

DR. SHARON L. MCKENNA (Orcid ID : 0000-0002-6764-6274)

Received Date : 17-Jun-2019

Revised Date : 29-Aug-2019

Accepted Date : 06-Dec-2019

Article type : Research Article

## **Inhibition of UBE2L6 attenuates ISGylation and impedes ATRA-induced differentiation of leukemic cells**

Nina Orfali,<sup>1,2,3</sup> Deborah Shan-Krauer,<sup>4</sup> Tracey R. O'Donovan,<sup>1</sup> Nigel P. Mongan,<sup>3,5</sup> Lorraine J. Gudas,<sup>3</sup> Mary R. Cahill,<sup>1,2</sup> Mario P. Tschan,<sup>4</sup> and Sharon L. McKenna<sup>1</sup>

1. Cork Cancer Research Centre & CancerResearch at UCC, 4<sup>th</sup> Floor Western Gateway building, University College Cork, Cork, Ireland;
2. Department of Hematology, Cork University Hospital, Cork, Ireland;
3. Department of Pharmacology, Weill Cornell Medical College, New York, NY, USA;
4. Division of Experimental Pathology, Institute of Pathology, University of Bern, Bern, Switzerland;
5. Faculty of Medicine and Health Science, School of Veterinary Medicine and Science, University of Nottingham, LE12-5RD, United Kingdom

### **Author for correspondence**

Dr. Sharon McKenna

s.mckenna@ucc.ie Telephone: +353 21 420 5710

### **Running title**

UBE2L6 mediated differentiation of AML cells

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/1878-0261.12614](https://doi.org/10.1002/1878-0261.12614)

Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

## Keywords

Differentiation, AML, APL, ISG15, UBE2L6, ATRA.

## Abbreviations

UBE2L6 - ubiquitin-conjugating enzyme E2L6, ISG15 - interferon-stimulated gene 15, AML - acute myeloid leukemia, APL - acute promyelocytic leukemia, ATRA - all-trans-retinoic acid, PTM - post-translational modification, DUBs - deubiquitinases, UBLs - ubiquitin-like modifiers, SUMO - small ubiquitin-like modifier, ATG8 - autophagy related protein 8, ATG12 - autophagy related protein 12, UBE1L - ubiquitin-like modifier activating enzyme 7, UBE2L6 - ubiquitin/ISG15-conjugating enzyme E2 L6, HERC5 - HECT and RLD domain containing E3 ubiquitin protein ligase 5, TRIM25 - tripartite motif containing 25, HHAR1 - Ariadne RBR E3 ubiquitin protein ligase 1, USP18 - ubiquitin specific peptidase 18, NBT - nitro blue tetrazolium, ISRE - interferon-stimulated response element, IFNAR - interferon relative receptor complex, RAR $\alpha$  - retinoic acid receptor alpha, IRF3 - interferon regulatory factor 3, CML - chronic myeloid leukemia.

## Abstract

Ubiquitin-conjugating enzyme E2L6 (UBE2L6) is a critical enzyme in ISGylation, a post-translational protein modification that conjugates the ubiquitin-like modifier, interferon-stimulated gene 15 (ISG15), to target substrates. Previous gene expression studies in acute promyelocytic leukemia (APL) cells showed that all-*trans*-retinoic acid (ATRA) altered the expression of many genes, including UBE2L6 (200-fold) and other members of the ISGylation pathway.

Through gene expression analyses in a cohort of 98 acute myeloid leukemia (AML) patient samples and in primary neutrophils from healthy donors, we found that *UBE2L6* gene expression is reduced in primary AML cells compared to normal mature granulocytes. To assess if UBE2L6 expression is important for leukemic cell differentiation – two cell line models were employed. The human APL cell line NB4 and its ATRA-resistant NB4R counterpart, as well as the ATRA sensitive human AML HL60 cells along with their ATRA-resistant subclone – HL60R.

ATRA strongly induced UBE2L6 in NB4 APL cells and in ATRA-sensitive HL60 AML cells, but not in the ATRA-resistant NB4R and HL60R cells. Furthermore, short hairpin (sh)RNA-mediated *UBE2L6* depletion in NB4 cells impeded ATRA-mediated differentiation, suggesting a functional role for UBE2L6 in leukemic cell differentiation. In addition, ATRA induced *ISG15* gene expression in NB4 APL cells, leading to increased levels of both free ISG15 protein and ISG15 conjugates. UBE2L6 depletion attenuated ATRA-induced ISG15 conjugation. Knockdown of ISG15 in NB4 APL cells inhibited ISGylation and also attenuated ATRA-induced differentiation.

In summary, we demonstrate the functional importance of UBE2L6 in ATRA induced neutrophil differentiation of APL cells and propose that this may be mediated by its catalytic role in ISGylation.

## 1.0 Introduction

Acute myeloid leukemia (AML) is a clonal disorder characterized by the accumulation of immature hematopoietic precursors in the bone marrow and peripheral circulation (Caceres-Cortes, 2013). Overall survival is poor, particularly for patients over 60 years of age,

who have an overall 5 year survival of approximately 10% (Kantarjian and O'Brien, 2010; Thein et al., 2013). Improved therapeutic strategies with tolerable toxicity profiles are needed.

Acute promyelocytic leukemia (APL) is a clinically, pathologically and molecularly distinct subtype of AML (Tallman and Altman, 2009). It is distinguished in 95% of cases by a translocation of chromosomes 15 and 17, which leads to the expression of a fusion oncoprotein PML-RAR $\alpha$  (Tallman and Altman, 2009). This protein disrupts functional retinoid signaling in APL cells, repressing gene transcription and halting myeloid maturation at the promyelocyte stage (Tang and Gudas, 2011). Therapeutic doses of all-*trans*-retinoic acid (ATRA) reactivate gene transcription and overcome this differentiation block allowing clinical remission (Tang and Gudas, 2011). As we have previously reviewed, ATRA also encourages the degradation of the PML-RAR $\alpha$  protein through cooperating pathways including proteasomal degradation and autophagy (Orfali et al., 2014).

Cellular protein activity and stability is regulated by post-translational modification (PTM) (Krishna and Wold, 1993). One such modification is 'ubiquitination', the reversible addition of ubiquitin protein (8.5kDa) to the lysine residues of target substrates (Komander and Rape, 2012). This is catalyzed by a series of enzymes: (i) Ubiquitin-activating enzymes (E1) use ATP to convert ubiquitin to a high-energy thioester; (ii) Ubiquitin-conjugating enzymes (E2) bind active ubiquitin on their cysteine residues; (iii) Ubiquitin-ligase enzymes (E3) interact with E2 enzymes and catalyze the formation of a covalent bond between ubiquitin and its target substrate. E3 ligases regulate substrate specificity. Ubiquitin can also be removed from target proteins through the action of deubiquitinases (DUBs) (Friend et al., 2014). Ubiquitin-like modifiers (UBLs) are proteins that share significant structural and some sequence homology with ubiquitin and modify substrates in a similar enzyme-controlled fashion (Hochstrasser, 2009). While protein ubiquitination is known to alter protein activity or target substrates for degradation by the 26S proteasome, the functional roles of UBL modifications are less well defined and remain under investigation (Hochstrasser, 2009). We now recognize at least ten UBLs, including Small Ubiquitin-like Modifier (SUMO), AuTophagy related proteins 8 and 12 (ATG8, ATG12), and Interferon-Stimulated Gene 15 (ISG15) – which is an important modification in the present study (Hochstrasser, 2009).

