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PBAF lacking PHD domains maintains transcription in human neutrophils

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ABSTRACT

The myeloid precursor cell differentiation requires an extensive chromatin remodeling. We show that the level of the PBAF chromatin remodeling complex decreases following the start of differentiation of myeloid precursors, becoming very low in the terminally differentiated peripheral blood (PB) neutrophils where it co-localizes with Pol II on the transcriptionally active chromatin. Previously, we have shown that the PHF10 subunit of the PBAF signature module has four isoforms, two of them (PHF10-P) contain a tandem of C-terminal PHD domains. We found that out of four PHF10 isoforms present in the myeloid precursor cells, only the PHF10-Ss isoform lacking PHD domains, is actively expressed in the PB neutrophils. In particular, the longest of the PHF10 isoforms (PHF10-Pl), which is essential for proliferation, completely disappears in PB neutrophils. In addition, in the myeloid precursors, promoters of neutrophil-specific genes are associated with the PHD-containing isoforms, together with PBAF and Pol II, when these genes are inactive and only during their activation stage. However, at the later stages of differentiation, when neutrophil-specific genes are actively transcribed, PHF10-P isoforms on their promoters are replaced by the PHF10-S isoforms. Evidently, PHD domains of PHF10 are essential for active chromatin remodeling during transcription activation, but are dispensable for the constantly transcribed genes.

1. Introduction

Renewal and differentiation of blood cells is a complex process, extensively utilized in the body for a constant renewal of different cell types. Neutrophils, which are formed during myeloid differentiation are the most abundant type of granulocytes and make up 65–70% of all white blood cells [1]. Neutrophils attack pathogens recognized by the innate immune system by phagocytosis, production of reactive oxygen species, and neutrophil extracellular traps [2]. One hundred billion neutrophils are destroyed every day [3], and need to be replaced through rapid differentiation from progenitor cells. Neutrophils are derived from lymphoid-primed multipotent progenitors in bone marrow, which give rise to granulocyte-monocyte progenitor cells that could differentiate in myeloid pathway from myeloblasts and promyelocytes to myelocytes and, finally, to mature neutrophils [2].

The maturation of hematopoietic precursors is associated with two fundamental processes: reduction in self-renewal potential and stepwise acquisition of a specific lineage identity. If a stem cell is triggered to begin differentiation, genes that maintain self-renewal are switched off, whereas genes that enforce differentiation are switched on.

This sequence of alterations in gene expression is orchestrated by stage-specific transcription factors and epigenetic remodelers and readers, which inhibit transcription of pro-proliferation genes and start transcription of lineage-specific genes, required for differentiation. If dysregulated or mutated, these transcription factors could fail to induce differentiation and cause uncontrolled continuation of proliferation. This might place the progenitors at a higher risk of developing a true leukemia, owing to the random accumulation of additional cooperating events.

Several transcription factors are involved in orchestration of the formation of myeloid cells (C/EBP family, PU.1, GFI1, Myc, RUNX1) [4–8]. Besides transcription factors, additional epigenetic regulatory complexes are required for myelogenesis. Intensive changes in gene expression during differentiation require extensive activity of chromatin remodeling complexes. SWI/SNF chromatin remodeling complexes are needed for both activation of specific myeloid genes, as well as cell-cycle specific genes [9]. For example inhibition of proliferation by C/EBPα, is abrogated when SWI/SNF components, like Brm1 or INI5/SNF5 are mutated, pointing to their function as inhibitors of cell cycle [10]. On the other hand PHF10 (BAF45a) and BAF53a are

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required for self-renewal of myeloid progenitor cells, their deletion leading to impaired renewal of stem cells and inhibition of cell cycle progression. BAF53a is required for expression of cell-cycle regulators such as Bmi1 and Hoxb4, while PHF10 effect on such genes remains unclear [11].

In mammals, there are two types of SWI/SNF complexes - BAF and PBAF, each consisting of core subunits, common for both types of SWI/SNF and several specific subunits [12,13]. The PBAF acts as a co-activator complex for transcription of a wide range of genes, in particular, of the proliferation-regulating genes [14]. The PBAF-specific subunits include BAF180, BAF200, BRD7, and PHF10 and form the module of the complex responsible for its interaction with the target genes [12]. The subunit composition of the PBAF complex may vary in cells of different types. Thus, the PHF10 subunit (BAF45a) was shown to be a component of the neural progenitor-specific SWI/SNF complex. It is essential for maintaining the proliferating state of murine neuroblasts but leaves the complex in the mature neurons [15]. PBAF complex, including PHF10/BAF45a is essential for maintaining proliferation of the hematopoietic cell precursors, its level decreases in the subsequent stages of differentiation [11].

Previously, we have demonstrated that mammalian PHF10 has four isoforms which are alternatively incorporated into the complex [16]. Of them, the longer isoform (PHF10-Pl) which corresponds to BAF45a described by others [11,15], was shown to activate genes responsible for proliferation [16]. The PHF10 is the most unstable subunit of PBAF and its isoforms demonstrate different stability in the complex, depending on their domain structure. Their stability is regulated by the CK-1 multiple phosphorylation cluster which protects PHF10 from the b-TrCP ubiquitin ligase-dependent degradation [17].

Here we investigated the role of PBAF and PHF10 in myeloid differentiation and activation of neutrophil-specific genes. For this study, we used human myeloblastic cell line HL-60 with the all-trans-retinoic acid (ATRA)-induced differentiation, which is a well-known model system [18], and primary human neutrophils, isolated from peripheral blood of healthy donors (PB neutrophils). We demonstrated that the level of the PBAF complex subunits strongly decreased in the promyelocytic leukemia cell line HL-60 following the start of differentiation, becoming very low in PB neutrophils. Still, the complex remained active, was localized at the actively transcribed chromatin, and participated in activation of the myeloid-specific genes. The subunit composition of the complex changed following differentiation. The level of the unstable PHF10-Pl isoform (corresponding to BAF45a), which is expressed in the undifferentiated precursor cells, was decreased upon differentiation. It completely disappeared in PB neutrophils where it was replaced by a stably expressed highly phosphorylated PHF10-Ss isoform. Thus, in PBAF of the terminally differentiated PB neutrophils, PHF10-Pl was replaced by the PHF10-Ss isoform. We also demonstrated that PHF10-P isoforms, together with the preassembled Pol II transcription complex, were present before differentiation on the promoters of neutrophil-specific genes, when these genes were inactive, and during the first days of differentiation, but later were replaced by the PHF10-Ss isoform.

2. Methods

2.1. Isolation of PB neutrophils

Neutrophils were isolated from freshly drawn citrate-anticoagulated donor peripheral blood by standard techniques, as previously described [19]. Leukocyte-rich plasma was prepared by sedimentation of red blood cells with 3% dextran T-500 (ThermoFisher Scientific), and granulocytes were purified by centrifugation through Ficoll-Paque (Merck, density 1.077 g/mL). All methods were performed in accordance with the relevant guidelines and regulations of the Ministry of Public Health Service of the Russian Federation and local laws.

2.2. Differentiation of the HL-60 cell line

The HL-60 cells were purchased from ATCC (Manassas, VA, USA). Cells were grown in the RPMI-medium containing 10% fetal bovine serum (FBS, Merck) with cell concentration not exceeding one to two million cells per mL, supplemented with 2 mM L-Glutamine (Merck) and penicillin/streptomycin. For induction of differentiation, all-trans-retinoic acid (ATRA, Merck) was stored under argon and diluted in DMSO before addition up to a final concentration of 2 μM. Media was changed once per 3 days in the control and differentiated samples. After 3 and 6 days, we assessed the extent of cell differentiation monitoring several parameters: the expression of CD66-specific markers, progression of the cell cycle, changes in nuclear morphology, and apoptosis.

2.3. Western Blotting and antibodies

For Western-Bblot analysis, HL-60 cells (about 3 million per sample) were harvested and lysed in RIPA Buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.5% Na-deoxycholate; 0.1% SDS; 150 mM NaCl; 2 mM EDTA; protease inhibitor cocktail (PIC, Roche)), centrifuged at 12000 rpm, +4 °C. Protein concentration was detected using Qubit Protein Assay Kit (ThermoFisher Scientific) and adjusted, samples were mixed with 4× Laemmli Buffer (200 mM Tris-HCl, pH 6.8; 4% SDS; 40% glycerol; 0.,01% Bromophenol blue; 100 mM DTT), and boiled for 10 min.

