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Unfolded protein response suppresses *CEBPA* by induction of calreticulin in acute myeloid leukaemia

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Abstract

The unfolded protein response (UPR) is triggered by the accumulation of misfolded proteins within the endoplasmic reticulum (ER). The role of the UPR during leukemogenesis is unknown so far. Here, we studied the induction of mediators of the UPR in leukaemic cells of AML patients. Increased expression of the spliced variant of the X-box binding protein 1 (*XBP1s*) was detected in 17.4% (16 of 92) of AML patients. Consistent with activated UPR, this group also had increased expression of ER-resident chaperones such as the 78 kD glucose-regulated protein (*GRP78*) and of calreticulin. Conditional expression of calreticulin in leukaemic U937 cells was found to increase calreticulin binding to the *CEBPA* mRNA thereby efficiently blocking translation of the myeloid key transcription factor *CEBPA* and ultimately affecting myeloid differentiation. Consequently, leukaemic cells from AML patients with activated UPR and thus increased calreticulin levels showed in fact suppressed CEBPA protein expression. We identified two functional ER stress response elements (ERSE) in the calreticulin promoter. The presence of *NFY* and *ATF6*, as well as an intact binding site for *YY1* within these ERSE motifs were essential for mediating sensitivity to ER stress and activation of calreticulin. Thus, we propose a model of the UPR being activated in a considerable subset of AML patients through induction of calreticulin along the *ATF6* pathway, thereby ultimately suppressing *CEBPA* translation and contributing to the block in myeloid differentiation.

Keywords: UPR • AML • Calreticulin • CEBPA • ATF6

Introduction

One of the transcription factors crucial for normal myeloid cell development is the CCAAT/enhancer binding protein alpha (*CEBPA*) [1–5]. Targeted disruption of *CEBPA* results in a block of granulocyte maturation [6], whereas expression of *CEBPA* in precursor cells is sufficient to trigger granulocytic differentiation [7]. In AML patients, deregulation of *CEBPA* function is a common event comprising genomic mutations [8–10], transcriptional suppression [11] and functional inactivation [12, 13]. Furthermore, CEBPA protein is suppressed in chronic myeloid leukaemia in blast crisis by the poly(rC)-binding protein hnRNP E2 [14]. Finally, the RNA-binding protein calreticulin can be specifically induced in core binding factor AML [15, 16].

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The unfolded protein response (UPR) is triggered by the accumulation of misfolded proteins in the endoplasmic reticulum (ER). It reduces the protein load entering the ER by lowering the global protein synthesis and by increasing the capacity to handle misfolded proteins through activation of ER chaperone molecules [17, 18]. If homeostasis cannot be achieved cell death is triggered [19, 20]. Three pathways of ER-stress transduction have been identified comprising the inositol-requiring protein-1 (*IRE1*), the activating transcription factor 6 (*ATF6*) and the protein kinase RNA-like ER kinase (*PERK*) [21]. In the case of *IRE1*, ER-stress induces the cleavage of the X-box binding protein 1 (*XBP1*) mRNA encoding a novel potent transcriptional activator of UPR target genes [22–25]. Other cellular targets of the UPR are calreticulin, *CHOP* and *GRP78*.

In this report, we found that the UPR is activated in 17.4% of AML patients as determined by the induction of the *XBP1* spliced variant and increased expression of *GRP78* and calreticulin. At the molecular level, we found that the activation of the calreticulin promoter following ER-stress was mediated by two copies of an ER-stress response element (ERSE) [27–30]. We identified *YY1*, the nuclear

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transcription factor Y (*NF-Y*) and the nuclear form of the activating transcription factor 6 (*ATF6n*) as ERSE-binding transcription factors. These results suggest a role of the UPR during leukaemogenesis including early induction of calreticulin, thereby suppressing CEBPA translation and inhibiting myeloid differentiation.

Materials and methods

Patients

Malignant cells were collected at the time of diagnosis before initiation of treatment. They were prepared from Ficoll-separated mononucleated cells of bone marrow aspirates from 92 consecutive AML patients seen at the Department of Medical Oncology, University Hospital, Bern, Switzerland between 2002 and 2004. Informed consent from all patients was obtained according to the Declaration of Helsinki, and the studies were approved by decisions of the local ethics committee of Bern, Switzerland. Conventional karyotype analysis of at least 20 metaphases was performed for each patient.

Cell lines and reagents

Human lung cancer H1299 and myeloid leukaemic K562 and U937 cells were grown in RPMI 1640 (SIGMA, Buchs, Switzerland) supplemented with 10% fetal bovine serum (FBS) (PAA, Pasching, Austria). For induction of ER-stress, cells were incubated with Thapsigargin (300 nM, final concentration), Calcimycin A23187 (7 μM, final concentration) or Tunicamycin (10 μg/ml), all obtained from SIGMA (Buchs, Switzerland) and dissolved in DMSO.

RT-PCR and assay to detect the spliced variant of *XBP1* mRNA (*XBP1s*)

RT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Taqman PCR Mix (Roche, Branchburg, NJ, USA). For *GRP78*, calreticulin, *NFYA* and *ABL*, assay-on-demand gene expression probes (Applied Biosystems) were applied. The relative quantization was indicated by Ct values, determined based on duplicate reactions for each target and internal control gene [31]. To assess the induction of *XBP1s* by PCR, the following primers were used: (s) 5'-GGGAATGAAGTGAGGCCAG-3' and (as) 5'-CAATACCGCCA-GAATCCATG-3' corresponding to nucleotides 412–431 and 834–853 of the *XBP1* cDNA generating two PCR products of 442 (*XBP1u*) and 416 (*XBP1s*) base pairs [23]. For statistical analysis, the Mann–Whitney U test, two-sided was applied.

Conditional calreticulin expression in myeloid cells

The U937-T cell line was kindly provided by Gerard Grosveld (Memphis, TN, USA). These cells were stably transfected with a plasmid encoding the tetracycline transactivator under the control of a tetracycline responsive element. A 4 kb Sca1-BamH1 fragment of the pcDNA3 vector, encoding the neomycin resistance gene, was ligated with the 0.95 kb fragment of the tet-off response plasmid pTRE to generate the pTRE-neo vector. A 1.4 kb EcoR1-Xho1 fragment encoding the 5'-haemagglutinine (HA)-tagged human calreticulin cDNA was then introduced into the pTRE-neo plasmid. The plasmid was stably transfected into U937-T cells by electroporation.