ISG15 is a 15kDa protein, which contains 2 UBL domains that share 33% and 32% homology with ubiquitin (Sgorbissa and Brancolini, 2012). The conjugation of ISG15 to substrate lysine residues, known as ISGylation, relies on a narrow range of E1, E2 and E3 enzymes (Supplementary Figure 1). Ubiquitin-like modifier activating enzyme 7 (UBA7/UBE1L) is the E1 enzyme of ISGylation (Jeon et al., 2010). Ubiquitin/ISG15-conjugating enzyme E2 L6 (UBE2L6) operates as an E2 enzyme for both ISGylation and ubiquitination (Jeon et al., 2010). Three known E3 ligases determine ISGylation targets, over 300 of which have been proposed (Jeon et al., 2010; Sgorbissa and Brancolini, 2012). HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase 5 (HERC5) is the dominant E3 for ISGylation and associates with ribosomes to target newly synthesized proteins in a non-specific manner (Durfee et al., 2010). Tripartite Motif Containing 25 (TRIM25) and Ariadne RBR E3 Ubiquitin Protein Ligase 1 (HHAR1) show specificity for 14-3-3 and 4EHP respectively (Sgorbissa and Brancolini, 2012). Ubiquitin Specific Peptidase 18 (USP18) removes ISG15 from its substrates and thus negatively regulates the ISGylation pathway (Malakhov et al., 2002). The precise functions of ISGylation remain under investigation and may be contextual. All components of the system are induced on Type I interferon stimulation suggesting a functional role in anti-viral responses (Sgorbissa and Brancolini, 2012). Free ISG15 has interferon-stimulating cytokine activity when secreted outside of the cell (Bogunovic et al., 2013).

*ISG15* expression and ISGylation are induced during erythropoiesis and primary erythroblasts harvested from *ISG15*<sup>-/-</sup> knockout mice show impaired differentiation in *ex vivo* culture (Maragno et al., 2011). Transcriptional profiling of human granulopoiesis has shown that *ISG15* expression is similarly induced during terminal neutrophil differentiation and a PU.1 binding site has been identified within the *ISG15* promoter region (Meraro et al., 2002; Theilgaard-Monch et al., 2005). To date however a functional role for ISGylation in granulopoiesis has not been proven.

Our work has found that *UBE2L6*, the gene encoding the E2 enzyme of ISGylation, is strongly upregulated following ATRA treatment of APL cells. Through a series of short hairpin (sh)RNA knockdown experiments, we have investigated for the first time, the functional importance of this enzyme in the ATRA-mediated granulocytic differentiation of APL cells. We report that inhibiting *UBE2L6* expression results in reduced ISGylation and

impaired APL cell differentiation. Interference with *ISG15* expression similarly impedes differentiation. Through improving our understanding of ISGylation and protein PTMs involved in ATRA-mediated differentiation of APL cells, we hope to identify ways of promoting differentiation therapy in other AML subtypes.

## **2.0 Materials and Methods**

### *2.1 Cell lines & culture conditions*

The human APL cell line NB4 and its ATRA-resistant NB4R counterpart were kindly gifted by Dr. B.E. Torbett and Prof. P. Paolo-Pandolfi respectively. ATRA-sensitive human M2 AML HL60 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). Their ATRA-resistant subclone, HL60R cells were kindly gifted by Dr. Mario Tschan. All cell lines were maintained in RPMI 1640 (Sigma R8758) medium supplemented with 10% fetal calf serum (Sigma F7524) and 1% Penicillin/Streptomycin (Gibco 15070-063) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. For differentiation experiments, cells were seeded at 0.2 x 10<sup>5</sup> cells/mL and treated for 4 days with 1µM ATRA (Sigma R2625) diluted from a 1mM stock in 100% ethanol.

### *2.2 Patient study*

A cohort of 98 AML patient samples, collected through the HOVON/SAKK (Dutch-Belgian Hematology-Oncology / Swiss Group for Clinical Cancer Research Cooperative Group) protocols 04, 04A, 29 and 42 between 1987 and 2006, were provided by Drs. P. Valk and B. Lowenberg. Patient characteristics have been previously outlined (Schlafli et al., 2012). Primary neutrophils from healthy donors were isolated using polymorphprep (AXIS-SHIELD, Switzerland). All patients provided written informed consent in accordance with the Declaration of Helsinki.

### *2.3 RNA extraction, quantitative real-time PCR (qPCR), Taqman low-density array*

Total cellular RNA was harvested using Trizol (Invitrogen 15596-018), according to the manufacturer's protocol. 1µg of RNA was reverse transcribed using qScript (Quanta Biosciences #95047) as per product protocol at a final reaction volume of 20µL and the resulting cDNA was diluted 1:10 in H<sub>2</sub>O. Subsequent qRT-PCR reactions were carried out using 2µL of template together with 1x SYBR Green Supermix (Quanta Biosciences #84091), forward and reverse primers at 0.25µM and 2.5µL H<sub>2</sub>O in a final reaction volume of 15µL. Reactions were run on a Bio-Rad MyiQ™ Single Color Real-time PCR detection system with each cycle including a 94°C x 20sec denaturation step, 60°C x 20sec annealing step and a 72°C x 30sec extension step. Primer pairs were designed to span distinct exons software to

avoid genomic DNA signaling and gene expression amplicons were validated with sequencing at the Genomics Resources Core Facility, WCMC. Sequences of specific primers were: UBE2L6\_F CTGGAAGCCTTGCACCAAGA, UBE2L6\_R GAACATGAGTTAGGAGGGCCG, ISG15\_F GGTGGACAAATGCGACGAAC, ISG15\_R TCGAAGGTCAGCCAGAACAG. The transcript levels in biological replicates (n=3) were normalized to *hPRT* transcript levels and relative differences were calculated using the Pfaffl method. Graphical displays and measurements of statistical significance were performed on GraphPad prism software.

#### 2.4 Lentiviral shRNA transduction

pLKO.1 lentiviral vectors expressing small hairpin shRNAs targeting both UBE2L6 and ISG15 were purchased from Sigma-Aldrich along with a non-targeting shRNA control (SCH002) in bacterial glycerol stocks. For each gene, five shRNAs were initially tested for efficiency by measuring mRNA levels by qPCR and two shRNAs were then selected for use in further experiments. (shUBE2L6\_499 = NM\_004223.3-499s1c1/ TRCN0000007284, shUBE2L6\_1082 = NM\_004223.3-1082s1c1/ TRCN0000007281, shISG15\_319 = NM\_005101.3-319s21c1/ TRCN0000237825 and shISG15\_352 = NM\_005101.3-352s21c1/ TRCN0000237824). Lentiviral production and transduction was performed as previously described (Tschan et al., 2003). All vectors contain a puromycin resistance gene and transduced cell clones were selected for 4 days using 1.5 µg/ml puromycin.

#### 2.5 Morphology examination

Cells were cytopun onto glass slides and stained with Rapi-Diff (Braidwood Laboratories 22007, 22008, 22009) according to product guidelines. Morphology was examined using an Olympus DP70 digital microscope at 400X magnification (Mason Technology, Ireland).

#### 2.6 Nitro Blue Tetrazolium (NBT) assay

Cells were incubated with 0.2% NBT (Sigma N5514) and 40ng/mL Phorbol 12-Myristate 13-acetate (PMA) (Sigma P8139) in RPMI for 20 min at 37°C, washed and cytopun onto glass slides on a base of Phosphate Buffered Saline (PBS)/1% FBS. Slides were then counterstained with 0.5% Safranin O (Sigma 84120) in 20% ethanol and coverslipped with



Entellan (Merck 1076910100). NBT positive cells were then examined, counted in triplicate and presented accordingly.

### *2.7 Western blotting*

Cellular protein extracts were lysed in modified RIPA buffer (50 mM TrisHCl - pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Igepal, 1 mM EDTA, 1x Pefabloc, 1x Protease inhibitor cocktail, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF). Protein samples were separated on NuPAGE 4-12%, Bis-Tris gels (Invitrogen NP0322) and electrophoretically transferred onto PVDF membranes (Invitrogen IB401001). Primary antibodies were: anti-UBE2L6 (Abgent AP2118A), anti-ISG15 (Proteintech 15981-1-AP) and anti-β-ctin (Sigma A5441). Proteins were visualized using relevant IR-DYE secondary antibodies and quantified on the Odyssey IR imaging system (Li-Cor, Cambridge, UK).

