# Characterization of ZC3H15 as a potential TRAF-2-interacting protein implicated in the NFκB pathway and overexpressed in AML

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**Abstract.** The gene product of the *zinc finger CCCH-type containing 15* (ZC3H15) gene, an immediate early erythropoietin response gene (synonymous: LEREPO4), was further characterized. ZC3H15 was expressed ubiquitously in all human tissues tested by northern blotting and showed mainly a diffuse cytoplasmic distribution by immune fluorescence microscopy and western blotting of subcellular protein fractions. The expression of ZC3H15 was downregulated effectively in HeLa cells to ≤13% of the control by transfection of specific small interfering RNA (siRNA). Subsequent Affymetrix microarray analysis revealed 202 differentially expressed genes including 114 induced (≥3-fold) genes and 88 suppressed (≤0.3-fold) genes. The gene ontology (GO) categories containing an over-representation of differentially expressed genes comprised cell growth, transcription, cell adhesion, regulation of NF-κB, regulation of MAPK, cell cycle arrest and immune response. ZC3H15 interacted with the signaling adapter protein tumor necrosis factor receptor associated factor 2 (TRAF-2) as shown by co-immunoprecipitation. ZC3H15 expression was found to be significantly increased in acute myeloid leukemia (AML) samples compared to MDS, CML, ALL and normal bone marrow samples using the Leukemia Gene Atlas (LGA) database. Based on these data, it is hypothesized that ZC3H15 may interact with TRAF-2 functionally within the NF-κB pathway, and may be explored as a potential target in AML.

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## Introduction

ZC3H15 (1) was discovered as an erythropoietin-induced gene. ZC3H15's synonymous name, likely ortholog of mouse immediate early response erythropoietin 4 (LEREPO4) gene (2), suggests that it was swiftly induced by erythropoietin as a growth factor. Another name, DRG family regulatory protein 1 (DFRP1) (3) indicates that it regulates developmentally regulated GTP-binding protein 1 (DRG1) suggesting a role in cellular signaling. We initially found ZC3H15 (LEREPO4) as a potential HIV-dependency factor (4).

In order to carry out a broader characterisation of ZC3H15, its expression, localization and effect on cellular gene expression were studied. Since ZC3H15 was originally described as an erythropoietin-induced early response gene, we explored a potential role in hematology by screening for a possible association with leukemias.

## Materials and methods

*Cell culture.* For siRNA knockdown experiments, HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany). All media were supplemented with 10% fetal calf serum (Gibco-BRL, Karlsruhe, Germany), 1% glutamine (Gibco-BRL) and 1% antibiotic solution (penicillin and streptomycin; Gibco-BRL). Transfection of the cells was carried out in the absence of any antibiotics.

*siRNA transfection.* Three different siRNAs specific to ZC3H15 and one non-silencing negative control siRNA, that has no relevant homology to mammalian genes, were employed as listed in Table I. All siRNAs were synthesized by Qiagen (Hilden, Germany) and were purchased as annealed RNA-duplexes. The non-silencing control siRNA was 5-prime labelled with Rhodamine. HeLa cells and P4-CCR5 cells were plated in 24-well plates (Corning, Kaiserslautern, Germany) 24 h before transfection at $5x10^4$ cells per well in Dulbecco's minimal essential medium containing 10% FBS without antibiotics. Transfections were performed with Lipofectamine 2000 transfection reagent (Invitrogen)

Table I. siRNA sequences.

| Name | siRNA (sense strand) | siRNA (antisense strand) |
|---|---|---|
| si-ZC3H15-1 | 5'-GGAGCAAAGCAACAGAAGUtt-3' | 5'-ACUUCUGUUGCUUUGCUCCtt-3' |
| si-ZC3H15-2 | 5'-GGCUGUCACACAUCAAGUUtt-3' | 5'-AACUUGAUGUGUGACAGCCtt-3' |
| si-ZC3H15-3 | 5'-GGAUGACAAGAAGAAAGAUtt-3' | 5'-AUCUUUCUUCUUGUCAUCCtt-3' |
| si-nons-Rho | 5'-UUCUCCGAACGUGUCACGUtt-3' | 5'-ACGUGACACGUUCGGAGAAtt-3' |

according to the manufacturer's recommendations with siRNA at a final concentration of 30 nM. After incubation for 6 h, the excess lipid/siRNA complexes were removed, and the medium was replaced with fresh medium. For further analysis, cells were removed from the culture dish by trypsinization with 100 $\mu$l of 0.25% trysin/0.02% EDTA in PBS (Cambrex, Verviers, Belgium) for 5 min at 37˚C, at different time points after transfection. Transfection efficiency was analysed by flow cytometry 24 h after transfection. Data were acquired and analyzed on FACScalibur with Cell Quest software (Becton Dickinson, Heidelberg, Germany). Effects on cellular viability after siRNA treatment were measured using the cell proliferation reagent WST-1 according to the manufacturer's instructions (Roche, Penzberg, Germany).

*Real-time PCR quantitation of cellular RNA*. RNA was extracted using the RNeasy mini kit including treatment with RNase-free DNase I (Qiagen). Synthesis of cDNA was carried out using random hexamer primers and Superscript-II RNase H-reverse transcriptase according to the manufacturer's specifications (Invitrogen).

Real-time PCR was performed in duplicate reactions employing ABI PRISM 7700 (Applied Biosystems, Darmstadt, Germany) with standard conditions (50˚C for 2 min, 95˚C for 10 min and 40 cycles at 95˚C for 15 sec and 60˚C for 1 min). The 25 $\mu$l PCR included 2.5 $\mu$l cDNA, 1X TaqMan® Universal PCR Master mix (Applied Biosystems), 0.2 $\mu$M TaqMan probe, 0.2 $\mu$M forward primer and 0.2 $\mu$M reverse primer. Primers and probes were designed using Primer Express v.1.0 software (Applied Biosystems) and were synthesized by Thermohybaid (Ulm, Germany). In order to quantify ZC3H15 and GAPDH cDNA, the primers and probes, as listed in Table II, were used. The probes were labeled with FAM at the 5' end and TAMRA at the 3' end. For normalization, GAPDH housekeeping gene expression was analyzed. In order to quantify transcript copy numbers precisely, standard curves were obtained after amplification of log step dilutions between 10 to $10^6$ copy numbers of purified plasmids carrying the amplicons of ZC3H15 (Table II) or human GAPDH (5), respectively.

