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CITED2 affects leukemic cell survival by interfering with p53 activation

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CITED2 (CBP/p300-interacting-transactivator-with-an-ED-rich-tail 2) is a regulator of the acetyltransferase CBP/p300 and elevated CITED2 levels are shown in a number of acute myeloid leukemia (AML). To study the in vivo role of CITED2 in AML maintenance, AML cells were transduced with a lentiviral construct for RNAi-mediated knockdown of CITED2. Mice transplanted with CITED2-knockdown AML cells (n = 4) had a significantly longer survival compared to mice transplanted with control AML cells (P < 0.02). In vitro, the reduction of CITED2 resulted in increased p53-mediated apoptosis and CDKN1A expression, whereas BCL2 levels were reduced. The activation of p53 upon CITED2 knockdown is not a direct consequence of increased CBP/p300 activity towards p53, since no increased formation of CBP/p300/p53 complexes was demonstrated and inhibition of CBP/p300 activity could not rescue the phenotype of CITED2-deficient cells. Instead, loss of CITED2 had an inhibitory effect on the AKT-signaling pathway, which was indicated by decreased levels of phosphorylated AKT and altered expression of the AKT-pathway regulators PHLDA3 and SOX4. Notably, simultaneous upregulation of BCL2 or downregulation of the p53-target gene PHLDA3 rescued the apoptotic phenotype in CITED2-knockdown cells. Furthermore, knockdown of CITED2 led to a decreased interaction of p53 with its inhibitor MDM2, which results in increased amounts of total p53 protein. In summary, our data indicate that CITED2 functions in pathways regulating p53 activity and therefore represents an interesting target for AML therapy, since de novo AML cases are characterized by an inactivation of the p53 pathway or deregulation of apoptosis-related genes.

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Acute myeloid leukemia (AML) is a genetically heterogeneous disease that is characterized by an accumulation of immature myeloblasts in the bone marrow. Despite the variety in the mutational background, the transcriptional regulator CITED2 (CBP/p300-interacting-transactivator-with-an-ED-rich-tail 2) is found to be upregulated in the majority of AML cases.1 As demonstrated by conditional knockout studies, CITED2 is essential for the maintenance of adult hematopoietic stem cells during normal haematoipoiesis, whereas it is dispensable in more committed cells.2 Notably, recent data strengthened the hypothesis that CITED2 has also critical functions in maintaining human leukemic cells, since knockdown of CITED2 in AML cells inhibited AML engraftment in vivo, when competed with control cells.1 These findings suggest that CITED2 might have an important role in the survival of the leukemic stem cell (LSCs) – those AML cells that fail to be targeted sufficiently and most likely cause disease relapse.

CITED2 was originally identified and characterized as a protein binding to CH1 domain of the acetyltransferase CBP/p300.3,4 Acetylation of histones and non-histone proteins is crucial for eukaryotic cells to regulate transcription. By binding to CBP/p300, CITED2 was shown to regulate the CBP/p300-mediated transcription of multiple transcription factors such as HIF1α,5 NF-κB,6 LHX2,5 TFAP2,7 MYC,8 PPARα9 and SMAD2.10 Presence of CITED2 can enhance or repress the target gene expression of these transcription factors. In case of HIF1α and NF-κB, CITED2 has been described to compete for p300 and thereby block HIF1α- and NF-κB-mediated transcription.3,5 For most other transcription factors, CITED2 acts as a co-activator of transcription by recruiting CBP/p300 to the transcription factor target sites. Whereas increased expression of CITED2 has been described to facilitate MYC- and PPARα-mediated cell-cycle progression,8 low expression of CITED2 is associated with an increase of cell death, for example by impaired SMAD2- and TFAP2 signaling.10 Interestingly, loss of CITED2 expression has been reported to lead to increased activation of the tumor suppressor protein p53,12 which is acetylated and co-activated by CBP/p300. Wu et al.12 suggested increased formation of p53-CBP/p300 complexes in the absence of CITED2. However, other studies reported decreased p300 function in CITED2-deficient cells,13 and therefore the role of CITED2 in p53 signaling is still unclear.

