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Inhibition of GATE-16 attenuates ATRA-induced neutrophil differentiation of APL cells and interferes with autophagosome formation

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Abstract

Autophagy is an intracellular bulk degradation process involved in cell survival upon stress induction, but also with a newly identified function in myeloid differentiation. The autophagy-related (ATG)8 protein family, including the GABARAP and LC3 subfamilies, is crucial for autophagosome biogenesis. In order to evaluate the significance of the GABARAPs in the pathogenesis of acute myeloid leukemia, we compared their expression in primary AML patient samples, CD34⁺ progenitor cells and in granulocytes from healthy donors. GABARAPL1 and GABARAPL2/GATE-16, but not GABARAP, were significantly downregulated in particular AML subtypes compared to normal granulocytes. Moreover, the expression of GABARAPL1 and GATE-16 was significantly induced during ATRA-induced neutrophil differentiation of acute promyelocytic leukemia cells. Lastly, knocking down GABARAPL2/GATE-16 in APL cells attenuated neutrophil differentiation and decreased autophagic flux. In conclusion, low GABARAPL2/GATE-16 expression is associated with an immature myeloid leukemic phenotype and these proteins are necessary for neutrophil differentiation of APL cells.

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Authorship

DB performed the experimental research and drafted the article. BET and JC provided primary cells and revised the article. MPT and MFF designed the project, wrote the manuscript and gave final approval of the submitted manuscript.

Keywords

GABARAP; GABARAPL1; GATE-16; AML; APL; neutrophil differentiation

1. Introduction

Autophagy is a process of cellular self-digestion involved in the degradation of protein aggregates and cell organelles. Autophagy can be observed in a variety of normal cellular processes such as proliferation, cell death and differentiation, as well as in pathological cellular processes including neurodegeneration, inflammatory diseases and cancer [1,2]. Autophagy is a ubiquitous intracellular process in eukaryotes characterized by the formation of double-membraned vesicles, the so-called autophagosomes. Autophagosomes engulf dispensable or harmful components and finally fuse with lysosomes for the degradation of their contents [3]. Attenuation of autophagy results in genomic instability indicating a tumor suppressor function promoted by autophagy. Conversely, cancer therapy related induction of autophagy frequently supports tumor cell survival [4]. In addition, it has been described that autophagy promotes cancer cell metastasis but, may also promote cancer cell death and inhibit metastasis in certain cases [5]. Therefore, the role of autophagy in tumorigenesis is still controversial and clearly depends on the type of tumor and the stage of disease progression [6].

The autophagy-related (ATG) proteins are the major molecular components of the autophagy machinery [3]. In yeast a single ATG8 protein is essential in promoting expansion of the autophagosomal precursor membrane, the autophagophore [7]. In contrast, an ATG8 protein family exists in human that can be divided in three sub-families: (a) the microtubule-associated protein 1 light chain (MAP1LC3, often referred to as LC3), (b) the γ -aminobutyric acid receptor-associated protein (GABARAP), and (c) the golgi-associated ATPase enhancer of 16 kDa GABARAPL2/GATE-16 (hereafter referred to as GATE-16) subfamily [8]. LC3 proteins have an essential function in elongating the autophagosomal membrane, whereas the GABARAP family members are required for closing the autophagosomal membrane [11]. Moreover, tissue or cell specific functions or different roles of ATG8 proteins in particular subtypes types of autophagy have been proposed [9,10].

The role of autophagy in hematopoietic development and function is still under investigation. Several reports showed a critical function for autophagy in the clearance of mitochondria during reticulocyte maturation [12,13]. Moreover, autophagy in macrophages and neutrophils is essential for effective innate immunity and contributes to the prevention of inflammatory diseases (reviewed in [4,14,15]). Recent findings highlighted an important role for autophagy during monocytic differentiation and acquisition of macrophage function [16]. A link to aberrant hematopoiesis was found in a hematopoietic tissue specific, ATG7 conditional knockout mouse model, where the mice developed invasive myeloproliferation, which strongly resembles acute myeloid leukemia (AML) [17]. Lastly, autophagy contributes to all-*trans* retinoic acid (ATRA) therapy-induced PML-RARA degradation in t(15;17) acute promyelocytic leukemia (APL) cell lines [18].

