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LEDGF/p75-mediated chemoresistance of Mixed-Lineage Leukemia involves cell survival pathways and super enhancer activators

RUNNING TITLE

LEDGF, survival pathways and SEs activators in MLL

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ABSTRACT

MLL is an aggressive subtype of leukemia with a poor prognosis that mostly affects pediatric patients. MLL-rearranged fusion proteins (MLLr) induce aberrant target gene expression resulting in leukemogenesis. MLL and its fusions are tethered to chromatin by LEDGF/p75, a transcriptional co-activator that specifically recognizes H3K36me2/3. LEDGF/p75 is ubiquitously expressed and associated with regulation of gene expression, autoimmune responses and HIV replication. LEDGF/p75 was proven to be essential for leukemogenesis in MLL. Apart from MLL, LEDGF/p75 has been linked to lung, breast and prostate cancer. Intriguingly, LEDGF/p75 interacts with Med-1, which co-localizes with BRD4. Both are known as co-activators of super-enhancers. Here, we describe LEDGF/p75-dependent chemoresistance of MLLr cell lines. Investigation of the underlying mechanism revealed a role of LEDGF/p75 in the cell cycle and in survival pathways and showed that LEDGF/p75 protects against apoptosis during chemotherapy. Remarkably, LEDGF/p75 levels also affected expression of BRD4 and Med1. Altogether, our data suggest a role of LEDGF/p75 in cancer survival, stem cell renewal, and activation of nuclear super enhancers.

INTRODUCTION

Mixed Lineage Leukemia (MLL) is an aggressive subtype of leukemia, which is driven by the rearrangement of the MLL gene (MLLr) by chromosomal translocation or partial tandem duplications, resulting in in-frame fusions with one of 80 different partner genes (1, 2). The rearrangement prompts to the aberrant expression of MLL target genes involved in leukemogenesis and tumor progression. Approximately 10% of leukemia

patients worldwide are affected by MLL rearrangements, the majority of them are pediatric patients characterized by a poor overall survival compared to patients with no MLL rearrangement (3). Tumor recurrence and lack of specific treatments determine the lower survival.

The establishment of a triple complex of MLL or MLLr with Lens epithelium-derived growth factor p75 (LEDGF/p75) and Menin (4) is necessary for the regulation of gene expression. LEDGF/p75 is a co-transcriptional activator and a stress inducible nuclear protein (5, 6), ubiquitously expressed in healthy and pathological tissues. LEDGF/p75 depletion causes the inhibition of disease progression in MLL mouse models, demonstrating that MLL critically depends on LEDGF/p75 for leukemogenesis (4, 7, 8). In solid tumors, LEDGF/p75 has been associated with chemoresistance (9), cell cycle regulation (10), and cell survival (11). Among the nuclear binding partners of LEDGF/p75 (12-14), JPO2 is involved in tumor invasion and survival (11, 12, 15), and Med-1 has been recently associated with the activation of nuclear super enhancers (SEs) (16).

Here we report for the first time LEDGF/p75 as a driver of chemoresistance in MLLr cell lines. Furthermore, our investigation revealed a crucial role of LEDGF/p75 for cell survival in MLLr. The underlying mechanisms involve cell cycle regulation and survival pathways. LEDGF/p75 also affected expression levels of Med-1 and BRD4, suggesting a role in the regulation of SEs. The SE, a cluster of enhancers with a high density of transcription factors is one of the most interesting recent discoveries in cancer biology. We conclude that LEDGF/p75 may constitute a novel therapeutic target for treatment of MLL.

METHODS

Cell lines and compound: MLL-AF9 (Thpl and Molm-13), and MLL WT Kasumi-1 cell lines were kindly provided by Prof. Schwaller (Basel University, Switzerland). The MLL WT Nalm6 cell line was generously provided by Prof. Noritaka Adachi (Yokohama City University, Yokohama, Japan). LEDGF/p75 overexpression and depletion was induced by lentiviral vector transduction as previously described (8). Cytarabine has been purchased from Sigma Aldrich.