Here we report that loss of CITED2 severely impairs AML cell survival by induction of p53-mediated apoptosis. Interestingly, loss of CITED2 activates the p53 pathway independent of CBP/p300-mediated acetylation of p53. Instead, reduction of CITED2 levels impairs cell survival by interfering with the AKT signal transduction pathway.

Results

Knockdown of CITED2 in CD34+ AML cells impairs leukemia development. In order to demonstrate that CITED2 truly impacts the survival and maintenance of

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leukemia initiating cells (LiCs, or LSCs), AML cells were transduced with a lentiviral GFP-vector expressing a short hairpin targeting CITED2 (shCITED2) or a control vector. The hairpin was selected based on previous data demonstrating that various shCITED2-constructs resulted in similar phenotypes. After transduction, GFP-positive cells were sorted and subsequently transplanted into NSG mice (n = 4) (Figure 1a). Mice which only received sorted shCITED2-transduced leukemic cells have a significantly longer survival as compared to mice that only received control-transduced leukemic cells (138 versus 256 days, P < 0.02, Figure 1b). Eventually, engraftment of human AML cells and leukemia development was observed in both control- and shCITED2 mice (Supplementary Figure S1); however, the progression in shCITED2 mice was delayed (Figure 1c). It appeared that the GFP-positive shCITED2-AML cells that eventually did engraft did not show a reduction of CITED2 levels and therefore most likely have escaped sufficient knockdown of CITED2 (Figure 1d), indicating that CITED2 knockdown impacts the survival and maintenance of leukemia initiating cells.

**Loss of CITED2 triggers apoptosis in leukemic cells.** Similar suppressive effects of CITED2 reduction were observed in the leukemic cell lines NB4 and MOLM-13 (Figure 2a, Supplementary Figure S2A). To gain a first insight into the cause of reduced cell expansion, the effects of CITED2 knockdown on apoptosis and cell-cycle distribution were analyzed in NB4 or MOLM-13 cells. A strong increase of Annexin V<sup>+</sup> cells was observed in shCITED2 cells compared to control cells (Figure 2b, Supplementary Figure S2B), whereas no significant changes in cell-cycle distribution could be demonstrated (Figure 2c, Supplementary Figure S2C).
shCITED2-mediated cell death is triggered by a p53-dependent pathway. Previously, microarray analysis of shCITED2-transduced versus control-transduced CD34+ cord blood cells was performed to identify the molecular pathways that are particularly dependent on CITED2 levels (GSE47218). Further analysis of this data set revealed a significant enrichment of p53 target genes among the upregulated genes after CITED2 knockdown (Figure 3a). Gene expression analyses by Q-PCR in shCITED2-transduced NB4 and MOLM-13 cells confirmed an upregulation of several p53 targets such as CDKN1A, BTG2, ABHD4 and PHLDA3, whereas the p53-regulated anti-apoptotic protein BCL2 was strongly down regulated (Figure 3b, Supplementary Figure S3A). Simultaneous knockdown of p53 and CITED2 rescued the increased apoptosis levels in NB4 and MOLM-13 cells (Figure 3c and d, Supplementary Figure S3A). Simultaneous knockdown of p53 and CITED2 rescued the increased apoptosis levels in NB4 and MOLM-13 cells (Figure 3c and d, Supplementary Figure S3A). Importantly, decreasing CITED2 levels had no impact on apoptosis levels of leukemic cell lines that are devoid of p53, such as K562 (Figure 3d, right panel).