Constructs

The human HA-tagged *ATF6* plasmid was provided by Ron Prywes [32]. The plasmid for the nuclear form of human *ATF6n* was a gift from Katzutoshi Mori [23]. The plasmid for *YY1* was from Yang Shi [33]. A fragment of the human calreticulin promoter spanning nucleotide –1175 to +72 was amplified from DNA of peripheral blood lymphocytes from a healthy volunteer and cloned into the KpnI-XhoI sites of the pGL3 luciferase vector.

Transfection conditions and reporter gene assays

H1299 cells were transfected with 80 ng of reporter plasmid and 100 ng of pCMV-ATF6–373 or pCMV empty vector together with 0.5 ng of the CMV-Renilla reference plasmid using Lipofectamine 2000® (Invitrogen, Carlsbad, CA, USA). Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). In Hela cells, 200 ng of reporter plasmid were transfected together with 0.5 ng of the CMV-Renilla reference plasmid. Eight hours after transfection, cells were treated with thapsigargin (300 nM), calcimycin A23187 (7 μM) or tunicamycin (10 μg/ml). Luciferase activities were measured after 16 hrs. Fold induction was defined as the ratio of induced levels of activity compared to the level of activity in cells treated with DMSO alone. Each transfection experiment was repeated at least three times.

Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotides were labeled using γ-P^{32}-ATP (GE Healthcare Ltd Amersham, Buckinghamshire, UK). EMSA was performed as previously described [8, 15]. For super-shift analyses, 4 μg of antibody (*ATF6*, *YY1*; all from Santa Cruz Biotech, Santa Cruz, CA, USA) were incubated with nuclear extracts. Sequences of the oligonucleotides are given in Table S1 as supplemental material.

Chromatin immunoprecipitation (ChIP) assay

The ChIP-IT kit (Active Motif, Rixensart, Belgium) was used according to the manufacturer's instructions. Isolated chromatin of K562 cells was incubated with antibodies against *NFY-A*, *YY1* or *ATF6*, all from Santa Cruz Biotechnology. PCR primers for calreticulin were: (s) 5'-CCTGGCCTGA GGTGCTCG-3'; and (as): 5'-AGGCTGCGCCTCGGAACG-3'; intron 7 (s): 5'-GTGCAGA GAACTGAAGTCAG-3' and intron 7 (as): 5'-ACCTGGGTGACA-GAGCAAG-3'.

Western blot analyses

The following antibodies were used against calreticulin (sc-6467), GRP78 (sc-13968), YY1 (sc-7341X), XBP1 (sc-7160X), CEBPA (sc-61) and SP1

(sc-59X) all from Santa Cruz, against ATF6 (IGENEX, IMG-273), actin (Chemicon, MAB1501) and the haemagglutinin epitope (HA) (Covance, MMS-101P).

UV cross-link assay for assessment of calreticulin mRNA-binding activity

A double-stranded RNA oligomer encoding a calreticulin binding site within the *CEBPA* mRNA was generated by annealing oligomers A: 5'-CCC-CACGGGCGGCGGCGGCGGCGGCGACUU-3' containing CGG repeats and B: 5'-UAACCAGCCGCCGCCGCCGCCGCCGCCGC CC-3' containing CCG repeats. The UV cross-link assay was previously described [15, 34].

Results

The *XBP1* spliced variant (*XBP1s*) is expressed in a subset of AML patients

The unfolded protein response (UPR) involves the cleavage of the *XBP1* mRNA generating a spliced form (*XBP1s*) [23]. Activated *IRE1* excises 26 nucleotides from the *XBP1* mRNA leading to a frame shift and thus to a novel potent transcription factor at a molecular weight of 54 kD. In order to assess the activation of the UPR in AML patients at diagnosis, we screened mRNA from mononucleated bone marrow samples of 92 patients of all subtypes. Using PCR primers spanning the critical 26 nucleotides of the *XBP1* cDNA, we determined the expression of the spliced and unspliced forms of *XBP1* mRNA (Fig. 1A). The threshold of the assay was determined by competitive PCR using constant amounts of *XBP1* wild-type plasmid combined with increasing amounts of the spliced form of *XBP1* (Fig. S1). The ratio observed with 30 ng of wild-type *XBP1* and 20 ng of the spliced form of *XBP1* was selected to separate AML patients with induced *versus* uninduced *XBP1s* mRNA. We found that the *XBP1s* mRNA variant was present (S+) in 16 of 92 patients (17.4%), whereas it was not detectable (S-) in mononuclear cells from bone marrow samples of 10 healthy individuals. Characteristics of S+ and S- patients are summarized in a supplemental Table S2. Bone marrow was available at remission from three patients with activated UPR in leukaemic cells at diagnosis. Consistently, induced *XBP1s* mRNA was undetectable at remission. In addition, selected CD34+ cells obtained at remission during stem cell collection in two patients, who had activated UPR at diagnosis, showed no evidence of *XBP1s* induction.

Relative expression levels of GRP78 and calreticulin are specifically induced in AML patients with activated UPR (S+)

To confirm activation of the UPR in AML patients with induced XBP1s, we determined mRNA expression levels of UPR target genes such as GRP78 and calreticulin. The mean ΔCt-value of patients diagnosed with FAB-subtype M0 served as reference (1-fold). In the group of S+ patients, median expression levels were increased for calreticulin (2.5-fold) and *GRP78* (2.3-fold) as compared to S- patients (Figs. 1B and C). The increased expression observed for these UPR target genes in S+ patients supports the concept that the UPR is activated in these AML patients. Remarkably, we found no difference in *CEBPA* mRNA levels between S(+) and S(-) patients (Fig. S2).

Induction of ER-stress transcriptionally activates calreticulin expression

Thapsigargin triggers the UPR by blocking the endoplasmatic Ca-ATPase leading to depletion of ER calcium stores. We treated adherent Hela and leukaemic K562 cells with 300 nM thapsigargin. We detected splicing of the *XBP1* mRNA after 2 hr of thapsigargin treatment in both cell lines (Fig. 2A). Thus, *XBP1* splicing is an early event during the UPR.