Proliferation assay: 20000 MLL cells were seeded in 96 well plates and treated after 12h with vehicle or with different concentrations of cytarabine for 72h. Cell proliferation was determined by WST-1 assay, and performed according to the manufacturer (Roche). IC₅₀ has been calculated online with IC50 tool kit (ic50.tk), and AAT Bioquest (<https://www.aatbio.com/tools/ic50-calculator>).

Western Blotting: Cell pellets were lysed using RIPA buffer supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentrations were determined by BCA assay (Thermo Fisher) and equal quantities of lysates were run on 4-20 % SDS-PAGE TGX gels (Bio-Rad). Proteins were transferred using turbo transfer apparatus to PVDF, and blotted for LEDGF, cleaved Caspase3, GAPDH, cCHK2, pCDC2, Gai, pAkt, Akt, pERKs, ERKs, Caspase1, cMyc (Cell Signaling), BRD4 (Bethyl Laboratories), MED1 (Abcam). Developing and imaging acquisition was performed using Las3000 mini (Fujifilm).

Flow Cytometry: The FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) has been adopted to evaluate the induction of apoptosis upon treatment with cytarabine. The staining on unfixed cells has been performed according to the instruction of the

manufacturer. The cell cycle of cells treated with cytarabine has been assessed through DNA quantification by propidium iodide (PI) staining (Sigma Aldrich). Briefly, after treatment cells were washed with PBS, fixed in cold absolute ethanol overnight at +4 °C, washed again in PBS and incubated for 30 minutes at 37 °C with 1ml of staining solution: 0.1% v/v Triton X-100, 200µg/ml DNase-free RNase, 30µg/ml propidium iodide (Sigma Aldrich). 15.000 cells per sample were acquired with a Gallios Cytometer (Beckman Coulter) at the Flow Cytometry core facility of KU Leuven, and a threshold on FSC has been set to reduce background, to avoid acquisition of cellular debris, and dead cells. Data have been analyzed with FCS Express for the investigation of the cell cycle, and Kaluza for the evaluation of apoptosis, and necrosis.

RT-qPCR: Total RNA from human cancer cells was extracted using Aurum Total RNA Mini Kit (BioRad), cDNA was synthesized using High Capacity Reverse Transcription Kit (Applied Biosystem). PCR was performed with the following primer sequences: LEDGF (Fwd: GGGCCAGAAGGAAGAAGATAAG; Rev: TCAGAATCGGAGGTTGAAGTAAC), Gai (Fwd: CTGAGGCCTGTTCTTTCTAC; Rev: GGCACTGCTCTAAGCATATCA), BRD4 (Fwd: ACCTCCAACCCTAACAAGCC; Rev: TTTCCATAGTGTCTTGAGCACC), cMyc (Fwd: CCTGGTGCTCCATGAGGAGAC; Rev: CAGACTCTGACCTTTTGCCAGG), β Actin (Fwd: CACTGCGCGAGGCTACAGC; Rev: TTGATGTCGCGCACGATTT).

RT-qPCR experiments were performed using SYBR Green and Light Cyclers 480 (Roche Diagnostics).

RNAseq: Total RNA from human cancer cells was extracted using Aurum Total RNA Mini Kit (BioRad). Analysis has been performed with HiSeq4000 (Illumina). Data have been normalized by the Genomics core facility (KU-Leuven): adapters were filtered with ea-

utils fastq-mcf v1.05, alignments were performed with HiSat2 and Samtools v1.5, quantification of reads per gene with HT-seq Count v2.7.14. p-values were calculated by two-tailed t-test distribution and genes with significant differential expression were clustered with Cluster 3. The heatmap has been created with TreeView, the gene enrichment analysis determined by IPA[®] software (Qiagen), and raw data have been deposited to the NCBI GEO (accession number GSE164751).