### *2.8 Flow cytometry*

Live cells were incubated for 30 min with PE-conjugated anti-CD11b antibody (eBioscience 12-0118 or Immunotools #21279114) in 1% albumin/phosphate buffered saline (PBS), and washed with PBS prior to analysis. Fluorescence was detected using a BD-LSRII flow cytometer (BD Biosciences, Oxford, UK). Data analysis and histogram overlays were performed on FlowJo software.

### 3.0 Results

#### 3.1 *UBE2L6* is induced during the neutrophil differentiation of leukemic cells

We have previously examined the gene expression changes induced by ATRA in APL cells by sequencing RNA extracted from NB4 cells treated with 1 $\mu$ M ATRA for 72h alongside untreated controls (Orfali N, 2019). These data showed that ATRA, induced a 200-fold increase in *UBE2L6* expression. Other members of the ISGylation pathway were also found to be co-regulated (RNAseq data reproduced in Table 1). As NB4 cells respond to ATRA by differentiating towards mature neutrophils, this prompted us to question whether *UBE2L6* expression is important for leukemic cell differentiation.

We first examined *UBE2L6* mRNA expression in 98 primary AML patient samples (M0-M4), 6 samples of normal CD34<sup>+</sup> (HSC) cells and in 24 donated mature granulocyte samples using a TaqMan Low Density Array. Relative *UBE2L6* mRNA levels are shown as differences in Ct-values as compared to mRNA levels for the housekeeping genes *HMBS* and *ABL1*. Expression was significantly lower in AML patient samples and HSC cells than in granulocytes, suggesting that increased expression may be important for the mature granulocyte phenotype (Mann Whitney U test, \*\*\*\*p  $\leq$  0.0001) (Fig. 1A).

To test this hypothesis further, we treated NB4 cells with ATRA along with their ATRA-resistant counterparts NB4R cells. We measured *UBE2L6* expression by quantitative real-time (q)PCR at 72h, assessing Ct values relative to the housekeeping gene *hPRT*. Validating our earlier RNA sequencing observations we detected a 180-fold increase in *UBE2L6* expression in differentiating NB4 cells (\*\*\*\*p  $\leq$  0.0001), but only a 0.23-fold difference in NB4R cells (\*\*p = 0.0021) (Fig. 1B). The HL60 cell line (human M2 AML), although it does not carry the PML-RAR $\alpha$  oncoprotein, also differentiates down a granulocytic lineage in response to ATRA therapy and can be used as a second model of leukemic cell differentiation. Following 96h of ATRA treatment we found a 189-fold increase in *UBE2L6* expression in HL60 cells (\*\*p = 0.0003). ATRA-resistant HL60R cells however failed to induce *UBE2L6* (Fig. 1C).

These results indicate that *UBE2L6* is prominently upregulated during leukemic cell differentiation rather than solely on ATRA treatment and that this effect is not restricted to APL cells carrying the PML-RAR $\alpha$  fusion oncoprotein.

### **3.2 Knockdown of UBE2L6 inhibits ATRA-induced neutrophil differentiation of NB4 APL cells**

In order to investigate whether UBE2L6 has a functional role in leukemic cell differentiation, we generated *UBE2L6* knockdown NB4 cells using a lentiviral delivery system to deliver target-specific shRNA. NB4 cells transduced with a non-targeting shRNA were used as a control (SHC). Functional knockdown was confirmed by detecting reduced UBE2L6 protein levels following ATRA treatment in two knockdown clones: shUBE2L6\_499 and shUBE2L6\_1082. Superior knockdown efficiency is evident in shUBE2L6\_1082 (Fig. 2A).

ATRA-induced neutrophil differentiation was reduced in *UBE2L6* knockdown NB4 clones compared to control cells. At a transcript level, we detected reduced expression of Granulocyte Colony Stimulating Factor Receptor (*GCSFR*), a marker of neutrophil differentiation, by qPCR at 72h (\* $p = 0.0123$ , \*\*\* $p = 0.0004$ ) (Fig. 2B). At a protein level, surface CD11b expression was reduced in ATRA treated knockdown cells when analyzed by flow cytometry at 72h. A direct correlation was observed between UBE2L6 knockdown efficiency and detectable CD11b levels, with both shUBE2L6\_499 and shUBE2L6\_1082 showing a significant reduction in CD11b (\*\* $p = 0.0017$  and  $p = 0.0067$ , respectively) (Fig. 2C). Morphologically, ATRA treated control cells displayed characteristic features of granulocytic differentiation with increased cytoplasmic volume and visible nuclear indentation (Fig. 2D lower left panel, arrows). This phenotype was stunted in knockdown clones (Fig. 2D lower middle and right panels). Finally we assessed functional differentiation using the Nitro Blue Tetrazolium (NBT) assay, which tests the reducing power of the neutrophil enzyme alkaline phosphatase. We observed and quantified a decreased NBT reduction in *UBE2L6* knockdown clones (\*\*  $p = 0.0069$ , \*  $p = 0.0354$ ), with a direct correlation again seen between knockdown efficiency and functional differentiation (Fig. 2E (i) lower panels & Fig. 2E (ii)).

Together these results demonstrate that UBE2L6 depletion impedes ATRA-mediated granulocytic differentiation of APL cells and prompted us to question the potential mechanism involved in this differentiation block.

### **3.3 UBE2L6 mediates protein ISGylation in ATRA treated APL cells**

As described earlier, UBE2L6 is an E2 ligase critical in the conjugation of ISG15 to target proteins during ISGylation, a process with an unknown role in leukemic cell differentiation. A search of UBE2L6 human protein interactions on the publicly available STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins)(www.string-db.org) (Franceschini et al., 2013) depicts a high confidence of interaction between UBE2L6 and ISG15, as well as interactions with the E1 and all E3 ligases of the ISGylation pathway (Fig. 3A).

We tested levels of both free and conjugated ISG15 protein in *UBE2L6* knockdown NB4 cells using western blot analysis. We found a prominent induction of free ISG15 protein following ATRA treatment in control cells and in knockdown clones. Notably, conjugated ISG15 was markedly increased after 72h of ATRA treatment in control cells but was impaired in *UBE2L6* knockdown cells, with the most efficient knockdown clone shUBE2L6\_1082 having the least amount of visible conjugates (Fig. 3B (i) & (ii)).

### **3.4 ISG15 is induced during the neutrophil differentiation of leukemic cells**

Our RNA sequencing data showed a 17.54-fold increase in *ISG15* gene expression in NB4 cells with ATRA treatment (Orfali N, 2019) (Table 1). We validated this finding by qPCR measurement of *ISG15* expression in ATRA treated NB4 cells at 72h, which showed a 23-fold increase in expression in differentiating cells (\*\*\*\* $p \leq 0.0001$ ). No significant change in expression was found in ATRA treated NB4R cells (Fig. 4A). HL60 cells undergoing differentiation with ATRA treatment showed a four-fold induction of ISG15 at 96h (\*\* $p = 0.0011$ ) whereas no induction was seen in HL60R cells (Fig. 4B). These findings associate ISG15 induction with leukemic cell differentiation rather than solely with ATRA treatment.

### **3.5 Knockdown of ISG15 inhibits ATRA-induced neutrophil differentiation of NB4 APL cells**

To investigate whether the induction of ISG15 during ATRA-mediated leukemic cell differentiation had functional significance, we knocked down *ISG15* in NB4 APL cells. We confirmed efficient ISG15 protein knockdown in two clones, shISG15\_319 and shISG15\_352, detecting reduced basal levels of free ISG15 in knockdown cells compared to controls and by detecting reduced induction of free ISG15 by 72h ATRA treatment. ISG15 conjugates were

not seen before or after ATRA treatment in knockdown cells consistent with the knockdown blocking ATRA-induced ISGylation (Fig. 5A).