Similarly, increased calreticulin mRNA expression was detectable after 2 hrs of UPR inducing treatment in Hela cells (Fig. 2B). Results were similar for three different UPR inducing compounds (A23187, thapsigargin and tunicamycin). After 16 hrs, the mean increase of calreticulin mRNA was 2.5-fold (SD $\pm$ 1.1) in K562 cells (Fig. 2C) and 4.1-fold (SD $\pm$ 1.1) in Hela cells (Fig. 2B). The induction of calreticulin protein in K562 cells following UPR activation is depicted in Fig. 2D. Expression of additional UPR chaperons such as *GRP78*, *CHOP* and the nuclear form of *ATF6* was similarly induced. We thus conclude that thapsigargin treatment induces mediators of the UPR in myeloid leukaemic cells.

Two elements mediate transcriptional activation of the calreticulin promoter following activation of the UPR

Our results previously mentioned suggest that induction of the UPR transcriptionally activates calreticulin. In order to define the elements mediating this activation, myeloid U937 cells were transfected with a series of deletion constructs of the calreticulin promoter cloned into the pGL3b-luciferase plasmid, and subsequently treated with 300 nM thapsigargin for 16 hrs, starting 8 hrs after transfection (Fig. 3A). We observed a two-step activation of the calreticulin promoter: A 2-fold increase of activity was measured for the –205 nt construct, and an additional increase was observed with the –298 nt construct, whereas larger constructs failed to mediate additional activity.

This region of the calreticulin promoter harbours two putative ERSE designated as ERSE1 and ERSE2 (Fig. 3B). ERSE1 has an inverse orientation of the ERSE consensus sequence CCAAT-N9-CCACG, whereas ERSE2 differs in the last 3' two nucleotides from the consensus sequence. We transfected U937 cells with specific mutation constructs of ERSE1, ERSE2 or both, again with subsequent

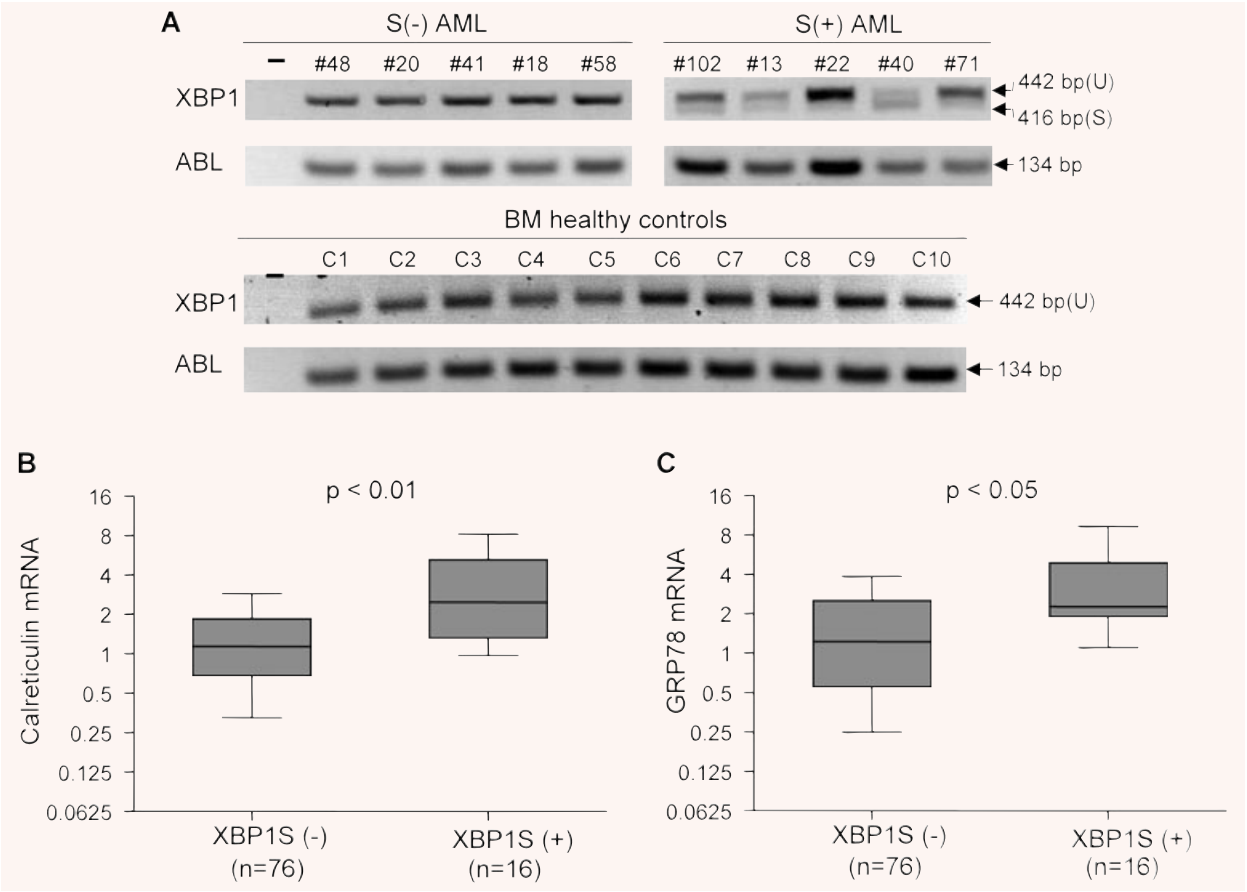

Fig. 1 The unfolded protein response (UPR) is activated in a subset of AML patients. (**A**) Representative examples of AML patients are depicted analyzed by semi-quantitative RT-PCR detecting only the unspliced (U) form of *XBP1* mRNA (S-; left upper panel) or both the spliced (S) and unspliced form in S+ AML patients (right upper panel). The expression of the *ABL1* gene is presented as a control. (**B** and **C**) Expression levels of the UPR target genes calreticulin (mean ± SD, 3.95 ± 3.6 *versus* 1.75 ± 1.9; $P = 0.0018$) and *GRP78* (mean ± SD, 3.33 ± 2.52 *versus* 2.03 ± 2.24; $P = 0.0224$) are increased in *XBP1S* (+) AML patients. Box plots of calreticulin and *GRP78* mRNA expression in AML patients are presented. N-fold expression levels are depicted in log 2 scale. Boxes represent interquartile ranges, horizontal bars represent medians and thin bars represent the 10^{th} and 90^{th} percentiles.