Purified polyclonal rabbit antibodies against the total PHF10, PHF10-PHD's, BRG1, BAF155, BAF200, BAF180, BRD7, and TAF5 were generated in our laboratory and described previously [16,17,20,21]. Antibodies for total RNA-polymerase II (4H8) were from Active Motif, for P-Ser2-polymerase II (5095) from Abcam, for p21 (12D1) and Histone-3 (D1H2) from Cell Signaling, and for β-tubulin (E7) from DSHB. The secondary antibodies were the HRP-conjugates of goat anti-rabbit IgG (DSHB) and goat anti-mouse IgG (DSHB).

2.4. Primers, RNA isolation, and cDNA synthesis

RNA was isolated from 3 million HL-60 cells and 10 million PB neutrophils using TriReagent (MRC) according to the manufacturer's protocol. cDNA was synthesised using oligo(dT) primers and MMLV reverse transcriptase (Thermo Fisher Scientific). Primers used for the measurement of gene expression are described in Supplemental materials (S1, primers). The values were normalized to a housekeeping gene RPLP0.

2.5. IP and ChIP experiments

For co-immunoprecipitation, 2 million ± ATRA HL-60 cells and 10 million PB neutrophils were lysed with a buffer containing 10 mM HEPES, pH 7.9; 5 mM MgCl; 0.5% Nonidet P-40; 0.45 M NaCl; 1 mM DTT; PIC (Roche); and 1% Phosphatase Inhibitor Cocktail 3 (PhIC) fromSigma-Aldrich: DNAse I (USB, 0.6 units/mL), and then diluted 4-fold with the same buffer, but without NaCl. Supernatants after centrifugation were mixed with appropriate antibodies (10 μg) and 15 μL of wet ProteinA sepharose (Sigma), incubated overnight and washed. Precipitated proteins were eluted with 4× LB (200 mM Tris-HCl, pH 6.8; 4% SDS; 40% glycerol; 0.01% Bromophenol blue; 100 mM DTT) followed by Western blotting.

For ChIP experiments, 15 million ± ATRA treated cells were collected and crosslinked for 10 min with 1% fresh formaldehyde, the reaction was stopped by 2.5 M Glycine, then washed three times with cold 1× PBS and resuspended in Sonication Buffer (50 mM HEPES-KOH, pH 7.9; 140 mM NaCl; 1 m M EDTA; 1% Triton X-100; 0.1% deoxycholate Na; 0.1% SDS with PIC (Roche)). DNA was sheared to about 500 bp by sonication (for 30 s with 30 s intervals between sonications, for 10 cycles) and then centrifuged for 15 min at 12000 rpm, at +4 °C. Approximately 2 million cells, 10 μg of an antibody, and 15 μL of wet ProteinA sepharose (Sigma) were used per precipitation. The primers

used for analysis are listed in the Supplemental materials (S1, primers). After ChIP, the recovered DNA was analyzed by qPCR with StepOnePlus (Applied Biosystems).

2.6. DAPI and immunostaining

HL-60 cells were prepared with Cytofunnel® (Thermo Fisher Scientific) and centrifugation. After sedimentation and DAPI staining (600 nM for 15 min) the cells were visualized using Leica TCS SP2 confocal microscope or the DMR/HC5 fluorescent microscope (Leica) with an HCX PZ Fluotar ×100/1.3 objective lens. Microscopic images were taken with a Leica DC350 F digital camera.

For immunostaining of the PBAF complex subunits, PB neutrophils were sedimented without centrifugation, fixed with formaldehyde and immunostained with the previously described primary antibodies (1:100) for 1 h at RT, washed three times with PBS and stained with the secondary anti-rabbit Alexa-488 Fluor-conjugated antibodies (Invitrogen) for 1 h at room temperature (RT). Stained preparations on glass slides were mounted in a mounting medium (Vector Laboratories) and examined under the same microscope.

2.7. Flow cytometry analysis of the CD66b surface marker

PB neutrophils and HL-60 cells (1 million per mL) were centrifuged and diluted in 100 μL of Hanks solution containing 2 μL of CD66b-PE antibodies (BD Pharmingen). Samples were incubated for 30 min on ice in the dark, then 250 μL of Hanks solution was added. 30,000 cells from each sample were analyzed on Cytoflex flow cytometer (Beckman Coulter) in the PE channel. Data were analyzed with the BD CytoExpert 2.0 software.

2.8. Cell cycle analysis

Differentiating and the control HL-60 cells were centrifuged and lysed in a buffer containing 0.1% sodium citrate; 0.3% NP-40; 50 μg/mL RNAse A; and 50 μg/mL of propidium iodide (BD Pharmingen). Lysates were mixed by vortexing and incubated for 30 min at RT in the dark. 30,000 cells from each sample were detected on a BD FACS Canto II flow cytometer in PE (linear) and PerCP (log) channels and analyzed using FlowJo cell cycle analysis software.

2.9. Detection of apoptosis

PB neutrophils, differentiating, and control HL-60 cells were washed in 1 mL of 1× PBS (MP Biomedicals), centrifuged and resuspended in 500 μL of Annexin Binding Buffer (Invitrogen). Cells were resuspended in concentration of one to five million cells per mL, and 100 μL of cell suspension was incubated with 5 μL of Annexin V-FITC (Invitrogen) for 15 min at RT in the dark. Cells were washed in 1 mL of Annexin Binding Buffer, incubated with 5 μL of Propidium Iodide Staining Solution (Merck) for 5 min at RT, assayed using Cytoflex flow cytometer (Beckman Coulter) in FITC and PE channels, and analyzed with CytExpert and FlowJo Software. 30,000 cells per sample were analyzed in every experiment.

2.10. Flow cytometry analysis of the PBAF subunits

PBAF subunits were analyzed as nuclear proteins according to the method we described previously [22]. PB neutrophils and HL-60 (2 million per mL) were centrifuged (6 min, 300*g*, +4 °C) and washed in 5 mL 1× PBS containing 0.05% BSA (Merck). Subsequently, the pellet was resuspended in 1 mL of PBS with 2–4% formaldehyde. Samples were incubated at 37 °C for 10 min, chilled to 4 °C, 5 mL of PBS with 0.05% BSA was added, and then centrifuged. Cells were resuspended in 1 mL of 70% ice-cold ethanol, placed on ice for 30 min, and centrifuged. For blocking of the non-specific antibody (Ab) binding, the cells were incubated for 30 min at RT in 3 mL of PBS with 1% BSA and 10% non-immune goat serum (Merck). After centrifugation, the pellet was re-suspended in 100 μL of PBS with 1% BSA (staining buffer) and primary antibody (1:1000). Samples were incubated for 30–40 min at RT and after addition of 2.5 mL of staining buffer, centrifuged again. The pellet was resuspended in 200 μL of staining buffer containing secondary antibodies. The solution was incubated for 30 min in the dark at RT, washed once in PBS and assayed using Cytoflex flow cytometer (Beckman Coulter) in PE channel. 30,000 cells per sample were analyzed in every experiment. Overlay histograms were made in CytExpert 2.0 software.

2.11. Statistical analysis

Results are reported on the diagrams as mean ± SD of at least three independent experiments. Statistical significance was evaluated using a one-way or two-way ANOVA with Holm-Sidak's multiple comparison test, GraphPadPrism6 software. P-values of < 0.05 were considered significant.

3. Results

3.1. HL-60 cells and PB neutrophils as the model system of blood cell differentiation via the myeloid pathway

To study the role of PBAF in the blood cell development via the myeloid pathway, we used the HL-60 cell line and neutrophils from the peripheral blood of healthy donors. The HL-60 cells are characterized as acute promyelocytic leukemia cells [23]. Presently, they are described as promyelocytes or myeloblasts and are well known as a model system for studying myeloid cell differentiation [24].

Treatment with all-trans retinoic acid (ATRA) induces differentiation of the HL-60 cells into myelocytes and neutrophils, accompanied by a decrease in proliferative activity, induction of apoptosis, and transcription of specific markers [24,25]. A continuous treatment of HL-60 cells with ATRA induces cell-cycle arrest at the G0 stage with concomitant differentiation into a granulocytic lineage [24,25]. We have characterized the differentiation of HL-60 cells following ATRA treatment, to compare it with the terminally differentiated PB neutrophils and to develop a correct model system for subsequent experiments.

The CD66a, CD66b, and CD66d are constitutively expressed on the neutrophil surface and were chosen as lineage-specific marker genes to track the correct induced myeloid differentiation in the HL-60 cell line [24,26]. The treatment of HL-60 with 2 μM ATRA for three days led to an increase in the CD66a, CD66b, and CD66d transcripts, the levels of the CD66a and CD66b mRNAs were comparable to their expression in the PB neutrophils (Fig. 1A). The increase of CD66d in the differentiated HL-60 was lower, but strong enough (about 7-fold) comparatively to the existing level in the untreated HL-60 cells. In line with these results, measurements of the CD66b protein by flow cytometry on the third and sixth days after the ATRA treatment, demonstrated a CD66b accumulation on the HL-60 membrane (Fig. 1B, Supplementary1 Fig. 1A), confirming a proper differentiation of the HL-60 cells.