ATRA-induced neutrophil differentiation was reduced in both ISG15 knockdown clones compared to control cells, analogous to UBE2L6-depleted cells. At the transcript level, we found reduced GCSFR mRNA expression in ATRA treated knockdown clones compared to ATRA treated control cells at 72h (\*\*\*\* $p \leq 0.0001$ , \*\* $p = 0.0002$ , respectively) (Fig. 5B). At the protein level, surface CD11b expression was reduced in both ATRA treated knockdown clones (\* $p = 0.0434$  in shISG15\_319 clone), but did not achieve significance in the shISG15\_352 knockdown clone (Fig. 5C). ISG15 knockdown cells failed to morphologically differentiate into mature myeloid forms after 72h of ATRA treatment (Fig. 5D lower middle and right panels) and their ability to reduce NBT was also diminished as is shown in Fig. 5E (i) lower middle and right panels. Decreased NBT reduction was quantified and is presented in Fig. 5E (ii) (\* $p = 0.0312$ , \*\* $p = 0.0031$ ).

Our findings suggest a functional role for ISGylation in the ATRA-mediated neutrophil differentiation of APL cells. In the context of our earlier results, ISGylation may be the prominent mechanism by which UBE2L6 regulates differentiation.

#### 4.0 Discussion

Our results demonstrate that *UBE2L6* is under-expressed in AML cells compared to their mature myeloid counterparts. Using two cell line models of AML cell differentiation, we show that cells undergoing ATRA-mediated neutrophil differentiation strongly induce *UBE2L6*. We show that shRNA-depletion of *UBE2L6* in leukemic cells impedes their ability to differentiate, reporting for the first time a functional importance for this enzyme in ATRA-mediated leukemic cell differentiation.

While *UBE2L6* was first identified as an E2 conjugating enzyme in ubiquitination, it is now thought to preferentially function as an E2 enzyme for ISG15 conjugation (Jeon et al., 2010; Kim et al., 2004; Zhao et al., 2004). We demonstrate that *UBE2L6* modulates ISGylation in leukemic cells and further show that genetic inhibition of *ISG15* strongly interferes with the neutrophil differentiation of ATRA treated APL cells. We hence propose that the effects of *UBE2L6* on leukemic cell differentiation are likely to involve its activity in ISGylation.

In addition to ISG15 and other elements of the cellular ISGylation machinery, *UBE2L6* is induced by Type 1 interferon signaling, and contains an Interferon-stimulated response element (ISRE) in its promoter region (Kim et al., 2004). ATRA has previously been shown to upregulate ISGylation machinery in NB4 cells and to stimulate ISGylation (Dao et al., 2006). This effect is thought to be due to ATRA stimulating the secretion of Type I interferon as antibody-mediated blockade of the interferon receptor complex (IFNAR) suppresses this ISGylation (Dao et al., 2006). Experiments by Pitha-Rowe and colleagues identified that *UBE1L*, the E1 activating enzyme of ISGylation, is induced by ATRA treatment in ATRA-sensitive but not ATRA-resistant APL cells (Kitareewan et al., 2002). Subsequently this group identified retinoic acid receptor alpha ( $RAR\alpha$ ) binding in a domain of the *UBE1L* promoter, which was repressed by the PML- $RAR\alpha$  oncoprotein (Kitareewan et al., 2002). While our observation that *UBE2L6* is induced only in ATRA-sensitive NB4 APL cells could be explained by direct repression by PML- $RAR\alpha$ , we observe equivalent *UBE2L6* expression levels in ATRA-sensitive and -resistant HL60 cells which do not carry the PML- $RAR\alpha$  oncoprotein. We thus speculate that *UBE2L6* is activated during the leukemic cell differentiation program with a functional purpose regardless of the presence of fusion oncoproteins.

Equally, *ISG15* induction has been reported following ATRA treatment only in differentiating NB4 APL cells and not in ATRA-resistant NB4R cells (Guo et al., 2010; Pitha-Rowe et al., 2004). Up until now, a similar finding in HL60 and HL60R cells has not been reported. Our findings again suggest that *ISG15* is activated during the cellular differentiation program rather than being regulated by the PML-RAR $\alpha$  oncoprotein. The *ISG15* promoter contains a PU.1 binding site in addition to two ISREs and it is possible that it is activated by this transcription factor during leukemic cell differentiation (Meraro et al., 2002).

The precise cellular functions of ISGylation remain under speculation. Proteomic studies in a range of cell lines have now collectively identified over 300 putative ISGylation targets, with no functional or compartmental class over-represented amongst them (Giannakopoulos et al., 2005; Malakhov et al., 2003; Zhao et al., 2005). HERC5, the primary E3 ligase of ISGylation, associates with ribosomes and broadly captures newly synthesized proteins of both endogenous and exogenous origin for ISGylation (Durfee et al., 2010). Induced by both Type I interferon and lipopolysaccharide, ISGylation is thought to play a role in our defense against viral pathogens but the exact mechanisms at play remain under investigation (Zhang and Zhang, 2011). While ubiquitination can modulate protein function or promote proteasomal degradation (depending on specific linkages)(Ebner and Versteeg, 2017), it is unclear whether ISGylation has analogous effects on target proteins and whether these effects may be contextual (Zhang and Zhang, 2011). ISGylation can stabilize proteins, competitively preventing their ubiquitination and subsequent degradation, as is seen with interferon regulatory factor 3 (IRF3) (Shi et al., 2010). It may also modify enzymes involved in ubiquitination impeding their function and thus negatively regulating proteasomal degradation of ubiquitin substrates (Takeuchi and Yokosawa, 2005; Zou et al., 2005). ISGylation might also inactivate or destabilize proteins through proteasomal channels. UBE1L-mediated ISGylation of Cyclin D1 in lung cancer cells reduces detectable protein levels with an anti-proliferative effect. The reduction in Cyclin D1 is reversed with the overexpression of the deISGylating enzyme USP18 (Feng et al., 2008). Further study is warranted into the context-dependent and possibly tumor-suppressive actions of ISGylation in physiologic and pathologic settings.

An inhibitory effect on ATRA-mediated neutrophil differentiation of NB4 APL cells as a direct result of shRNA depletion of either *UBE2L6* or *ISG15* has not been previously reported. Our findings propose a functional role for these genes in differentiation. We have previously reviewed the effects of ATRA on the PML-RAR $\alpha$  protein in APL cells (Orfali et al., 2014). Briefly, in addition to de-repressing transcription, ATRA induces the degradation of the PML-RAR $\alpha$  oncoprotein through caspase-3 mediated cleavage, ubiquitin/proteasome mediated proteolysis and through lysosomal-mediated autophagy (Orfali et al., 2014). Investigating a temporal correlation between the reduction in PML-RAR $\alpha$  and an induction of UBE1L in ATRA treated NB4 cells, Shah et. al. reported ISGylation of the PML domain of PML-RAR $\alpha$  with subsequent repression. This repression was opposed by the overexpression of USP18 (Shah et al., 2008). Subsequent work showed that knockdown of USP18 destabilized PML-RAR $\alpha$  and promoted apoptosis in NB4 APL cells but did not have an effect on differentiation (Guo et al., 2010). A similar destabilizing effect of ISGylation on a leukemic oncoprotein is suggested for the BCR-ABL kinase that drives chronic myeloid leukemia (CML). The expression of BCR-ABL in mouse bone marrow cells results in splenomegaly and an abnormal myeloproliferation resembling CML. Bone marrow cells harvested from USP18<sup>-/-</sup> mice and transfected with BCR-ABL prior to transplantation into wild-type recipient mice, developed a CML-like state in only 40% of cases, whereas all mice transplanted with USP18<sup>+/+</sup> BCR-ABL expressing cells developed disease (Yan et al., 2007). Degradation of PML-RAR $\alpha$  may be one mechanism through which UBE2L6 and ISGylation contribute to ATRA-mediated APL cell differentiation as we have observed. However we speculate that this pathway may have broader functions in this process. This is supported by a proposed role for ISGylation machinery in the differentiation of other hemopoietic cells. High levels of USP18 in murine hematopoietic cells block the cytokine-induced terminal differentiation of monocytic cells (Liu et al., 1999). ISG15 along with UBE1L and UBCM8 (the murine orthologue of UBE2L6) are induced during erythroid development in mice and erythroblasts cultured *ex vivo* from ISG15<sup>-/-</sup> mice show impaired differentiation.(Maragno et al., 2011) Future work will examine the effects of modulating ISGylation in non-APL models of leukemic differentiation.