treatment with 300 nM thapsigargin. For ERSE1, the mutations were cloned into the –298 nt construct (Fig. 3C), and for ERSE2 into the –205 nt construct (Fig. 3D). We found that activation following ER-stress was completely abolished when the CAAT motifs of both ERSE1 and ERSE2 were mutated (construct M4 in Fig. 3C). Specific mutation of a putative *ATF6* binding site in ERSE1 (M2), as well as mutation of the CAAT motif (M1) reduced the activation potential to that seen with the wild-type ERSE2 alone (–205 nt construct). Mutation of the putative *YY1*-binding site in the ERSE1 motif (M3) had no effect on activation.

The ERSE2 motif encodes no obvious *ATF6* binding site. Mutation of the CAAT motif (M5) or the putative binding site of *YY1* (M6) completely abolished the activation potential of the ERSE2 motif (Fig. 3D). Reduction of the 9 nucleotides spacer sequence between the CAAT motif and the YY1-binding site to 7 nucleotides (M8) also inactivated the promoter construct following induction of ER-stress. In contrast, exchanges of two nucleotides *per se* within the spacer sequence (M7) had no effect. In summary, both ERSE sequences appear to be essential for transcriptional activation of calreticulin following UPR induction.

ATF6n activates the calreticulin promoter through both ERSE1 and ERSE2

The nuclear form of *ATF6* orchestrates the induction of target genes during the UPR [35]. The presence of a putative *ATF6*-binding site in ERSE1 suggested *ATF6n* to be involved in calreticulin

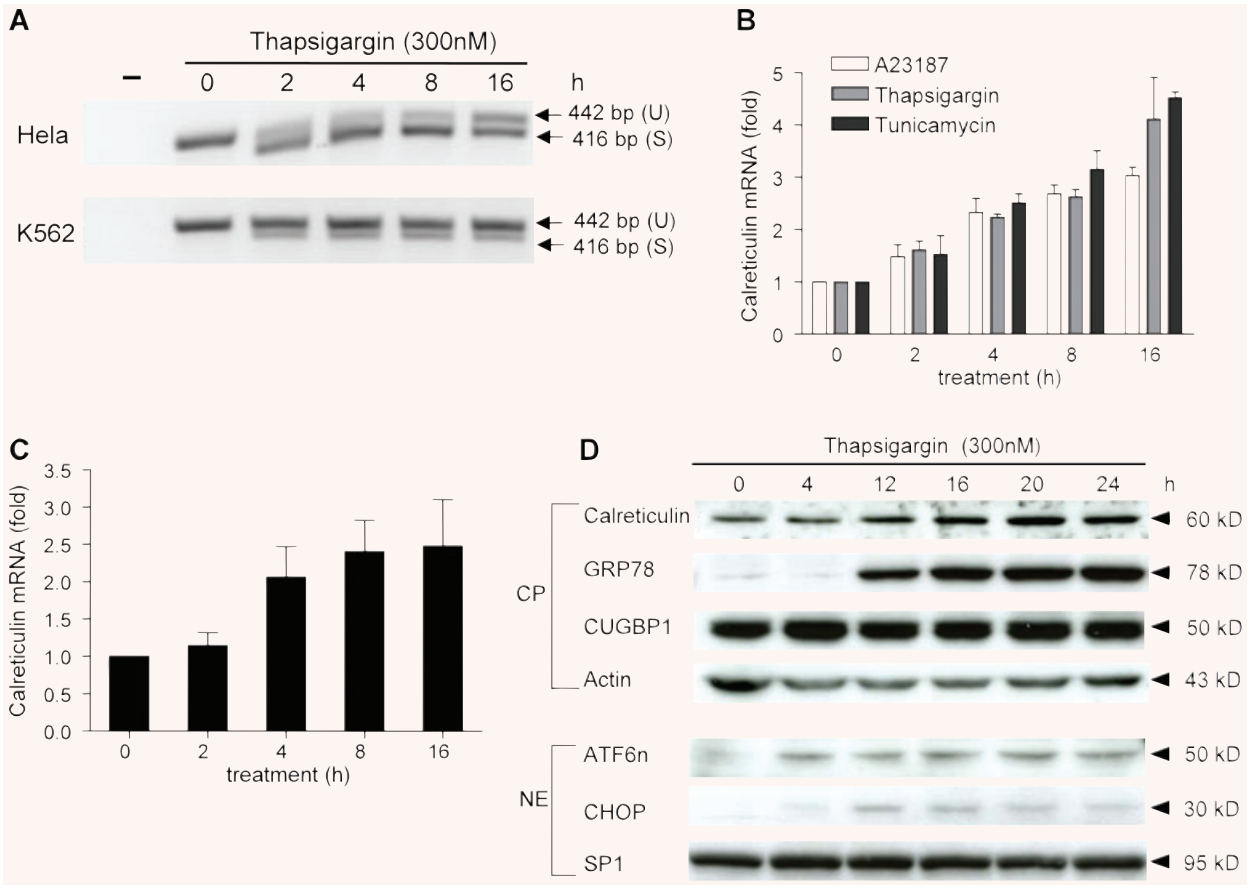

Fig. 2 ER-stress induces *XBP1* mRNA splicing and calreticulin expression. (**A**) Adherent Hela and leukaemic K562 cells were treated with thapsigargin (300nM). The spliced (S) and unspliced (U) form of *XBP1* mRNA were detected by RT-PCR. (**B**) Calreticulin mRNA was induced in Hela cells after treatment with the ER-stress inducing agents A23187 at 7 μM, thapsigargin 300 nM or tunicamycin 5 μg/ml. Relative expression levels of calreticulin were measured by quantitative RT-PCR and normalized to *7s* RNA. (**C**) Treatment of K562 cells with thapsigargin (300nM) increased expression of calreticulin mRNA. Each experiment was repeated at least three times. Mean values and standard deviation (SD, error bars) are depicted. (**D**) Western blot analyses for calreticulin, GRP78, CHOP, the nuclear form of ATF6 (ATF6n) and CUGBP1 (as negative control) is shown using cytoplasmic (CP, upper panel) and nuclear (NE, lower panel) protein fractions of K562 cells treated with 300 nM thapsigargin. Actin and Sp1 protein served as loading control.