Copy numbers of the individual transcripts were calculated by plasmid standard curves and normalized by GAPDH housekeeping gene transcripts.

*Northern blotting*. A 2.1 kb long cDNA sequence of ZC3H15, which had been cloned from a full-length PCR product was labeled as a probe for northern blot hybridization using 50 ng DNA template (1 $\mu$l) with 20 $\mu$l random oligonucleotide primers and 47 $\mu$l $H_2O$, which was incubated at 95˚C for 5 min. Subsequently, 10 $\mu$l of [$\alpha$-$^{32}$P]dATP at 3000 Ci/mmol, 20 $\mu$l of 5X dATP primer buffer and 2 $\mu$l of Exo(-) Klenow enzyme (5 U/$\mu$l) were added. After 10 min at 37˚C 4 $\mu$l of stop buffer was added. The labeled probe was purified using a Stratagene NucTrap probe purification column (Stratagene, La Jolla, CA, USA). The labeled ZC3H15 probe was hybridized at standard conditions with a Human Multiple Tissue Northern (MTN) Blot purchased from Clontech (Mountain View, CA, USA). The blot was generated by loading 2 $\mu$g of polyA+ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas per lane, respectively.

*Gene expression analysis by microarrays*. The microarray analysis of the effect of ZC3H15 suppression by siRNA was performed using the HG-U133 Plus 2.0 microarray of Affymetrix (Santa Clara, CA, USA) per manufacturer's instructions (GeneChip® Expression Analysis Manual). This chip contains 47,000 transcripts which represent 39,000 annotated genes. The data analysis was carried out according to established standards for Affymetric microarrays using GeneChip Operating Software (GCOS; Affymetrix) und GeneSpring (Agilent Technologies, Böblingen, Germany).

*Western blotting*. Proteins in hydrophilic solution were precipitated by addition of trichlor acetate (TCA), centrifuged (at 20,000 x g for 30 min), washed twice with ice-cold acetone (TCA and acetone from Sigma-Aldrich, Taufstein, Germany) and subsequently resuspended in SDS sample buffer at 95˚C for 5 min (ImmunoPure; Pierce, Rockford, IL, USA). Proteins were separated by discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli with 15% acrylamide, 0.8% bisacrylamide, 385 mM Tris-HCl (pH 8.8), 0.05% APS, 0.035% TEMED (Roth, Karlsruhe, Germany) and 0.1% SDS (Sigma-Aldrich). The SDS running buffer (pH 8.9) consisted of 3% Tris-base, 1% SDS (Sigma-Aldrich) and 14.4% glycin (Merck, Darmstadt, Germany). Blue Plus2 Pre-Stained Standard (Invitrogen) was used in order to determine protein sizes. The electrophoresis was carried out at 30 mA employing the Mini-Protean 3 Electrophoresis System (Bio-Rad, München, Germany). Proteins were transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad) in the HEP-1 Semi Dry Electroblotting System (OWL Separation Systems, Portsmouth, NH, USA) with 1 mA/cm$^2$ for 60 min. The membrane was blocked with 3% (w/v) BSA (Sigma-Aldrich) in PBST (Gibco-BRL, Paisley, UK) at pH 7.5 (0.05% (w/v) Tween-20 (Roth) for 30 min, followed by incubation with the primary polyclonal rabbit anti-ZC3H15 antibody (dilution: 1:200) for 30 min. This antibody had been

Table II. Primers and probes for quantitative PCR.

| Name | Forward-Primer | Backward-Primer | TaqMan Probe |
|---|---|---|---|
| ZC3H15 | GGTGCCATCTGTCTCCGC | TCTGTTGCTTTGCTCCTTTCTTATT | TGCCCCCCAAGAAACAGGCTCA |
| GAPDH | GAAGGTGAAGGTCGGAGTC | GAAGATGGTGATGGGATTTC | CAAGCTTCCCGTTCTCAGCC |
| ZC3H15 amplicon | CTCGTCCTGCCGCAGGGCCAGA | GTTGTTTTCAATAGCTTCCAGGAAAT | |

generated by vaccination of rabbits with a C-terminal peptide of ZC3H15 (NH2-CDELEEELNTLDLEE-COOH) which was linked to keyhole limpet haemocyanin (KLH) generated by Pineda Antibody Service, Berlin, Germany for us. The specificity of this antibody had been validated by a recombinant ZC3H15 with a strep-tag. After 3 washes in PBST for 5 min, the blot was incubated with the secondary mouse anti-rabbit IgG (RG-96, γ-chain specific) conjugated to HRP in a dilution of 1:200.000 (Sigma-Aldrich, Hamburg, Germany). The binding of the secondary antibody was detected by ECL solution and autoradiography (Cronex 5; AGFA, Mortsel, Belgium).

*Immune fluorescence microscopy*. Intracellular localization of proteins and analysis of siRNA oligonucleotide transfection efficiency were accomplished by fluorescence microscopy. Therefore $1x10^4$ to $5x10^4$ adherently growing cells were seeded onto a slide overnight (diagnostic slide with adhesive epoxy layer; Roth) in culture dishes. Cells were gently washed in PBS (37˚C), fixed for 10 min in 3% (v/v) formaldehyde in PBS at room temperature (RT) and permeabilized for 10 min in 0.1% (v/v) Tritonx-100 in PBS. Before incubating with antibodies, cells were blocked with Image-iT FX Signal Enhancer reagent (Molecular Probes, Eugene, OR, USA) for 30 min at RT and after washing incubated with primary antibody (diluted in 3% (w/v) BSA in PBS) for 90 min at RT. After washing in PBS, cells were incubated with the respective fluorophor-coupled secondary antibody for 90 min at RT. Then nuclei were stained with DAPI (4',6-Diamidino-2-Phenylindol; Molecular Probes, Life Technologies GmbH, Darmstadt, Germany) in 1:300 PBS dilution for 5 min at RT followed by overlaying cells with cover medium (ProLong mounting medium; Molecular Probes). Slides were evaluated with the Axioplan II-imaging microscope and Axiovision Software (Zeiss, Göttingen, Deutschland) and displayed by Adobe Photoshop 6.01 Software (Adobe Systems GmbH, Munich, Germany). Polyclonal rabbit anti-ZC3H15 antibody (1:100) or mouse IgG2a anti-protein disulfide isomerase (PDI) (Acris Antibodies, Herford, Germany) at 1:200 were used as primary antibodies and goat anti-rabbit IgG (1:500) labeled with Alexa 488 or goat anti-mouse IgG (1:500) labeled with Alexa 594 (Molecular Probes) were employed as secondary antibody, respectively.