Next, we examined the cell cycle characteristics by measuring DNA content and detected an accumulation of G0/G1 cells, starting on Day 1, with a gradual decrease in the number of replicating cells, from the first to the fifth day of differentiation (Fig. 1C), with the majority of cells exiting the cycle on the first and second days of treatment. We have also measured the mRNA levels of several key cell-cycle regulatory proteins, CyclinE1 (*CCNE1*), CyclinD1 (*CCND1*), p21 (*CDKN1A*), and p27 (*CDKN1B*). Cyclins D1 and E1, the major cyclins involved in the G1/S cell-cycle checkpoint progression, decreased on the third day (Fig. 1D). The mRNA levels of the cyclin-dependent kinase inhibitors, p21 and p27, which are important for the prevention of transition into the G1 phase and through the G1/S cell-cycle checkpoint, increased

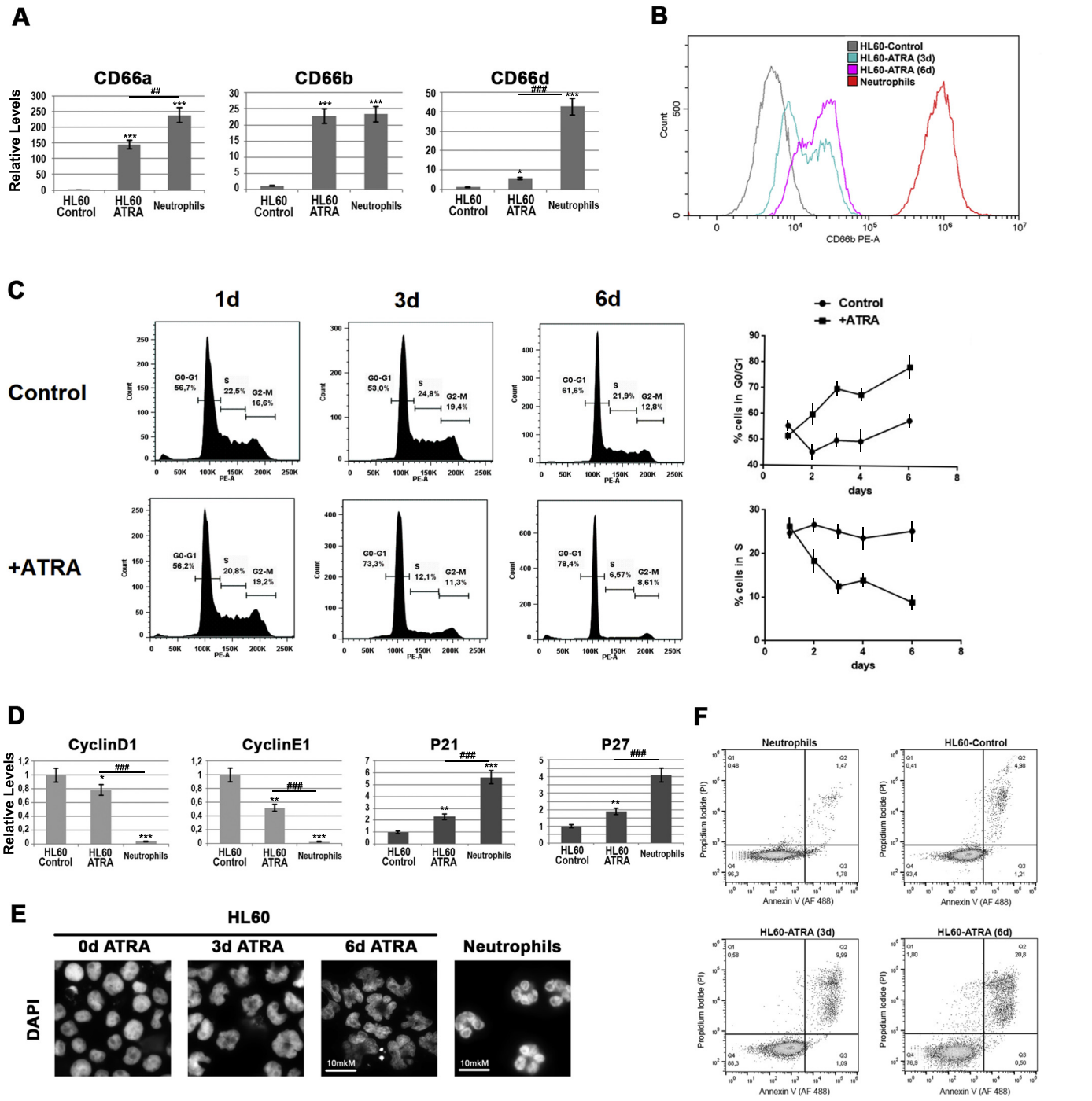

Fig. 1. Induction of the HL-60 cell culture differentiation with ATRA.

(A) Transcription levels of the CD surface specific genes in HL-60 cells (± ATRA, on the third day of differentiation) and PB neutrophils. Values indicate the mean ± SD from four independent experiments and were normalized to RPLP0. Here and in other similar experiments, Y-axis shows RNA-fold change. * $p < 0.05$, *** $p < 0.005$ compared to the control; ## $p < 0.01$, ### $p < 0.005$ for the pair of data compared (1-way ANOVA, Holm-Sidak's multiple comparison test).

(B) Overlay histogram representing the level of CD66b surface marker in control (HL-60), HL-60 treated with ATRA for three or six days, and in neutrophils analyzed by flow cytometry. Cells were stained with PE-labeled mouse anti-human CD66b antibodies.

(C) Differentiation of HL-60 cells leads to changes in cell cycle, measured by DNA content. Typical flow cytometry histograms of control (HL-60) and HL-60/ATRA treated cells are shown. Cells were permeabilized, stained with PI and analyzed on every day of differentiation. On the right is the percentage of HL-60 cells in G0/G1 (upper panel) and S (lower panel) phases measured during 6 days of differentiation compared to control HL-60 samples. Values indicate the mean ± SD of three independent experiments.

(D) The transcription levels of cell cycle promoting genes CyclineD1, CyclineE1 (CCND1, CCNE1) and inhibitors of cell cycle genes p21, p27 (CDKN1A, CDKN1B) in control (HL-60), HL-60 treated for three days with ATRA, and in PB neutrophils, measured by qRT-PCR. Values indicate the mean ± SD from four independent experiments and were normalized to RPLP0. ** $p < 0.01$, *** $p < 0.005$ compared to the control; ### $p < 0.005$ for pairs of data compared (1-way ANOVA, Holm-Sidak's multiple comparison test).

(E) The nuclei of HL-60 cells, the HL-60 treated with ATRA for three or six days, and of neutrophils stained with DAPI.

(F) The level of apoptosis analyzed using annexin V and PI staining is 1.78% for early apoptotic cells (annexin V-positive, PI-negative) in neutrophils and 1.09% and 0.5% in HL-60 on the third and sixth days of differentiation, respectively. The number of late apoptotic cells (double-positive cells) steadily increased through the

treatment period reaching 20.8% by the sixth day. Analyzed populations are shown in Supplementary1 Fig. 1A.

concurrently (Fig. 1D). The levels of these cell cycle regulatory proteins in HL-60 following differentiation were not as low as in the fully differentiated PB neutrophils. Taken together, our results show cell-cycle arrest in the HL-60 in the course of the ATRA-dependent differentiation.

The increasing changes in the nuclear shape during HL-60 differentiation were also detected. On the third day, the nuclei lost their oval shape and acquired invaginations, on the sixth day, nuclei became lobulated, with 3–5 segments, their shape became similar to the shape of the PB neutrophil nuclei (Fig. 1E).

We have also detected an accumulation of apoptotic bodies on the sixth day of differentiation comparatively to the third day (Supplementary1 Fig. 1B). These data is in accordance with previous reports [24]. We measured the number of apoptotic HL-60 cells following the ATRA treatment (Fig. 1F). ATRA-induced differentiation of HL-60 cells also led to an apoptosis level increase [24]. In line with these data, the number of the late apoptotic cells was low, but had steadily increased through the treatment period reaching 20.8% by the sixth day (Fig. 1F).

Majority of the cells exited the cycle on the first and the second days of ATRA treatment. This indicates that the main changes in the gene expression pattern would also occur during the first two days of differentiation. Indeed, the expression of the key cell-cycle regulatory proteins decreased while the levels of p21 and p27 mRNAs increased. The genes encoding neutrophil-specific markers were already actively expressed on the third day.

