Transglutaminase 2 programs differentiating acute promyelocytic leukemia cells in all-trans retinoic acid treatment to inflammatory stage through NF-κB activation

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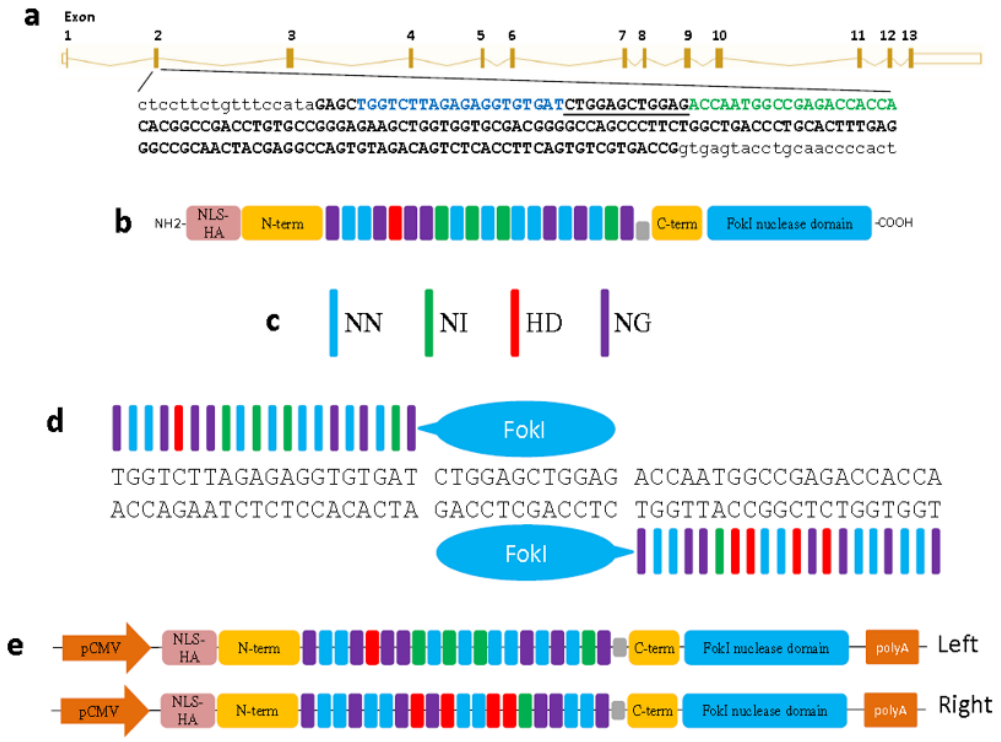
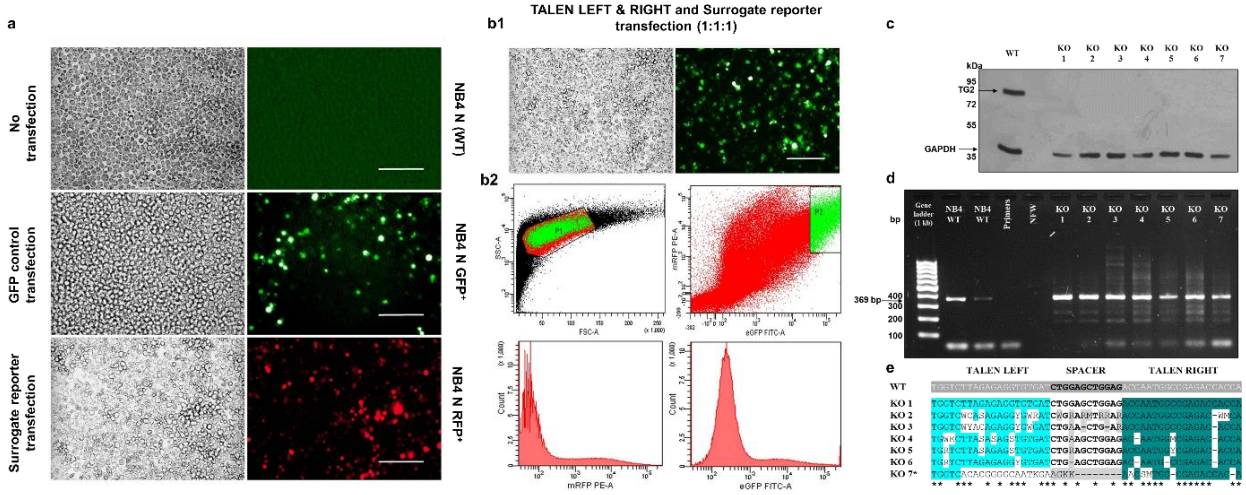
Supplementary figures