*Co-immunoprecipitation*. Potential interaction partners of ZC3H15 were identified by the ProFound Co-immunoprecipitation System (Pierce) according to manufacturer's instructions. The polyclonal anti-ZC3H15 antibody was covalently bound to the aldehyde-activated matrix (AminoLink Plus Gel, Pierce). Co-immunoprecipitation was carried out in centrifugable spin columns (Handee Spin Cup Columns, Pierce). Cell lysates were transferred to columns and incubated for 3 h in a rotator at RT. Unspecifically bound proteins were removed by 4 washes (Modified Dulbecco's PBS, Pierce). The elution of specifically bound protein complexes was accomplished by stark changes of pH (ImmunoPure IgG Elution Buffer, Pierce) and subsequent neutralization of retrieved fractions (1 M Tris, pH 9.5; Ambion). The elution fractions were analyzed by subsequent SDS gel electrophoresis and western blotting.

*Analysis of ZC3H15 expression on Leukemia Gene Atlas (LGA) platform*. The Leukemia Gene Atlas is a public platform which provides leukemia related gene expression data sets together with various analysis and visualization tools (6). First, the data set published by Haferlach *et al* (7) was evaluated for differential ZC3H15 expression among different leukemias. ZC3H15 expression in AML (n=542 samples), CML (n=76 samples), ALL (n=134 samples) and MDS (n=207 samples) samples were compared to a control group of non-leukemia samples (n=73 samples) using Wilcoxon tests. Next, the data set published by Verhaak *et al* (8) was employed to compare ZC3H15 expression between different FAB classes of AML (FAB M0: n=16; M1: n=95; M2: n=106; M3: n=24; M4: n=79; M4E: n=5; M5: n=104; M6: n=6). The same data set was used to assess differences in ZC3H15 expression between AML samples with different karyotypes (+8: n=20; complex: n=14; del(5q)+del(7q): n=16; del(7q): n=6; idt(16): n=12; inv(16): n=18; normal: n=129; t(11q23)/MLL: n=15; t(15;17): n=23; t(6;9): n=6; t(8;21): n=33). The Kruskal-Wallis rank sum test was applied to detect differences between the FAB classes as well as between different karyotypes. Both data sets were generated on the Affymetrix HG-U133Plus2 platform that contains two probe sets measuring the expression of ZC3H15. The results presented here are based on probeset 201595_s_at. Highly similar results were obtained for probeset 201593_s_at (data not shown).

## Results

*Expression of ZC3H15 in normal human tissue*. In order to characterize ZC3H15, the expression of ZC3H15 was determined in various human tissues. A human tissue northern blot exhibited a ubiquitous expression in all examined tissues. The expression level was relatively similar among different tissues, except for lung tissue, which showed a lower expression level for ZC3H15 mRNA, and except for skeletal muscle tissue, which showed a relatively strong expression in relation to the L32 housekeeping gene loading control (Fig. 1).

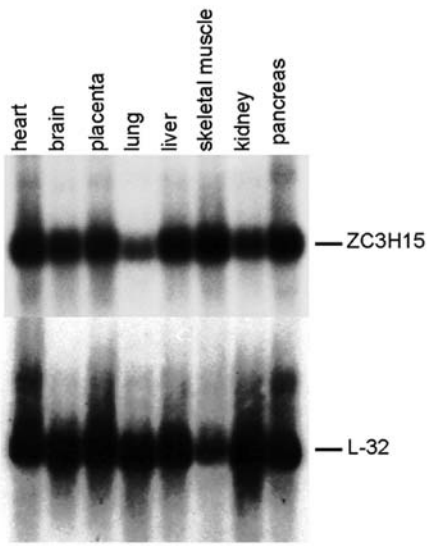


Figure 1. Northern blot of ZC3H15 expression in human tissues. mRNA extracted from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas tissue was hybridized with specific probes for ZC3H15 or housekeeping gene L32, respectively.

*Subcellular localization of ZC3H15*. In order to screen for a possible function of ZC3H15, the subcellular localization of this protein was studied. Western blot analysis revealed a band at ~50 kD in the cytosolic fraction of Jurkat and HeLa cells, respectively, which corresponded to the complete protein of ZC3H15 (Fig. 2A). In addition, HeLa cells showed a weak 50 kD band in the nucleus fraction of cellular proteins. Furthermore, Jurkat and HeLa cells revealed a protein of 30 kD which was specifically stained by the ZC3H15-specific polyclonal antibody. The 30 kD band may indicate a shorter fragment of ZC3H15 after processing or a very homologous protein.

Immune fluorescence microscopy was also employed to determine the subcellular localization of ZC3H15. Staining with the specific polyclonal anti-ZC3H15 followed by a secondary Alexa-488 labeled antibody exhibited a diffuse staining of the cytoplasm with no preference for specific compartments (Fig. 2B). The nuclei were contrasted with DAPI dye showing no staining with the ZC3H15-specific antibody. Thus, in contrast to the western blot, there was no nuclear localization detected by immune fluorescence. However, the nuclear band in the western blot was relatively weak indicating a low level of protein in the nucleus, which may have been below the threshold of detection by immune fluorescence.

*Suppression of ZC3H15 expression by siRNA*. HeLa cells were transfected with siRNAs directed against ZC3H15 or a non-silencing siRNA, which was 5-prime labeled with Rhodamine. Flow cytometry analysis of non-silencing siRNA-transfected cells at 24 h post-transfection revealed transfection efficiencies of ~90% in all samples (Fig. 3A). Quantitative real-time PCR revealed significantly reduced ZC3H15 gene transcript levels in cells transfected with specific siRNA compared to the cells transfected with non-silencing siRNA (Fig. 3B). A reduction of ZC3H15 expression by 87, 88 and 88% was obtained with 3 different siRNAs, respectively.