Activation of p53 signaling in CITED2 knockdown cells is not due to direct CBP/p300-mediated p53 acetylation. As activated p53 signaling turned out to be a crucial factor in the shCITED2-mediated cell death and CITED2 has been described to co-regulate binding of CBP/p300 to its targets, we questioned whether CBP/p300-mediated acetylation on C-terminal lysine 382 of p5314 is involved. In shCITED2-transduced NB4 cells, increased acetylation of p53 on the C-terminal lysine 382 was observed compared to control cells (Figure 4a). Next, it was investigated whether CITED2 directly interferes with p53 acetylation by either (i) recruiting deacetylases (HDACs, SIRTs) to the p300/p53 complex, or (ii) by blocking the binding of CBP/p300 to p53. By performing co-immunoprecipitation (IP) assays with lysates from transduced NB4 cells, binding of CBP/p300 to CITED2 as well as binding of CBP/p300 to p53 was observed (Supplementary Figure S2).
Figure S4A), but no evidence was obtained for a CBP/p300/CITED2/p53 interaction (Figure 4b). In addition, no interaction of CITED2 with HDAC1, HDAC2, or the acetyltransferase P/CAF could be demonstrated (Figure 4b, Supplementary Figure S4B). The knockdown of CITED2 did not lead to an increased binding of p53 to CBP or p300 (Supplementary Figure S4C). Finally it was shown that treatment of leukemic cells with the CBP/p300 inhibitor C646 for 24 h did not rescue
the shCITED2-mediated effects on p53 acetylation, or PHLDA3- and BCL2 expression (Figure 4c). These results suggest that the effects of shCITED2 are independent of direct CBP/p300-mediated p53 activation. This was confirmed by simultaneous transduction of shCITED2 cells with short hairpins against p300, CBP or both, which did not affect levels of p53 acetylation (Figure 4d). Furthermore, knockdown of CITED2 in CBP/p300-deficient cells still led to an approximately twofold increase in apoptosis (Figure 4e). These data propose that p53 activation in shCITED2 cells is not a direct consequence of interfering with its acetylation state. This hypothesis was further supported by the fact that
interference with other acetyltransferases that target p53 (P/CAM,15 GCN5,16 TRRAP,17 TIP6018,19 or MOZ20) did not rescue the phenotype of CITED2-low cells. There was no increase in GCN5 binding to ac-p53 in NB4 cells with CITED2 knockdown (Supplementary Figure S4D) and treatment of shCITED2-transduced MOLM-13 cells with the TIP60/MOZ inhibitor MG-149 could not rescue the apoptotic phenotype of cells upon CITED2 knockdown (Supplementary Figure S4E).
CITED2 knockdown mediates a p53/PHLDA3/BCL2-dependent decrease in AKT signaling. As depicted in Figure 5a, knockdown of CITED2 also leads to an increased amount of total p53 protein. The ubiquitin ligase MDM2 has a central role in regulating p53 stability by binding to p53 and thereby either directly inhibiting its transcriptional activity or promoting proteasome-mediated degradation of p53 (reviewed in Kruse and Gu21 and Manfredi22). By performing IP in MOLM13 cells, it was demonstrated that knockdown of CITED2 results in decreased interaction between MDM2 and p53 (Figure 5a). In line with these data, shCITED2-transduced cells had an increased sensitivity to treatment with Nutlin3a, an inhibitor of the MDM2/p53 interaction (Figure 5b). The pro-survival AKT signaling pathway23,24 is promoting p53-inhibiting functions of MDM2 (Figure 5c). Interestingly, knockdown of CITED2 resulted in strong increase in mRNA expression of PHLDA3 (Figure 3b), a known repressor of AKT signaling.25 PHLDA3 interferes with binding of AKT to second messenger molecules at the cell membrane and thus prevents AKT phosphorylation and activation (Figure 5c). In line with these data, decreased AKT phosphorylation, and thus decreased AKT activation was demonstrated that reduction of CITED2 levels results in a strong increase in mRNA expression of PHLDA3 (Figure 3b), a known repressor of AKT signaling.25 PHLDA3 interferes with binding of AKT to second messenger molecules at the cell membrane and thus prevents AKT phosphorylation and activation (Figure 5c). In line with these data, decreased AKT phosphorylation, and thus decreased AKT activation was observed in CITED2-knockdown cells by western blotting (Figure 5d). Besides upregulation of PHLDA3, upregulation of the AKT-inhibitor TXNIP26 and downregulation of the AKT-activator SOX427 was observed (Figure 5e). Interestingly, simultaneous knockdown of p53 rescued the shCITED2-mediated increase in PHLDA3 expression, whereas shCITED2-mediated alteration of BCL2, SOX4 and TXNIP was found to be p53-independent (Figure 5e). To further investigate the involvement of the AKT-signaling pathway in the shCITED2-mediated phenotype, transduced shCITED2 cells were simultaneously transduced with a short hairpin against PHLDA3 or a lentiviral construct overexpressing BCL2 (Supplementary Figure S5). Simultaneous knockdown of CITED2 and PHLDA3 partially rescued the shCITED2-mediated induction of cell apoptosis (Figure 5f upper panel), indicating that the increased expression of PHLDA3 contributed to the CITED2-mediated effects on cell survival. Strikingly, simultaneous upregulation of BCL2 in CITED2 knockdown cell could almost completely rescue the apoptotic phenotype (Figure 5f lower panel). These data demonstrate that both upregulation of PHLDA3 and downregulation of BCL2 are critical events in shCITED2-mediated apoptosis in AML cells.

