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GCN5 Regulates the Superoxide-Generating System in Leukocytes Via Controlling gp91-phox Gene Expression

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†

The superoxide anion (O$_2^-$)-generating system is an important mechanism of innate immune response against microbial infection in phagocytes and is involved in signal transduction mediated by various physiological and pathological signals in phagocytes and other cells, including B lymphocytes. The O$_2^-$-generating system is composed of five specific proteins: p22-phox, gp91-phox, p40-phox, p47-phox, and p67-phox, and a small G protein, Rac. Little is known regarding epigenetic regulation of the genes constituting the O$_2^-$-generating system. In this study, by analyzing the GCN5 (one of most important histone acetyltransferases)-deficient DT40 cell line, we show that GCN5 deficiency causes loss of the O$_2^-$-generating activity. Interestingly, transcription of the gp91-phox gene was drastically downregulated (to ~4%) in GCN5-deficient cells. To further study the involvement of GCN5 in transcriptional regulation of gp91-phox, we used in vitro differentiation system of U937 cells. When human monoblastic U937 cells were cultured in the presence of IFN-$\gamma$, transcription of gp91-phox was remarkably upregulated, and the cells were differentiated to macrophage-like cells that can produce O$_2^-$

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The online version of this article contains supplemental material. Abbreviations used in this article: CBP, CREB-binding protein; CGD, chronic granulomatous disease; ChIP, chromatin immunoprecipitation; CL, chemiluminescence; HAT, histone acetyltransferases; H2BK16, Lys$^4$ of H2B; H3K9, Lys$^9$ of H3; O$_2^-$, superoxide anion; PCAF, p300/CREB-binding protein-associated factor; SOD, superoxide dismutase.
to early embryonic lethality with increased apoptosis in mesodermal lineages (36–38). Recently, it was reported that loss of GCN5 in mouse embryonic stem cells invoked a cell-autonomous pathway of cell death (39). However, its functions remain poorly defined in vertebrate cells.

Using gene-targeting techniques (40), in contrast, we generated homozygous chicken DT40 mutant cell line, GCN5\(^{-/-}\), devoid of two GCN5 alleles (41). The GCN5 deficiency not only caused delayed growth rate and suppressed cell cycle progression at G1/S phase transition, but also caused down- or upregulation of various G1/S phase transition-related genes. Further, GCN5 was shown to induce premature B cell apoptosis by collaboration with BCR signaling (42). In this study, we show that the depletion of GCN5 drastically inhibits the O\(_2^+\)-generating activity in DT40. In addition, to further examine the role of GCN5 in the O\(_2^+\)-generating system, monoblastic leukemia U937 cell line was used as a model. Based on findings obtained in this study, we clarified the participation of GCN5 in epigenetic regulation of the O\(_2^+\)-generating system in leukocytes via controlling gp91-phox gene expression through elevation in acetylation of H2BK16 and H3K9 surrounding its promoter region.

**Materials and Methods**

**Materials**

PMA (Calbiochem), human IFN-\(\gamma\) (Roche), Diogene (National Diagnostics), normal rabbit serum (Vector Laboratories), PMSF (Wako), bovine aprotinin, laminin, normal rabbit IgG, and superoxide dismutase (SOD) (Sigma-Aldrich) were obtained. Anti-CREB–binding protein (CBP), anti-GCN5, anti-p300, and anti-p300/CBP-associated factor (PCAF) Abs (Santa Cruz Biotechnology), all anti-acetylated histone Abs (Millipore), monoclonal anti-gp91-phox Ab (BD Bioscience), anti-\(\beta\)-actin Ab (Abcam), HRP-conjugated goat anti-\(\beta\)-actin Ig, and HRP-conjugated rabbit anti-mouse Ig (DakoCytomation) were used.

**Cell cultures and monoclonal expression of U937 cells with IFN-\(\gamma\)**

Generation of GCN5\(^{-/-}\) was described in our previous report (41). DT40 cells and all subclones were cultured essentially as described (41, 42).