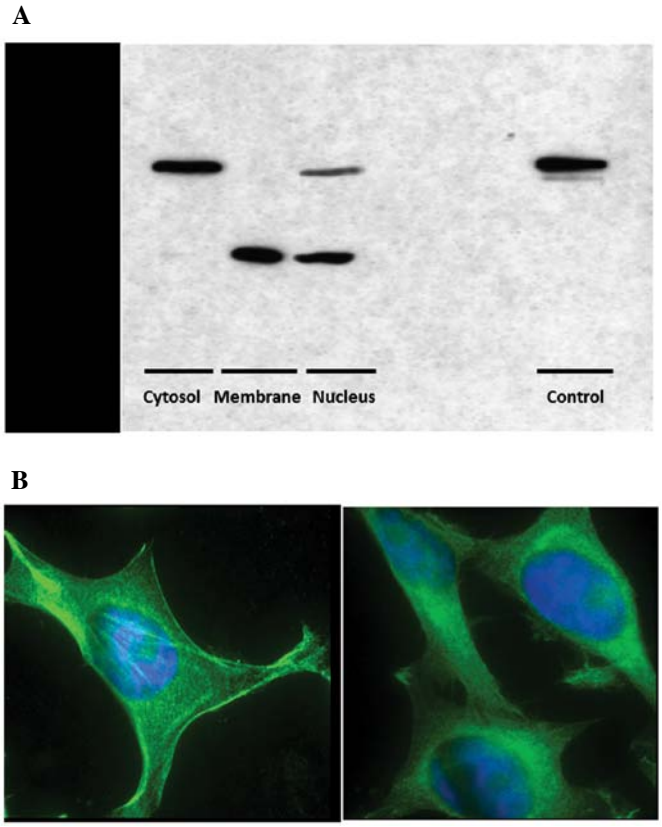


Figure 2. Western blot analysis of ZC3H15 subcellular localization (A). Subcellular membrane, cytosol and nucleus fractions of HeLa cells were electrophoresed, blotted and incubated with polyclonal rabbit anti-ZC3H15 antibody followed by secondary HRP-conjugated anti-rabbit antibody. Cellular localization of ZC3H15 in immune fluorescence microscopy (B). Polyclonal rabbit anti-ZC3H15 and goat anti-rabbit IgG linked to fluorochrome Alexa 488 were employed to display the subcellular localization of ZC3H15. Cell nuclei were contrasted by DAPI in blue.

The reduction of ZC3H15 expression was confirmed on the protein level. Total protein was extracted from controls and si-ZC3H15 transfected HeLa cell cultures 48 and 72 h post-transfection. A western blot displayed a strong specific band at 48 kD representing ZC3H15 in the negative control and markedly weaker bands for the si-ZC3H15 transfected samples (Fig. 3C). In order to control the protein quantity loaded onto the gel, tubulin was determined on the same western blot. The protein level of ZC3H15 was lower at 48 h after transfection of si-RNAs than at 72 h suggesting that the effect of the siRNA specific to ZC3H15 was slowly fading. However, the ZC3H15 protein at 72 h was still considerably lower than in the negative control.

Viability of cells transfected with siRNAs against ZC3H15 or with the non-silencing siRNA remained unchanged as determined by WST-1 cell proliferation assay (Fig. 4). Both shape and growth rate of cells with ZC3H15 knockdown were normal. Thus, siRNAs against ZC3H15 efficiently and specifically reduced the expression of ZC3H15 without affecting cell viability.

*Cellular effects of ZC3H15 suppression by microarray screen*. In order to elucidate the role of ZC3H15 in cellular processes and signaling, the effect of ZC3H15 knockdown on the cellular gene expression pattern was investigated. Two independent

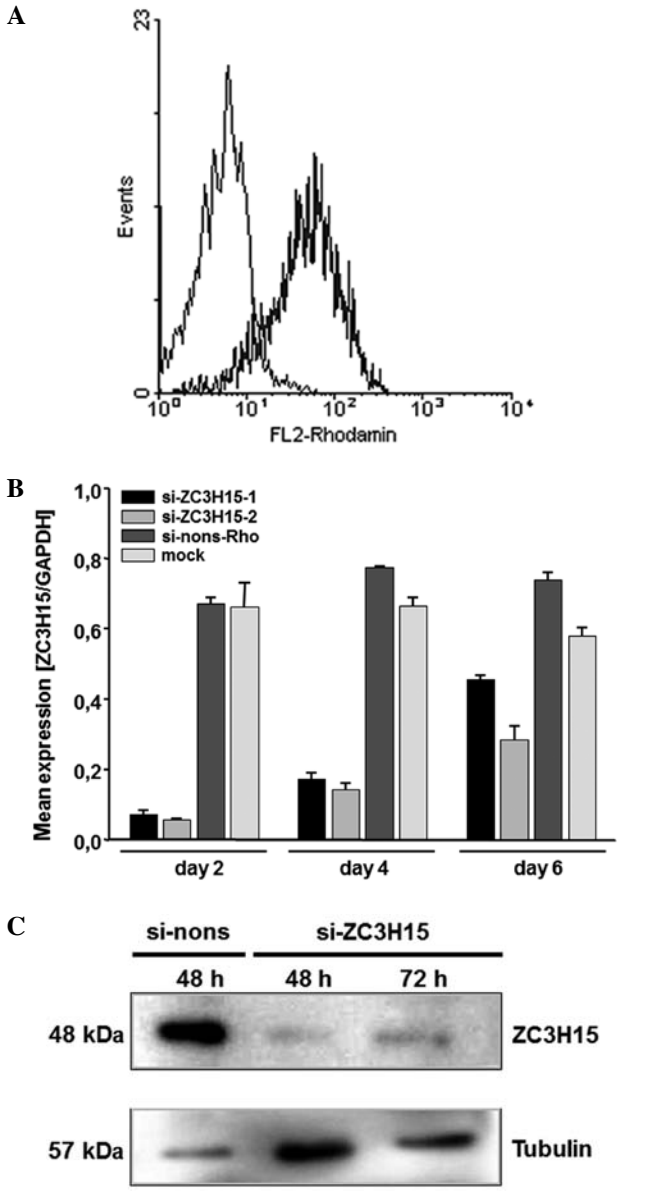


Figure 3. Transfection efficiency of siRNA (A). HeLa cells were transfected with non-silencing control siRNA oligonucleotide labeled with rhodamine and analyzed by flow cytometry 18 h post-transfection. Efficacy of siRNA-mediated suppression of ZC3H15 mRNA level (B). Quantitative PCR analysis of ZC3H15 expression in HeLa cells which were transfected with 2 different siRNAs against ZC3H15 or the control non-silencing siRNA (labeled with rhodamine). Bars represent the standard deviation of the mean of triplicate determinations. Efficacy of siRNA-mediated suppression of ZC3H15 protein level (C). Western blot of ZC3H15 expression in HeLa cells, which were transfected with specific siRNAs against ZC3H15 or the control non-silencing siRNA for 48 or 72 h. Tubulin was determined as a gel loading control.