In this study, we aimed at analyzing ATG8 family expression in normal and leukemic myeloid tissue as well as during neutrophil differentiation of APL cells. Further, the requirement for ATG8 proteins for ATRA-induced neutrophil differentiation and autophagy was determined.

2. Materials and Methods

2.1 Patient samples and primary cells

A cohort of 98 AML patient samples (Supplementary Table 1), provided by Drs. P.J.M. Valk and B. Löwenberg, was enrolled on HOVON/SAKK (Dutch-Belgian Hematology-Oncology/Swiss Group for Clinical Cancer Research Cooperative group) protocols -04, -04A, -29, and -42 (available at "<http://www.hovon.nl>" www.hovon.nl) between 1987 and 2006 [20-24].

Primary neutrophils from healthy donors were isolated using polymorphprep (AXIS-SHIELD Baden-Dattwil, Switzerland). *In vitro* differentiation of CD34⁺ progenitor cells was done as previously described [25].

2.2 Cell lines and culture conditions

NB4 APL cells and their all-*trans* retinoic acid (ATRA)-resistant NB4-R2 subclone and HEK-293T cells were cultured as described [26]. ATRA-induced autophagy was blocked using Bafilomycin A1 (BML-CM110, Enzo Life Science, Lausen, Switzerland) at a concentration of 100nM.

2.3 TaqMan low-density array (LDA) and quantitative real-time RT-PCR (qPCR)

RNA extraction, RT-PCR, LDA measurements as well as data analysis were performed as described [4,27]. Gene Expression Assays for GABARAPL1, GATE-16, MAP1LC3B and CSF3R used in a 96 well format on the ABI 7500 Sequence detection system were Hs00744468_s1, Hs00371854_m1, Hs00797944_s1 and Hs00167918_m1, respectively (Applied Biosystems, Rotkreuz, Switzerland). *HMBS* primer and probes have been described previously [28].

2.4 Western blotting

Westernblot was performed as described [26]. Primary antibodies used were anti-LC3B (NB600-1384; Novus Biologicals, Cambridge, England and anti-GAPDH (MAB374; Milipore, Zug, Switzerland).

2.5 Lentiviral transductions

pLKO.1 lentiviral vectors expressing small hairpin (sh)RNAs targeting GATE-16 (shGATE16_247: "NM_007285.6-247s1c1/TRCN0000048287" and shGATE16_359: NM_007285.6-359s1c1/TRCN0000048285) as well as a non-targeting shRNA control (SHCOO2) were purchased from Sigma-Aldrich, Buchs, Switzerland Lentiviral production and transduction of NB4 cells was done as described [29].

2.6 Fluorescent microscopy

Cells were fixed and permeabilized in methanol (-20°C) for 4 min, further washed once with PBS and incubated with the first antibody (LC3B, Cell signalling, Cat. no. 3686; ATG5, Cell signalling, Cat. no. 2630S) for one hour at room temperature. Then, cells were washed twice with PBS-Tween and once with PBS followed by the incubation with the secondary antibody (FITC conjugated anti-rabbit, Jackson Immunoresearch (Cat. no. 111-096-045) for 1 hour at room temperature. Fluorescence labeled cells were mounted (*SlowFade*® Gold Antifade Reagent with DAPI, Invitrogen, Cat. no. S36938) and covered with a glass slide prior to analysis. Images were taken with an Olympus Fluoview FV1000-IX81 confocal laser scanning microscope, using a 60x oil immersion objective. Image processing and analysis were done with ImageJ 1.45s and Adobe Photoshop CS5.

2.7 Proteolysis assay

NB4 cells were treated with or without ATRA for 4 days. 0.3 uCi ¹⁴C-Valine (L-(U-¹⁴C)Valine, Code CFB.75, Amersham) per ml/well was added after 2 days of ATRA treatment. Addition of BafilomycinA1 was performed 24h prior analysis. Assay was further performed as described in [30]. Exception: second incubation period which was performed during 5 hours.