activation. We transfected various constructs of the calreticulin promoter together with a plasmid encoding human *ATF6n* into U937 cells (Fig. 3E, F, G). We found that *ATF6n* activated the –298 nt construct up to 6.2-fold. *ATF6n*-mediated activation again showed a two-step pattern (Fig. 3E). Specific mutation of the CAAT motifs in both ERSE1 and ERSE2 (M4) completely abolished *ATF6n*-mediated activation (Fig. 3F). Mutation of the putative binding site for *ATF6* in ERSE1 (M2) reduced the activating potential to that observed for ERSE2 alone. Mutation of the putative *YY1*-binding site in ERSE1 (M3), again, had no effect on *ATF6n*-mediated activation. In contrast, mutation of the *YY1* site in ERSE2 (M6) abolished *ATF6n*-mediated activation of ERSE 2 (Fig. 3G). *XBP1s* and *TFII-I*, transcription factors reported to activate promoters through ERSE motifs, failed to affect *ATF6n*-mediated activation (data not shown). These results indicate that the two ERSE sequences of the calreticulin promoter are targeted by *ATF6*, by at least one CAAT binding protein, and (at least for ERSE2) by YY1.

YY1 is required for *ATF6* binding to ERSE 2, but not to ERSE 1 of the calreticulin promoter

We performed EMSA in H1299 cells to identify the factors binding to the two ERSE motifs. We observed two binding activities (complex 1 and 2) in each motif (Fig. 4A). Analyses with specific antibodies identified complex 1 as *YY1* (lanes 7 and 16 in Fig. 4A), and complex 2 as *NFYA*, the DNA-binding subunit of the tripartite transcription factor *NFY* (lanes 3 and 12). Specific mutations of