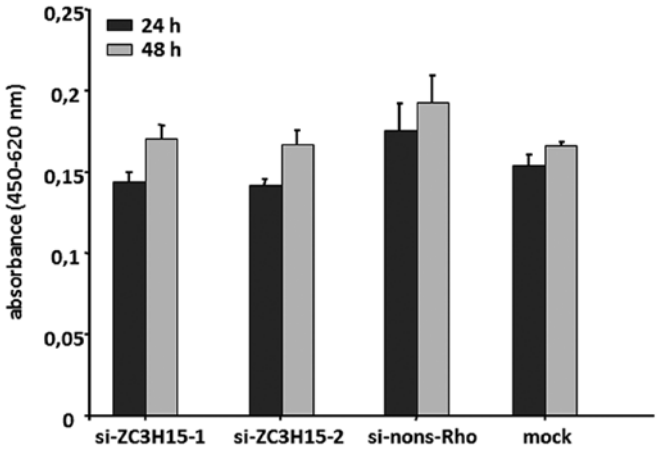


Figure 4. WST-1 cytotoxicity assay. HeLa cells were transfected with siRNAs specific to ZC3H15, non-silencing control si-RNA labeled with rhodamine or without siRNA. Potential cytotoxic effects and impairment of viability and proliferation were tested by WST-1 assay.

transfections with siRNA against ZC3H15 or non-silencing siRNA were performed and analyzed in parallel on microarrays. The specific siRNA reduced the expression of ZC3H15 by 93 and 92%, which was on average a 13.5-fold decrease, as measured by quantitative RT-PCR. This measurement correlated very well with the determination by micro-array analysis showing a 14.6-fold mean reduction of ZC3H15 expression. In order to derive hints about the functional involvement of ZC3H15, differentially expressed genes were grouped according to the Gene Ontology Databank categories. The Gene Ontology Databank is structured hierarchically according to 3 different criteria: subcellular localization, type of biological process, molecular function. Each of the differentially regulated genes was annotated to one or more specific Gene Ontology (GO) categories. Subsequently, it was evaluated, which GO categories showed an over-representation of the differentially expressed genes compared to a theoretical random annotation. Relevant GO term categories were displayed with their upper terms.

Fig. 5 shows the relevant categories with an over-representation according to the main criterion biological processes. The knockdown of ZC3H15 revealed 202 differentially expressed genes including 114 induced genes and 88 suppressed genes (≥3-fold increase or ≤0.3-fold decrease). The main GO categories that contained an over-representation of differentially expressed genes comprised development, growth, physiological process, cellular process, regulation of biological process and response to stimulus (Fig. 5). In the end of these GO paths, there were seven categories with particular over-representation including negative regulation of cell growth, mRNA transcription from RNA polymerase II promoter, cell cycle arrest, regulation of I-κB cascade/NF-κB cascade, cell adhesion, regulation of MAPK activity and immune response (Fig. 5). The genes that are allocated to these GO categories are listed in Table III according to the magnitude of induction and suppression. The categories mRNA transcription from RNA polymerase II promoter (7 genes) and immune response (10 genes) contained only induced genes associated with knockdown of ZC3H15, while the other categories contained both induced and suppressed genes in various ratios.

*Interaction of ZC3H15 with cellular signaling proteins.* Our microarray results and the fact that the mouse homolog of ZC3H15 had been identified and named as TRAF-C terminal interacting factor (TCIF) (GenBank accession no.: BD173935) (9) lead us to investigate whether ZC3H15 interacted with human TRAF-2 as well. The physical interaction of ZC3H15 with human TRAF-2 was studied by co-immunoprecipitation. The ZC3H15-specific antibody was used to extract ZC3H15 containing protein complexes. The subsequent western blot analysis of the ZC3H15 containing

Table III. Differentially expressed genes in over-represented GO categories following ZC3H15 knockdown.

| GO categories | Gene ID | Up ↑/down ↓ regulation |
|---|---|---|
| Negative regulation of cell growth | PPP2R1B (16.6) | ↑ |
| | PML (-3.2); SIPA1 (-5.5) | ↓ |
| Transcription from RNA polymerase II promoter | HSF2 (16.4); NFYA (13.0); MTF1 (11.0); ZNF174 (3.8); SMARCA5 (3.7); KLF5 (3.2); POLR2D (3.1) | ↑ |
| Cell cycle arrest | DDIT3 (7.3) | ↑ |
| | DST (-3.1); PML (-3.2); CDKN1C (-3.3) | ↓ |
| Regulation of I-κB/NF-κB cascade | PPP2R1B (16.6) | ↑ |
| | TSPAN6 (-3.9) | ↓ |
| Cell adhesion | PPP2R1B (16.6); COL21A1 (5.6); KITLG (4.2); CD84 (4.2); PPFIA1 (3.8); PKP2 (3.7); THBS1 (3.5) | ↑ |
| | DST (-3.1); FARP1 (-3.3); SSX2IP (-3.3); WISP2 (-3.5); CTNND1 (-3.5); PKD1 (-3.5); NINJ1 (-3.7); LAMB2 (-4.8); SIPA1 (-5.5) | ↓ |
| Regulation of MAPK activity | PPP2R1B (16.6); HIPK3 (5.4); TRIB3 (3.3) | ↑ |
| | MAP4K5 (-3.0) | ↓ |
| Immune response | IFI6 (7.2); GBP1 (5.6); AOX1 (4.6); PTGER4 (4.6); NCF2 (4.2); ZNF3 (3.6); NR4A2 (3.3); BCL2 (3.3); IFIT5 (3.1); IFIT1 (3.0) | ↑ |

The genes are allocated in the last category of each of the seven GO paths over-represented by differentially expressed genes in this respective category. The upward arrows illustrate that these genes were induced in HeLa cells following knockdown of ZC3H15, while downward arrows illustrate that these genes were suppressed. The positive numbers in parentheses indicate the magnitude (e.g. 16.6-fold) of induction. The numbers with a negative sign indicate the magnitude of suppression (e.g. control expression divided by 3.2 equals expression in experimental culture).

eluate revealed that TRAF-2 was associated with ZC3H15 in a protein complex (Fig. 6).