RESULTS

LEDGF/p75 confers chemoresistance in MLLr cells. Refractory AML patient blasts display increased levels of LEDGF/p75 and resistance to apoptosis (17). Hence, we investigated whether LEDGF/p75, apart from being a molecular chromatin-tether for MLLr, also plays a more general role in cell survival and apoptosis in MLL. Therefore, we examined the protective role of LEDGF/p75 in Thp1 cells, characterized by the expression of the MLL-AF9 fusion variant, by adding increasing concentrations of the chemotherapeutic agent cytarabine and evaluating cell proliferation and apoptosis. Expression levels of LEDGF/p75 indeed increased upon treatment (Supplementary figure 1A). The depletion of LEDGF/p75 in Thp1 cells (Figure 1B) sensitized the cells to cytarabine, resulting in decreased proliferation (Figure 1A), and increased apoptosis (Figure 1C, D) when compared with control cells. A similar trend has been also observed in vitro on another type of AML and ALL leukemias, not driven by MLL rearrangement (Supplementary figure 1C). Interestingly, Molm-13 cells (MLL-AF9) that naturally express barely detectable levels of LEDGF/p75 (Figure 1D) possibly due to the peculiar nature of the MLL recombination (18), were also characterized by a higher sensitivity to cytarabine

(Figure 1). Of note, the sensitivity to the drug treatment was not related to the FLT3 mutational status in AML cell lines. In fact, the IC_{50} values of cytarabine in Molm-13 (FLT3-IDT, Figure 1A) and Kasumi-1 (FLT3 not mutated, Supplementary figure 1C) with normal LEDGF/p75 expression were respectively 0.11 and 0.10 μ M (Supplementary figure 1D). In line with the results obtained in proliferation assay, flow cytometry (Figure 1C) and Western Blotting (Figure 1D) revealed lower levels of apoptosis in cells with higher LEDGF/p75 expression after treatment with cytarabine. Recently, it has been reported that LEDGF/p75 regulates proteins involved in the cell cycle of breast cancer (10). Based on these findings, we evaluated the cell cycle distribution in Thpl cells at variable LEDGF/p75 expression levels and after treatment (Figure 1E). As expected, Thpl control cells showed a typical cell cycle distribution and cytarabine caused a block in the S phase of the majority of cells. Contrarily, cells depleted for LEDGF/p75 revealed an aberrant cell cycle distribution, with the majority of events blocked in G2+M phase. After treatment, cells were more present in G2+M and in subG0G1 phases. This abnormal pattern provided the rationale to investigate the expression of the more crucial cell cycle checkpoints (Figure 1F). We discovered that the activation of CDC2 (19), which is involved in G2 to mitosis transition, was lower after cytarabine treatment in MLLr cells depleted for LEDGF/p75, suggesting that the upregulation of LEDGF/p75 in response to chemotherapy (Supplementary figure 1A) may stimulate cell division in cancer cells. On the other hand, the activation of CHK2 (20), associated with triggering of the apoptotic process in case of DNA damage, was higher after treatment in cells depleted for LEDGF/p75. When depletion of LEDGF/p75 was rescued by back complementation in control experiments, drug resistance was restored, as well as characteristics of

proliferation, apoptosis and cell cycle distribution in comparison to Thpl control cells (Fig. 1A-F). Surprisingly, the ectopic upregulation of LEDGF/p75 in Thpl cells (Supplementary figure 1A, $F_c=4.5$) was not associated with improved chemoresistance to cytarabine compared with control cells (Supplementary figure 1B). Similar results have been obtained in Thpl cells with LEDGF/p75 expression levels restored by back complementation (Figure 1A-B, $F_c=2.3$). Therefore, we believe that the normal LEDGF expression in Thpl cells confers the maximum resistance to the chemotherapeutic agent cytarabine, while the loss of expression is associated with increased chemosensitivity.