3. Results

3.1 Regulation of the GABARAP subfamily in primary AML patient samples

Based on the role of autophagy in normal myeloid development, we investigated whether autophagy gene expression is deregulated in primary AML patient samples. To this end, we first determined the mRNA expression levels of the GABARAP ATG8 subfamily (GABARAP, GABARAPL1 and GATE-16) in clinical AML samples, CD34⁺ progenitor cells and in mature neutrophils from healthy donors. We were able to detect GABARAP, GABARAPL1 and GATE-16 mRNA expression in 95/98, 95/98 and 94/97 AML patient samples, respectively. GABARAP family expression was found in all granulocyte (n=14) and CD34⁺ progenitor cell (n=3) samples. Compared to normal granulocytes GABARAPL1 was downregulated in the AML subtypes t(8;21), t(15;17) and complex karyotypes, whereas GATE-16 expression was significantly lower only in t(8;21) and t(15;17) AML. Interestingly, no significant differences in GABARAP expression were detected (Figs. 1A-C), suggesting that low expression of this GABARAP subfamily member is not associated with a differentiation block in AML. Together, GABARAPL1 and GATE-16 mRNA expression is significantly downregulated in particular AML subtypes.

3.2 GABARAPL1 and GATE-16 expression is associated with ATRA-induced neutrophil differentiation of APL cells

Given the low GABARAPL1 and GATE-16 expression in AML blast cells we surmised that these genes are involved in myeloid differentiation of leukemic cells. Firstly, to determine if GABARAPL1 and GATE-16 are associated with neutrophil differentiation of APL cells, we measured mRNA expression levels during all-*trans* retinoic acid (ATRA)-induced neutrophil differentiation of NB4 cells (Fig. 2A and B). As a control we used the ATRA-

resistant NB4-R2 APL cells to rule out a direct effect of ATRA on *GABARAPL1* or *GATE-16* mRNA expression. Successful neutrophil differentiation of NB4 cells was confirmed by increased CD11b surface and *CSF3R* mRNA expression (data not shown). A significant 2-fold increase in *GABARAPL1* and *GATE-16* mRNA levels in NB4 cells at day 4 of ATRA treatment compared to control cells was seen. At day 6, *GABARAPL1* and *GATE-16* expression increased 9- and 4-fold, respectively (Fig. 2A). In contrast, *GABARAPL1* and *GATE-16* mRNA levels did not significantly change in ATRA-resistant NB4-R2 cells upon neutrophil differentiation (Fig. 2B). Consistent with our findings in the NB4 neutrophil differentiation model, primary CD34⁺ progenitor cells showed markedly increased *GABARAPL1* and *GATE-16* mRNA levels upon *in vitro* neutrophil differentiation using G-CSF (Fig. 2C). Taken together, our data demonstrate that induction of *GABARAPL1* and *GATE-16* is clearly associated with neutrophil differentiation.

3.3 Knocking down *GATE-16* significantly impaired ATRA-induced neutrophil differentiation of APL cells

To evaluate whether *GATE-16* is functionally involved in ATRA-induced neutrophil differentiation of APL cells, we inhibited *GATE-16* expression in NB4 cells. We generated two different NB4 *GATE-16* knockdown cell lines using lentiviral vectors expressing two independent small hairpin (sh) RNAs targeting *GATE-16*. *GATE-16* knockdown efficiency compared to NB4 SHC002 control cells was 65% and 90% at day 4 and 6 of neutrophil differentiation, respectively (Fig. 3A). Knocking down *GATE-16* resulted in impaired differentiation levels, as evidenced by a significant reduction of CD11b levels in NB4 sh*GATE-16*_247 (50%) and NB4 sh*GATE-16*_359 (70%) knockdown cell lines as compared to SHC002 control cells (Fig. 3B). Similarly, the expression of the neutrophil marker *CSF3R* was markedly downregulated in both NB4 sh*GATE-16* knockdown cells for both time points analyzed (Fig. 3C). These findings indicate that *GATE-16* is functionally involved in ATRA-induced neutrophil differentiation of APL cells.