*Expression pattern of ZC3H15 in leukemias*. Since ZC3H15 expression had been found to be induced by erythropoietin, we wondered, whether ZC3H15 expression is associated with specific leukemia types. The analysis was performed *in silico*. A published data set based on gene expression microarrays of bone marrow samples of hematological patients and controls (7) was employed. Bone marrow (BM) samples (n=542) of patients with AML revealed a significantly increased ZC3H15 expression level ($p<0.001$, fold change 1.3) compared to controls (n=73) of subjects without leukemia (Fig. 7). Furthermore, 134 bone marrow samples of acute lymphoblastic leukemias (ALL) at diagnosis prior to therapy had a similar level of ZC3H15 expression as the control samples from normal bone marrows.Subsets of patients with chronic myeloid leukemia (CML, n=76) and myelodysplastic syndrome (MDS, n=207) had similar median expression levels compared to these controls as well (Fig. 7).

Furthermore, we were interested in whether ZC3H15 expression levels were associated with a specific subgroup of AML. A screen in the dataset of Verhaak *et al* (8) revealed only small and non-significant ($p>0.1$) variations of the median ZC3H15 expression in different FAB classes. In addition, ZC3H15 expression did not significantly ($p>0.1$) differ between AML specimens with different karyotypes in the data set from Verhaak.

## Discussion

ZC3H15 as a zinc finger containing protein with a putative role in cell signaling intriegued us to investigate its expression pattern, subcellular localization and function in cellular processes. ZC3H15 showed ubiquitous expression in various normal human tissues. Immune fluorescence microscopy revealed a diffuse cytosolic localization that was confirmed by western blotting in cytoplasmic extracts. Additionally, a weak expression was found in the nuclear fraction of HeLa cells by Western blotting. Microarray experiments in cells with siRNA suppression of ZC3H15 revealed an over-representation of differentially expressed genes involved in cell growth, transcription, cell cycle regulation, cell adhesion, immune response and regulation of the NF-κB pathway and the MAPK activity. ZC3H15 was shown to interact physically with adapter protein TRAF-2.

ZC3H15 has been published in GenBank (accession no.: NM_018471) as coding sequence for a protein with 2 CCCH-type zinc fingers suggesting a DNA binding capacity and a putative transcription factor role. Its gene locus is at chromosome 2q32.1. There are several matching GenBank entries of open reading frames, which were derived from various tissues including $CD34^+$ stem cells, hypothalamus, HSPC303, HT010, MSTP012, and PP730. The broad tissue expression of this gene is in agreement with our own findings showing ubiquitous expression in a Human Multiple Tissue Northern Blot

Table IV. The genes with modified expression following ZC3H15 knockdown in HeLa cells.

| Gene | Fold change | Gene | Fold change | Gene | Fold change | Gene | Fold change |
|---|---|---|---|---|---|---|---|
| SLC7A11 | 36.50 | CENPJ | 3.81 | GRSF1 | -3.01 | OSBPL1A | -4.46 |
| OGFRL1 | 24.58 | PPFIA1 | 3.80 | SH3GLB1 | -3.03 | HD | -4.51 |
| ZNF588 | 18.30 | CCHCR1 | 3.78 | MAP4K5 | -3.04 | RAP2A | -4.56 |
| PPP2R1B | 16.60 | ZNF174 | 3.77 | RPL22 | -3.09 | ATXN10 | -4.62 |
| HSF2 | 16.42 | ATF4 | 3.71 | CXorf53 | -3.11 | KIAA1102 | -4.63 |
| ZNF227 | 13.14 | PKP2 | 3.69 | DST | -3.12 | UBE2W | -4.67 |
| NFYA | 13.05 | ATF4 | 3.71 | RPL15 | -3.17 | LAMB2 | -4.8 |
| TLK1 | 11.82 | PKP2 | 3.69 | CENPF | -3.18 | RAP140 | -4.9 |
| MTF1 | 11.01 | LOC253982 | 3.69 | JAK3 | -3.19 | PAFAH1B1 | -4.94 |
| FAM29A | 10.54 | RAB3IL1 | 3.69 | PML | -3.21 | FLJ21687 | -4.94 |
| ARG2 | 9.27 | C16orf34 | 3.68 | SNAP23 | -3.23 | KLK5 | -5.26 |
| HES2 | 9.23 | SMARCA5 | 3.66 | SFRS7 | -3.24 | PEX10 | -5.41 |
| IFIT5 | 9.00 | FBXO2 | 3.61 | C5orf13 | -3.24 | NFYB | -5.44 |
| FGFR2 | 8.92 | MLH3 | 3.60 | FARP1 | -3.25 | SIPA1 | -5.51 |
| WBP4 | 7.71 | ZNF3 | 3.59 | CDKN1C | -3.26 | SDC2 | -5.6 |
| VAMP4 | 7.59 | ZNF556 | 3.59 | SNAP23 | -3.27 | STAU2 | -5.77 |
| MCF2L | 7.58 | DYRK4 | 3.58 | NAB1 | -3.27 | RBL2 | -5.88 |
| DDIT3 | 7.31 | FHL1 | 3.55 | SSX2IP | -3.31 | ATG5 | -6.1 |
| G1P3 | 7.22 | KIAA0241 | 3.55 | RNASET2 | -3.38 | HCRT | -6.18 |
| DCUN1D4 | 7.10 | RNF170 | 3.53 | COQ6 | -3.43 | PFAAP5 | -6.22 |
| S100A14 | 6.65 | THBS1 | 3.49 | WISP2 | -3.47 | C10orf116 | -6.38 |
| ZNF273 | 6.36 | AOC2 | 3.47 | MRS2L | -3.47 | SPINK4 | -6.62 |
| TNFRSF8 | 6.34 | KIF1B | 3.37 | CTNND1 | -3.5 | SCP2 | -6.65 |
| RNASE2 | 6.19 | HPS4 | 3.36 | BF | -3.51 | GPM6B | -6.72 |
| UST | 5.95 | SLC2A3 | 3.35 | PKD1 | -3.51 | **LEREPO4** | **-7.42** |
| COL21A1 | 5.95 | LOC388335 | 3.35 | SUB1 | -3.52 | CYLD | -10.23 |
| GBP1 | 5.64 | RABL4 | 3.33 | FYN | -3.57 | ANKRD46 | -10.29 |
| ARHGAP19 | 5.50 | NR4A2 | 3.33 | DAB2 | -3.59 | TPD52 | -12.83 |
| HIPK3 | 5.37 | SLC7A1 | 3.32 | IGFBP6 | -3.62 | **LEREPO4** | **-14.61** |
| CDS2 | 5.28 | TRIB3 | 3.32 | MRPS30 | -3.64 | ABHD5 | -15.97 |
| ZNF473 | 5.25 | SLD5 | 3.30 | IL1RAP | -3.68 | YIPF6 | -18.04 |
| LOC283970 | 5.23 | CASP7 | 3.28 | PARD6A | -3.68 | | |
| TFF1 | 4.76 | PLA2G12A | 3.26 | DOCK6 | -3.69 | | |
| RAB11FIP1 | 4.73 | BCL2 | 3.25 | SPIN | -3.7 | | |
| FGFR2 | 4.70 | ASNS | 3.25 | KIAA1644 | -3.71 | | |
| SEC24D | 4.61 | KLF5 | 3.23 | USP14 | -3.72 | | |
| AOX1 | 4.59 | ETV5 | 3.22 | NINJ1 | -3.72 | | |
| PTGER4 | 4.55 | PPP3CC | 3.20 | SAMD4 | -3.76 | | |
| --- | 4.55 | MPDZ | 3.19 | KIAA1102 | -3.83 | | |
| KIF1C | 4.54 | DYRK3 | 3.18 | HEMK1 | -3.83 | | |
| GNAS | 4.50 | RGS2 | 3.15 | CENTD1 | -3.86 | | |
| TMEM16B | 4.40 | AKR7A3 | 3.15 | PTP4A2 | -3.88 | | |
| SLIT1 | 4.37 | TWSG1 | 3.15 | VDP | -3.89 | | |
| UBAP2L | 4.36 | ACACA | 3.14 | TSPAN6 | -3.91 | | |
| FGFR1 | 4.25 | ZNF165 | 3.13 | CLASP2 | -3.92 | | |
| YLPM1 | 4.22 | SMOX | 3.10 | UBE2N | -3.93 | | |
| KITLG | 4.21 | SLC2A3 | 3.09 | WIG1 | -3.95 | | |
| NCF2 | 4.20 | POLR2D | 3.08 | TXNRD1 | -4.01 | | |
| CD84 | 4.17 | NR4A2 | 3.06 | TSFM | -4.11 | | |
| RCP9 | 4.15 | IFIT5 | 3.05 | UBL3 | -4.22 | | |
| TPM4 | 4.11 | ADAMTS1 | 3.05 | RBM8A | -4.32 | | |
| IFRD1 | 3.98 | PIGL | 3.04 | KYNU | -4.34 | | |
| SS18L1 | 3.94 | IFIT1 | 3.03 | MARCKSL1 | -4.36 | | |
| MFAP5 | 3.90 | PTBP2 | 3.03 | SHOX2 | -4.38 | | |
| C10orf95 | 3.88 | TBPIP | 3.02 | ATP2C1 | -4.45 | | |
| IFI44 | 3.83 | FLJ12443 | 3.00 | NASP | -4.46 | | |