LEDGF/p75 regulates MLLr cell renewal and survival. RNAseq analysis of the whole transcriptome in Thpl cells depleted for LEDGF/p75, followed by differential expression analysis identified 540 significantly deregulated genes (Figure 2A, Supplementary Table 1: 198 with $F_c<-2$, 199 with $F_c>2$, $p\text{-value}<0.05$). Gene enrichment analysis performed by IPA[®] software pointed out that the Sphingosine-1 Phosphate pathway was the most significantly down-regulated (Figure 2B, $p\text{-value}=0.042$, Z score -1.000). This pathway is crucial in leukemia and in several other kinds of cancer, controlling survival, and migration (21-23). Among the major downstream players of this pathway in cancer (Gai, PI3K, Plc, Akt, ERKs, and Caspase-1 (21-23)), gene expression analysis by RT-qPCR and protein expression by western blotting confirmed the significant downregulation of Gai (Figure 2C), activated ERKs (Figure 2D), activated and total Akt (Figure 2E) in MLLr cells upon LEDGF/p75 depletion. Of note, the restoration of expression levels of LEDGF/p75 in knocked down Thpl cells by back complementation was associated with a partial recovery of activated Akt. (Figure 2E). On the other hand, evidence was provided for the upregulation of Caspase-1 (Figure 2F), which is involved in apoptosis and usually

inhibited by Akt. Remarkably, while depletion of LEDGF/p75 in Thp1 cells resulted in a significant downregulation of genes involved in cancer and survival (Figure 2G), upregulation of genes involved in hematopoiesis, leukopoiesis and stem cell differentiation was considerable. Furthermore, in support of our experimental results on LEDGF and chemoresistance, the downregulation of LEDGF/p75 in MLL in vitro is also associated with significant deregulation of genes involved in cellular development, proliferation, cell cycle, and survival (Supplementary figure 1E).

LEDGF/p75 is involved in the post-transcriptional regulation of SEs activators. The upregulation of genes involved in hematopoiesis and stem cell differentiation after LEDGF/p75 depletion, as well as the downregulation of genes involved in cancer (Figure 2F), suggested us to investigate more in detail the fate of some critical oncogenes. Moreover, LEDGF/p75 is known to tether Med-1, an essential component of SEs, to the chromatin (14). In a recent study, Sabari et al. (16) reported that BRD4 and Med-1 are key elements for the activation of SEs. Although Med-1 has recently been identified as a binding partner of LEDGF/p75 (14), the association between LEDGF/p75 and SEs, has not been explored before. BRD4 is crucial in leukemia and other cancers (3, 24, 25) and essential for the control of RNA polymerase II elongation, cMyc expression, and stem cell renewal. Therefore, we investigated if BRD4 and Med-1 expression are regulated by LEDGF/p75. Although variable in extent, we reproducibly observed a downregulation of BRD4 (Figure 3A, 43.8%±33, p-value=0.0003) and Med-1 (Figure 3C, 24.2%±13.7, p-value<0.0001) protein levels in Thp1 KDp75 and in Molm-13 cells. The BRD4 downregulation was post-transcriptional since no effect on RNA levels was detected (data not shown), implying a potential impact of LEDGF/p75 on the BRD4 protein stability. Of

note, the inhibition of the BRD4 activity by JQ1 did not influence LEDGF/p75 expression (Figure 3B, Supplementary figure 1F).

DISCUSSION

In summary, we report the essential role of LEDGF/p75 in chemoresistance of MLLr cell lines. LEDGF/p75 impacts the regulation of cell cycle check points, survival pathways and stem cell renewal (Figure 3D). We also observed that LEDGF/p75 affects BRD4 and MED-1 expression levels. Downregulation of MED-1 and BRD4 in depleted LEDGF/p75 cells may influence the stability and activity of the nuclear SEs, which would constitute a novel mechanism. Furthermore, LEDGF/p75 may represent a potential novel prognostic factor for disease and treatment. Our findings on LEDGF expression and chemosensitivity were also confirmed in MLLr-independent leukemic cell lines, thus extending the significance beyond this rather rare form of mixed lineage leukemia and providing the rationale for the development of novel therapeutic strategies targeting LEDGF/p75. The indirect effect of LEDGF/p75 on BRD4 expression and SEs activity can be relevant to MLL and other malignancies driven by BRD4 (24).