3.4 Inhibition of *GATE-16* attenuates ATRA-induced autophagy in APL cells

Next, we asked whether the reduced neutrophil differentiation in NB4 *GATE-16* knockdown cells is linked to decreased autophagic activity. As a first indicator of reduced autophagy in NB4 *GATE-16* knockdown cells, we found a significant 2-fold reduction of LC3B mRNA during ATRA-induced neutrophil differentiation in *GATE-16* knockdown cells (Suppl. Fig. 1). Moreover, we found increased LC3B puncta formation for control- as well as ATRA-treated *GATE-16* knockdown cells as compared to SHC002 transduced control cells, suggesting either an increase, or a block in autophagic activity (Figs. 4 A and B). Using the autophagosome-lysosome fusion inhibitor Bafilomycin A1 to determine autophagic flux, we found that BafilomycinA1 treatment resulted in slightly higher LC3-II levels in *GATE-16* knockdown as compared to ATRA only treated cells (Fig. 4 C). This is clearly not as pronounced as for SHC002 control cells, thus suggesting an autophagy block after *GATE-16* knockdown. Supporting our above findings, reduced long-lived protein degradation was observed in ATRA-treated *GATE-16* knockdown, as compared to SHC002 control cells, indicating a block in autophagy in *GATE-16* APL knockdown cells during neutrophil differentiation (Fig. 4 E). Furthermore, we observed increased ATG5 puncta formation in ATRA-treated *GATE-16* NB4 knockdown cells. The accumulation of ATG5 puncta in these

cells further indicates that the autophagosomes are not yet fused with the lysosome in GATE-16 impaired cells, since ATG5 dissociates from the membrane upon completion of autophagosome formation [33] (Fig. 4 D). In order to exclude an APL cell specific role for GATE-16 in autophagosomal membrane formation during ATRA-treatment, we knocked down GATE-16 in HEK 293T cells and treated them with ATRA. We observed an increase in LC3B puncta formation upon ATRA treatment in SHC002 control cells, which was paralleled by autophagic induction. This demonstrated for the first time that ATRA promotes autophagy in non-APL cells. Similar to NB4 cells, endogenous LC3B puncta accumulated in HEK293T GATE-16 knockdown cells in control as well as in ATRA-treated cells further supporting that attenuated GATE-16 expression blocks autophagy (Suppl. Figs. 2 A and B). Moreover, autophagic flux activation was seen in HEK293T cells upon ATRA treatment using the proteolysis assay (Suppl. Fig. 2C). In addition, we measured reduced proteolysis levels for GATE-16 knockdown cells upon starvation, a classical stimulus to induce autophagy, further supporting an essential role for GATE-16 in autophagy (Suppl. Fig. 2 D).

In summary, knocking down GATE-16 attenuates autophagic flux during ATRA-mediated differentiation of APL and in ATRA-treated 293T cells.

4. Discussion

Overall, we found that GABARAPL1 and GATE-16 mRNA levels were significantly lower expressed in APL, t(8;21) and complex karyotype AML than in mature neutrophils. The low levels of both autophagy genes in APL and t(8;21) positive AML samples may be attributed to transcriptional repression by the leukemic fusion proteins PML-RARA and AML1-ETO, respectively. Recently, the myeloid transcription factor GATA-1 was described as direct regulator of the ATG8 family in erythropoiesis [34]. We found that GABARAPL1 and GATE-16 expression is associated with neutrophil differentiation and that inhibiting GATE-16 expression attenuates development. We therefore speculate that particular ATG8 family members are involved in myeloid differentiation.

GABARAPL1 functions in selective autophagy as a cargo adaptor and may recruit harmful protein aggregates to the autophagic machinery for degradation [8,9]. Previous studies linked autophagy to early neutrophil differentiation of APL cells by contributing to PML-RARA degradation [18]. Together with our findings of low GABARAPL1 expression in APL cells we now propose a role for GABARAPL1 in recruiting aggregated PMLRARA proteins to the autophagosome. Furthermore, GABARAPL1 as well as GATE-16 associate with the heat shock protein (HSP) 90 resulting in protection from proteosomal degradation [35]. Recently, published findings revealed that heat shock protein inhibitors, already used in clinical trials for AML therapies, preferentially eliminate AML stem cells, [36]. One explanation for the killing of AML stem cells might be that these cells display increased autophagic activity [37] making them vulnerable to HSP90 inhibitors that attenuate autophagy via increased degradation of ATG8 proteins. Conversely, HSP90 inhibitors might interfere with differentiation therapy in APL patients due to the depletion of GABARAPL1 and GATE-16 proteins, possibly causes reduced differentiation. Our findings suggest that

novel treatment strategies for AML need to take into consideration drugs that target autophagy function.