Our previous work has proposed that promoting autophagy may enhance the differentiating effects of ATRA on leukemic cells (Orfali et al., 2015). With further study,



promoting ISGylation may prove to have similar benefits. Inhibiting USP18, the negative regulator of ISGylation, has been shown in both cell line and *in vivo* systems to enhance ISG15 conjugation (Ketscher et al., 2015). A small molecule inhibitor of this isopeptidase is awaited and will greatly advance study in this arena (Basters et al., 2012).

## **5.0 Conclusions**

We have identified a novel function of UBE2L6 in the granulocytic differentiation of APL cells, mediated by ISGylation. Our work contributes to the growing area of study of post-translational protein alteration by ubiquitin-like modifiers. A greater understanding of the protein handling that occurs during leukemic cell differentiation might allow us to modulate these processes and broaden the application of differentiation therapy for the improved treatment of AML.

## **6.0 Authors' contributions**

N.O., D.S-K., T.R.O'D., N.P.M., L.J.G., M.R.C., M.P.T., and S.L.M., conceived, designed, and conducted the experiments; interpreted the results; and wrote the manuscript.

## **7.0 Acknowledgements**

The authors would like to thank Dr. Michelle J. Nyhan, along with members of the Cork Cancer Research Centre, the Gudas laboratory at Weill Cornell New York, Mongan Laboratory at University of Nottingham and the Tschan laboratory at the University of Bern, for review of data and critical feedback. M.P.T., T.R.O'D. and S.L.M are members the TRANSAUTOPHAGY COST ACTION CA15138 and acknowledge support for collaboration.

## **8.0 Funding sources and disclosure of conflict of interest**

This research was supported by the National Institutes of Health (CA043796) (L.J.G.) and by Weill Cornell funds to LJG. N.O. was funded by the Haematology Education and Research Trust (H.E.R.O), and Breakthrough Cancer Research (BCR), with unrestricted educational support from Pfizer, Merck Sharp & Dohme, Bristol Myers Squibb, Novartis and Amgen. M.P.T. was supported by Swiss Cancer Research (KFS-3409-02-2014) and by the Swiss

National Science Foundation (31003A\_143739). The financial support of the University of Nottingham to N.M is gratefully acknowledged.

All authors declare no conflict of interest.

Accepted Article

## References

- Basters, A., Ketscher, L., Deuerling, E., Arkona, C., Rademann, J., Knobloch, K.P., Fritz, G., 2012. High yield expression of catalytically active USP18 (UBP43) using a Trigger Factor fusion system. *BMC biotechnology* 12, 56.
- Bogunovic, D., Boisson-Dupuis, S., Casanova, J.L., 2013. ISG15: leading a double life as a secreted molecule. *Experimental & molecular medicine* 45, e18.
- Caceres-Cortes, J.R., 2013. Blastic leukaemias (AML): a biologist's view. *Cell biochemistry and biophysics* 66, 13-22.
- Dao, C.T., Luo, J.K., Zhang, D.E., 2006. Retinoic acid-induced protein ISGylation is dependent on interferon signal transduction. *Blood cells, molecules & diseases* 36, 406-413.
- Durfee, L.A., Lyon, N., Seo, K., Huijbregtse, J.M., 2010. The ISG15 conjugation system broadly targets newly synthesized proteins: implications for the antiviral function of ISG15. *Molecular cell* 38, 722-732.
- Ebner, P., Versteeg, G.A., 2017. Ubiquitin enzymes in the regulation of immune responses. 52, 425-460.
- Feng, Q., Sekula, D., Guo, Y., Liu, X., Black, C.C., Galimberti, F., Shah, S.J., Sempere, L.F., Memoli, V., Andersen, J.B., Hassel, B.A., Dragnev, K., Dmitrovsky, E., 2008. UBE1L causes lung cancer growth suppression by targeting cyclin D1. *Molecular cancer therapeutics* 7, 3780-3788.
- Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., Simonovic, M., Roth, A., Lin, J., Minguez, P., Bork, P., von Mering, C., Jensen, L.J., 2013. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic acids research* 41, D808-815.
- Friend, S.F., Deason-Towne, F., Peterson, L.K., Berger, A.J., Dragone, L.L., 2014. Regulation of T cell receptor complex-mediated signaling by ubiquitin and ubiquitin-like modifications. *American journal of clinical and experimental immunology* 3, 107-123.
- Giannakopoulos, N.V., Luo, J.K., Papov, V., Zou, W., Lenschow, D.J., Jacobs, B.S., Borden, E.C., Li, J., Virgin, H.W., Zhang, D.E., 2005. Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. *Biochemical and biophysical research communications* 336, 496-506.
- Guo, Y., Dolinko, A.V., Chinyenetere, F., Stanton, B., Bomberger, J.M., Demidenko, E., Zhou, D.C., Gallagher, R., Ma, T., Galimberti, F., Liu, X., Sekula, D., Freemantle, S., Dmitrovsky, E.,

2010. Blockade of the ubiquitin protease UBP43 destabilizes transcription factor PML/RARalpha and inhibits the growth of acute promyelocytic leukemia. *Cancer research* 70, 9875-9885.
- Hochstrasser, M., 2009. Origin and function of ubiquitin-like proteins. *Nature* 458, 422-429.
- Jeon, Y.J., Yoo, H.M., Chung, C.H., 2010. ISG15 and immune diseases. *Biochimica et biophysica acta* 1802, 485-496.
- Kantarjian, H., O'Brien, S., 2010. Questions regarding frontline therapy of acute myeloid leukemia. *Cancer* 116, 4896-4901.
- Ketscher, L., Hanns, R., Morales, D.J., Basters, A., Guerra, S., Goldmann, T., Hausmann, A., Prinz, M., Naumann, R., Pekosz, A., Utermohlen, O., Lenschow, D.J., Knobloch, K.P., 2015. Selective inactivation of USP18 isopeptidase activity in vivo enhances ISG15 conjugation and viral resistance. *Proceedings of the National Academy of Sciences of the United States of America* 112, 1577-1582.
- Kim, K.I., Giannakopoulos, N.V., Virgin, H.W., Zhang, D.E., 2004. Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation. *Molecular and cellular biology* 24, 9592-9600.
- Kitareewan, S., Pitha-Rowe, I., Sekula, D., Lowrey, C.H., Nemeth, M.J., Golub, T.R., Freemantle, S.J., Dmitrovsky, E., 2002. UBE1L is a retinoid target that triggers PML/RARalpha degradation and apoptosis in acute promyelocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America* 99, 3806-3811.
- Komander, D., Rape, M., 2012. The ubiquitin code. *Annual review of biochemistry* 81, 203-229.
- Krishna, R.G., Wold, F., 1993. Post-translational modification of proteins. *Advances in enzymology and related areas of molecular biology* 67, 265-298.
- Liu, L.Q., Ilaria, R., Jr., Kingsley, P.D., Iwama, A., van Etten, R.A., Palis, J., Zhang, D.E., 1999. A novel ubiquitin-specific protease, UBP43, cloned from leukemia fusion protein AML1-ETO-expressing mice, functions in hematopoietic cell differentiation. *Molecular and cellular biology* 19, 3029-3038.
- Malakhov, M.P., Kim, K.I., Malakhova, O.A., Jacobs, B.S., Borden, E.C., Zhang, D.E., 2003. High-throughput immunoblotting. Ubiquitin-like protein ISG15 modifies key regulators of signal transduction. *The Journal of biological chemistry* 278, 16608-16613.

Malakhov, M.P., Malakhova, O.A., Kim, K.I., Ritchie, K.J., Zhang, D.E., 2002. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *The Journal of biological chemistry* 277, 9976-9981.

Maragno, A.L., Pironin, M., Alcalde, H., Cong, X., Knobloch, K.P., Tangy, F., Zhang, D.E., Ghysdael, J., Quang, C.T., 2011. ISG15 modulates development of the erythroid lineage. *PloS one* 6, e26068.