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AUTHORSHIP CONTRIBUTIONS

AC, SVB, TB and MSC performed experiments. AC contributed substantially to the design of experiments, and analysis of data. GN contributed to the RNA-seq data analysis and interpretation. AC, FC, and ZD interpreted the data and wrote the manuscript. All the authors were involved in the final editing and ZD approved the final manuscript.

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FIGURE LEGENDS

Figure 1. LEDGF/p75 is involved in chemoresistance and cell cycle regulation in MLL-AF9 cell lines. (A) WST-1 proliferation assay of MLL-AF9 cells incubated with different concentrations of cytarabine for 72h. Solid lines: Thpl WT (black), mock (blue), LEDGF/p75 depleted “KD p75” (red), LEDGF/p75 depleted and restored by back complementation “KD+BC” (green). Dashed line: Molm-13 WT (black) (n=3, mean $\pm$ SD, unpaired two-tailed Student’s t-test compared with Thpl mock data, * indicates p-value <0.05). **(B)** RT-qPCR for the quantification of LEDGF/p75 expression in the cell lines of panel A, represented with the same color code as in (A). Data are expressed as fold change compared to levels in Thpl WT cells (n=3, mean $\pm$ SD. Unpaired two-tailed Student’s t-test: KD p75 p-value <0.0001, KD+BC p= 0.0007, Molm-13 p-value<0.0001). **(C)** Thpl cells with different LEDGF/p75 expression levels and Molm-13 cells were treated for 24h with 2 μ M cytarabine, stained with AnnexinV-FITC/PI, and induction of apoptosis/necrosis was evaluated by flow cytometry. In red are the % of events in necrosis (UL), late apoptosis (UR), early apoptosis (LR) (n=3). **(D)** LEDGF/p75, activated caspase-3 (cCas3) and GAPDH (as loading control) protein expression analyzed by immunoblotting of Thpl and Molm-13 cell line extracts after 24h of treatment with 2 μ M

cytarabine (n=3). **(E)** Flow cytometry analysis of cell cycle distribution of MLL-AF9 Thpl cells treated with 2 μ M cytarabine for 24h (n=3). **(F)** Immunoblot analysis of the activation of CDC2 and CHK2 after 24h of treatment with 2 μ M cytarabine. GAPDH was run as loading control (n=2).

Figure 2. LEDGF/p75 regulates the sphingosine-1P survival pathway in MLL-AF9 cell lines. **(A)** RNA samples isolated from Thpl mock or LEDGF/p75 depleted cells at passage 3 (n=4/experimental group) were analyzed by RNA sequencing, and genes with a significant differential expression (p-value<0.05), and Fc >2, or <-2, are displayed in the heat map. Color indicates row-normalized expression from +1.5 (red) to -1.5 (blue). **(B)** Ingenuity pathway analysis (IPA) was used to evaluate significantly different canonical pathways. The Sphingosine-1P pathway proved the most significantly downregulated pathway (p-value=0.042, Z score -1.000), as shown by the IPA pathway diagram. The genes of the pathway significantly downregulated after LEDGF/p75 depletion are encircled. **(C)** RT-qPCR (left panel, n=2, p-value=0.0142, unpaired two-tailed t-test) and immunoblot (right panel, n=3) analysis of Gai expression in Thpl cells with normal (mock) or downregulated (KD p75) expression of LEDGF/p75. **(D)** Immunoblot analysis of the protein expression of activated pERKs, total ERKs and GAPDH in Thpl cells with normal (mock) or downregulated (KD p75) expression of LEDGF/p75 (n=3). **(E)** Immunoblot analysis of activated pAkt and total Akt expression in Thpl cells with normal (mock), restored by back complementation (KD+BC) or downregulated (KD p75) expression of LEDGF/p75. (n=3) **(F)** Immunoblot analysis of Caspase-1 (Cas1) expression in Thpl cells with normal (mock) or downregulated (KD p75) expression of LEDGF/p75. (n=3) **(G)** IPA

analysis of genes significantly deregulated and grouped by "diseases and cellular functions". MNL: mononuclear leukocytes, HPCs: hematopoietic progenitor cells.