LC3 and GABARAP subfamilies have unique functions at different steps during autophagosomal membrane formation [11] and also differ in cargo recruitment [38]. We link for the first time GATE-16 to neutrophil differentiation of APL cells, presumably through an increase in autophagy. The observed increase in LC3B-II levels upon ATRA or starvation treatment in GATE-16 impaired APL cells, indicates a block in autophagic turnover rather than increased autophagy. The reduced turnover of ¹⁴C-labeled proteins in GATE-16 knockdown APL cells further supports our finding. This is in line with a report by Weidberg et. al. [11] describing that knocking down GATE-16 interferes with the autophagic machinery. Increased endogenous LC3B and the accumulation of endogenous ATG5 puncta in GATE-16 knockdown cells suggests that GATE-16 acts downstream of LC3. Moreover, GATE-16 may have a rather unique function in neutrophil differentiation that is not compensated by other GABARAP family members during autophagosomal biogenesis.

ATRA and other retinoid derivatives are promising agents not only in the treatment of APL but also in solid cancers (reviewed in [39]). In breast cancer, for example, retinoids can trigger cell cycle arrest, apoptosis and differentiation (reviewed in [40-42]). Based on our findings that ATRA induces autophagy in solid cancer cells, the process needs to be studied in more detail in order to understand if retinoid therapies of solid cancers may profit from autophagy activation or inhibition.

In conclusion, we have found that decreased expression of the ATG8 family members GABARAPL1 and GATE-16 is associated with immature AML blast cells of particular subtypes. Furthermore, we found that GATE-16 expression is necessary for ATRA-induced differentiation and autophagy in APL cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Low *GAPARAPL1* and *GATE-16* mRNA expression is associated with immature AML blast cells of particular subtypes

A unique role for GATE-16 during ATRA-mediated neutrophil differentiation APL cells

Gate-16 contributes to autophagosomal membrane formation downstream of LC3 during ATRA-induced autophagy

ATRA induces autophagy in non-hematopoietic cells

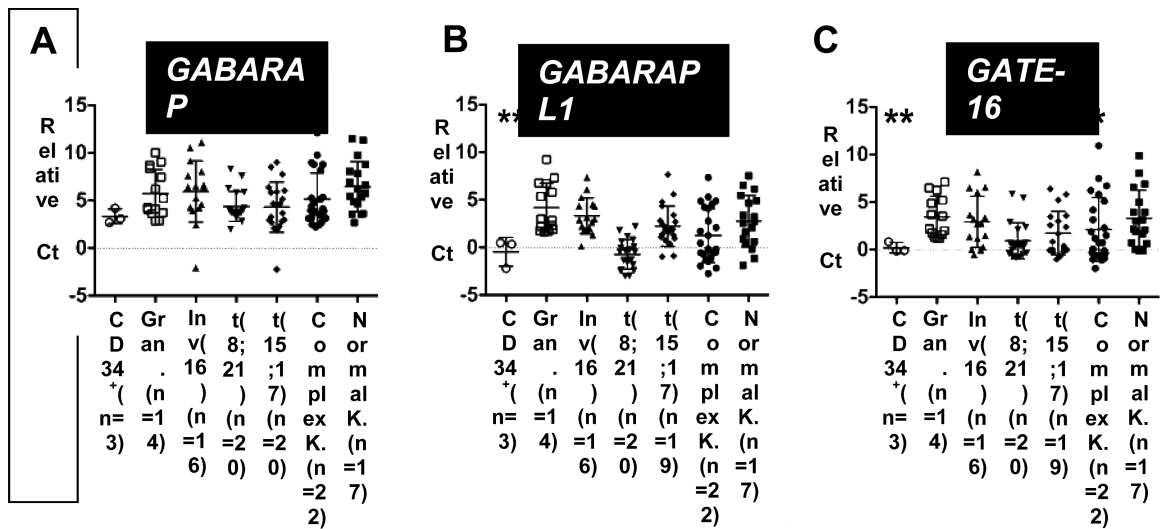


Figure 1. Significantly decreased GABARAPL1 and GATE-16 expression in primary AML patient samples

GABARAP (A), GABARAPL1 (B) and GATE-16 (C) mRNA levels of granulocytes from healthy donors, CD34⁺ progenitor cells and AML patient samples were quantified utilizing qPCR. The relative Ct expression was calculated by the difference of GABARAP, GABARAPL1 and GATE-16 expression to the housekeeping genes *HMBS* and *ABL*. M.W.U, *p<0.01. **p<0.001, *** p<0.0001.