**Abbreviation of superoxide generation**

O\(_2^+\) was quantified by measuring SOD-sensitive chemiluminescence (CL) using GCN5 expression vector (Supplemental Fig. 2). Unfortunacy, PMA-stimulation for 24 h was carried out at 96°C for 20 s, 55°C for 30 s, and 72°C for 30 s, for 30–40 cycles, using sense (forward) primer 5'-TCGGATGAGGACATTCAGTCG-3' and antisense (reverse) primer 5'-TCATCTCCTGCACTTCCACG-3' (Supplemental Fig. 1). PCRs were stopped before reaching the plateau. PCR products were subjected to 1.5% agarose gel electrophoresis and analyzed by Image Gauge software Profile mode (densitometrical analysis mode) using a luminescent image analyzer, LAS-1000plus (Fujifilm).

**Results**

**Loss of superoxide-generating activity and drastic downregulation of gp91-phox gene expression in GCN5\(^{-/-}\) cells**

We examined effects of GCN5 deficiency on the O\(_2^+\)-generating activity (Fig. 1A). As expected, DT40 cells generated CL when stimulated with PMA, and it was completely abolished by SOD (100 \(\mu\)g/ml). These results suggested that CL generated by PMA-stimulated DT40 cells was preferentially derived from O\(_2^+\). In contrast, PMA-stimulation for GCN5\(^{-/-}\) (three independent clones tested) showed a negligible level of O\(_2^+\) generation, indicating that depletion of GCN5 completely prevented the O\(_2^+\)-generating system. Next, to know the effects of GCN5 deficiency on the O\(_2^+\)-generating activity, four genes responsible for CGD (p22-phox, gp91-phox, p47-phox, and p67-phox), we carried out semi-quantitative RT-PCR on total mRNAs prepared from DT40 and three independent GCN5\(^{-/-}\) clones (Fig. 1B). Interestingly, transcription of the gp91-phox gene was drastically downregulated in GCN5\(^{-/-}\) (to ~4%). Transcription levels of p22-phox and p67-phox genes were decreased (to ~65 and ~80%), whereas p47-phox mRNA level was increased (to ~170%). These results revealed that the drastic effect of GCN5 deficiency on the gene expression of gp91-phox probably resulted in the loss of O\(_2^+\)-generating activity. Next, to confirm the role of GCN5 in the gene expression of gp91-phox, re-expression study was carried out using GCN5 expression vector (Supplemental Fig. 2). Unfort-
and three independent GCN5−/− clones (1 × 10⁵ cells/ml) were stimulated by 200 ng/ml PMA (at time indicated by arrow) at 37°C, and PMA-induced CLs were monitored by LB9505C. CL was completely abolished by the addition of SOD (100 μg/ml). B, Total RNAs were extracted from DT40 and three independent GCN5−/− clones, and mRNA levels were determined by semiquantitative RT-PCR using appropriate primers. Chicken GAPDH gene was used as internal control. Numbers under the panels indicate cycle numbers of PCR. The gel images obtained were analyzed by Image Gauge Software Profile 6 SD) obtained from DT40 (wild-type) at the right of each panel.

FIGURE 1. Influences of GCN5 deficiency on the O₂⁻-generating activity and gene expressions of the O₂⁻-generating system-related factors. A, DT40 and three independent GCN5−/− clones (1 × 10⁵ cells/ml) were stimulated by 200 ng/ml PMA (at time indicated by arrow) at 37°C, and PMA-induced CLs were monitored by LB9505C. CL was completely abolished by the addition of SOD (100 μg/ml). B, Total RNAs were extracted from DT40 and three independent GCN5−/− clones, and mRNA levels were determined by semiquantitative RT-PCR using appropriate primers. Chicken GAPDH gene was used as internal control. Numbers under the panels indicate cycle numbers of PCR. The gel images obtained were analyzed by Image Gauge Software Profile 6 SD) obtained from DT40 (wild-type) at the right of each panel.

