assays for *RBM38*, *DND1, p21*^{CIP1} and *G-CSF-R* used in a 96-well format were Hs00250139_m1, Hs00832091_s1, Hs00355782_m1 and Hs00167918_m1 (Applied Biosystems®), respectively. Specific primers and probes for *HMBS* and data analysis were used as described.[41] We calculated the n-fold mRNA induction upon ATRA-treatment using the ddCt method of relative quantification. All data are shown as mean ± the standard error of the mean (SEM) of at least three independent experiments. Nonparametric Mann-Whitney-U tests (MW.) were applied to compare the difference between two groups using the program GraphPad Prism 4 (Graph Pad Software, San Diego, CA). P-values <0.05 were considered to be statistically significant.

2.4. Western Blotting

Whole cell extracts were prepared using Urea lysis buffer, supplemented with 25X proteinase-inhibitors. Total protein was loaded on a 10% denaturing polyacrylamide gel. Blots were incubated with the primary antibodies in TBS 0.05% Tween-20/2% milk overnight at 4°C, incubated with secondary antibodies goat anti-rabbit IRDye 800CW and goat anti-mouse IRDye 680LT (LI-COR Biosciences, Lincoln, NE) at 1:5000 for 1 h at room temperature, and analyzed using the Odyssey infrared imaging system detection (LI-COR Biosciences). Primary antibodies used were anti-RBM38 1:500, anti-p21 1:200 (Santa Cruz Biotechnology, CA), and anti-GAPDH 1:5000 (Millipore, Darmstadt, Germany).

3. Results and Discussion

3.1 RBM38 and *DND1* mRNA expression levels are down-regulated in clinical AML samples

In a first attempt to identify a potential role for *RBM38* and *DND1* in AML (molecular) pathogenesis, we quantified gene expression in a cohort of 98 M0-M4 clinical AML patient samples. As compared to granulocytes from healthy donors, *RBM38* AML transcript levels were significantly decreased by 7-fold in AML patients, by 4-fold in macrophages from healthy individuals and 10-fold in $CD34^+$ cells. Of note, we also found a trend of lower RBM38 mRNA expression in $CD34^+$ cells as compared to AML patient blast cells (Fig. 1, upper panel). Similarly, *DND1* mRNA expression was significantly decreased in primary AML samples by 18-fold as compared to granulocytes and by 8-fold as compared to macrophages. Interestingly, *DND1* expression in AML patient samples is significantly increased by 3-fold as compared to $CD34^+$ cells (Fig. 1, lower panel). Comparing the different AML translocations as well as complex and normal karyotype AML, we found that RBM38 is also significantly upregulated in normal karyotype AML samples as compared to the other subtypes. Moreover, cell samples from AML patients characterized by the expression of the AML-ETO fusion protein t(8;21), expressed significantly lower mRNA levels of both RBM38 and DND1 as compared to the other AML subtypes. Consistently, we found markedly lower RBM38 protein expression in t(8:21) positive Kasumi cell line as compared to t(8:21)-negative HL60 and NB4 cell lines (Additional file 2: Figure S1). Of interest, we found that yet another RNA binding protein regulated by p53, the zinc finger, matrin-type 3 (ZMAT3) is significantly repressed in primary AML as compared to healthy granulocytes (Additional file 3: Figure S2a).

Our findings indicate that *RBM38* and *DND1* expression associates with a mature neutrophil phenotype and that both RBPs are repressed in the presence of AML1-ETO. Low RBM38 expression in AML may also reflect a defective p53 pathway as RBM38 is a transcriptional target of p53.[22] Although NB4 and HT93 APL cells express a mutant p53, p73 may partially replace p53 activity during ATRA-mediated AML differentiation allowing for activation of RBM38 transcription.[30],[42] Together, AML1-ETO expression as well as impaired p53 family signaling in AML may provide a possible explanation for low RBP expression in particular AML subtypes.

3.2 RBM38 and *DND1* expression is significantly induced during neutrophil differentiation and knocking down either of these genes interferes with APL differentiation

Since *RBM38* and *DND1* mRNA expression was lower in AML patient samples than in healthy, mature granulocytes, we asked if these two genes are involved in neutrophil differentiation, specifically of the APL cell lines NB4 and HT93. These two cell lines can be differentiated *in vitro* towards neutrophil-like cells by treating them with ATRA. After 6 days of treatment, we observed a 9-fold

induction of RBM38 message, and a marked upregulation of RBM38 protein expression (Figs., 2a-b). Consistently, we found an 8-fold upregulation of RBM38 message in HT93 APL cells upon ATRA treatment (Fig. 2c). Similarly, although to a lesser extend, *DND1* mRNA expression levels were significantly induced 2.5-fold in NB4 and HT93 APL cells in response to ATRA-treatment for 6 days (Figs. 2d-e). Importantly, the induction of *RBM38* and *DND1* is not due to unspecific ATRA toxicity since their expression was not induced in the ATRA-resistant control cell line NB4-R2 (Figs. 2a and d, right panels).