For further experiments, we used non-differentiated HL-60, PB neutrophils, and HL-60 cells on the third day of differentiation. The last were chosen since they are at a “middle point” between non-differentiated HL-60 cells and PB neutrophils, because of clear changes associated with the start of myeloid development. We did not use the 6 day ATRA treated HL-60 cells due to a high level of apoptosis.

3.2. The level of PBAF complex subunits decreases upon the beginning of myeloid differentiation and becomes very low in PB neutrophils

Specialization of the neutrophilic cells during myeloid differentiation is accompanied by global changes in the pattern of transcribed genes and the chromatin structure. PBAF complex participates in the maintenance of open chromatin structure and activation of some specific genes responsible for proliferation, reviewed in [13]. We were interested in studying functions of PBAF during myeloid differentiation. The levels of transcription for genes encoding PBAF subunits in HL-60 cells, HL-60 cells treated with ATRA (HL-60/ATRA), and in PB neutrophils, were measured by RT-qPCR (Fig. 2A). We observed a modest decrease in transcripts encoding core (Brg1, BAF155), and specific (BAF200, BRD7, and PHF10) PBAF subunits (Fig. 2A) in HL-60/ATRA cells comparatively to the untreated HL-60 cells on the third day of treatment. In PB neutrophils, the transcription levels dropped dramatically (more than ten times) comparatively to the HL-60 (Fig. 2A). Interestingly, the level of the RNA-polymerase II transcript did not substantially decrease in PB neutrophils comparatively to HL-60 (Fig. 2A). Similar results were observed when the levels of PBAF subunits were measured by flow cytometry (FACS) (Fig. 2B, C; Supplementary1 Fig. 1C). In line with RT-qPCR data, the levels of all the subunits decreased during differentiation of HL-60, and were significantly lower in human PB neutrophils.

In summary, in line with the decreased level of chromatin remodeling, the PBAF level dramatically dropped in the differentiated PB neutrophils comparatively to the undifferentiated myeloid precursors.

3.3. PBAF complex maintains its integrity in the course of differentiation and localizes in transcriptionally active chromatin in PB neutrophils

Some of the PBAF subunits may leave the complex following the start of differentiation [15,27]. We verified the integrity of the specific PBAF module and its association with core subunits in co-immunoprecipitation experiments. Antibodies against BRG1 and BAF155 core subunits co-precipitated BAF200, BAF180, BRD7, and PHF10-specific subunits from extracts of control and HL-60/ATRA cells. Antibodies against specific subunits precipitated each other and the core subunit BAF155, indicating that the PBAF complex maintains its integrity during differentiation (Fig. 3A, Supplementary 1, Fig. 2A). Only a small amount of total BRG1 ATPase was co-precipitated with antibodies against BAF200, BAF180, BRD7, and PHF10, both from control and HL-60/ATRA cell extracts. The reason could be a disruption of interaction with BRG1 by antibodies against specific subunits.

During differentiation, the chromatin of neutrophils becomes more condensed, and their nuclei changed their shape and become segmented [28]. To identify the regions of active transcription, we stained PB neutrophils with antibodies against Ser2 phosphorylated CTD of RNA-polymerase II, (PS2-Pol II) which marks the elongating Pol II and co-stained the nuclei with DAPI. The DAPI staining revealed the segmented PB neutrophil nuclei including several spherical lobes with a more condensed chromatin on the periphery of the lobes (Fig. 3B). The anti-PS2 Pol II antibodies marked active chromatin in the central area of the lobes which was less intensively stained with DAPI (Fig. 3B).

The PB neutrophils were also stained with antibodies against PBAF subunits (BRG1, BAF155, BAF200, BAF180, BRD7, and PHF10) and co-stained with DAPI (Fig. 3B). Similar to PS2-Pol II, all PBAF subunits were detected in the centric chromatin which was less intensively stained with DAPI (Fig. 3B). The 3-color staining of PBAF subunits, PS2-PolII, and DAPI (Supplementary 1 Fig. 2B) demonstrated that PBAF co-localized with PS2-PolII. Thus, our results indicate that in PB neutrophils, PBAF complex was localized in the actively transcribed regions of chromatin.

3.4. The PHF10-P and PHF10-S transcription levels are reversed during myeloid differentiation

As was shown previously, the PHF10 subunit of PBAF complex has four isoforms (PHF10-Pl, PHF10-Sl, PHF10-Ps and PHF10-Ss) which are functionally different, two of them have C-terminal PHD domains (PHF10-P isoforms), while in other isoforms PHDs are replaced by a motif for SUMO modification (PHF10-S isoforms) [16]. Two of the isoforms, PHF10-Pl and PHF10-Sl, also contain additional 46 N-terminal amino acids (Fig. 4A). We examined expression and activity of different PHF10 isoforms during myeloid differentiation.

Similar to other PBAF subunits, the levels of transcripts for PHF10-P isoforms rapidly decreased (Fig. 4B). Unexpectedly, the levels of PHF10-S transcripts only slightly decreased in the differentiating HL-60 and PB neutrophils (Fig. 4B). Normalization of the PHF10-P and PHF10-S transcript levels to the amount of the total PHF10 demonstrated that PHF10-S content is growing relative to PHF10-P, with their expression becoming equal to PHF10-P in HL-60-ATRA, and PHF10-S being the predominant PHF10 isoform in PB neutrophils (Fig. 4C). Effectively, the levels of expression are reversed during myeloid differentiation.

3.5. The PHF10-Ss isoform replaces the PHF10-Pl isoform in the PB neutrophil PBAF complex

Next, we compared the expression levels of PHF10 isoforms and other PBAF subunits in the HL-60 and in the HL-60/ATRA cells and in PB neutrophils with Western-blot analysis (Fig. 5A, B). In line with the