Unfortunately, re-expression of GCN5 could not complement both the decreases in the gp91-phox gene expression and the loss of O₂⁻-generating activity (Supplemental Fig. 2B). The gene expression of PCAF that was extremely increased in GCN5−/− (41) was further upregulated by re-expression of GCN5 (to ~200% of GCN5−/−) (Supplemental Fig. 2C). These results suggested that PCAF could play, in part, complementary roles in chromatin dynamics linked to the acetylation state of core histones in absence of GCN5 (41), and the altered epigenetic state caused by GCN5 deficiency may not be restored by re-expression of GCN5. Its molecular mechanism remains to be resolved. In addition, we examined effects of PCAF deficiency on the O₂⁻-generating activity using PCAF−/− [homozygous DT40 mutant cell line devoid of two PCAF alleles (41)] (Supplemental Fig. 3A). PMA stimulation for PCAF−/− (three independent clones tested) induced about a half level of O₂⁻ generation, indicating that the depletion of PCAF partly repressed the O₂⁻-generating system. To determine effects of PCAF deficiency on transcriptions of p22-phox, gp91-phox, p47-phox, and p67-phox genes, we carried out semiquantitative RT-PCR on total mRNAs prepared from DT40 and three independent PCAF−/− clones (Supplemental Fig. 3B). Transcriptions of the gp91-phox and p22-phox genes were downregulated (to ~45 and to ~80%), and the decreases were less when compared with those in GCN5−/− clones. The deficiency showed insignificant influence on transcription of the p47-phox gene, whereas the transcription level of the p67-phox gene was considerably increased (to ~150%). These results suggested that in regulation of gp91-phox gene expression, GCN5 acts as a supervisor, and PCAF plays a supporting role.

Effects of IFN-γ treatment on gene expression of gp91-phox and GCN5 in U937 cells

To clarify molecular mechanisms for regulation of the O₂⁻-generating system via controlling the gp91-phox gene expression by GCN5, human monoblastic U937 cells were used as an in vitro model for monocytic differentiation (43, 44). When U937 cells are cultured in the presence of various agents, they differentiate to macrophage-like cells that can produce O₂⁻. IFN-γ, one of the effective inducers for monocytic differentiation, induces the gp91-phox gene expression, and the induction mechanisms mediated by IFN-γ have been well studied (20, 21, 24, 27, 28, 30, 46, 47).

Undifferentiated U937 cells had no activity of O₂⁻ generation when stimulated with PMA. After 48 h incubation with IFN-γ, they acquired the O₂⁻-generating activity as judged by CL assay (43, 44) (Fig. 2A). Remarkable increases in gp91-phox gene expression (to ~850%) and also in protein level (to ~1200%) were observed in parallel with induction of the O₂⁻-generating activity, whereas insignificant changes in the GCN5 gene expression and also in protein level were detected (Fig. 2B, 2C). In addition, the IFN-γ-induced O₂⁻-generating activity and increased gene expression and protein level of gp91-phox in U937 cells were completely inhibited by addition of 50 μM GCN5 inhibitor CPTH2 (48) (Fig. 3, Supplemental Fig. 4). Interestingly, CPTH2 remarkably downregulated protein levels of p47-phox and p67-phox (Supplemental Fig. 4B), although it showed insignificant effects on gene expressions of these two factors (Fig. 3B, Supplemental Fig. 4A). We also examined inhibition properties of CPTH2 (Supplemental Fig. 5). CPTH2 inhibited the growth of U937 cells (Supplemental Fig. 5A), induction of O₂⁻-generating activity (Supplemental Fig. 5B), and gene expression of gp91-phox (Supplemental Fig. 5C) in a dose-dependent manner, whereas it showed an insignificant effect on cell viability (data not shown).

In addition, CPTH2 showed a high specificity against GCN5 family, especially GCN5 (Supplemental Fig. 5D). These data, together with the findings obtained by the gene targeting study using DT40 (Fig. 1), supported the important participation of GCN5 in regulating the O₂⁻-generating activity via controlling gp91-phox gene expression.

IFN-γ enhances interaction of GCN5 with gp91-phox gene promoter and acetylation levels of H2BK16 and H3K9 residues surrounding the promoter