To further confirm our findings, we analyzed *RBM38* and *DND1* mRNA expression during G-CSF mediated neutrophil differentiation of primary $CD34^+$ progenitor cells *in vitro*. Both genes were significantly upregulated at 3 and 6 days of G-CSF treatment compared to day 0 of treatment (Fig. 2f).

Next, we aimed at investigating if RBM38 and DND1 are necessary for successful neutrophil differentiation. To address this question, we generated two independent NB4 RBM38 and DND1 knockdown cell lines using lentiviral vectors expressing shRNAs targeting *RBM38* (shRBM38_317 and shRBM38_1312) or *DND1* (shDND1_249 and shDND1_1084), respectively. To exclude non-specific effects caused either by the viral infection itself or the puromycin selection treatment, we used scrambled shRNA (SHC002) transduced NB4 cells as a control. RBM38 knock-down efficiency was measured by qPCR after 6 days of ATRA treatment, and showed 75% and 70% downregulation for shRBM38_317 and shRBM38_1312, respectively (Fig 3a, upper panel). Lower RBM38 mRNA expression was paralleled by markedly decreased RBM38 protein levels (Fig. 3a, lower panel). Knocking down RBM38 resulted in impaired neutrophil differentiation of the NB4 cells as evidenced by significantly reduced expression of the differentiation markers G-CSF-R and CD11b, up to 50 and 40%, respectively (Figs. 3b-c). DND1 mRNA knockdown experiments paralleled the RBM28 findings, with a knockdown efficiency after 6 days of ATRA treatment was 68% and 64% for NB4 shDND1_249 and shDND1_1084 cells, respectively (Fig. 4a). Both DND1 knockdown lines displayed significantly lower CD11b surface (reduced by 32% and 39%, respectively) as well as *G-CSF-R* mRNA expression (reduced by 66% and 87%, respectively) (Fig. 4b-c).

Our data clearly show that *RBM38* and *DND1* expression is significantly induced upon neutrophil differentiation of an APL cell line, as well as in $CD34^+$ cells. Furthermore, both RNA binding proteins function in APL differentiation, since knocking down either RBM38 or DND1 diminishes ATRA-induced neutrophil differentiation.

3.3 $p21^{CIP1}$ (CDKN1A) and LATS2 mRNA levels are significantly decreased in RBM38 and DND1 NB4 knockdown cells

Based on reports of enlarged mRNA stability by RBM38 and DND1 binding of cell cycle regulators, which identified the cell cycle regulator $p21^{CIP1}$ mRNA as an mRNA protected by RBM38[32],[28] and DND1[9],[34], as well as the described role of $p21^{CIP1}$ in ATRA-induced differentiation of APL cells[43], we asked if inhibiting RBM38 or DND1 expression affects $p21^{CIP1}$ mRNA levels during neutrophil differentiation. Therefore, we measured $p21^{CIP1}$ message in control and ATRA-treated NB4 RBM38 and DND1 knockdown cells. $p21^{CIP1}$ mRNA levels were significantly decreased in both knockdown cell lines under control conditions and upon ATRA treatment (Figs. 5a-b). These findings provide insights as to how inhibiting RBM38 or DND1 attenuates APL differentiation as it has been shown that reduced $p21^{CIP1}$ expression is results in reduced differentiation.[23] Moreover, previous studies indicated that a loss of $p21^{CIP1}$ facilitates AML1-ETO-induced leukemogenesis.[44] In line with this study, the particularly low RBM38 and DND1 expression in this AML subtype may contribute to decreased $p21^{CIP1}$ stability. However, the effects RBM38 and possibly DND1 of blocking ATRA-mediated neutrophil differentiation are most likely not solely based on reduced $p21^{CIP1}$ stability. Another previously described RBM38 binding RNA, the serine/threonine kinase LATS2 might be an interesting candidate as well. LATS2 is a member of the Hippo signaling pathway that is of outmost importance during development and early reports associated this pathway with hematopoiesis.[45] Consistently, we found that similar to $p21^{CIP1}$ also LATS2 mRNA expression is significantly decreased in NB4 RBM38 knockdown cells upon ATRA treatment (Additional file 3: Figure S2b). Thus, decreased LATS2 expression due to reduced RBM38 levels may also contribute to an immature myeloid phenotype. In line, significantly lower LATS2 mRNA expression was measured in AML patient samples as compared to mature neutrophils (Additional file 3: Figure S2c).

3.4 Conclusions

Although microRNAs are globally down-regulated in AML[46], specific oncogenic miRNAs such as miR-9[47] and miR-17 family members[48] are often found highly expressed in AML. Induction of RBM38 and DND1 during neutrophil differentiation may antagonize the activity of these oncomiRs by protecting mRNAs, including $p21^{CIP1}$ that are important for myeloid differentiation. Our data strongly suggest a novel function for the RNA binding proteins RBM38 and DND1 in AML differentiation possibly providing novel targets in AML therapy. In general, the role of RNA binding proteins during hematopoiesis and leukemic progression warrants further studies.