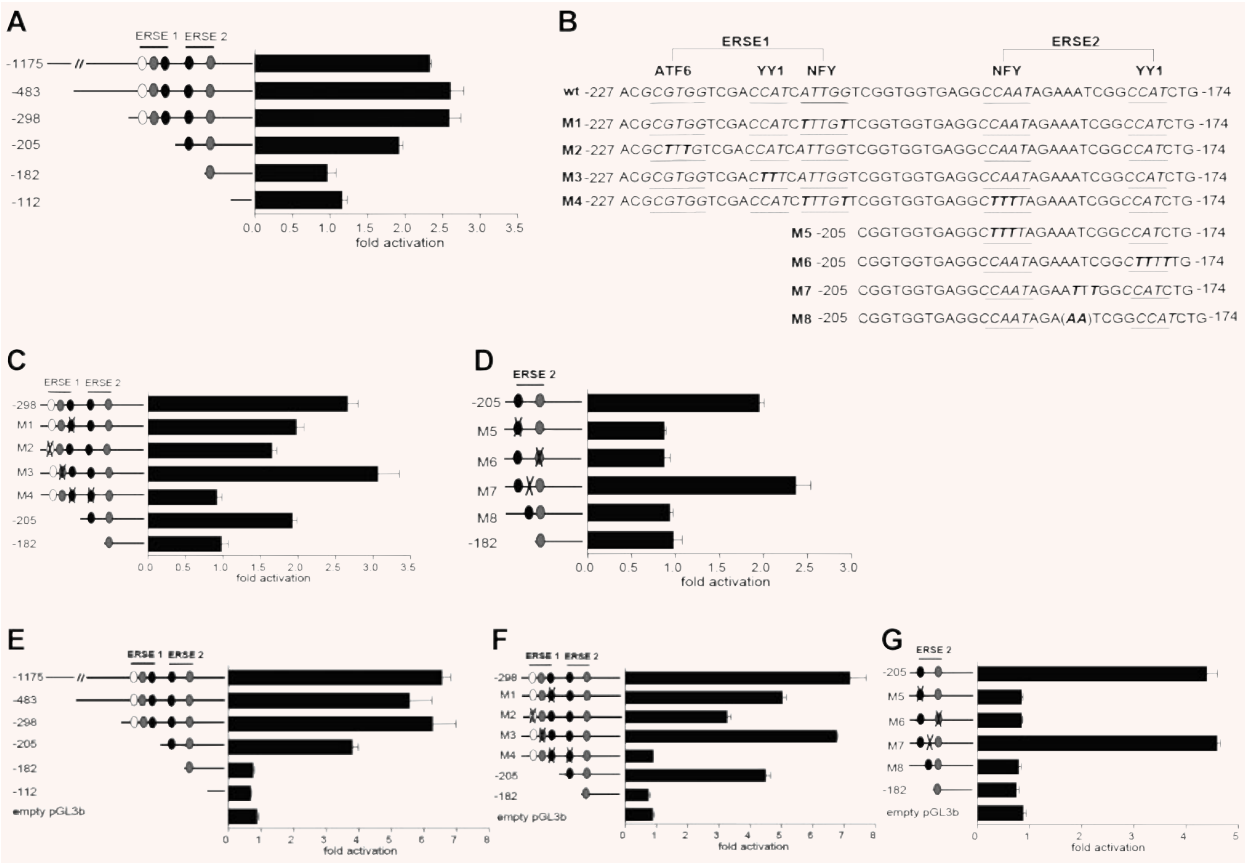

Fig. 3 Two ERSE mediate induction of calreticulin by ATF6 following ER-stress. (A, C and D) Myeloid U937 cells were treated with thapsigargin (300 nM) starting 8 hrs after transfection. After 16 hrs, cells were harvested. Each transfection experiment was repeated at least three times. (**A**) Using promoter deletion constructs, two ERSE motifs (ERSE1 and ERSE2) were identified. Fold induction was the ratio of activity compared to cells treated with DMSO (carrier) alone. Mean values and SD (error bars) are depicted. (**B**) Schematic presentation of both ERSE motifs (ERSE1 and ERSE2). M1 to M8 encode mutations within a fragment of the calreticulin promoter cloned into the pGl3 luciferase vector. (**C**) Mutations of the binding sites of *NFY* (M1) and *ATF6n* (M2), but not *YY1* (M3) within ERSE1 reduced activation of the calreticulin promoter following ER-stress. M4 indicates mutations of both *NFY* sites. (**D**) Mutations of the binding sites for *NFY* (M5), *YY1* (M6) or deletion of 2 bp between the two sites (M8) decreased the activation of the ERSE2 motif. (E-G) U937 cells were transfected with pCMV-ATF6–373 (*ATF6n)* or pCMV empty vector. (**E**) Calreticulin promoter deletion constructs transfected together with *ATF6n* led to the identification of two *ATF6n* responsive elements. (**F**) Mutations of the binding sites of *NFY* (M1) or *ATF6n* (M2), but not of *YY1* (M3) affected the activation of ERSE1 by *ATF6*. (**G**) Mutating the binding sites of *NFY* (M5) or *YY1* (M6), as well as a 2 bp deletion between these sites (M8) led to the complete loss of ERSE2 activation by *ATF6*. The exchange of 2 bp between these sites (M7) had no effect.

the CAAT-motif (M1 in ERSE 1 and M5 in ERSE 2) abolished *NFYA* binding, and no binding activity of complex 2 was observed with mutations of the *YY1*-binding site (M3 in ERSE 1 and M6 in ERSE 2). These results suggest that both NFYA and YY1 are directly binding to both ERSE sequences.

The demonstration of *ATF6* binding by EMSA is technically challenging [30, 35]. Using nuclear extracts from H1299 cells transfected with the expression plasmid encoding *ATF6n*, a third complex was detectable binding to both ERSE motifs of the calreticulin promoter (Figs. 4B and C). The addition of a specific antibody against ATF6 shifted complex 3 in ERSE 1 (lane 4 in Fig. 4B), and abolished binding of complex 3 to ERSE 2 (lane 4 in Fig. 4C). *ATF6n* binding to ERSE 1 was undetectable when the CAAT-motif (M1) or the putative *ATF6* binding site (M2) were mutated. Interestingly, mutating the *YY1* binding site (M3) had no effect on *ATF6n* binding. Binding of *ATF6n* to ERSE 2 was abolished, when the CAAT-motif (M5) or the *YY1* binding site (M6) were mutated. Reducing the nine nucleotides spacer sequence of the tripartite ERSE motif to 7 nucleotides (M8) also prevented *ATF6n* binding. Mutation of two nucleotides within the spacer region disrupting *YY1* binding (M7) also decreased *ATF6n* binding. We thus conclude that *NFYA* (and also *YY1* for ERSE2) are essential for *ATF6n* binding to the two ERSE sequences in the calreticulin promoter.

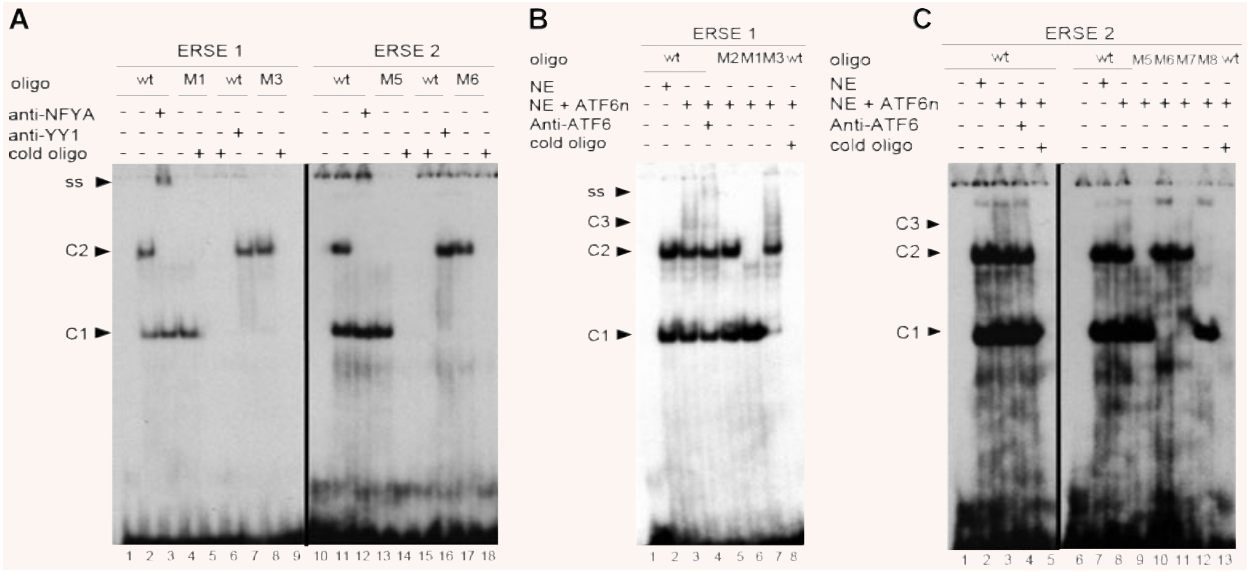

Fig. 4 *NFY*, *YY1* and *ATF6n* are binding to ERSE1 and ERSE2. The mutant oligonucleotides (M1–M8) are corresponding to the promoter constructs in Figure 3B. (**A**) In nuclear extracts from H1299 cells, two complexes (C1–2) were detectable by EMSA. In M1 and M5, the binding sites for *NFY* are mutated. M3 and M6 indicate mutations of the *YY1*-binding sites. 'cold' = unlabeled oligo. (**B**) Nuclear extracts (NE) from H1299 cells were transfected with *ATF6n*. Three complexes (C1–3) binding to ERSE1 were detectable. M2 indicates mutation of the *ATF6* binding site. The *NFY* site is mutated in M1, and *YY1* binding is blocked by mutant M3. (**C**) Incubating NE with an oligonucleotide encoding ERSE2 led to the formation of three complexes (C1–3). The addition of *ATF6* antibody abolished complex 3. Mutations of the *NFY* (M5) or the *YY1* (M6) binding site or a 2 bp deletion between the *NFY* and *YY1* binding sites (M8) also abrogated complex 3. M7 indicates the mutation of 2 bp within the *NFY* and *YY1* binding site (M7).