Many transcription factors are involved in regulation of the gp91-phox gene expression through their interaction with its promoter region (16–31). As is well known, HATs play critical roles in modulation of chromatin topology and thereby regulation of gene expression through acetylation of core histones. Such acetylation neutralizes positive charges to enhance hydrophobicity of core histones, resulting in the reduced affinity of acetylated N-terminal
tails of core histones to DNA (32, 33). The attenuated histone–DNA interaction is believed to change chromatin configuration for transcription activation through promoting transcription factor–DNA interaction, but molecular mechanisms of the acceleration of the gp91-phox gene expression by any HATs remain to be resolved. Because GCN5 deficiency caused a drastic decrease in the gp91-phox gene expression in DT40 cells resulting in loss of the O$_2^-$-generating activity (Fig. 1), GCN5 is expected to be the most important HAT in controlling the gene expression. To clarify molecular mechanisms for the gp91-phox gene expression regulated by GCN5, we investigated interaction of GCN5 and other major HATs (PCAF, CBP, and p300) with gp91-phox promoter by ChIP assay in U937 cells (Fig. 4). Cross-linked chromatin were coprecipitated from the lysates of U937 cells (48 h treated or untreated with IFN-γ) with Abs specific for GCN5, PCAF, CBP, and p300 or irrelevant normal IgG as a negative control. Precipitated chromatins were amplified by PCR using primers flanking the critical cis-element (Hox/Pbx consensus-like sequence) and inverted PU.1 binding site in the proximal gp91-phox gene promoter (Supplemental Fig. 1) (25, 30, 46). The data obtained showed not only that GCN5 and CBP associated with the gp91-phox gene promoter (Fig. 4A), but also that association of GCN5 or CBP with the promoter was remarkably increased or unchanged in U937 cells by IFN-γ treatment (Fig. 4A). In contrast, associations of PCAF and p300 with the gp91-phox gene promoter could not be detected by ChIP assay using several available Abs during cultivation with IFN-γ (0, 12, 24, and 48 h) (data not shown). Time-course study also showed that in IFN-γ–treated U937 cells, the interaction of GCN5 with the gp91-phox gene promoter was dramatically upregulated (to ∼300% by 48 h), although IFN-γ treatment showed an insignificant effect on the interaction of CBP with the promoter (Fig. 4B). Further, we investigated histone acetylation levels surrounding the gp91-phox gene promoter during cultivation of U937 cells with IFN-γ by ChIP assay using various anti-acetylated histone Abs (Fig. 5). Cross-linked chromatins were coprecipitated from the lysates of U937 cells (48 h treated or untreated with IFN-γ) with various antisera specific for acetylated Lys residues of histones or irrelevant normal serum as a negative control. PCR was carried out as described above. The IFN-γ treatment showed significant influences on acetylation levels of Lys$^16$ of H2B (H2BK16) and Lys$^9$ of H3 (H3K9) among the Lys residues of core histones H2A, H2B, H3, and H4 tested (Fig. 5A, 5B). Acetylations of H2BK16 and H3K9 surrounding the gp91-phox gene promoter were remarkably increased to ∼220 and ∼350% by 48 h, respectively, in a time-course manner during cultivation of U937 cells with IFN-γ (Fig. 5C), whereas IFN-γ treatment showed insignificant effects on acetylations of other Lys residues tested by 24 (data not shown) and 48 h (Fig. 5A). In addition, the increases in acetylation levels of both H2BK16 and H3K9 by IFN-γ were completely inhibited by addition of CPTH2 (Fig. 5D). We also examined interaction of PU.1 with the gp91-phox gene promoter during cultivation with IFN-γ (Supplemental Fig. 6). ChIP assay using anti-PU.1 Ab revealed that interaction of PU.1 with the promoter was upregulated during cultivation with IFN-γ (Supplemental Fig. 6A, 6B), and the increase was completely inhibited by addition of CPTH2 (Supplemental Fig. 6C). These results indicated not only that the association of GCN5 with the gp91-phox gene promoter was significantly accelerated in U937 cells with IFN-γ, but also that GCN5 preferentially acetylated H2BK16 and H3K9 surrounding the promoter, resulting in the activation of gp91-phox gene expression.