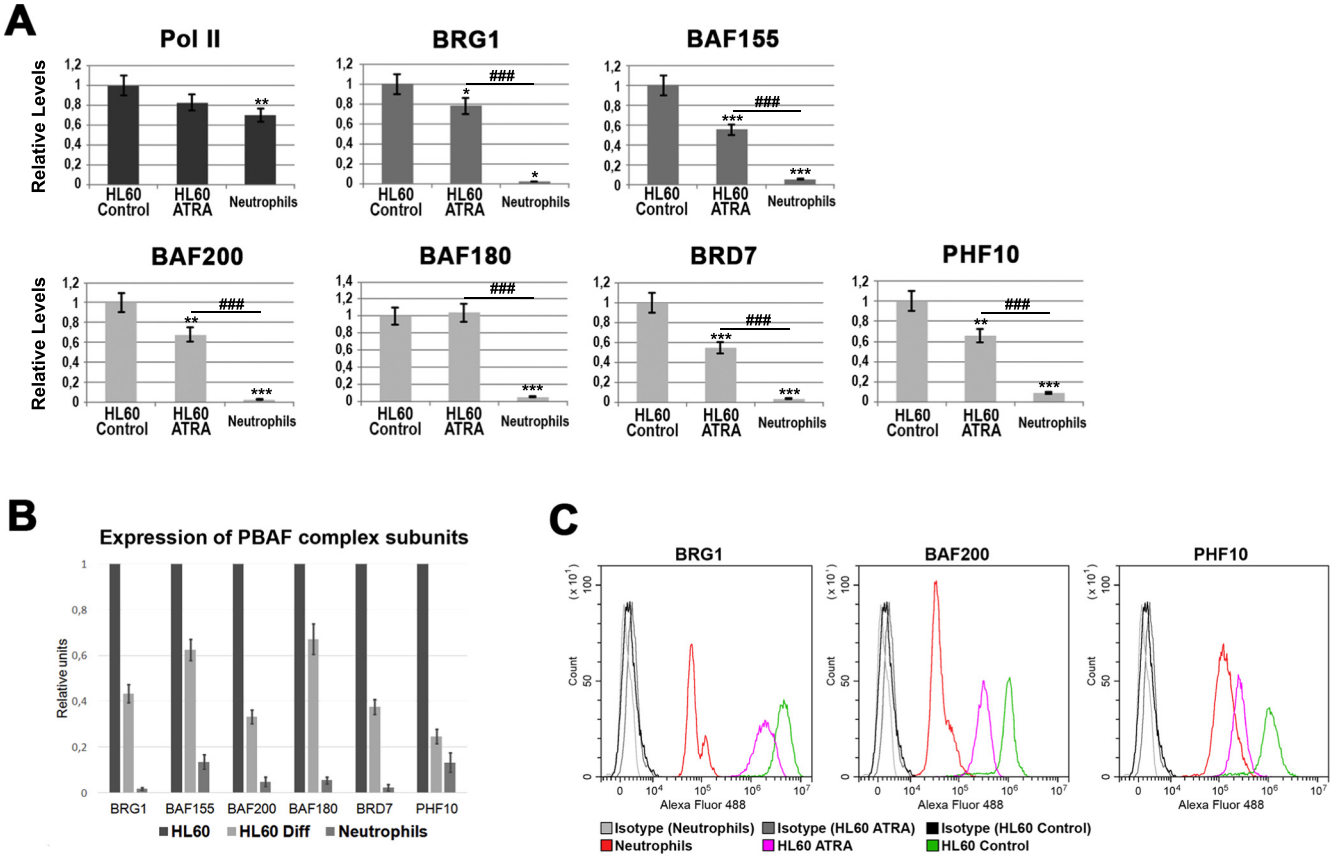

Fig. 2. The level of PBAF drops down following the start of HL-60 differentiation becoming very low in PB neutrophils.
(A) The transcription levels of genes encoding different PBAF subunits in control (HL-60), HL-60 treated for three days with ATRA, and in PB neutrophils measured by qRT-PCR. Values indicate the mean ± SD from five independent experiments and were normalized to RPLP0. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to the control; ### $p < 0.005$ for pairs of data compared (1-way ANOVA, Holm-Sidak's multiple comparison test).
(B) The levels of PBAF subunits in control (HL-60), HL-60 treated with ATRA for three days, and in neutrophils analyzed by flow cytometry, using protocol for intracellular protein analysis, described in Methods. Average data for all analyzed subunits is shown as X mean + SD of five samples pooled from three independent experiments. All data were compared in pairs with 2-way ANOVA, Holm-Sidak's multiple comparison test and have $p < 0.01$ or less.
(C) Fluorescence intensity of intracellular BRG1, BAF200, and PHF10 subunits and isotypic controls, measured by flow cytometry, analyzed populations are shown in Supplementary1 Fig. 1A. Cells were stained with primary antibodies for each protein and secondary antibodies conjugated with Alexa Fluor 488 according to protocol described in Methods.

decrease of corresponding transcripts the levels of BRG1, BAF200, BAF155, BAF180, and BRD7 proteins decreased upon differentiation (Fig. 5A, compare lanes 1 and 2, 3 and 4, Supplementary 1 Fig. 3A).

The PBAF subunits demonstrated the same pattern of bands in HL-60 and HL-60/ATRA, and in PB neutrophils (Fig. 5A). The only exception was the PHF10 subunit. In HL-60 cells, we detected all four PHF10 isoforms, including PHF10-Pl (Fig. 5A). The levels of the majority of the PHF10 isoforms significantly decreased following differentiation (Fig. 5A, compare lanes 1 and 2, 3 and 4; PHF10-P isoforms are indicated by arrowheads, PHF10-S isoforms are indicated by brackets, Supplementary 1 Fig. 3B), except for the PHF10 band which corresponded to the highly phosphorylated PHF10-Ss isoform (Fig. 5A, indicated by a dot). Moreover, while in the ATRA treated HL-60, its level was comparable with other isoforms, in PB neutrophils it substantially increased and this isoform became predominant (Fig. 5B, phosphorylated PHF10-Ss form is indicated by a dot). The PHF10-Ss lacks PHDs and is the smallest of the PHF10 isoforms, as it also lacks the 46 N-terminal amino acids [16].

The immunoprecipitations from the HL-60 and PB neutrophil extracts confirmed the results of the Western-blot analysis (Fig. 5C). It was performed with antibodies against total PHF10 or with antibodies against PHD domain of PHF10, which recognized only the PHF10-P isoforms [16]. The antibodies against total PHF10 precipitated a significant portion of all PHF10 isoforms from the HL-60 cell extract, including the phosphorylated forms (Fig. 5C, lane 2). Antibodies against PHD-domains precipitated both of the PHF10-P isoforms (Fig. 5C, lane 4). Comparison of lines 2 and 4 on Fig. 5C indicates that a significant amount of PHF10-S isoforms were also present in HL-60.

On the contrary, in PB neutrophils, antibodies against the total PHF10 protein predominantly precipitated the highly phosphorylated PHF10-Ss form (Fig. 5C, lane 7). There were also trace amounts of PHF10-Sl, and PHF10-Ps isoforms, but no PHF10-Pl isoform. Also, no PHF10-Pl was found in the precipitates made with the anti-PHD antibodies (Fig. 5C, lane 8).

Previously, we demonstrated that PHF10-Pl which contains C-terminal PHD domains and the N-terminal extension is the largest PHF10 isoform [16]. We have also demonstrated that it activates the proliferative genes [16]. In studies by other authors, PHF10-Pl is described as BAF45a [11,15], and shown to be essential for proliferation of undifferentiated cells [29]. Our data demonstrated that expression of PHF10-Pl started to decrease following the beginning of myeloid differentiation and PHF10-Pl was completely absent in PB neutrophils where it was replaced by highly phosphorylated PHF10-Ss. The PHF10-Ps and PHF10-Sl isoforms were expressed in PB neutrophils at an extremely low level compared to PHF10-Ss. Thus, in fully differentiated PB neutrophils, the PHF10-Pl with the PHD-domains is replaced by the PHF10-Ss isoform which lacks PHDs. We have previously shown that the hyperphosphorylated PHF10-Ss is a tightly bound subunit of the

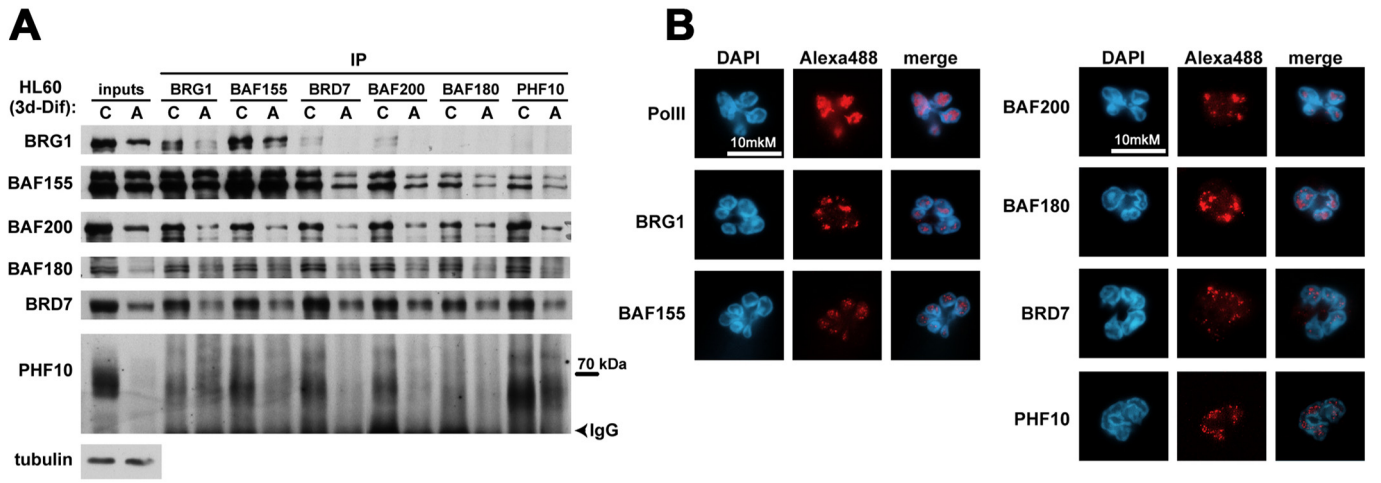

Fig. 3. PBAF maintains its integrity during myeloid differentiation and is localized at transcriptionally active chromatin of PB neutrophils.
(A) The results of co-immunoprecipitation demonstrate the integrity of the PBAF complex. The affinity purified antibodies against PBAF subunits (indicated on top) were used to co-precipitate the PBAF complex from the extract of control HL-60 (C) or HL-60/ATRA (A) cells. The precipitates were fractionated by SDS PAGE and Western-blot membrane was stained with indicated antibodies. Equal numbers of control and differentiated cells were taken for the lysate preparations. Tubulin was used as a loading control.
(B) The nuclei of PB neutrophils stained with antibodies (Alexa 488, red) against Pol II or PBAF subunits (indicated on the left) and co-stained with DAPI (blue) and the merged images.

PBAF complex, and is more stable than the non-phosphorylated form [17,20].