Discussion

The results of our study demonstrate that reduction of CITED2 levels impairs the survival of leukemic cells, in part due to induction of p53-mediated apoptosis. Notably, AML cells are characterized by elevated CITED2-expression and the present study highlights the importance of CITED2 levels in regulating cell survival pathways. It appeared that the activation of the p53 pathway is not due to increased CBP/p530-mediated p53 acetylation, which can stabilize p53 protein expression. Although an overall increase of acetylated p53 in CITED2-deficient cells was detected, treatment of cells with the CBP/p530 inhibitor C646 as well as RNAi-mediated knockdown of CBP/P300 could not rescue the effect on p53-mediated apoptosis and target gene expression, indicating that p53 activation upon CITED2 knockdown is not due to increased activity of CBP/p530 towards p53. This is in contrast to a previous study12 suggesting an increased formation of CBP/p530/p53 complexes in CITED2-deficient cells. Also increased deacetylation by SIRT1 or HDAC1 and HDAC2 in CITED2-knockdown cells could not be demonstrated, suggesting that the detected increase in acetylated p53 is mainly a result of an increased amount of total p53 protein.

Importantly, reactivation of p53 function represents a relevant strategy for AML therapy, since approximately 90% of de novo AML cases do not show a mutation in the p53 gene,28 but are rather characterized by an inactivation of the p53 pathway or deregulation of apoptosis-related genes.29 We demonstrated that reduction of CITED2 levels results in a decreased interaction between p53 and its inhibitor MDM2. Disruption of the MDM2–p53 interaction has been reported to serve as a promising tool in AML therapy30,31 and various small molecules targeting p53 are currently implemented in the treatment of hematological malignancies to improve the efficiency of conventional cytotoxic drugs.32 One of the molecules used for targeting p53 is the MDM2–p53 inhibitor Nutlin3a and in this study we demonstrated that reduction of CITED2 expression sensitizes AML cells to Nutlin3a treatment.

Inactivation of p53 signaling can be caused by aberrant activation of the AKT signaling pathway,33 which controls proliferation and differentiation of hematopoietic cells. Notably, constitutive activation of AKT (indicated by increased phosphorylation of AKT on Ser473 and Thr308) is found in 50–70% of AML patients.34 Multiple studies in the course of the last years demonstrated that aberrant activation of AKT signaling plays a critical role for the survival and maintenance of AML cells (reviewed in Fransecky et al.35 Park et al.36 and Birkenkamp et al.37). Whereas mutations of components involved in AKT downstream signaling are rarely found in AML, mutations of membrane-bound proteins upstream of AKT that trigger its activation are common.28 A frequent source of AKT deregulation in AML are mutations in receptor tyrosine
kinases such as presence of FLT3-ITD or aberrant IGF1-R signaling. Compounds that interfere with AKT signaling are currently used for AML therapy; however, further understanding of this pathway in order to improve drug development is still needed.