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Figure Legends

Fig1. Significantly decreased *RBM38* and *DND1* mRNA expression in AML patient samples. qPCR analysis of *RBM38* (upper panel) and *DND1* (lower panel) in primary AML patient samples (FAB M0-M4), granulocytes (G) as well as macrophages (M) from healthy donors, and $CD34^+$ progenitor cells. Data represent log2 expression levels and were normalized to the expression levels of the housekeeping genes *HMBS* and *ABL1*. For better readability we multiplied the results by (-1) and excluded Ct values higher than 40 ($\Delta Ct = 40- Ct^{WIPI-1} - (Mean\ Ct^{HMBS}$ and $Ct^{ABL1}) * (-1)$). Patient characteristics are shown in Supplementary Table 1. Mann-Whitney-U-test (MWU): *p<0.05. **p<0.01, *** p<0.001, ns: not significant.

Fig. 2. Induction of RBM38 and DND1 expression during neutrophil differentiation of APL and $CD34^+$ progenitor cells. **a** NB4 and NB4-R2 ATRA-resistant cells were differentiated with 1µM ATRA for four days. *RBM38* mRNA levels were quantified by qPCR and values were normalized to *HMBS*. Results are given as n-fold changes compared to untreated control cells. **b** NB4 and NB4-R2 cells were treated as in a. RBM38 protein levels were determined by Western blotting. The housekeeping gene GAPDH was used as a loading control. **c** HT93 APL cells were treated as in a. *RBM38* mRNA expression levels were quantified and analyzed as in a. **d** DND1 mRNA expression levels of NB4 and NB4-R2 cells differentiated and analyzed as in a. **e** *DND1* mRNA expression levels of HT93 cells treated and analyzed as in a. **f** $CD34^+$ progenitor cells were differentiated towards granulocytes *in vitro* using G-CSF for 3 and 6 days, respectively. *RBM38* (left panel) and *DND1* (right panel) mRNA expression levels were quantified by qPCR. Values were normalized to *HMBS* and compared to day 0 of G-CSF treatment. MWU: *P<0.05, ns: not significant.

Fig. 3. Knocking down *RBM38* significantly impairs APL differentiation. NB4 cells stably expressing shRNAs targeting *RBM38* (shRBM38_317 and shRBM38_1312) or a non-targeting shRNA (SHC002) were treated with 1µM ATRA for four days. **a** RBM38 knockdown efficiency of control and RBM38 knockdown NB4 cells upon ATRA treatment was determined by qPCR (upper panel) and western blotting (lower panel). Analysis as in 2a and b. **b, c** Neutrophil differentiation of NB4 SHC002 control and RBM38 knockdown NB4 cells was assessed by granulocyte colony stimulating factor receptor (*G-CSF-R*) qPCR (b) and CD11b FACS analysis (c). MWU: *p<0.05. **p<0.01.

Fig. 4. Knocking down *DND1* significantly impairs APL differentiation. NB4 cells stably expressing shRNAs targeting *DND1* (shDND1_249 and shDND1_1084) or a non-targeting shRNA (SHC002) were treated with 1µM ATRA for four days. **a** DND1 knockdown efficiency of control and DND1 knockdown NB4 cells upon ATRA treatment was determined by qPCR. Analysis as in 2a. **b, c** Neutrophil

differentiation of NB4 SHC002 control and DND1 knockdown NB4 cells was assessed as in 3b and c. MWU: *p<0.05. **p<0.01 , ***P<0.001.

Fig. 5. Knocking down *RBM38* or *DND1* decreases $p21^{CIP1}$ mRNA stability. **a, b** NB4 SHC002 control and RBM38 (shRBM38_317) as well as DND1 (shDND1_1084) knockdown cells were treated for 6 days with 1μM ATRA. $p21^{CIP1}$ mRNA levels were measured by qPCR and analyzed as in 2a.