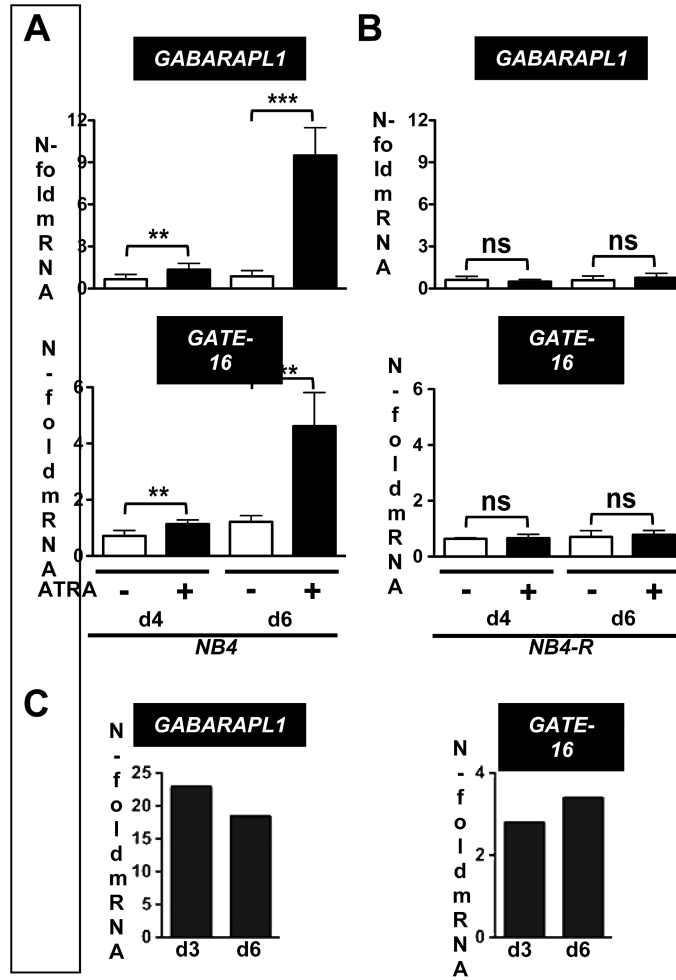


Figure 2. GABARAPL1 and GATE-16 expression during neutrophil differentiation of NB4 APL and CD34⁺ primary cells

(A,B) mRNA levels of GABARAPL1 (upper panels) and GATE-16 (lower panels) were measured in control and ATRA-treated NB4 (A) and NB4-R2 APL (B) cell lines at day 4 and 6 using qPCR. Values were normalized to the housekeeping gene *HMBS* and are given as n-fold mRNA expression relative to the control of day 4 and 6, respectively. M.W.U, *p<0.01. **p<0.001, *** p<0.0001. (C) Primary CD34⁺ progenitor cells showed markedly increased GABARAPL1 and GATE-16 mRNA levels upon *in vitro* neutrophil differentiation using G-CSF over a 12 day period. Values were normalized to the housekeeping gene *HMBS* and given as n-fold mRNA expression relative to the levels at day 0 upon *in vitro* neutrophil differentiation of CD34⁺ using G-CSF.