The minus sign before the factor in the column 'Fold change' indicates a decrease such that the respective expression level was the control expression level divided by the following number. LEREPO4 (bold) is ZC3H15 and represented by 2 microarray oligonucleotides.

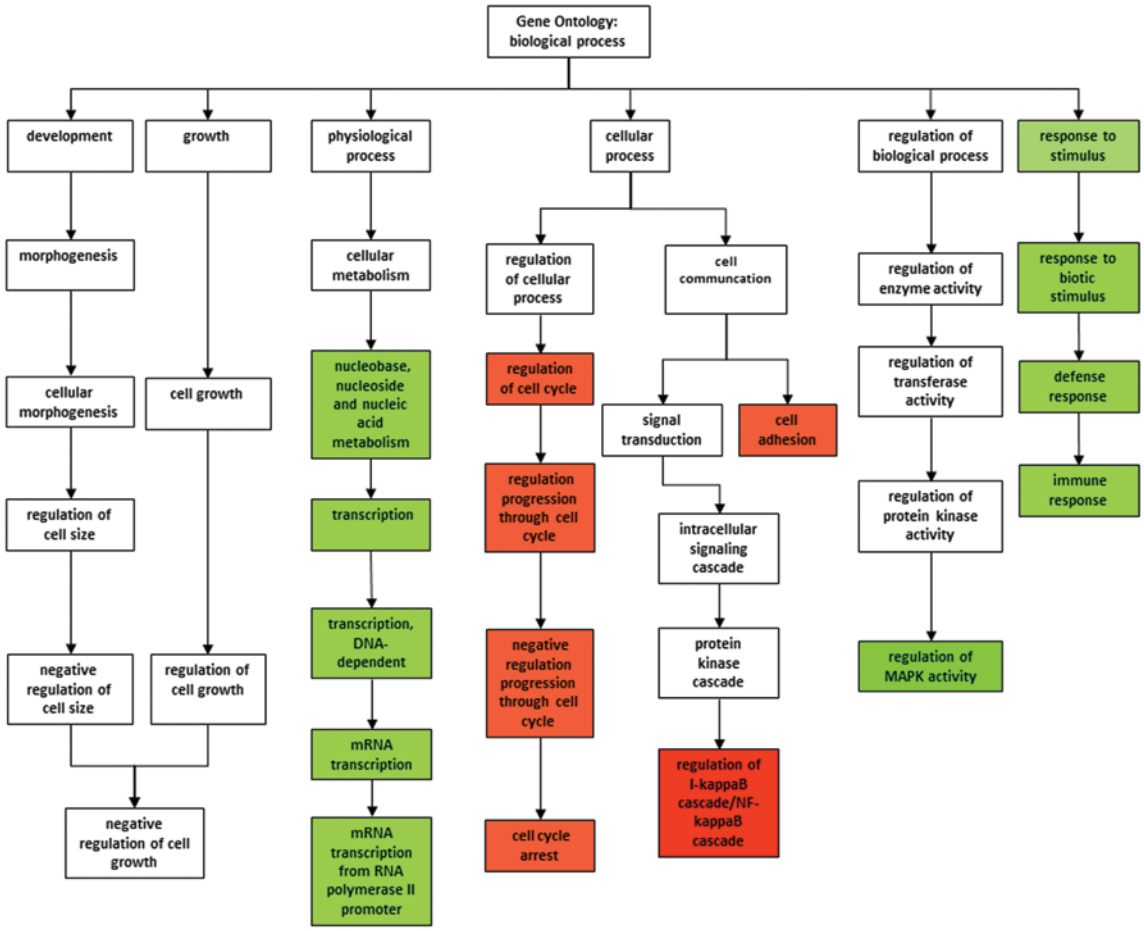


Figure 5. Gene Ontology (GO) classification of differentially expressed genes by ZC3H15 knockdown. GO-pathways showing significant over-representation of differentialy expressed genes were found by MAPPFinder 2.0 and NetAffx software. GO-pathways or GO-categories, in which predominantly suppressed genes were allocated, are highlighted in red, GO-categories, in which predominantly induced genes were allocated, are highlighted in green.