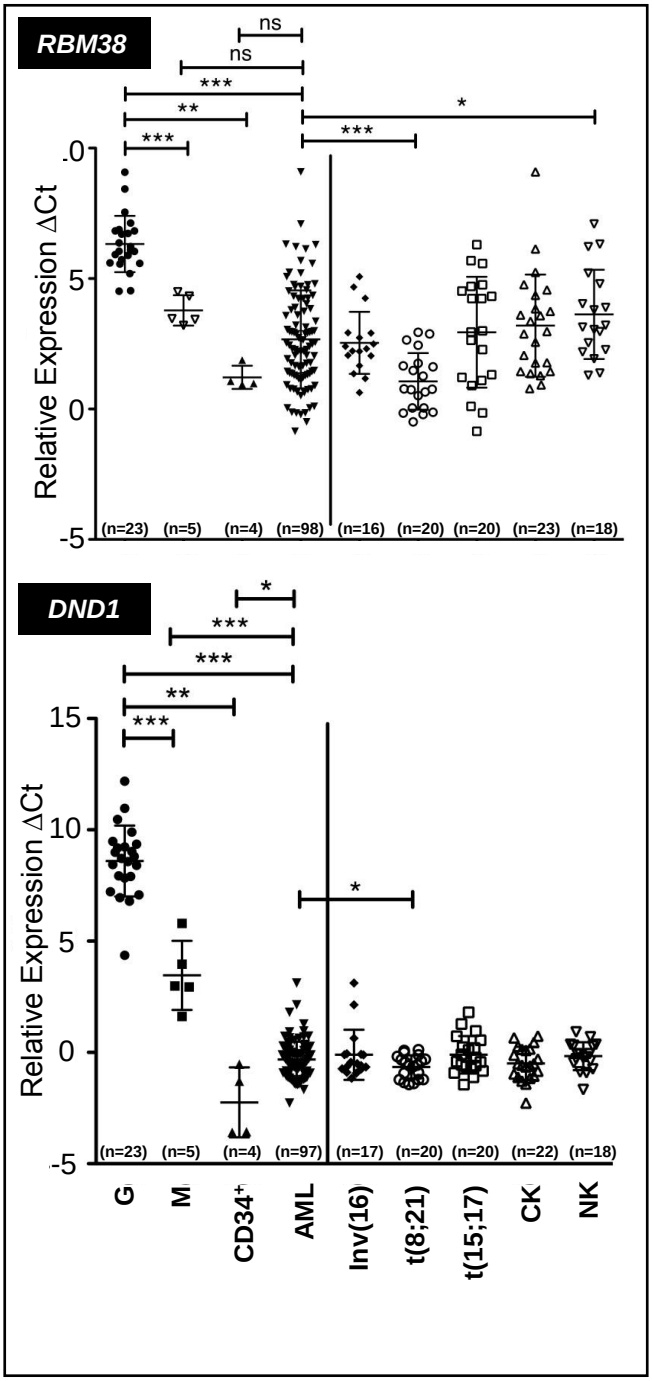
Supplementary Figure 1

RBM38 Western blotting analysis of AML1-ETO positive Kasumi compared to AML-ETO negative NB4 and HL60 cells. GAPDH was used as loading control.

Supplementary Figure 2

Reduced *ZMAT3* and *LATS2* mRNA expression in AML patient samples and decreased LATS2 expression in RBM38 knockdown NB4 cells. **a** qPCR analysis of *ZMAT3* in primary AML patient samples (FAB M0-M4), granulocytes (G) as well as macrophages (M) from healthy donors. Data represent log2 expression levels and were normalized to the expression levels of the housekeeping genes *HMBS* and *ABL1*. **b** *LATS2* mRNA expression levels were measured by qPCR in NB4 RBM38 knockdown (shRBM38_317) and SHC002 control cells upon treatment for 6 days with 1μM ATRA.. **c** qPCR analysis of *LATS2* in primary samples analyzed as described in a. MWU: **p<0.01, *** p<0.001, ns: not significant.