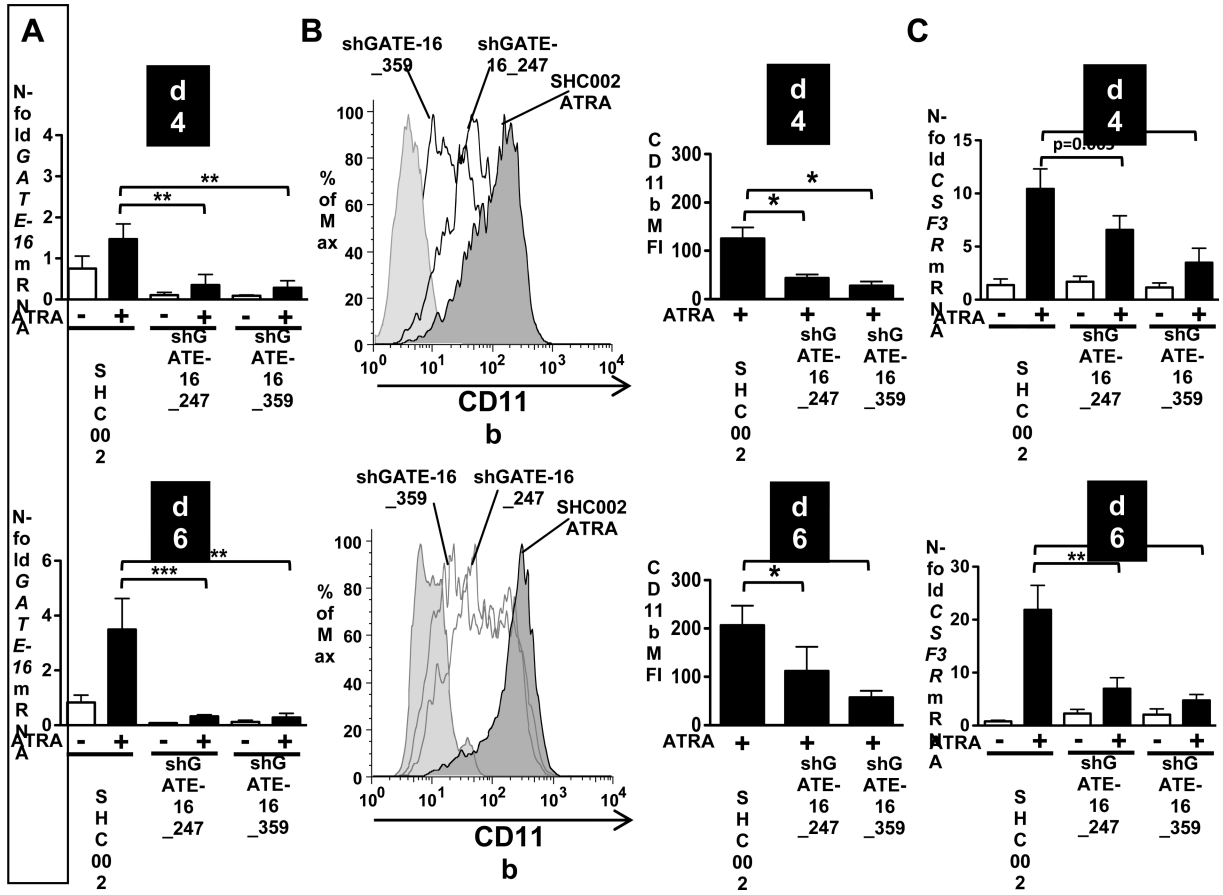


Figure 3. Impaired neutrophil differentiation in NB4 GATE-16 knockdown cells
 (A) GATE-16 mRNA expression was measured upon ATRA (1µM) administration in NB4 cells expressing non-targeting shRNA (SHC002) or shRNAs targeting GATE-16 (shGATE-16_247 and shGATE-16_359) at day 4 and 6, respectively. Values were calculated as described in Fig.1. (B) SHC002, shGATE-16_247 and shGATE-16_359 expressing NB4 cells were differentiated for 4 and 6 days and neutrophil differentiation was assessed by measuring CD11b surface expression. A representative CD11b histogram is shown in the upper panel. Bar graphs of the mean fluorescence intensity of 4 independent experiments are shown. (C) *CSF3R* mRNA was measured using qPCR in SHC002, shGATE-16_247 and shGATE-16_359 expressing NB4 cells upon 1µM ATRA treatment for 4 and 6 days, respectively. M.W.U, *p<0.01. **p<0.001, *** p<0.0001.