Discussion

We demonstrated that GCN5, one of the most important HATs, regulates the O$_2^-$-generating system in leukocytes via controlling the gp91-phox gene expression. Recently, by analyzing the GCN5-deficient DT40 cells, GCN5$^{-/-}$, we showed not only that GCN5 is preferentially involved as a supervisor in the normal cell-cycle progression through comprehensive control of expressions of various cell cycle-related genes (41), but also that it controls BCR-mediated apoptosis in immature B lymphocytes through regulation of several apoptosis-related genes (42). First, we studied the participation of GCN5 in induction of the O$_2^-$-generating activity by exposing GCN5$^{-/-}$ to PMA. Among various NOX proteins (NOX1$^{-/-}$ and DUOX1, 2) belonging to the NOX family in vertebrates, gp91-phox (NOX2) and NOX5 are expressed in B cells and lymphoid tissues, respectively (15, 49). Although we carried out RT-PCR using chicken NOX5 primers, the gene expression of...
NOX5 could not be detected in both DT40 and GCN5<sup>−/−</sup> (data not shown), indicating that the PMA-induced O<sub>2</sub><sup>−</sup>−generating activity in DT40 mostly depends on gp91-phox. Therefore, we focused on gp91-phox in the NOX family in this study. Interestingly, GCN5 deficiency completely inhibited the O<sub>2</sub><sup>−</sup>−generating activity (Fig. 1A) and reversely caused a drastic decrease in mRNA levels of gp91-phox (to ~4%), whereas it had minor influences on those of p47-phox, p22-phox, and p67-phox (i.e., the elevation [to ~170%] for the former and the slight suppression [to ~65 and 82%] for the latter two) (Fig. 1B). In contrast, the deficiency of PCAF belonging to the GCN5 family slightly suppressed both the O<sub>2</sub><sup>−</sup>−generating activity (to ~50%) and gp91-phox gene expression (to ~45%) compared with those in GCN5<sup>−/−</sup> (Supplemental Fig. 3). These results suggested that GCN5 mainly regulates gene expression of gp91-phox as a supervisor, and PCAF plays a supporting role in regulation of the gene expression.

To confirm this hypothesis, we used an in vitro differentiation system of U937 cells. The gp91-phox gene is a major gene regulated by IFN-γ (46, 47). When U937 cells were treated with IFN-γ, the gp91-phox gene was remarkably expressed (to ~800%), whereas the expression of GCN5 gene was not affected (Fig. 2A). Many groups have reported that GCN5 catalyzes acetylation of several specific Lys residues and causes transcriptional activation (50–57). In this study, we clarified that CPTH2, an inhibitor of GCN5 (48), strongly inhibited IFN-γ−induced O<sub>2</sub><sup>−</sup>−generating activity (Fig. 3A), gene expression of gp91-phox (Fig. 3B), and acetylation of H2BK16 and H3K9 (Fig. 5D) in U937 cells. Further, it is worth noting that CPTH2 can inhibit GCN5 strongly and PCAF moderately (Supplemental Fig. 5D). The binding of GCN5 to gp91-phox gene promoter was remarkably upregulated during cultivation with IFN-γ (Fig. 4A), whereas the association of PCAF with the promoter could not be detected by ChIP assay using

**FIGURE 3.** Effects of GCN5 inhibitor CPTH2 on the IFN-γ−induced O<sub>2</sub><sup>−</sup>−generating activity and gene expressions of five O<sub>2</sub><sup>−</sup>−generating system-related factors in U937 cells. A, O<sub>2</sub><sup>−</sup>−generating activity. U937 cells were treated with or without 50 μM CPTH2 in the presence of IFN-γ for 48 h. PMA-induced CLs were measured at 10 min poststimulation by TD-20/20 luminometer. Data represent the average of three separate experiments; error bars indicate SD. B, Gene expressions of five O<sub>2</sub><sup>−</sup>−generating system-related factors. Total RNAs were extracted from untreated (open bars), IFN-γ−treated (solid bars), and IFN-γ plus CPTH2−treated (striped bars) U937 cells, and mRNA levels were determined by semiquantitative RT-PCR using appropriate primers as in Fig. 1B. Human GAPDH gene was used as an internal control. The gel images obtained were analyzed by Image Gauge Software Profile mode using LAS-1000plus (Fujifilm). Quantitative data are indicated as percentages of control values obtained from untreated U937 cells and represent the average of three separate experiments with errors indicated by SD.