Figure 1


RBM38
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ns
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*
***
***
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(n=4)
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(n=18)
DND1
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10
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-5
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Relative Expression ΔCt
(n=23)
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M
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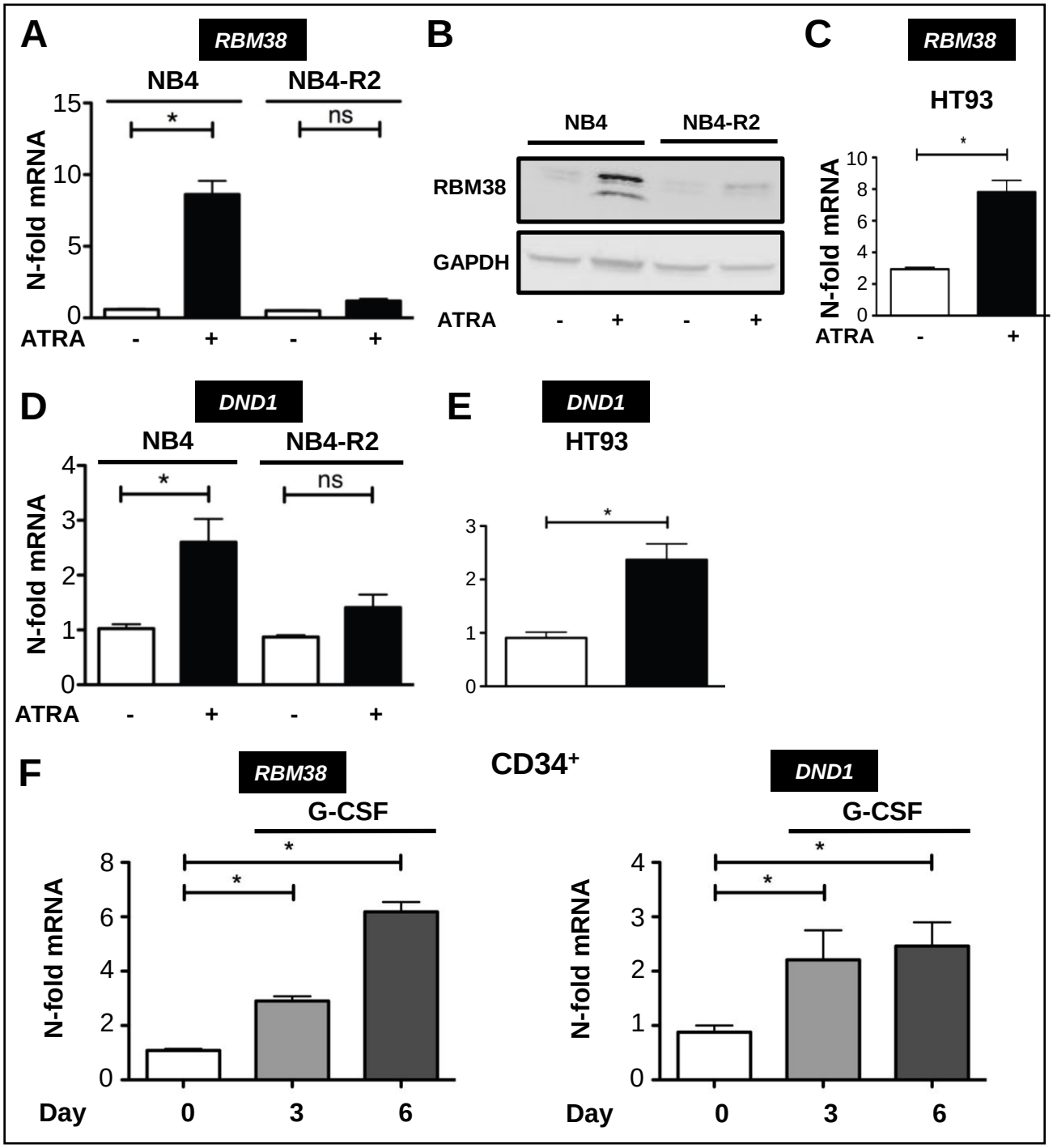
Inv(16)
t(8;21)
t(15;17)
CK
NK