Supplementary Fig. 1. Transcription activator-like effector nucleases-based targeting, engineered endonuclease architecture, and modification of the transglutaminase 2 gene. (**a**) TALEN Library Net H27143 transcription activator-like effector nucleases (TALEN) pair target site (chr20:36223360-36223392) in exon 2 of the transglutaminase (TGM2) gene (37.99 kb, 13 exons) on chromosome 20 (q11.23). The exon 2 sequence is shown in boldface capital letters, to differentiate it from surrounding intron sequences. Left and right TALEN target sites are marked with blue and green, respectively. The 12-base pair spacer sequence is underlined. (**b**) The main elements of the TALEN Library Net engineered endonuclease complex: an NLS-HA tag, the N-terminal deletion of *Xanthomonas* TALEs, followed by the repeat variable diresidues (RVD) domains and the half-domain, and a C-terminal sub-region fused to the catalytic domain of the FokI nuclease. (**c**) RVD base recognition. The RVDs NN, NI, HD and NG bind G, A, C and T nucleotides, respectively, and are colour-coded. (**d**) A TALEN pair binding to the TG2 gene target sequence. Coloured bars represent the target-specific RVDs which follow the RVD: the DNA nucleotide recognition code shown in (**c**). TALEN expression platforms. (**e**) DNA plasmids with TALEN arrays in trans with gene expression controlled by the cytomegalovirus promoter and the bovine growth hormone polyadenylation signal.



Supplementary Fig. 2. Transfection efficiency in NB4 cells with transcription activator-like effector nucleases and surrogate reporter plasmids and selection and sorting of RFP$^+$/GFP$^+$ double transfected potential NB4 TG2 KO cell population

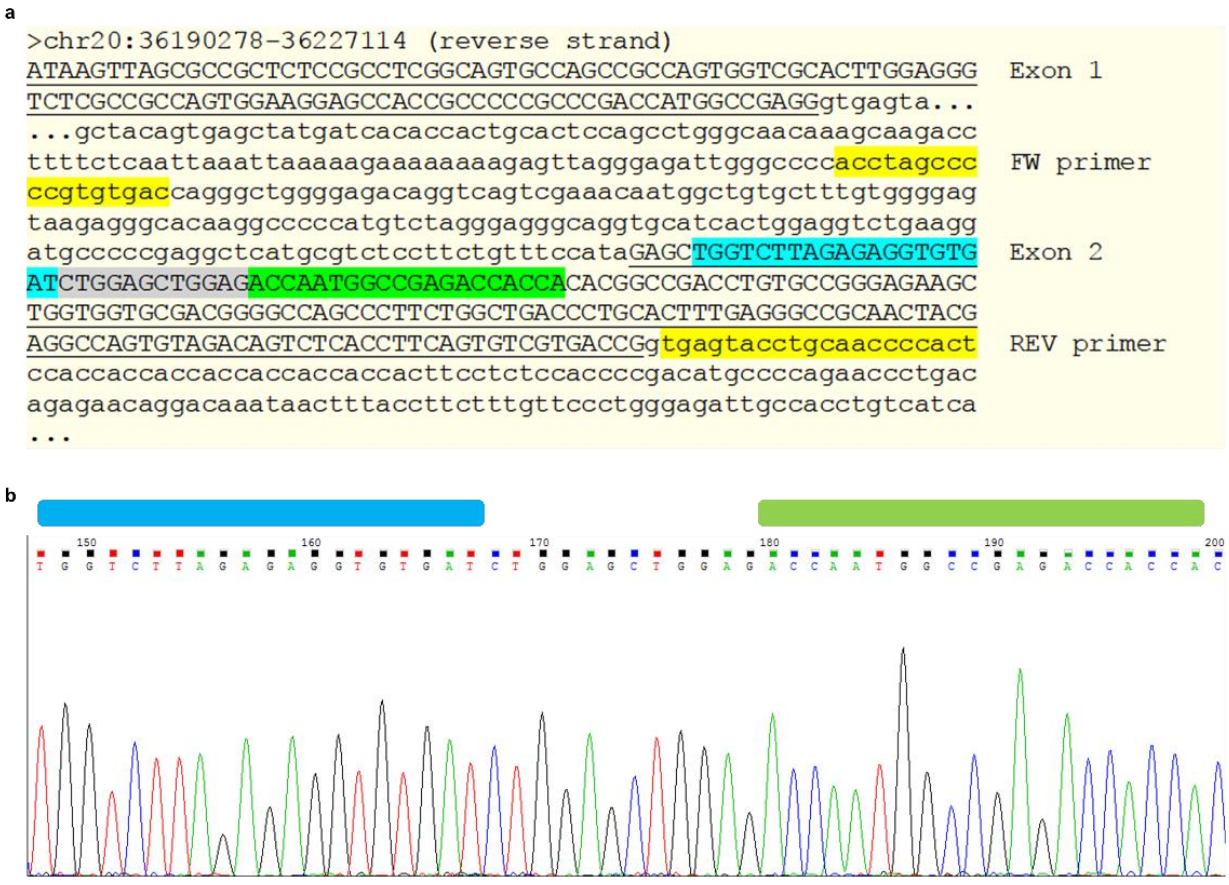
(**a**) Cell morphology and green fluorescent protein (GFP/RFP) positivity after transfection (day 3). Pictures and documentation were obtained using the FLoid® Cell Imaging Station (Life Technologies) instrument with the magnification ratio: 200 µm.