Notably, our present study indicates that CITED2 is acting in p53-dependent, as well as in p53-independent pathways. Reduction of CITED2 levels was shown to interfere with AKT signaling on multiple levels (summarized in Figure 6). Whereas upregulation of the p53-target gene and AKT-inhibitor PHLDA3 appeared to be a direct consequence of increased p53 protein levels, upregulation of the AKT-inhibitor TXNIP and down-regulation of the AKT-activator SOX4 upon CITED2 knockdown occurred in a p53-independent manner. These findings are in line with p53-ChIP-Seq data indicating that there is no p53-binding at the TXNIP- and SOX4 promoter region. The transcription factor SOX4 binds to the promoter region of multiple components of the AKT signaling pathways and facilitates their transcription, whereas TXNIP has been shown to play a role in activating PTEN—an important antagonist of AKT activation—which can directly interfere with MDM2. Interestingly, SOX4 and CITED2 expression are both induced by TGF-β signaling, and the CITED2-interaction partner SMAD2 was found to bind to the SOX4 promoter region, indicating that SOX4 and CITED2 might indeed function in the same signaling pathway. Further evidence for a potential CITED2-SOX4 axis came from the finding that decreased levels of the CITED2 inhibitor PU.1 had a synergistic effect with SOX4 overexpression in promoting murine myeloid leukemia.

Moreover, we identified the p53-independent downregulation of BCL2 to be a crucial factor in the apoptotic phenotype of CITED2-knockdown cells. BCL2 overexpression is a hallmark of AML and does not only contribute to AML pathogenesis but also plays an important role in therapy resistance (reviewed in Tzifi et al.). Previous in vitro studies demonstrated that AML cells are very sensitive to BCL2 inhibition. Notably, AKT signaling is closely interconnected with members of the BCL2 family and a combination of AKT- and BCL2-inhibitors in AML treatment could represent a beneficial combination. Therefore, components that regulate these pathways represent potential therapeutic targets.

In summary, we identified that reduction of CITED2 expression interferes with signaling pathways commonly deregulated in AML. Therefore, CITED2 represents a novel, potential target for AML therapy and it will be of interest to study the detailed mechanisms of CITED2-mediated interference with AKT/BCL2/p53 signaling.

Materials and Methods
Isolation of primary cells. Cord blood (CB) CD34+ cells were derived from neonatal cord blood from healthy full-term pregnancies after informed consent from the Obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands. AML blasts from peripheral blood cells or bone marrow cells from untreated patients with AML were studied after informed consent and the protocol was approved by the Medical Ethical Committee. CB and AML mononuclear cells were isolated by density gradient centrifugation and CD34+ cells were selected using the MACS CD34 microbead kit on autoMACS (Miltenyi Biotec, Amsterdam, the Netherlands).

Cell cultures. The NB4 and MOLM-13 leukemic cell-lines (DSMZ: ACC 207 and 554) were cultured in RPMI1640 (Lonza, Leusden, the Netherlands, containing l-glutamine) supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. CD34+ selected CB cells were cultured in HPGM (Lonza) supplemented with SCF, FLT3 ligand and N-Plate (Amgen, Breda, the Netherlands) (100 ng/ml each) prior to transductions. AML cells were cultured in HPGM (Lonza) supplemented with 20 ng/ml IL-3, G-CSF and Nplate prior to transductions. 23ST cells were cultured in DMEM (Lonza) supplemented with 10% heat-inactivated FCS and 100 U/ml penicillin/streptomycin.