3.6. PHF10-P isoforms are present at promoters of myeloid-specific genes during their activation, but are replaced by PHF10-Ss at the continuously transcribed genes

The data presented in Fig. 2A, indicated that the level of Pol II

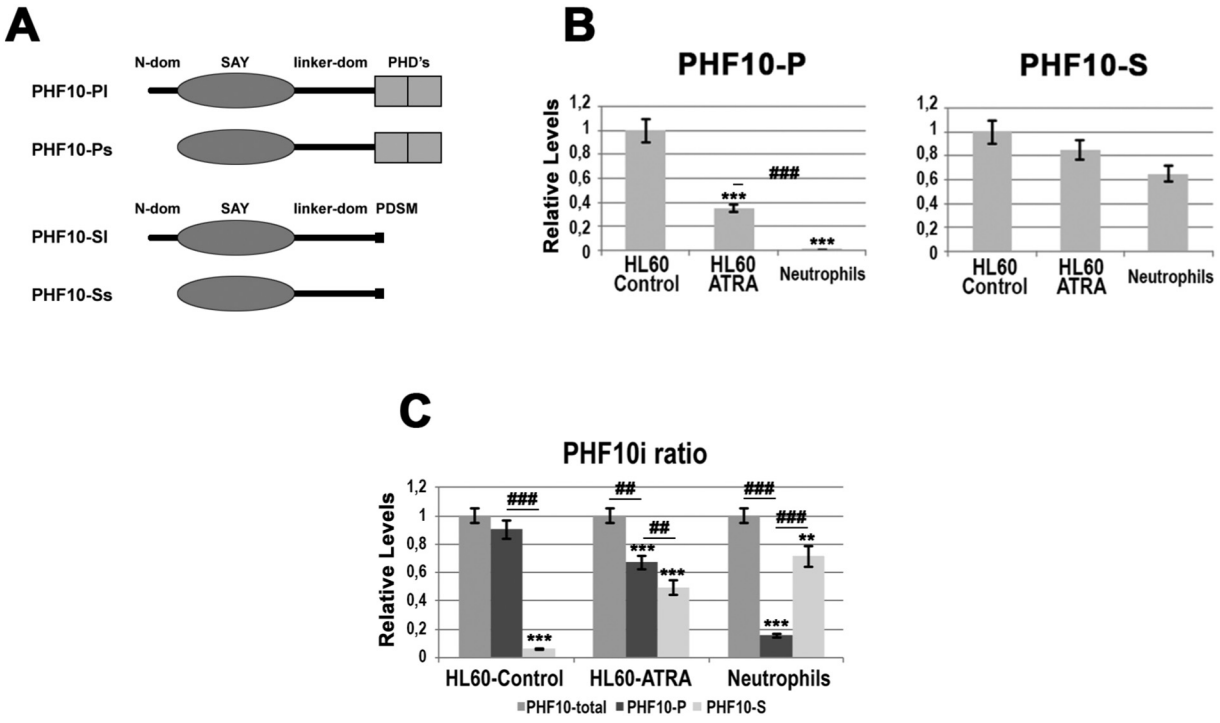

Fig. 4. The transcription levels of PHF10-P, but not PHF10-S isoforms decrease following differentiation.
(A) A schematic of the PHF10 isoforms: dark-gray ovals indicate SAY domains, light-gray boxes indicate PHD fingers of PHF10-P isoforms, black small boxes indicate PDSM motifs for SUMO I conjugation of PHF10-S isoforms. The N-domains are present in the long PHF10 isoforms.
(B) The transcription levels of PHF10–P and PHF10-S isoforms in the control (HL-60), HL-60 treated for three days with ATRA, and in PB neutrophils, measured by qRT-PCR. Values indicate the mean ± SD from five independent experiments and were normalized to RPLP0. * $p < 0.05$, *** $p < 0.005$ comparatively to the control; $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.005$ for pairs of data compared (1-way ANOVA, Holm-Sidak's multiple comparison test).
(C) The ratio of transcripts encoding PHF10–P and PHF10-S isoforms in the control (HL-60), HL-60 treated for three days with ATRA, and in PB neutrophils. The levels of PHF10-P and PHF10-S transcripts are shown relative to the total amount of PHF10. Values indicate the mean ± SD from five independent experiments. ** $p < 0.01$, *** $p < 0.005$ compared to the control; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.005$ for pairs of data compared (2-way ANOVA, Holm-Sidak's multiple comparison test).

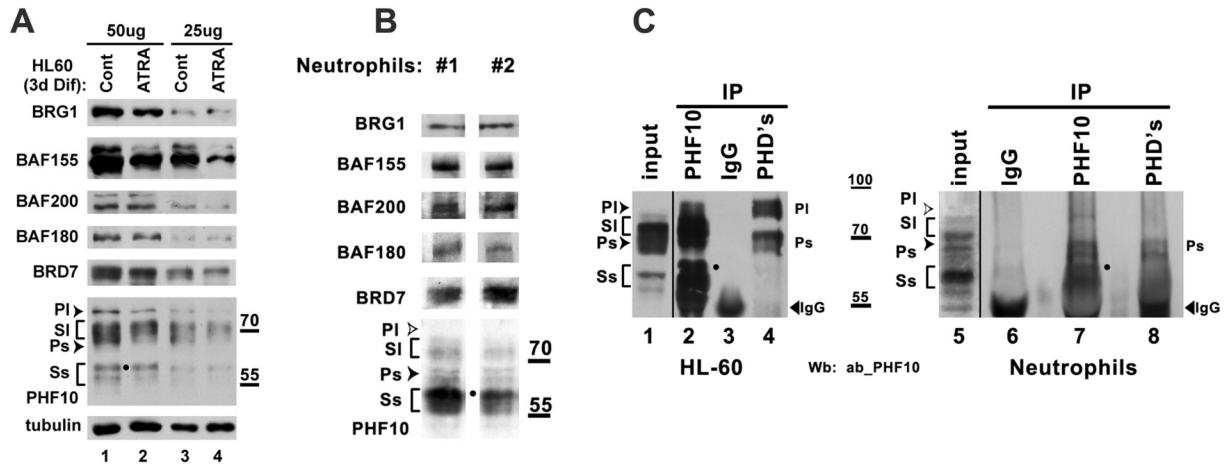

Fig. 5. The PHF10-Pl isoform is replaced by the PHF10-Ss isoform during myeloid differentiation.
(A) The PBAF subunits in HL-60 (control) and HL-60 after three days of ATRA treatment (ATRA), detected by Western-blot analysis. The protein extracts from corresponding cells were resolved by SDS PAGE. Different amounts of protein extract were loaded on a gel (lane 1 and 2, 50 μg; lane 3 and 4, 25 μg) for better visualization of PBAF subunits. Tubulin was used as a loading control.
(B) The PBAF subunits in PB neutrophils from two different donors detected by Western-blot analysis. On panels A and B the antibodies used for staining are shown on the left. The PHD-containing isoforms PHF10-Pl (Pl) and PHF10-Ps (Ps) are marked by arrowheads. Empty arrowhead indicates PHF10-Pl isoform, which is absent in PB neutrophils. Two patterns of bands corresponding to differently phosphorylated PDSM-containing isoforms, PHF10-Sl (Sl) and PHF10-Ss (Ss), are marked by brackets. The protein band corresponding to the highly phosphorylated PHF10-Ss is marked by a dot.
(C) The immunoprecipitation of different PHF10-isoforms from the extracts of HL-60 (left panel) and PB neutrophils. The antibodies against total PHF10 (PHF10) and its PHD domains (PHDs) were used for immunoprecipitation. The PHF10 isoforms are marked as on the previous panels.