Figure 2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NB4-R2
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ATRA
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B
NB4
NB4-R2
RBM38
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HT93
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8
6
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2
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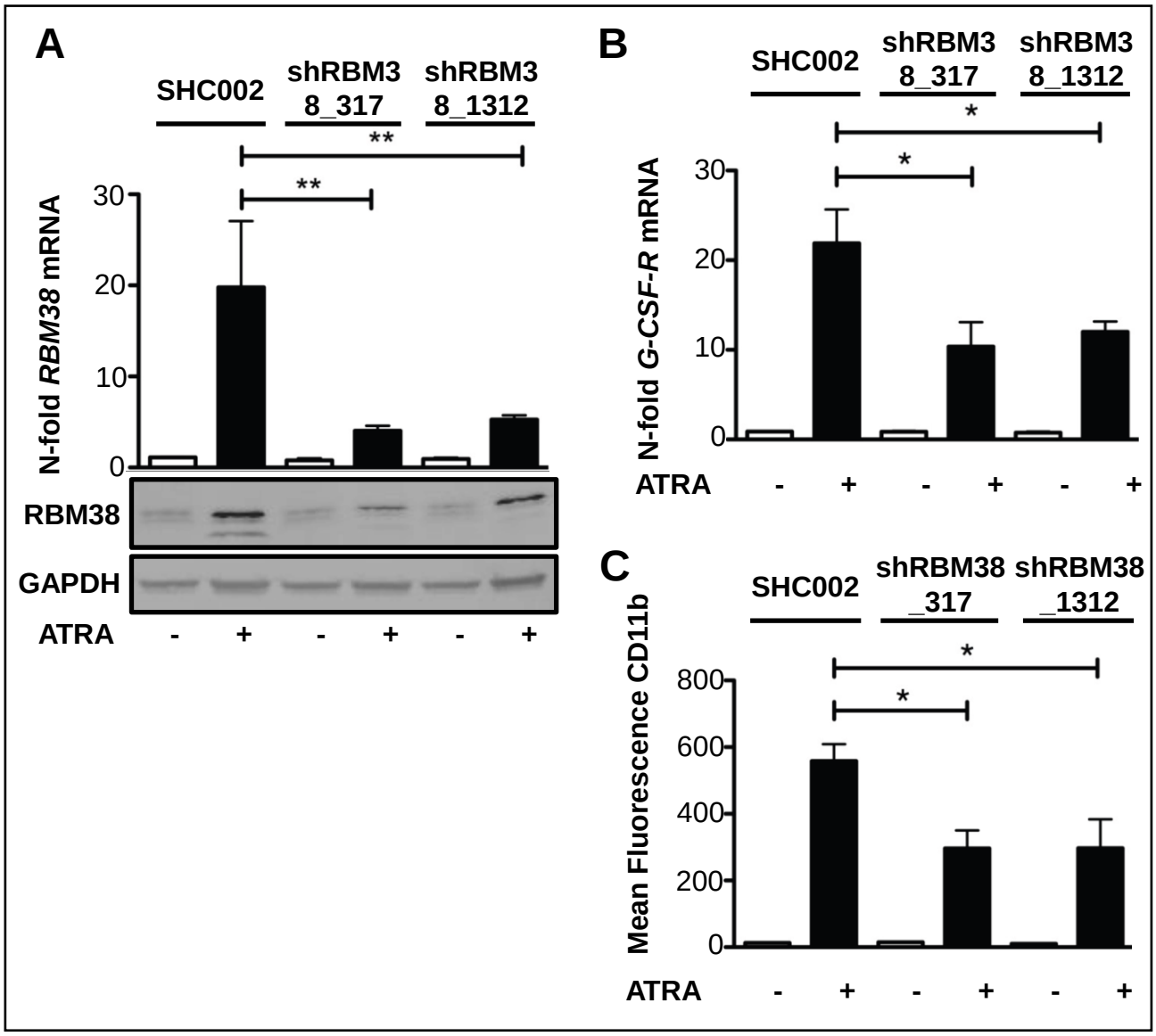
Figure 3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shRBM38_317
shRBM38_1312
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GAPDH
ATRA - + - + - +
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SHC002
shRBM38_317
shRBM38_1312
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*
N-fold G-CSF-R mRNA
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ATRA - + - + - +
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SHC002
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Mean Fluorescence CD11b
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200
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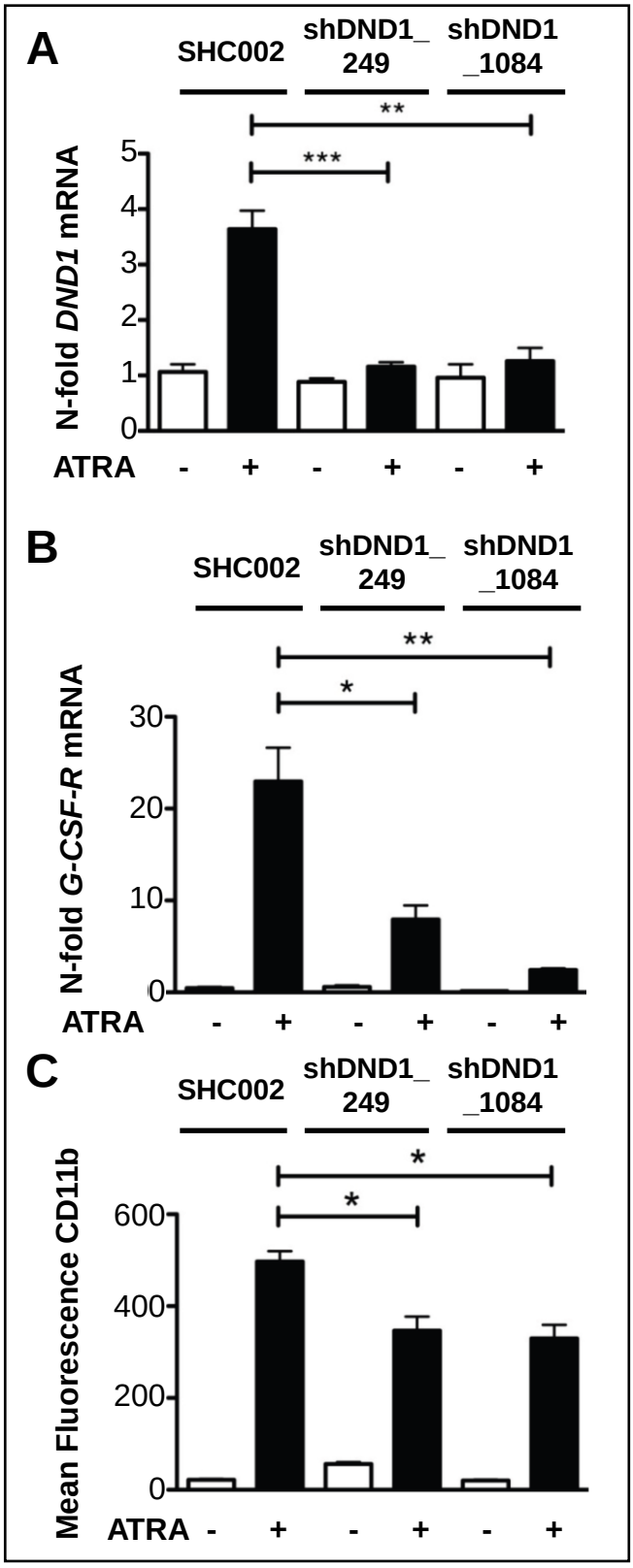
ATRA - + - + - +