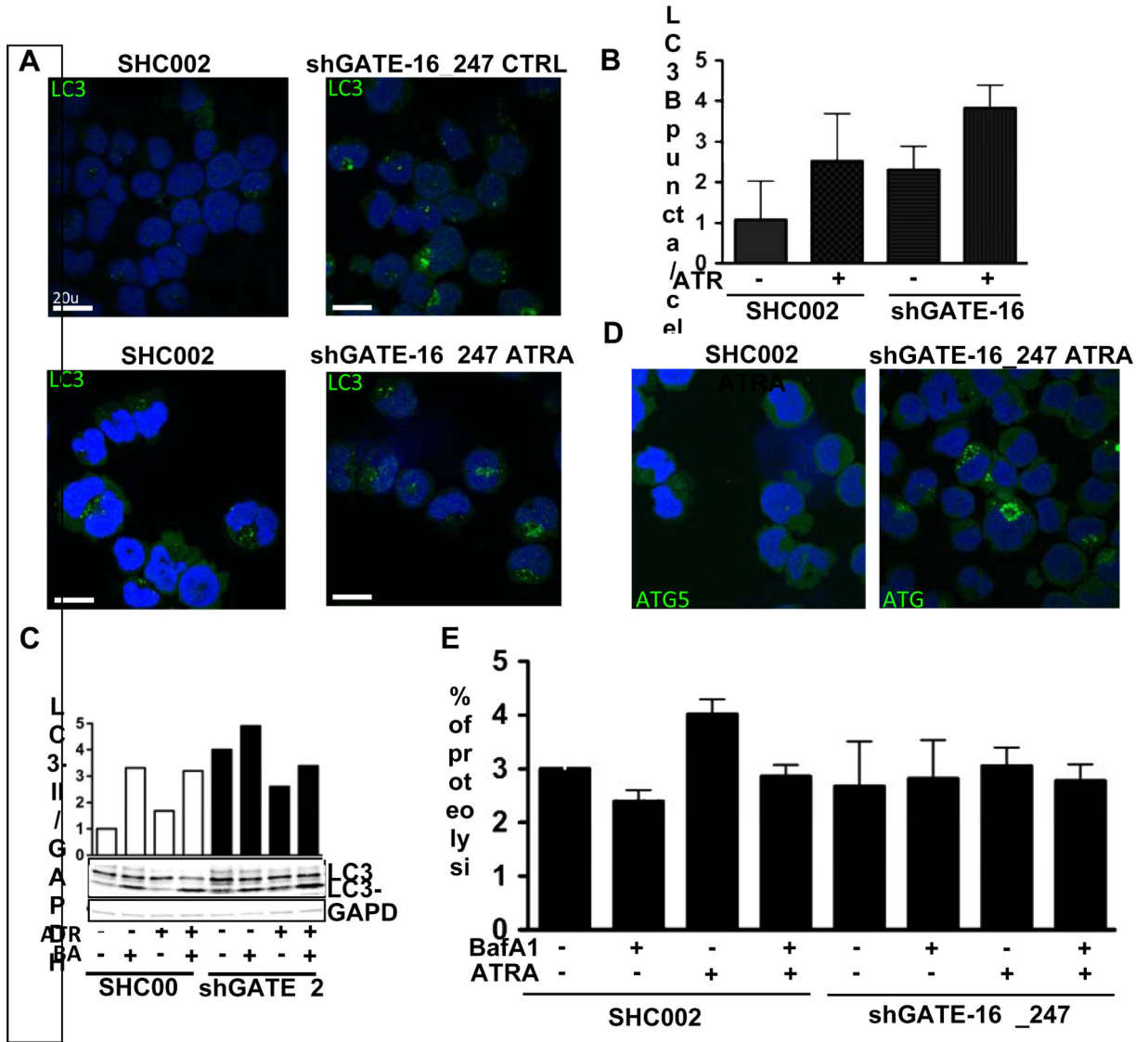


Figure 4. Knocking down GATE-16 impairs ATRA-induced autophagy in NB4 cells
 (A) Endogenous LC3B protein levels were analyzed using confocal microscopy at day 4 of ATRA only treatment or in combination with Bafilomycin A1 in SHC002 control transfected cells or GATE-16 knockdown cells. (B) LC3B puncta were quantified using ImageJ software. (C) Western blot analysis of LC3-II expression in SHC002 or shGATE-16_247 expressing NB4 cells after 4 days of ATRA administration. Total protein was extracted and 30ng of protein was loaded. Immunoblots were incubated with LC3 antibody. GAPDH represents the loading control. (D) Endogenous ATG5 protein levels were analyzed in ATRA-treated NB4 cells using confocal microscopy at day 4. (E) The proteolysis rate in NB4 cells upon ATRA treatment was measured in 4 independent experiments. Cells were either incubated with complete medium or medium supplemented with ATRA, in the presence or absence of BafilomycinA1.