**FIGURE 4.** GCN5 interacts with gp91-phox gene promoter. A. Upper panel: ChIP assay for HATs (GCN5 and CBP). The cross-linked chromatin from cell lysates of U937 cells untreated (−) and treated (+) with 100 U/ml IFN-γ for 48 h were coprecipitated by anti-human HATs (GCN5 and CBP) Abs. After decross-linking, coprecipitated chromatin were amplified by PCR with primers flanking the critical cis-element (Hox/Pbx consensus-like sequence) (30, 46) and inverted PU.1 binding site (25) in the proximal gp91-phox gene promoter (Supplemental Fig. 1). PCR products were analyzed by Image Gauge Software Profile mode (densitometrical analysis mode). Irrelevant normal IgG or Input was used as negative or positive control. Typical patterns are shown. Lower panel: Quantitative data of ChIP assays for GCN5 and CBP. Data represent the average of three separate experiments (including that in A) without (None) or with 100 U/ml IFN-γ and are indicated as percentages of control values obtained from untreated U937 cells with errors indicated by SD. B. Time courses of interactions of GCN5 and CBP with the gp91-phox gene promoter after IFN-γ treatments. U937 cells were incubated with 100 U/ml IFN-γ for indicated times. ChIP assay was performed as described in A. Data (the average of three separate experiments) are indicated as percentages of control values obtained from untreated U937 (0 h) cells with errors indicated by SD.
several available anti-PCAF Abs (data not shown). Therefore, our data suggested that the major target of CPTH2 could be GCN5. After all, ChIP assay revealed not only that the binding of GCN5 to gp91-phox gene promoter was remarkably upregulated without its increased transcription, but also that GCN5 promoted acetylation of H2BK16 and H3K9 surrounding the promoter, resulting in recruitment of various transcription factors (e.g., PU.1) during cultivation of U937 cells with IFN-γ (Figs. 4, 5, Supplemental Fig. 6) (25, 30, 46). These data, together with the findings obtained for the GCN5-deficient DT40 cells (Fig. 1), suggested the participation of GCN5 in regulating the O2−-generating activity via controlling the gp91-phox gene expression. In contrast, interestingly, GCN5 deficiency in DT40 cells also led to decreased acetylation levels of only two Lys residues in core histones: H2BK16 and H3K9 (41). It has been reported that the acetylation of H3K9 catalyzed by GCN5 participates in the activation of transcription (51–55). In addition, GCN5 is required for the acetylation of H2BK16, resulting in transcriptional activation in yeast (52). Therefore, acceleration of acetylation of H3K9 (and probably also H2BK16) may cause a remarkable increase in gp91-phox gene expression during cultivation of U937 cells with IFN-γ, although acetylation levels of other possible GCN5-catalyzed acetylation sites in core histones—H3K14, H3K18, H3K23, H3K27, H3K56, H4K8, and H4K16 (50–57)—remained unchanged (Fig. 5A). Previous studies showed that transcription factors may be newly expressed or modified, leading to interaction with DNA followed by gp91-phox gene expression (16). For IFN-γ–induced expression of the gp91-phox gene, PU.1 is thought to first bind to the promoter and participate in recruitment of other factors (28). Our data in this study, together with previous findings (28, 53, 54, 56), suggested that GCN5 (probably as HAT complex including GCN5) catalyzes the acetylation of H3K9 (and also H2BK16) residue surrounding the gp91-phox gene promoter; the increased histone acetylation accelerates binding of PU.1 to the promoter sequence; PU.1 recruits other activators to the region and finally promotes the gp91-phox gene expression during cultivation with IFN-γ (Supplemental Fig. 6).

Epigenetic mechanisms can define alterations in cellular phenotypes without altering genotypes (58). According to this theory, epigenetic control of transcriptional activation or inactivation is mostly influenced by the intricately and timely modifications of chromatin-bound histones mediated by acetylation, methylation,
phosphorylation, ADP-ribosylation, and/or ubiquitination known as histone code (58). It is well known that acetylation and deacetylation catalyzed reversibly by HATs and histone deacetylases play critical roles in the modulation of chromatin topology and thereby regulation of gene expression. In conclusion, our results obtained in this study indicate not only that GCN5 probably takes part in transcriptional regulation of the gp91-phox gene through alterations in the chromatin structure surrounding its promoter region, but also that as a result, GCN5 plays a key role in mechanisms of epigenetic regulation of the O2−-generating system. We also revealed the moderate participation of PCAF in regulating the O2−-generating activity via controlling gp91-phox gene expression (Supplemental Fig. 3); its molecular mechanisms remain to be resolved. In addition, the epigenetic regulation within U937 cells may be different from that in normal monocyte/macrophage because U937 cells are lymphoma. Therefore, the participation of GCN5 in the O2−-generating system in development of normal cells should be elucidated in the future. Anyhow, our results, together with enormous previous data, may significantly help in the understanding of CGD and epigenetic regulation of leukocyte differentiation.

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Disclosures
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