 Lentiviral vectors and transductions. Lentiviral vectors for shRNA expression: shRNA against CITED2 was obtained from Open Biosystem (Cambridge, UK, Clone IDs: TRCN0000015654 and TRCN0000015655) and cloned into a plKO.1 GFP (kind gift from Prof. Larson, Lund University) and plKO.1 mCherry vector (obtained by swapping GFP with mCherry). The CITED2 hairpins were chosen based on previous studies demonstrating that these hairpins had similar effects when targeting CITED2. The P300 PGK-TurboRFP shRNA vector was obtained from GE Healthcare (Cambridge, UK, #V3SH11243-00EG2033). The PHLDA3 oligomers were obtained from Invitrogen (Thermo Fisher Scientific, Landsmeer, the Netherlands) and cloned into the ‘mir-E’ backbone. The plKO.1 mCherry shCBP construct was kindly provided by Waldenik Zomerman (UMCG, University of Groningen). Short hairpin sequences used in this study are listed in Supplementary table 2. A lentiviral vector expressing BCL2 was constructed by inserting BCL2 cDNA into the lentiviral expression vector pRRL-IRES-blueberry. Lentiviral particles were produced as described before. After 24 h, medium was changed to HPGM and after 12 h, supernatant containing lentiviral particles was harvested and stored at −80 °C. Cells were transduced with lentiviral particles in the presence of 4 μg/ml polybrene. For AML and cord blood cells, virus particles were concentrated using CentriPrep Ultralres YM-50 Filter Units (Merck Millipore, Billerica, MA, USA) and cells were transduced in two consecutive rounds of 12 h.

 FACs analysis. All FACs analyses were performed on an LSRll (Becton Dickinson, Franklin Lakes, NJ, USA) or BD Accuri (BD Biosciences, Vlaken, the Netherlands) flow cytometer and data were analyzed using FlowJo software. Cells were sorted on a MoFlo XDP or Aria II (DakoCytomation, Carpinetia, CA, USA). For Annexin V staining, cells were harvested 4–7 days after lentiviral transduction, washed in calcium buffer (10 mm HEPES/NaOH, pH 7.4, 140 mm NaCl, 2.5 mm CaCl2), and stained with 3 μl Annexin V antibody (IgG-products, Groningen, the Netherlands) in 60 μl calcium buffer for 20 min at 4 °C. Cells were subsequently
washed and resuspended in calcium buffer for analysis. For cell cycle analysis, cells were resuspended in 200 μl PI-working solution (4 mM trisodium citrate, 20 μg/ml propidium iodide, 1% v/v Triton X-100, 0.1 mg/ml RNaseA); incubated for 30 min dark at 4 °C, and analyzed (Ex/Emission: 488/610). For some experiments, cells were treated with following inhibitors as indicated in the figures: C646 (BioVision, Uithoorn, the Netherlands), 4488-3a (Sigma-Aldrich, St. Louis, MO, USA), SML0580, MG-149 (kindly provided by FJ Dekker, University of Groningen).

Immunoblotting. Equal amounts of sorted cells were boiled in Laemmli sample buffer for 10 min and separated by SDS-PAGE (7.5–10% gels) according to standard protocols. Proteins were transferred onto Immobilon-P PVDF membrane (Millipore) by wet blotting overnight. Membranes were blocked with 5% BSA/PBS (Bio-Rad) and further analyzed by immunoblotting as described above. Primary antibodies for Co-IP: acetyl-p53 (Lys382; Cell Signaling Technology; catalog number 9293S), RPL27 or HPRT. Primer sequences are listed in Supplementary information table 1.

RNA isolation and Q-PCR. Total RNA was isolated using the RNeasy Micro Kit (QIAGEN, Venlo, the Netherlands) following the manufacturer's instructions. RNA was reverse transcribed using thescript cDNA synthesis kit (Bio-Rad). Real-Time PCR was performed on a CFX Connect System (Bio-Rad) using the SsoAdvanced SYBR Green Supermix (Bio-Rad). Data were quantified by the 2-ΔΔCT method using GAPDH as the reference gene.

Conflict of Interest

The authors declare no conflict of interest.
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