NFYA, YY1 and *ATF6* binding to the calreticulin promoter is activated after induction of ER-stress

In order to assess changes in transcription factor binding to the ERSE sequences following induction of ER-stress, we performed chromatin immunoprecipitation experiments (Fig. 5). Again, K562 cells were treated with 300 nM thapsigargin. We observed that binding of *NFY* and *YY1* to the ERSE sequences of the calreticulin promoter was detectable in untreated cells, whereas *ATF6* binding was not present. After activation of ER-stress, *NFY* and *YY1* binding was induced, and *ATF6* became detectable. We conclude that binding to the calreticulin promoter of all three factors is induced following ER-stress.

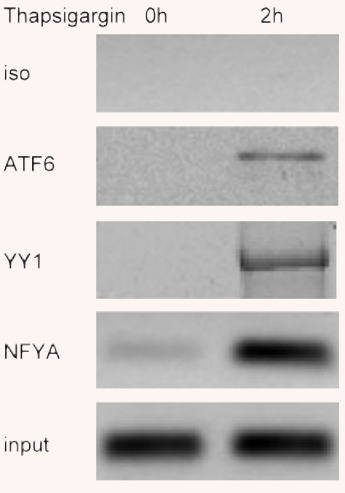

Fig. 5 *ATF6* together with *NFY* and *YY1* are recruited to the calreticulin promoter under ER-stress. K562 cells were treated with 300 nM thapsigargin for 2 hrs and immune-precipitation was performed using antibodies against *ATF6*, *YY1*, *NFYA* or an isotype control (iso). PCR primers were spanning the two ERSE motifs of the calreticulin promoter.

Conditional expression of calreticulin in myeloid cells suppresses *CEBPA* translation

We have reported that calreticulin *in vitro* leads to a translational block of the myeloid key transcription factor *CEBPA* through enhanced binding to a stem loop structure within the *CEBPA* mRNA [15]. To assess the effect of calreticulin activation on CEBPA protein levels following ER-stress, we established a cell line model conditionally expressing HA-tagged calreticulin from a

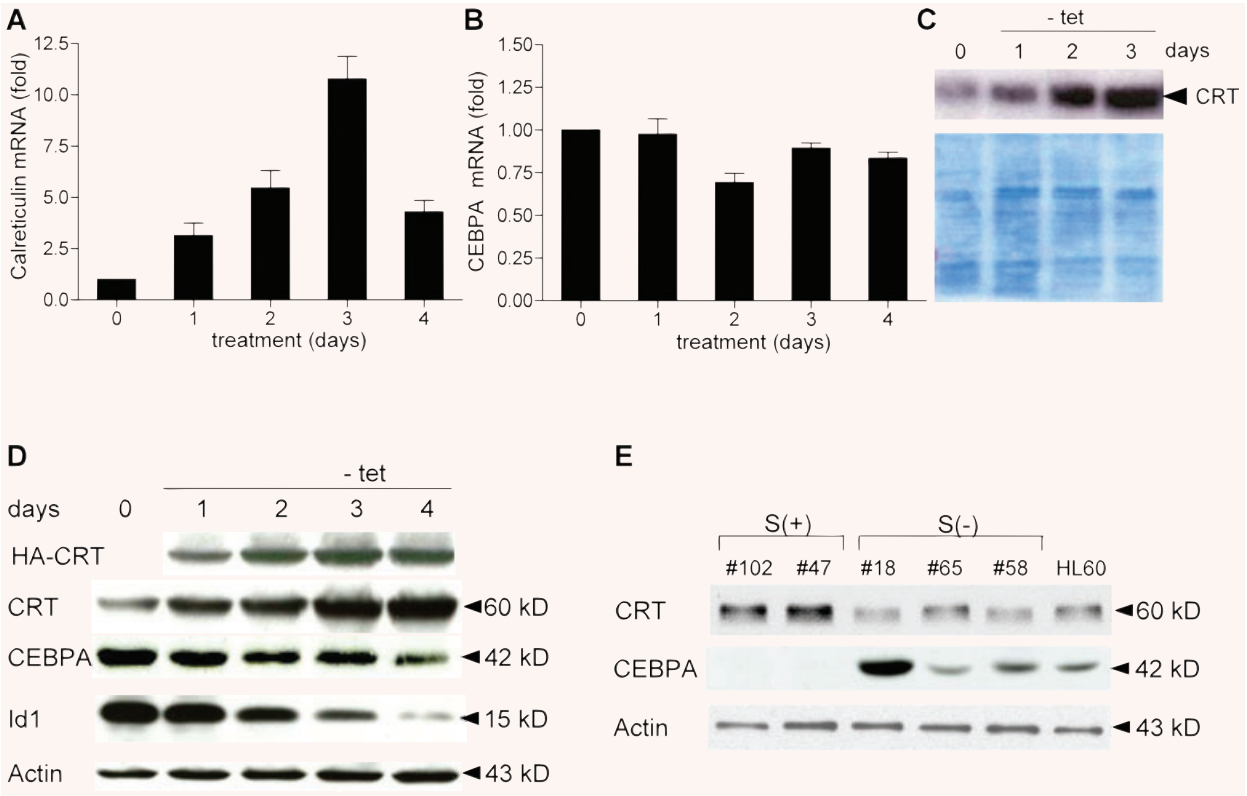

Fig. 6 Calreticulin expression suppresses CEBPA protein in a conditional cell line model and in *XBP1S* (+) patients. U937 cells were analyzed after withdrawal of tetracycline and thus induction of calreticulin. RT-PCR analyses for calreticulin (**A**) and *CEBPA* mRNA (**B**) expression were normalized to *7s* RNA and expressed as *n*-fold up/down-regulation compared to day 0. Results of three independent experiments are demonstrated with mean values and standard deviations (SD). (**C**) UV cross-linking analysis visualized the induction of the interaction between calreticulin protein and the *CEBPA* mRNA following withdrawal of tetracycline in U937 cells. (**D**) Conditional calreticulin expression in U937 cells suppressed CEBPA protein and its target gene *Id1* by Western blot analyses. A haemagglutin (HA) antibody identified induced HA-tagged calreticulin protein. (**E**) Representative Western blot analyses are presented for *CEBPA* and calreticulin in leukaemic cells from patients expressing *XBP1S* mRNA (*XBP1S* (+)) or from *XBP1S* (-) patients. HL60 cells served as positive control.