Figure 3. LEDGF/p75 regulates super enhancers in MLL-AF9 cell lines. (A) Upper panel: LEDGF/p75, BRD4 and GAPDH immunoblot. GAPDH was used in immunoblot assays as loading control. **Lower panel:** Whisker plot analysis of relative BRD4 expression in LEDGF/p75 depleted compared with Thpl mock cells calculated by densitometry (n=8, 43.8%±33, p-value=0.0003, unpaired two-tailed t-test). **(B)** Immunoblot analysis of BRD4, LEDGF/p75, cMyc and GAPDH in Thpl and Molm-13 cells after 24h of treatment with JQ1 250nM (n=3). GAPDH was used in immunoblot assays as loading control. **(C) Upper panel:** Immunoblot analysis of Med-1, BRD4 and GAPDH expression in Thpl and Molm-13 cells (n=3). GAPDH was used in immunoblot assays as loading control. **Lower panel:** Whisker plot analysis of relative Med-1 expression in LEDGF/p75 depleted compared with Thpl mock cells calculated by densitometry (n=4, 24.2%±13.7, p-value<0.0001, unpaired two-tailed t-test). **(D)** Schematic representation of the role of LEDGF/p75 in MLL-AF9 cell lines in chemotherapy, with respect to survival, cell cycle regulation and Med-1/BRD4 expression (orange: downregulation/inhibition, green: upregulation/activation).

Figure 1

A

B

C

D

E

F

Figure 2

Figure 3

A

B

C

D

Supplementary figure 1. (A) Expression levels of LEDGF/p75 after 48h of treatment with 2 μ M of cytarabine assessed by RT-qPCR in Thpl mock, knockdown (KD) of p75, ectopically expressing LEDGF/p75 “OV p75”, and in Molm-13 WT cells. Data are expressed as a fold change of LEDGF/p75 mRNA expression compared with Thpl mock cells (n=3, mean $\pm$ SD, unpaired two-tailed Student’s t-test compared with untreated data, * indicates p-values <0.05). **(B)** WST-1 proliferation assay of Thpl cells incubated with different concentrations of cytarabine for 72h. Solid lines: Thpl WT (black), mock (blue), and with ectopic expression of LEDGF/p75 “OV p75” (orange) (n=3, mean $\pm$ SD, unpaired two-tailed Student’s t-test compared with Thpl mock data). **(C)** WST-1 proliferation assay of AML cell line Kasumi-1 (left panel), and ALL cell line Nalm6 (right panel) incubated with different concentrations of cytarabine for 72h. Blue and red lines indicate mock and LEDGF/p75 depleted “KD p75” cells respectively (n=3, mean $\pm$ SD, unpaired two-tailed Student’s t-test compared with mock data, * indicates p-values <0.05). **(D)** IC₅₀ (μ M) values calculated after 72h of treatment with cytarabine on Thpl, Kasumi-1, Nalm6 and Molm-13 cell lines. **(E)** IPA analysis of genes significantly deregulated and grouped by “molecular and cellular functions”. **(F)** Expression levels of BRD4, LEDGF/p75, cMyc in Thpl and Molm-13 cells after 24h of treatment with JQ1 250nM (n=2, mean $\pm$ SD. cMyc: Thpl + JQ1 p-value=0.0065, Molm-13 + JQ1 p-value=0.0164, unpaired two-tailed Student’s t-test).

Supplementary Table 1. Genes significantly and differentially expressed in Thpl KD LEDGF/p75 compared with Thpl WT cells assessed by RNA sequencing (p-value < 0.05, 199 genes with Fc > 2, 198 genes with Fc < -2, unpaired two-tailed Student’s t-test).