Figure 4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shDND1_249
shDND1_1084
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N-fold DND1 mRNA
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ATRA - + - + - +
B
SHC002
shDND1_249
shDND1_1084
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*
N-fold G-CSF-R mRNA
30
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ATRA - + - + - +
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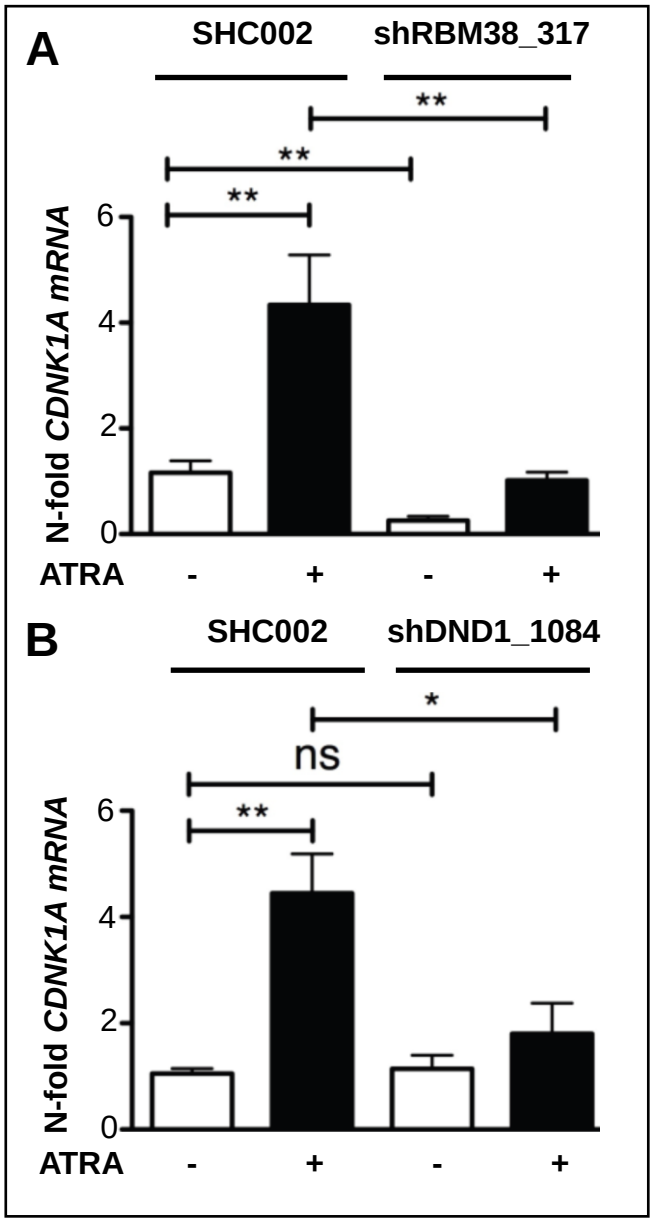
Figure 5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SHC002
shDND1_1084
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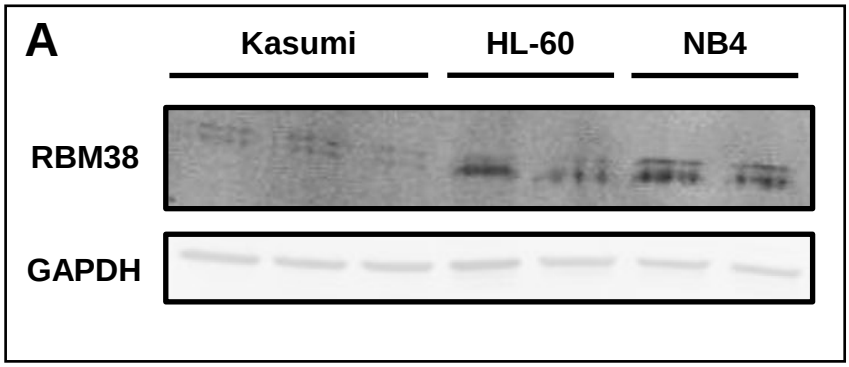
Supplementary Figure 1