(**b1-2**) The mixed cell population was then analysed based on monomeric red fluorescent protein (mRFP) and enhance green fluorescent protein (EGFP) positivity, followed by the sorting process of the mRFP EGFP double-positive cells using FACSAria III™ technology. The first population (P1) marked the viable NB4 cells selected from debris and the dead cells, and in the secondary analysis, only the GFP- or mRFP-positive population were sorted (P2). NB4 wild-type cells transfected with both plasmids. The P2 population shows the double-positive cells sorted from the mixed population

(**c**) Western blot analysis of TG2 in potential all-trans retinoic acid -treated NB4 TG2-KO cells. Following clonal selection, cells were treated with 1 µM all-trans retinoic acid (ATRA) for 72 hour. Total cell lysate (approximately 1x 10^6 cells) was prepared, followed by loading of 20 µg protein in each lane. Blots were probed with a CUB7402 monoclonal antibody against transglutaminase (TG) 2 (Neomarkers) and with a GA1R monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (Covalab) at a 1:7,000 dilution ratio and anti-mouse secondary antibodies at a 1: 5,000 dilutions were used. Enhanced chemiluminescence exposure times of 20 min were used for all the blots

(**d**) PCR amplification of the transcription activator-like effector nucleases (TALEN) target region from genomic templates. Genomic DNA was isolated from both wild-type and TG2 KO1-7 NB4 cells. PCR reactions were carried out using forward and reverse sequencing primers (5'- ACCTAGCCCCCGTGTGAC, 5'- AGTGGGGTTGCAGGTACTCA, respectively), resulting in a calculated 369 base-pair fragments for the wild-type genomic sequence