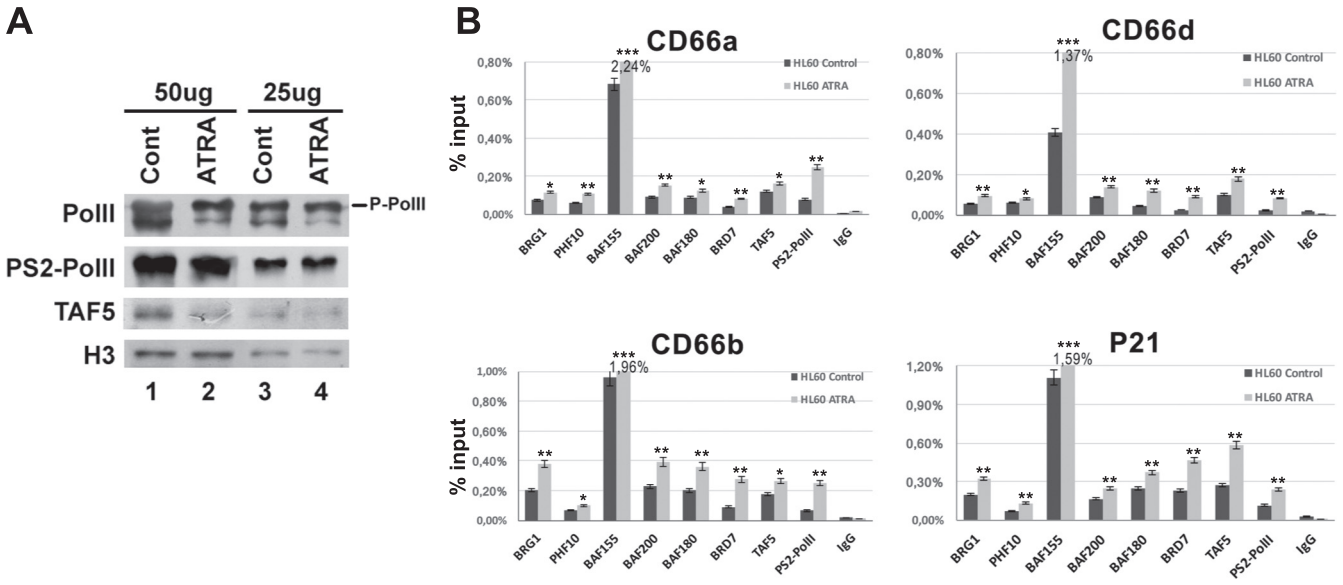

Fig. 6. The PBAF is present as a part of the preassembled Pol II transcription initiation complex on promoters of inactive myeloid-specific genes.
(A) The levels of total Pol II, PS2-Pol II, and TAF5 subunit of TFIID in HL-60, and HL-60/ATRA cells. The same amounts of protein extracts from HL-60 (Control) and HL-60/ATRA (ATRA) cells were resolved by SDS PAGE. The membrane was developed with antibodies against proteins indicated on the left of the panels. The antibodies against Pol II recognize both non-phosphorylated and phosphorylated forms of the protein. H3 was used as loading controls (10 times less of the amount was loaded).
(B) ChIP analysis of recruitment of PBAF subunits, phosphorylated Pol II (PS2-PolII) and TAF5 on promoters of CD66a, CD66b, CD66d, and p21 genes in HL-60 and HL-60/ATRA cells. Here and in Fig. 7, ChIP data are presented as a percentage of the input. Values indicate the mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.005$ compared to the control; (2-way ANOVA, Holm-Sidak's multiple comparison test).

encoding transcript did not change significantly during myeloid differentiation. Western-blot analysis with antibodies against total Pol II (Fig. 6A, upper panel, Supplementary 1 Fig. 3C) which recognizes both non-phosphorylated (lower band) and phosphorylated (upper band) forms of Pol II, also did not reveal a strong decrease in the total Pol II level (Fig. 6A, compare lane 1 and 2, or 3 and 4). The level of active PS2-Pol II did not change (Fig. 6A). At the same time, the level of non-phosphorylated inactive Pol II significantly decreased (Fig. 6A, upper panel, lower band), indicating that most of Pol II present in differentiated HL-60, is active. This must reflect significant changes in the mode of total transcription and is likely connected to the constant transcription of the lineage-specific genes in the mature neutrophils. The active transcription following differentiation is also confirmed by the level of TAF5, the subunit of preinitiation complex of Pol II, which decreased about twofold in HL-60/ATRA cells (Fig. 6A, compare lane 1 and 2, or 3 and 4).

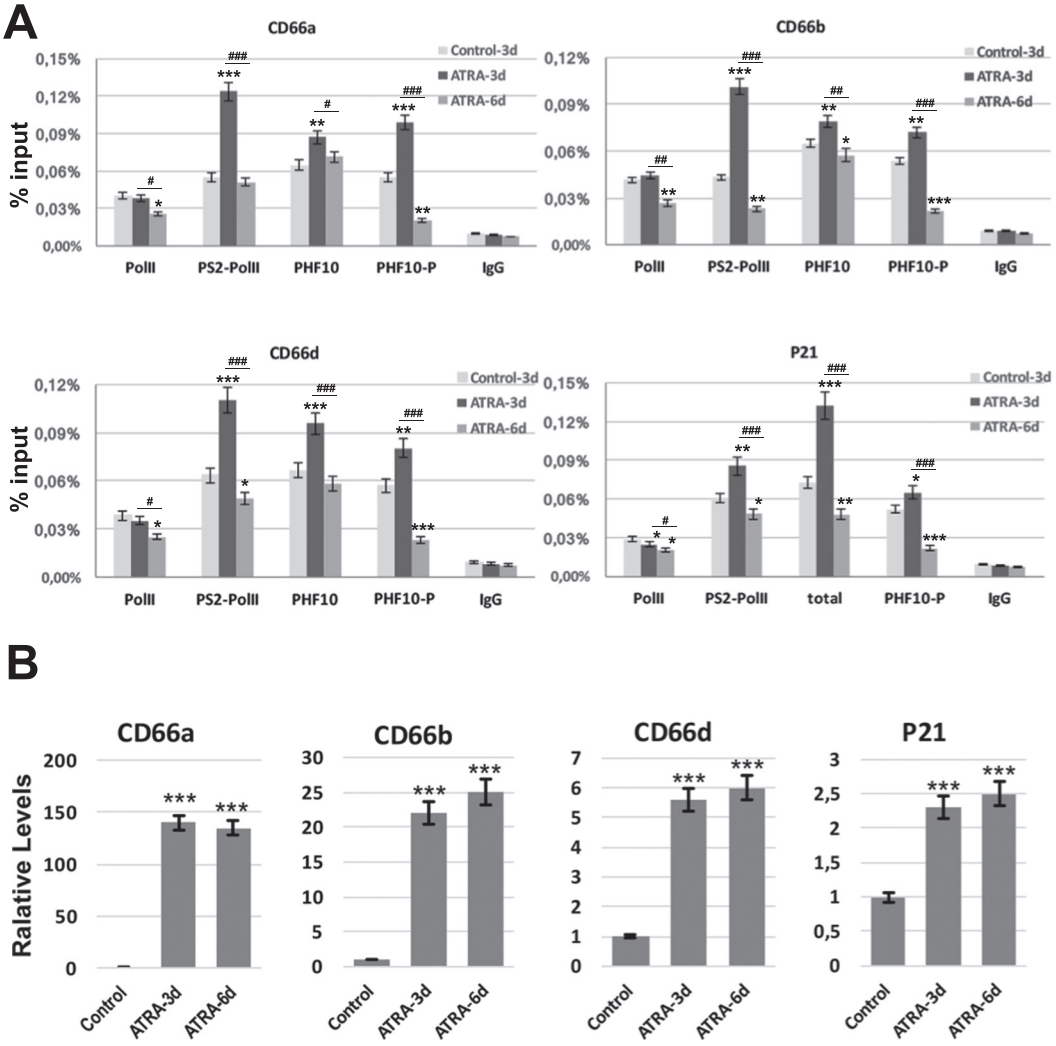

Fig. 7. The occupancy of promoters of genes activated following the start of myeloid differentiation by the PHF10 isoforms and Pol II.
(A) ChIP analysis of recruitment of the PHF10 isoform subunits, phosphorylated Pol II (PS2-PolII), and TAF5 on the promoters of CD66a, CD66b, CD66d, and p21 genes in HL-60 and HL-60/ATRA cells on the third and sixth day following the start ATRA treatment. ChIP data are presented as a percentage of the input. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to the control; $^{\#}$ $p < 0.05$ $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.005$ for pairs of data compared (2-way ANOVA, Holm-Sidak's multiple comparison test).
(B) The transcription levels of CD66a, CD66b, CD66d, and p21 in HL-60 and HL-60/ATRA treated cells on the third and sixth day following the start of ATRA treatment, measured by RT-PCR. The data shown for HL-60 and HL-60/ATRA cells on the third day are the same as in Fig. 1A. *** $p < 0.005$ compared to the control (1-way ANOVA, Holm-Sidak's multiple comparison test).

Indeed, the expression of neutrophil-specific genes, like CD66a, CD66b, and CD66d or genes responsible for the exit from the cell cycle progression, like p21, was strongly increased (Fig. 1A; D). As verified by chromatin immunoprecipitation (ChIP), Pol II together with TAF5 was found on CD66a, CD66b, CD66d, and p21 promoters in HL-60, suggesting that the preassembled Pol II complex was present on these genes before activation (Figs. 6B, 7A). The level of total Pol II did not change following activation on these promotors (Fig. 7A). However, in line with results presented on Fig. 6A the strong increase of phosphorylated Pol II (PS2-Pol II) on promoters was detected after day 3 (Figs. 6B, 7A).

The PBAF subunits were also present on studied gene promoters before their active transcription (Fig. 6B). We detected core (BRG1, BAF155) and specific (BAF200, BAF180, BRD7, and PHF10) PBAF subunits on CD66a/b/d promoters during differentiation (Fig. 6B). Thus, PBAF complex may be a part of the co-activator complex which regulates the expression of the myeloid-specific genes CD66a/b/d and p21.