Supplementary Figure 2

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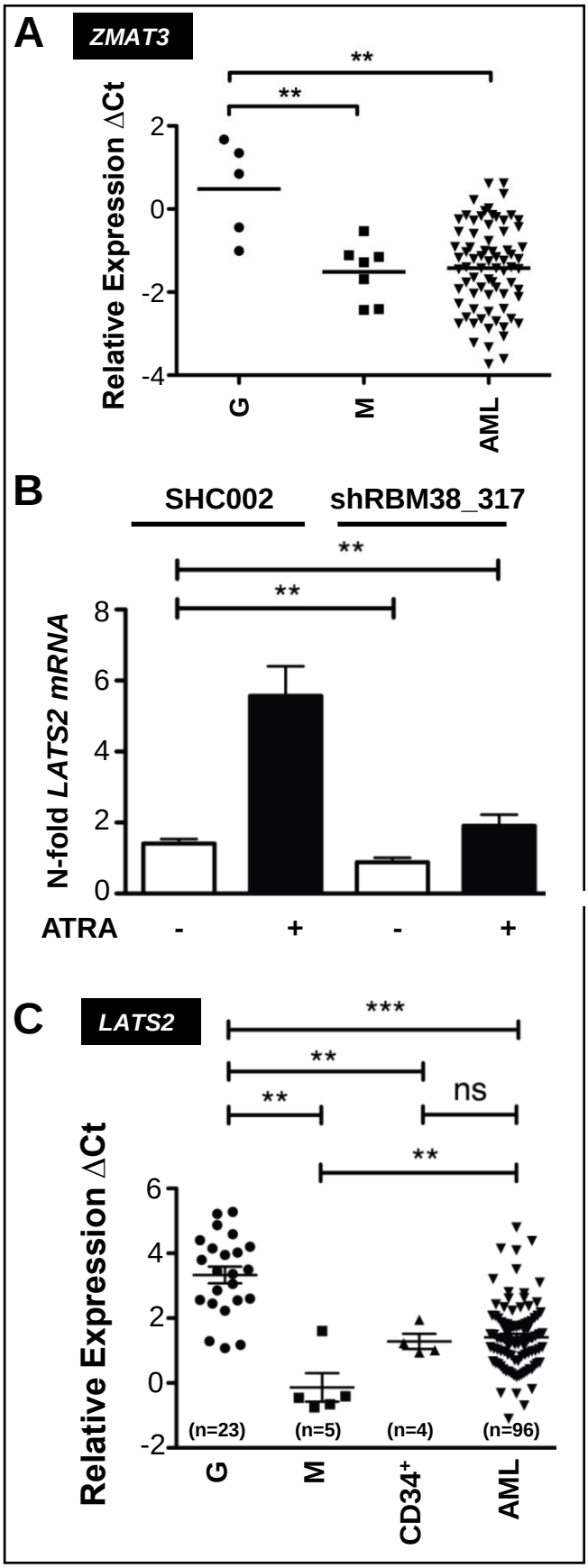

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Supplementary Table 1. AML patients characteristics from the HOVON/SAKK cohort.

RBM38

		Patient characteristics			FAB classification						Cytogenetics						
Cohort	**Variables**	Age (y)	Sex (female/male)	**Total**	M0	M1	M2	M3	M4	ND	t(8;21)	inv (16)	t(15;17)	CK	NK	Others	ND
HOVON/ SAKK	**Range**	15-74	-	**-**													
	Mean/median or %	43.2/43 (mean/median)	43.1/43	**100**	4.1	16.3	32.7	19.4	26.6	0	20.4	17.3	20.4	23.5	18.4	0	0
	No. of patients		58/40	**98**	4	16	32	19	27	0	20	17	20	23	18	0	0

DND1

		Patient characteristics			FAB classification						Cytogenetics						
Cohort	**Variables**	Age (y)	Sex (female/male)	**Total**	M0	M1	M2	M3	M4	ND	t(8;21)	inv (16)	t(15;17)	CK	NK	Others	ND
HOVON/ SAKK	**Range**	15-74	-	**-**													
	Mean/median or %	43.2/43 (mean/median)	43.1/43	**100**	4.1	16.5	33.0	18.6	27.8	0	20.6	17.5	20.6	23.5	18.6	0	0
	No. of patients		57/40	**97**	4	16	32	18	27	0	20	17	20	22	18	0	0

FAB, French-American-British; CK, complex karyotype; NK, normal karyotype; others, up to two chromosomal aberrations (deletions or aneuploidies) in the absence of t(8;21), inv(16)/t(16;16), t(15;17), or t(9;11); ND, not determined.

Conflict of interest statement

The authors declare no conflict of interest.

Authors' contributions

JW performed the research and drafted the manuscript. EAF analyzed patient data and performed $CD34^+$ neutrophil differentiation experiments. BET and MFF instigated the experimental design and revised the drafted article. MPT designed the project, wrote the paper and gave final approval of the submitted manuscript. All authors read and approved the final manuscript.

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*Statement of conflict of interest

Conflict of Interest Form

Manuscript Name:	The RNA binding proteins RBM38 and DND1 are repressed in AML and have a novel function in APL differentiation
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