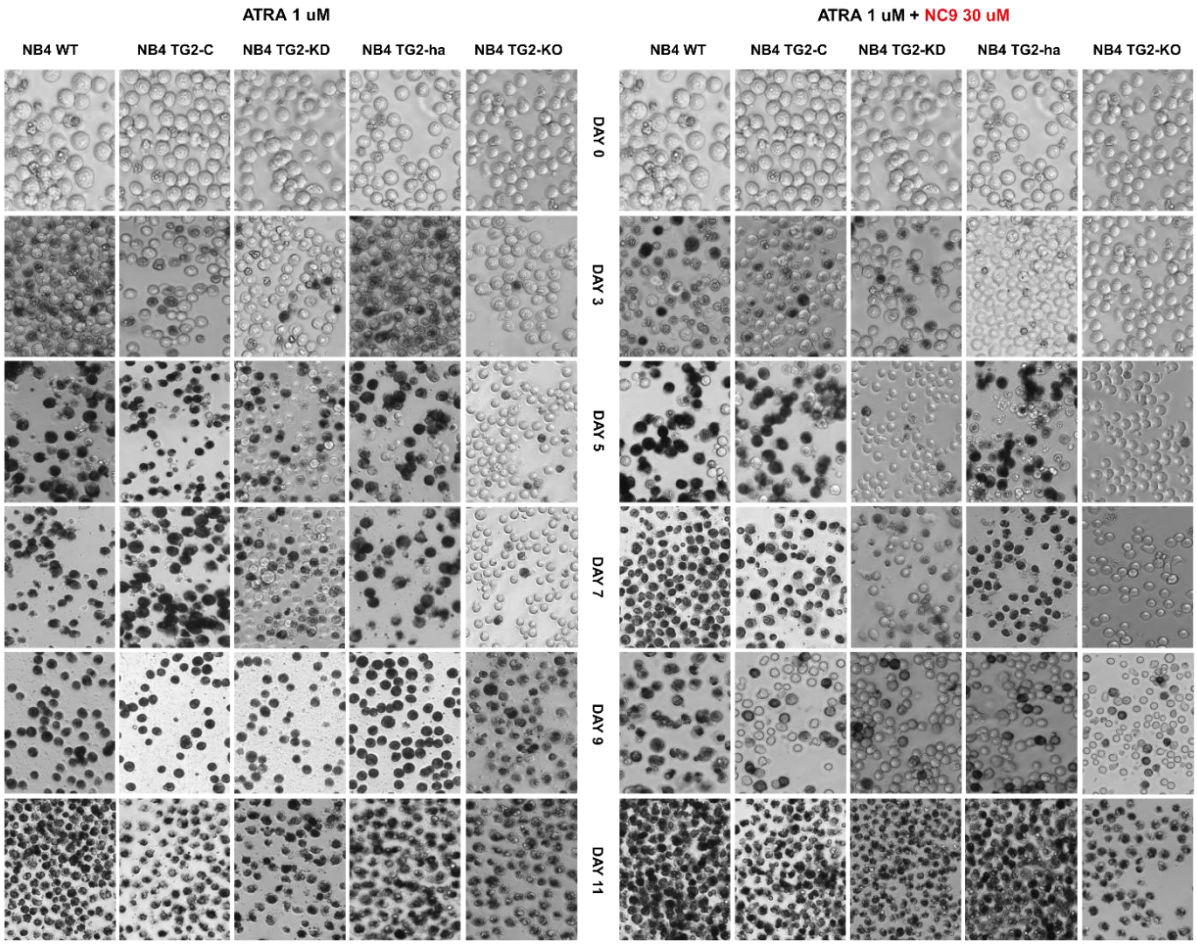
(**e**) Multiple sequence alignment Clustal W2 alignment of genomic sequences in the wild-type and NB4 TG2-KO cells at the TALEN pair binding sites Sequence changes or uncertainties due to sequences running in parallel (marked W, Y, R, M or S) are represented as white boxes in the TALEN binding sites and grey boxes in the spacer sequence. Nucleotides are in boldface mark wild-type sequences in the spacer region. Asterisks show the retention of wild-type nucleotides among the clones as conservation, excluding KO7, where the sequence of the region is highly divergent from that of the wild-type NB4 genomic sequence.