Next, we examined the levels of PHF10 isoforms on the CD66a/b/d and p21 promoters in HL-60 and in HL-60/ATRA cells, on the third and sixth days of differentiation (Fig. 7A). The antibodies against total PHF10 and against PHD domains were used for this ChIP. The dynamics of the PHF10 association was similar for all the studied genes. The levels of PHD-containing isoforms on p21, CD66a, CD66b, and CD66d promoters increased on the third day and strongly diminished (close to IgG control) on the sixth day of differentiation (Fig. 7A). However, the levels of total PHF10 on the promoters at day 6 did not change significantly. This indicates that at the later stages of differentiation, nearly all of the PHF10 on the promoters of the studied genes was represented by the PHF10-S isoforms. In the terminally differentiated PB neutrophils, this is expected to be the PHF10-Ss isoform, since it is the predominant isoform at this stage of myelogenesis (Fig. 5B).

Transcription levels of p21, CD66a, CD66b, and CD66d on the third day after the induction of differentiation were still growing, while on the sixth day the transcription levels became constant (Fig. 7B). Thus, PHD-containing isoforms were present on promoters of myeloid-specific genes when their transcription was rapidly activated, and were replaced by PHF10-Ss when genes were constantly transcribed in the terminally differentiated cells.

4. Discussion

The normally short-lived intermediate myeloid progenitors undergo rapid proliferation before they differentiate into functional immune cells. The balance between proliferation and differentiation is tightly controlled by the stage-specific transcription factors and epigenetic modifications.

Here we demonstrate that in the course of myeloid differentiation the total transcription level (the level of PS2-Pol II) did not change significantly but the content of the PBAF chromatin remodeling complex proteins strongly decreased. In addition, in the terminally differentiated neutrophils, the PHD-containing PHF10-Pl isoform of the PHF10 subunit was replaced by the PHF10-Ss isoform which lacks PHD domains. Previously, we have demonstrated that the PHF10-Pl isoform activates cell cycle progression and attracts Pol II to the promoters of the cell cycle regulating genes [16]. In line with these results, in this study we have demonstrated that PHF10-Pl is expressed in the actively proliferating precursor cells and its level drops following the start of differentiation, and it is completely absent from the terminally differentiated PB neutrophils.

Earlier, the BAF45a (PHF10-Pl isoform) together with BAF53a was shown to be a component of SWI2/SNF2 in the murine neural stem cells, and to leave the complex as the neural progenitors exit the cell cycle, and are replaced by the homologous BAF45b, BAF45c, and BAF53b[15] The murine BAF45a (PHF10-Pl isoform) is also essential for maintaining the adult hemopoietic stem cells [11]. In line with this data, we demonstrated the expression of human PHF10-Pl in the proliferating myeloid cells. However, as the myeloid progenitors exited the cell cycle and PHF10 subunit did not leave the PBAF complex, their ratio did change. Following a decrease in the expression of other isoforms, the PHF10-Ss became the predominant form of PHF10 expressed in the mature PB neutrophils. All the PHF10-Ss detected in the mature PB neutrophils were highly phosphorylated. As phosphorylation of PHF10-Ss ensures its association with PBAF [17], we conclude that in PB neutrophils we observed only the functional form of PHF10-Ss.

The chromatin of precursor cells undergoes multiple rounds of remodeling which accompany activation and repression of genes during the cell cycle progression. Following the exit from cell cycle and the start of differentiation, the chromatin of repressed myeloid-specific genes should be also remodeled to start their expression, which then is constantly maintained in the mature PB neutrophils. On the other hand, the mature PB neutrophils do not require high level of chromatin remodeling as the pattern of expressed genes is mostly stable. In line with this suggestion, we observed a strong drop in the PBAF proteins in PB neutrophils. Still, PBAF was localized at the actively transcribed chromatin and was found on promoters of myeloid-specific genes. We have also found that the majority of Pol II present in the differentiated myeloid cells, was highly phosphorylated on Ser2 of the CTD domain. The high level of the phosphorylated active Pol II likely reflected constant transcription of myeloid-specific genes in the differentiating cells.

Our study demonstrates that the preassembled Pol II transcription complex, including the PBAF, binds to promoters of myeloid-specific genes in the proliferating precursor cells when these genes are inactive. As shown by ChIP, the PHD-containing isoforms of PHF10 were present together with other PBAF subunits on the myeloid-specific genes before the start of their transcription and during their activation. It is likely, that of the two PHF10-P isoforms, only the short one (PHD-Ps which lacks N-terminal amino acids) was present on the promoters of myeloid-specific genes, as PHF10-Pl was shown to counteract differentiation [11]. At the later stages of differentiation, when myeloid-specific genes were constantly transcribed, the level of the PHD-containing isoforms on their promoters strongly diminished and they were replaced by the PHF10-Ss isoform. We suggest that the PHD-containing isoforms must be important for the extensive chromatin remodeling at the initial stages of transcription, when a gene rapidly changes its transcription status. This type of remodeling should be a characteristic attribute of a significant number of genes in the proliferating precursor cells, as well as for the myeloid-specific genes during their activation.

The difference in the structure of the PHF10-P and PHF10-S isoforms, is the presence of two PHD domains at the C-terminus of the PHF10-P isoforms. PHF10-S isoforms do not have PHD domain, but contain a C-terminal PDSM motif. Interestingly, both types of isoforms are conserved in mammals [16] suggesting their important function in gene regulation. Taking into account that PHF10 is a subunit of the specific PBAF module, which participates in target gene recognition [15,16], it could be suggested that the replacement of PHF10 isoforms leads to certain alterations in the mechanism of chromatin remodeling which accompanies different transcription status of target genes. Indeed, PHD domains of different proteins were shown to interact with a wide range of histone modifications [30,31]. Importantly several subunits of BAF chromatin remodeling complex BAF45B (DPF1), BAF45C (DPF3), and BAF45D (DPF2) also contain C-terminal tandem PHD domains that show significant sequence conservation with PHF10 [32–35]. The DPF2 was shown to inhibit the myeloid differentiation of hematopoietic stem/progenitor and acute myelogenous leukemia cells [33,35]. Moreover, the important role of PHD-dependent histone binding of DPF2 in regulation of myeloid differentiation was demonstrated [35]. The double PHD-containing PBAF subunits were shown to be implicated in the acute myelogenous leukemia and a wide range of other human cancers [33,36–38].

Our study demonstrates that on the already activated genes, which maintain the constant transcription levels the PHD-containing isoforms, are replaced by PHF10-S forms. This indicates that the PHD domains of PHF10 must be important for nucleosome remodeling required to activate transcription, but are dispensable for the maintenance of the constant transcription of already activated genes.

The other difference we found between the isoforms, is their different stability in the cell which is dependent on the different post-translational phosphorylation [17]. The unstable PHF10-P isoforms have a short half-life in the cell [17], and thus, their level may rapidly decrease following the start of differentiation. On the other hand, the stable PHF10-Ss isoform is expressed in the terminally differentiated cells.

The SWI/SNF complex acts as a dual regulator of the general cell cycle genes and the cell type-specific genes. Expression of genes coding for p21, p16, Cyclin D1, and Cyclin E1 is directly controlled by the subunits of SWI/SNF [39,40], and different SWI/SNF subunits are frequently mutated in different cancers [41].

Several components of SWI/SNF were shown to participate in myelopoiesis. BAF60b is required for myeloid differentiation, and its knockout leads to a block in differentiation of LSK (lineage negative, Sca1 positive, c-kit negative) bone marrow cells [9]. The chromatin remodeling function of Brg1 is necessary for the G-CSF dependent differentiation of myeloid cells towards the granulocytic lineage. This dependency on Brg1 may reflect a stringent requirement for chromatin remodeling at a critical stage of hematopoietic cell maturation [5].

In summary, the replacement of PHF10 isoforms in PBAF complex which accompanies the transition from proliferating to the committed myelogenic lineages may represent one of the mechanisms ensuring the global changes in transcription and chromatin organization in the course of differentiation.

Ethics statement

The authors prepared neutrophils from the blood of healthy volunteers. Blood was collected via venous puncture, as approved by the Ministry of Public Health Service of the Russian Federation. Experimental and the subject consent procedures were approved by the Institutional Ethics Committee of the A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University. Fully informed consent was obtained, and all investigations were conducted according to the principles laid down in the Declaration of Helsinki.

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Author contributions

G.M.V., V.V.T., A.A.Sh. and Eu.V.T. performed the experimental work. G.M.V., V.V.T., G.F.S. and N.V.S. analyzed data, G.M.V., S.S.G., and N.V.S. planned the experiments and wrote the paper.

Transparency document

The Transparency document associated with this article can be found, in online version.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its Supplementary information file).

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2019.118525.

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