Meraro, D., Gleit-Kielmanowicz, M., Hauser, H., Levi, B.Z., 2002. IFN-stimulated gene 15 is synergistically activated through interactions between the myelocyte/lymphocyte-specific transcription factors, PU.1, IFN regulatory factor-8/IFN consensus sequence binding protein, and IFN regulatory factor-4: characterization of a new subtype of IFN-stimulated response element. *Journal of immunology* 168, 6224-6231.

Orfali, N., McKenna, S.L., Cahill, M.R., Gudas, L.J., Mongan, N.P., 2014. Retinoid receptor signaling and autophagy in acute promyelocytic leukemia. *Experimental cell research*.

Orfali, N., O'Donovan, T.R., Nyhan, M.J., Britschgi, A., Tschan, M.P., Cahill, M.R., Mongan, N.P., Gudas, L.J., McKenna, S.L., 2015. Induction of autophagy is a key component of all-trans-retinoic acid-induced differentiation in leukemia cells and a potential target for pharmacologic modulation. *Experimental hematology* 43, 781-793.e782.

Orfali N, O.D.T., Cahill M, Benjamin D, Nanus D, McKenna S, Gudas L, Mongan N. , 2019. All-trans retinoic acid (ATRA) induced TFE3 expression is required for myeloid differentiation in acute promyelocytic leukemia (APL). *European Journal of Haematology*. In Press.

Pitha-Rowe, I., Hassel, B.A., Dmitrovsky, E., 2004. Involvement of UBE1L in ISG15 conjugation during retinoid-induced differentiation of acute promyelocytic leukemia. *The Journal of biological chemistry* 279, 18178-18187.

Schlafli, A.M., Torbett, B.E., Fey, M.F., Tschan, M.P., 2012. BIRC6 (APOLLON) is down-regulated in acute myeloid leukemia and its knockdown attenuates neutrophil differentiation. *Experimental hematology & oncology* 1, 25.

Sgorbissa, A., Brancolini, C., 2012. IFNs, ISGylation and cancer: Cui prodest? *Cytokine & growth factor reviews* 23, 307-314.

Shah, S.J., Blumen, S., Pitha-Rowe, I., Kitareewan, S., Freemantle, S.J., Feng, Q., Dmitrovsky, E., 2008. UBE1L represses PML/RAR{alpha} by targeting the PML domain for ISG15ylation. *Molecular cancer therapeutics* 7, 905-914.

- Shi, H.X., Yang, K., Liu, X., Liu, X.Y., Wei, B., Shan, Y.F., Zhu, L.H., Wang, C., 2010. Positive regulation of interferon regulatory factor 3 activation by Herc5 via ISG15 modification. *Molecular and cellular biology* 30, 2424-2436.
- Takeuchi, T., Yokosawa, H., 2005. ISG15 modification of Ubc13 suppresses its ubiquitin-conjugating activity. *Biochemical and biophysical research communications* 336, 9-13.
- Tallman, M.S., Altman, J.K., 2009. How I treat acute promyelocytic leukemia. *Blood* 114, 5126-5135.
- Tang, X.H., Gudas, L.J., 2011. Retinoids, retinoic acid receptors, and cancer. *Annual review of pathology* 6, 345-364.
- Theilgaard-Monch, K., Jacobsen, L.C., Borup, R., Rasmussen, T., Bjerregaard, M.D., Nielsen, F.C., Cowland, J.B., Borregaard, N., 2005. The transcriptional program of terminal granulocytic differentiation. *Blood* 105, 1785-1796.
- Thein, M.S., Ershler, W.B., Jemal, A., Yates, J.W., Baer, M.R., 2013. Outcome of older patients with acute myeloid leukemia: an analysis of SEER data over 3 decades. *Cancer* 119, 2720-2727.
- Tschan, M.P., Fischer, K.M., Fung, V.S., Pirnia, F., Borner, M.M., Fey, M.F., Tobler, A., Torbett, B.E., 2003. Alternative splicing of the human cyclin D-binding Myb-like protein (hDMP1) yields a truncated protein isoform that alters macrophage differentiation patterns. *The Journal of biological chemistry* 278, 42750-42760.
- Yan, M., Luo, J.K., Ritchie, K.J., Sakai, I., Takeuchi, K., Ren, R., Zhang, D.E., 2007. Ubp43 regulates BCR-ABL leukemogenesis via the type 1 interferon receptor signaling. *Blood* 110, 305-312.
- Zhang, D., Zhang, D.E., 2011. Interferon-stimulated gene 15 and the protein ISGylation system. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 31, 119-130.
- Zhao, C., Beaudenon, S.L., Kelley, M.L., Waddell, M.B., Yuan, W., Schulman, B.A., Huibregtse, J.M., Krug, R.M., 2004. The Ubch8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN-alpha/beta-induced ubiquitin-like protein. *Proceedings of the National Academy of Sciences of the United States of America* 101, 7578-7582.
- Zhao, C., Denison, C., Huibregtse, J.M., Gygi, S., Krug, R.M., 2005. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse

cellular pathways. Proceedings of the National Academy of Sciences of the United States of America 102, 10200-10205.

Zou, W., Papov, V., Malakhova, O., Kim, K.I., Dao, C., Li, J., Zhang, D.E., 2005. ISG15 modification of ubiquitin E2 Ubc13 disrupts its ability to form thioester bond with ubiquitin. Biochemical and biophysical research communications 336, 61-68.

### Supporting Information

**Supplementary Figure 1** ISGylation – In the first step of ISGylation ISG15 is activated by the E1 enzyme UBE1L in an ATP dependent reaction. ISG15 is then transferred to the E2 UBE2L6 and subsequently to a target through an E3 enzyme such as HERC5. USP18 is an ISG15 specific isopeptidase which reverses this coupling.

### Figure Legends

**Figure 1. *UBE2L6* expression is increased during leukemic cell differentiation. (A)** *UBE2L6* mRNA levels of primary AML patient samples, normal CD34<sup>+</sup>(HSC) cells and mature granulocytes from healthy donors were quantified using qPCR. The relative  $\Delta$ Ct expression was calculated by the difference of *UBE2L6* expression to the housekeeping genes *HMBS* and *ABL* (Mann Whitney U Test \*\*\*\*p  $\leq$  0.0001). **(B)** NB4 and NB4R cells were seeded at  $0.2 \times 10^5$  cells/mL and treated with 1 $\mu$ M ATRA for 72h. Successful differentiation was confirmed in NB4 cells by flow cytometric analysis of CD11b expression. ATRA-resistant NB4R cells did not differentiate (data not shown). Total RNA was extracted and *UBE2L6* mRNA expression was quantified by qPCR. Values are given as n-fold induction compared to untreated cells and normalized to housekeeping gene *hPRT* (n=3) (t-test \*\*\*\*p  $\leq$  0.0001, \*\*p  $\leq$  0.01). **(C)** HL60 and HL60R cells were treated with 1 $\mu$ M ATRA for 96h. Successful HL60 differentiation was confirmed by qPCR measurement of GCSFR expression. ATRA-resistant HL60R cells failed to differentiate (data not shown). Total RNA was extracted and *UBE2L6* expression was quantified by qPCR. Values are given as n-fold induction compared to untreated cells and normalized to housekeeping gene *HMBS* (n=3) (t-test \*\*\*p  $\leq$  0.001).