Supplementary Fig. 3. ***Human transglutaminase 2 (TGM2) gene knock-out transcription activator-like effector nucleases target sequence in exon 2 and arrangement of repeat variable diresidue domains pair (TALEN Library Net, HGNC:11778, TGM2, H27143)***

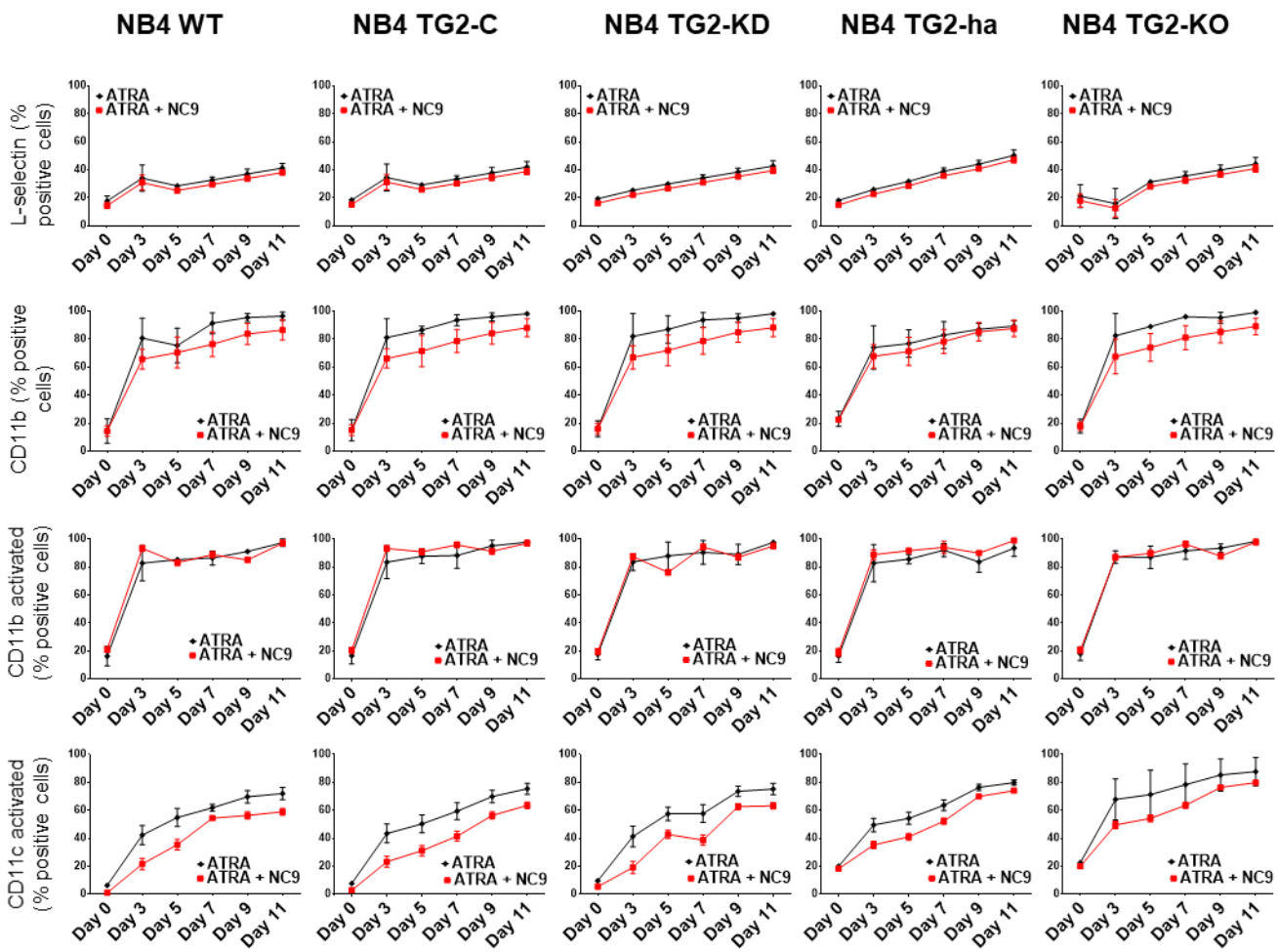
(**a**) The transglutaminase 2 gene sequence on the reverse strand (partial). Exon sequences 1 and 2 are in capital letters and are underlined. Left and right transcription activator-like effector nucleases (TALEN) target sites (TALEN L, TALEN R) are shown in boldface, the spacer is highlighted in grey, and the genomic sequencing primers are marked in italics.