tetracycline-responsive promoter in the myeloid U937 cell line. Twenty-four single cell clones were tested, and clone #8 was selected. All experiments mentioned subsequently were repeated with at least one different clone. We found a 10.8-fold increase of calreticulin mRNA after 3 days of tetracycline withdrawal (Fig. 6A). In contrast, *CEBPA* mRNA remained unchanged (Fig. 6B). Induction of calreticulin expression strongly increased binding of calreticulin protein to a ribooligonucleotide probe encoding the stem loop sequences of the *CEBPA* mRNA in a UV-cross link assay (Fig. 6C). Increased binding of calreticulin protein to *CEBPA* mRNA induced a decrease of CEBPA protein to below 30% (Fig. 6D). Finally, AML patients with activated UPR (S+) and thus induced calreticulin expression had hardly detectable CEBPA protein expression as compared to S- patients which expressed higher levels of CEBPA and lower calreticulin protein (Fig. 6E). These data indicate that increased expression of calreticulin in leukaemic cells blocks translation of *CEBPA* mRNA.

Discussion

In this report, we identified activation of the unfolded protein response (UPR) in a subgroup of AML patients. Our observation is based on the detection of the spliced variant of *XBP1* mRNA in 16 out of 92 AML patients. *XBP1s* is produced after activation of the *IRE1* protein through sensing of unfolded proteins in the lumen of the ER [23, 36, 37], and the detection of *XBP1s* was identified as a sensitive marker for activated UPR in AML patients.

We found that patients with activated UPR also expressed higher levels of calreticulin and *GRP78*. Induction of these ER chaperones is pivotal for cell survival by re-establishing correct folding of proteins and preventing their aggregation. *GRP78* expression is up-regulated in a variety of human tumours including breast, lung, liver and prostate cancer, whereas its expression in haematological malignancies has not been investigated so far.

The conditions activating the UPR in leukaemic cells remain to be elucidated. Cancer cells in general are susceptible to ER-stress because of intrinsic or extrinsic factors. In solid tumours, an environment is often created characterised by glucose deprivation, acidosis and hypoxia leading to the accumulation of underglycosylated and misfolded proteins in the ER, which triggers the UPR [38]. In contrast, intrinsic factors initiating the UPR might be more relevant in AML cells. Candidate events are mutations in secretory proteins, which lead to their misfolding as reported in patients with severe congenital neutropenia [39].

We confirmed calreticulin as a key target of the UPR. Beside its role in proper folding of glycoproteins in the ER [40], calreticulin was previously identified as an RNA-binding protein thereby blocking the translation of specific target mRNAs such as *CEBPA*, *CEBPB* and *CDKN1A* [34, 41]. In fact, we have previously found that calreticulin is activated in certain subtypes of AML with consecutive suppression of CEBPA protein [15, 16]. Consistent with this model, we were able to demonstrate the translational block of CEBPA protein through increased calreticulin expression in a conditional cell line model. Given the importance of suppressed CEBPA function for leukemogenesis [2, 4–16], we thus conclude that activation of the UPR with induction of calreticulin and thus CEBPA suppression may represent a significant event in AML with activated UPR.

The increased expression of calreticulin under ER-stress is mediated through the *ATF6* pathway of the UPR. Two cis-acting ERSE in the proximal calreticulin promoter are crucial for promoter activation following ER-stress. *NFYA* binding to both ERSE motifs was essential for *ATF6n*-mediated activation of the calreticulin promoter. Interestingly, *ATF6n* bound directly to ERSE1 independent of *YY1*, whereas ERSE 2 has no direct binding site for *ATF6n*. An intact YY1 binding site was identified to be necessary for *ATF6n* binding to ERSE 2. *YY1*, *NFYA* and *ATF6* binding to the calreticulin promoter is increased after induction of ER-stress in ChIP experiments. This is consistent with a report on activation of the *GRP78* promoter under ER-stress conditions, where *ATF6n* is recruited to the ER-stress response element via interaction with *YY1* [30].

Misfolded proteins in the ER are usually translocated to the cytoplasm, ubiquitinated and targeted to proteasomal degradation. However, accumulation of proteins in the cytoplasm blocks the efflux of unfolded proteins from the ER and leads to an increase of misfolded proteins in the ER lumen inevitably inducing the UPR and ultimately apoptosis of the cell. Our findings of activated UPR in malignant cells of AML patients might provide a basis for treatment strategies in AML. Multiple myeloma cell lines with activated UPR are especially prone to undergo apoptosis after treatment with the proteasome inhibitor bortezomib [42]. Also, high expression of calreticulin in patients with light chain amyloidosis predicts responsiveness to treatment with the alkylating agent melphalan [43]. Moreover, toxicity of melphalan appears to be increased *in vitro* in cells pretreated with ER-stress inducing agents [44]. These reports suggest increased responsiveness to chemotherapy in malignant cells with activated UPR. In particular, this study might provide a rational for the use of bortezomib alone or together with standard regimens in AML patients presenting with an activated UPR in the leukaemic cells at diagnosis.

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Disclosures

All authors declare that they have no competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The sensitivity of the PCR assay was determined using pcDNA3 plasmids encoding wild-type human *XBP1* (U) or the spliced form of *XBP1 (S).* A competitive PCR is depicted using constant amounts of wild-type *XBP1* plasmid combined with increasing amounts of the spliced form of *XBP1*. The ratio observed with 30 ng of wild-type *XBP1* plasmid and 20 ng of the spliced form *of XBP1* was determined to separate AML patients with induced (S) *versus* uninduced (U) *XBPls* mRNA.
Fig. S2 No difference was observed by RT-PCR of *CEBPA* mRNA expression between UPR positive AML patients and UPR negative AML patients (median 1.4 *versus* 1.9; $P = 0.27$). The median of AML-MO patients served as a reference (1-fold). Boxes represent interquartile ranges, horizontal bars represent medians and thin bars represent the 10^{th} and 90^{th} percentiles.
Table S1 Sequences of the oligonucleotides used for EMSA.
Table S2 Clinical characteristics of patients

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