**Figure 2. UBE2L6 inhibition attenuates APL cell differentiation.** NB4 cells expressing non-targeting shRNA (SHC) or shRNA targeting *UBE2L6* (shUBE2L6\_499 and shUBE2L6\_1082) were seeded at  $0.2 \times 10^5$  cells/mL and treated for 72h with  $1\mu\text{M}$  ATRA. **(A)** Functional knockdown efficiency was tested by measuring UBE2L6 protein levels in whole cell lysates by immunoblot at 72h.  $\beta$ -actin was used as a loading control. **(B)** Total RNA was extracted and differentiation was assessed by measuring *GCSFR* mRNA expression by qPCR. Values are given as n-fold induction compared to untreated cells and normalized to housekeeping gene *HMBS* (n=3) (t-test \*\*\* $p \leq 0.001$ , \* $p \leq 0.05$ ). **(C)** Surface CD11b protein expression on live cells was measured by flow cytometry as a second assay of differentiation. Median fluorescence intensities (MFIs) are shown at 72h (n=3) (t-test \*\* $p \leq 0.01$ ). **(D)** Morphologic appearance of treated cells at 72h. Neutrophil differentiation evidenced by increased cytoplasmic volume and nuclear lobulation, indicated with arrows. **(E)** Neutrophil function was tested using NBT at 72h **(i)** Differentiated cells reduce NBT to a blue colour. **(ii)** NBT positive cells were counted in triplicate and presented as mean  $\pm$  S.E.M (t-test \*\* $p \leq 0.01$ , \* $p \leq 0.05$ )(Magnification 400X).

**Figure 3. UBE2L6 regulates ATRA-induced ISGylation.** **(A)** Proteins known with a high confidence to interact with UBE2L6 are shown. Image created using the STRING proteomics database ([www.string-db.org](http://www.string-db.org)). **(B)** Levels of free and conjugated ISG15 was measured by immunoblot in whole cell lysates extracted from NB4 cells expressing either non-targeting shRNA (SHC) or shRNA targeting UBE2L6 (shUBE2L6\_499 and shUBE2L6\_1082) following a 72h treatment with  $1\mu\text{M}$  ATRA.  $\beta$ -actin was used as a loading control.

**Figure 4. ISG15 expression is induced during leukemic cell differentiation.** **(A)** NB4 and NB4R cells were seeded at  $0.2 \times 10^5$  cells/mL and treated with  $1\mu\text{M}$  ATRA for 72h. Successful differentiation was confirmed in NB4 cells by flow cytometric analysis of CD11b expression. ATRA-resistant NB4R cells did not differentiate (data not shown). Total RNA was extracted and *ISG15* mRNA expression was quantified by qPCR. Values are given as n-fold induction compared to untreated cells and normalized to housekeeping gene *hPRT* (n=3) (t-test \*\*\*\* $p \leq 0.0001$ ). **(B)** HL60 and HL60R cells were treated with  $1\mu\text{M}$  ATRA for 96h. Successful HL60 differentiation was confirmed by qPCR measurement of *GCSFR* expression. ATRA-resistant



HL60R cells failed to differentiate (data not shown). Total RNA was extracted and *ISG15* expression was quantified by qPCR. Values are given as n-fold induction compared to untreated cells and normalized to housekeeping gene *HMBS* (n=3) (t-test \*\*p ≤ 0.01).

**Figure 5. Inhibition of *ISG15* impedes APL cell differentiation.** NB4 cells expressing non-targeting shRNA (SHC) or shRNA targeting *ISG15* (shISG15\_319 and shISG15\_352) were seeded at  $0.2 \times 10^5$  cells/mL and treated for 72h with 1 $\mu$ M ATRA. **(A)** Functional knockdown efficiency was tested by measuring protein levels of both free and conjugated *ISG15* in whole cell lysates by immunoblot at 72h.  $\beta$ -actin was used as a loading control. **(B)** Total RNA was extracted and differentiation was assessed by measuring *GCSFR* mRNA expression by qPCR. Values are given as n-fold induction compared to untreated cells and normalized to housekeeping gene *HMBS* (n=3) (t-test \*\*\*\*p ≤ 0.0001, \*\*\*p ≤ 0.001). **(C)** Surface CD11b protein expression on live cells was measured by flow cytometry as a second assay of differentiation. Median fluorescence intensities (MFIs) are shown at 72h (n=3) (t-test \*p ≤ 0.05). **(D)** Morphologic appearance of treated cells at 72h. Neutrophil differentiation evidenced by increased cytoplasmic volume and nuclear lobulation, indicated with arrows. **(E)** Neutrophil function was tested using NBT at 72h. **(i)** Differentiated cells reduce NBT to blue colour. **(ii)** NBT positive cells were counted in triplicate and presented as mean  $\pm$  S.E.M (t-test \*\*p ≤ 0.01, \*p ≤ 0.05) (Magnification 400X).

**Table 1. ATRA-induced expression changes in ISGylation genes**

<b>Gene</b>	<b>Name</b>	<b>Fold Change in Expression</b>
<i>UBE2L6</i>	Ubiquitin-conjugating enzyme E2L6	200.93
<i>ISG15</i>	Interferon-stimulated gene 15	17.54
<i>USP18</i>	Ubiquitin specific peptidase 18	12.53
<i>UBE1L</i>	Ubiquitin-like modifier activating enzyme 7	7.77
<i>HERC5</i>	HECT and RLD domain containing E3 ubiquitin protein ligase 5	3.62
<i>TRIM25</i>	Tripartite motif containing 25	2.16