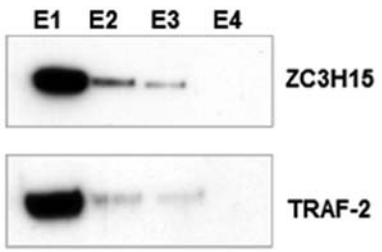


Figure 6. Co-immunoprecipitation of ZC3H15 and TRAF-2. Four eluation fractions were subjected to electrophoresis (E1-E4). Western blot analysis showed the presence of ZC3H15 predominantly in E1 with low decreasing amounts in E2 and E3. In parallel, TRAF-2 was detected by western blot predominantly in E1 with low amounts in E2 and E3.

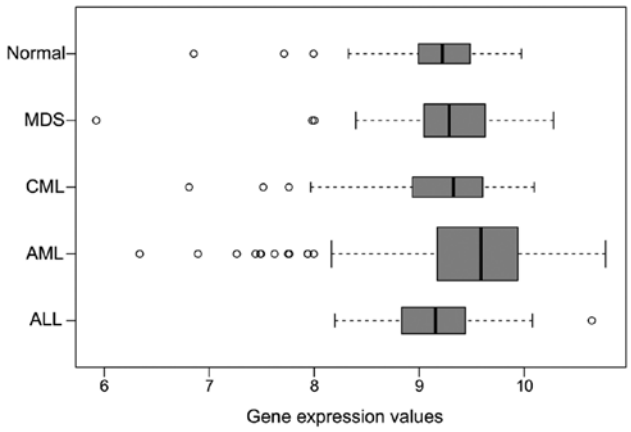


Figure 7. ZC3H15 expression in leukemias. ZC3H15 expression was analyzed in the Leukemia Gene Atlas (LGA) platform (6) using a dataset published by Haferlach *et al* (7). ALL (n=134), AML (n=542), CML (n=76) and MDS (n=207) specimens were compared to a reference of normal/non-leukemia bone marrow specimen (n=73). The normalized and log2 transformed expression values are shown according to the scale of the X-axis. The expression of ZC3H15 was significantly ($p<0.001$) increased in AML compared to the reference. No significant ($p>0.1$) differences were detected between the ALL, CML or MDS group and the reference group, respectively.

including 8 different tissue types. ZC3H15 has been reported as a likely ortholog of mouse immediate early response erythropoietin 4 (2) indicating that it was swiftly induced by erythropoietin. Furthermore, ZC3H15 has the synonym DRG family regulatory protein 1 (DFRP1) (3). It has been reported that DFRP1 regulates the developmentally regulated GTP-binding protein 1 (DRG1). Human DRG1 associates with SCL (TAL-1) oncogenic protein and stimulates the co-transforming activity of c-Myc and Ras (10). DRG proteins, a subfamily of the GTPase superfamily (11) play a critical role in cell growth such that their aberrant expression triggers the disruption of normal growth control. DFRP1 was reported to have a nuclear export

signal (NES) at the C terminus (3). It was shown that DFRP1 binds to DRG1 specifically thereby blocking the DRG1 poly-ubiquitination and proteolysis and thus upregulating the level of DRG1. Immunofluorescence microscopy demonstrated that DFRP1 and DRG1 co-localized throughout the whole cytosol without associating with the endoplasmic reticulum, Golgi or mitochondria (3). In summary, these data suggest that ZC3H15 (DFRP1) is part of one or several signaling pathway(s) that are important for cell proliferation.

Our microarray data suggest that ZC3H15 affects the NF-κB signaling pathway. We have shown that ZC3H15 protein associates with TRAF-2 protein. Others have shown that the murine homologue of ZC3H15 interacts with murine TRAF-2 and TRAF-6, respectively. Therefore, it was called TCIF (TRAF-C terminal interacting factor) (9). TCIF has a 91% homology with human ZC3H15. TRAF-2 and TRAF-6 are members of a family of adaptor proteins, which participate in signal transduction of TNF receptors and IL-1/Toll-like receptors (12). TRAF proteins bind to the cytoplasmic domain of these receptors and recruit various TRAF-interacting effector proteins, which may activate the c-Jun N-terminal kinase (JNK) or the NF-κB signal cascade (13). Many of the molecules involved in NF-κB activation also play a role in JNK activation, one of the MAPK subpathways (14). Considerable cross-talk between these two pathways has been reported (15). Of note, we found that ZC3H15 knockdown was associated with modified gene expressions in the MAPK pathway. Future research may elucidate the precise role of ZC3H15 in both the NF-κB and the MAPK pathway.

Our initial *in silico* screen revealed a clear overexpression of ZC3H15 in AML. However, we could not detect different ZC3H15 expression levels among the different subtypes of AML. Our own gene expression analysis after ZC3H15 knockdown showed a strong induction of protein phosphatase 2 regulatory subunit Aβ (PPP2R1B) by more than 16-fold. Moreover, GO analyses of the microarray data allocated PPP2R1B to 4 over-represented subcategories, which was unique in our analysis. The whole protein conglomerate, protein phosphatase 2A (PP2A), of which PPP2R1B is part, is a tumor suppressor and the inactivation of PP2A is a common event in AML (16). While ZC3H15 knockdown has been found to be associated with strong induction of PPP2R1B, conversely overexpression of ZC3H15, as found in AML, may be associated with suppression of PPP2R1B and thus decreased PP2A activity. Therefore, it may be hypothesized that ZC3H15 overexpression may provide a target in AML. Others have demonstrated that the reactivation of PP2A by FTY720 exerted a cytotoxic effect on AML cells, especially on AML cells with C-KIT mutations (17). Therefore, it may be worthwhile to examine whether ZC3H15 is a functionally important and specific target in AML.

In conclusion, our data suggest that ZC3H15 is a mainly cytoplasmic TRAF-2-interacting protein associated with the NF-κB signal pathway showing increased expression in AML. Further experimental studies are required in order to dissect the precise role of ZC3H15 in AML.

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