Figure 1

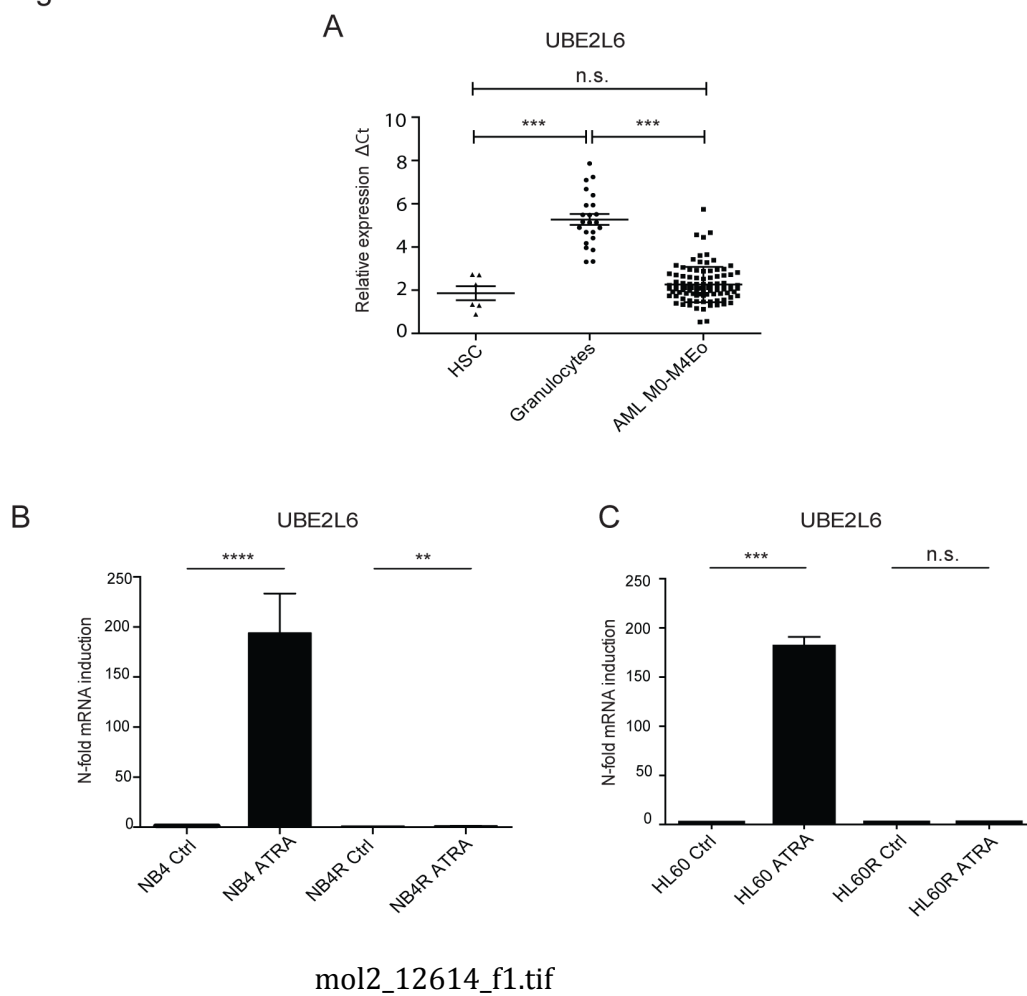


Figure 2

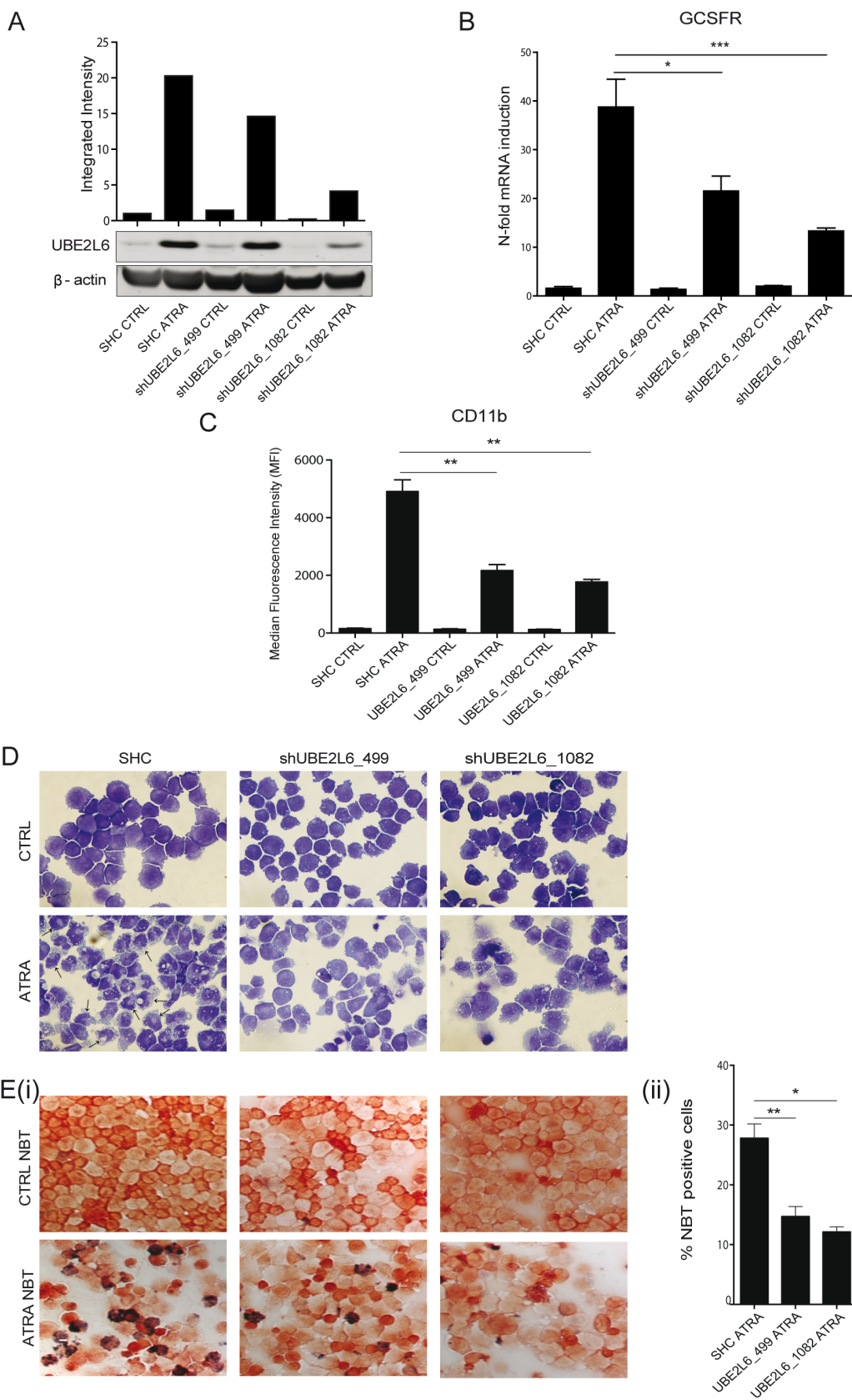
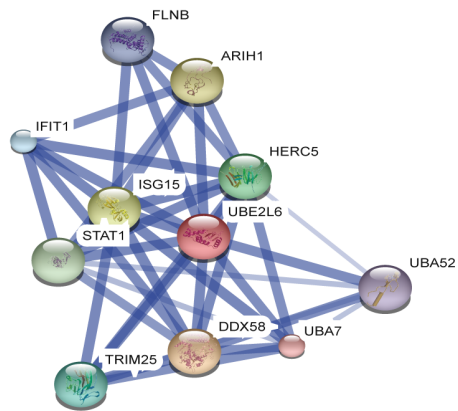
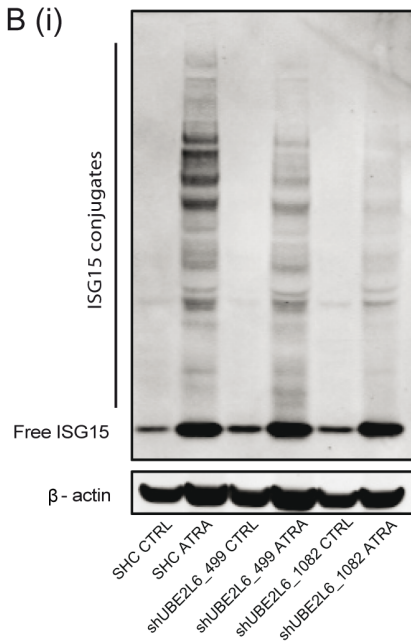


Figure 3

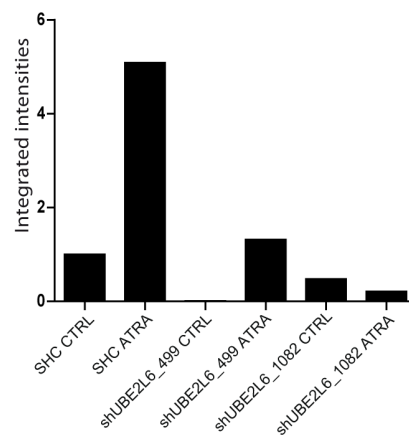
A



B (i)

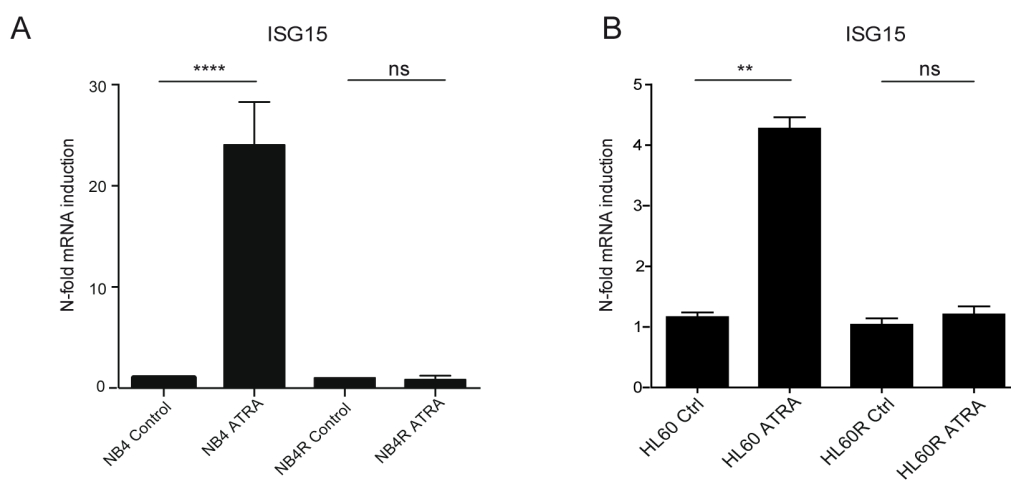


(ii)



mol2\_12614\_f3.tif

Figure 4



mol2\_12614\_f4.tif

Figure 5