(**b**) Transglutaminase 2 knockout transcription activator-like effector nucleases genomic target site sequencing in non-transfected NB4 cells

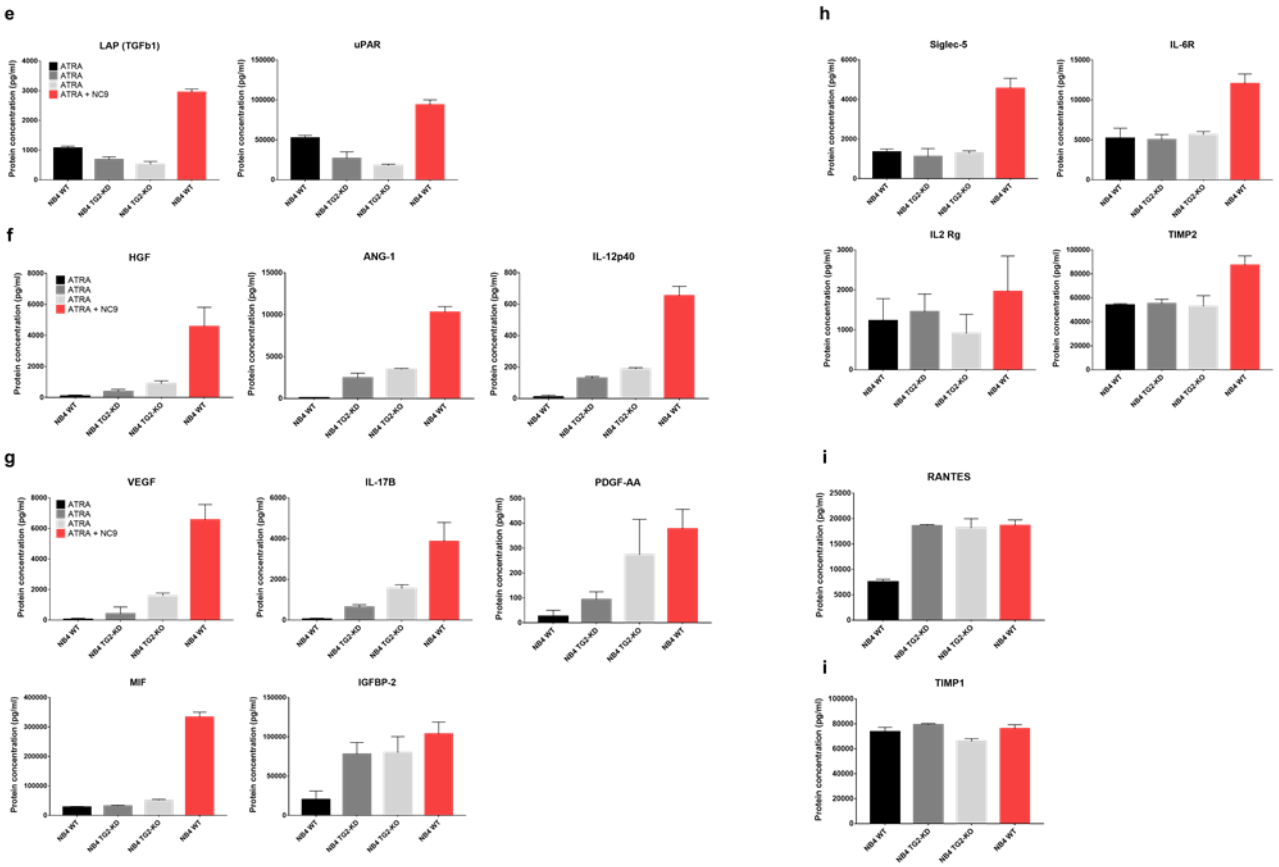


Supplementary Fig. 4. TG2 accelerates the phagocytotic and microbial killing functions of differentiating NB4 cell lines

NB4 cell lines were cultured in the presence of 1 μM ATRA (left) and the presence of 1 μM ATRA together with 30 μM NC9 (right) for 11 days. NBT reduction assays were performed at the indicated time points in triplicates. Light microscopic images and documentation were obtained using the FLoid® Cell Imaging Station (Life Technologies) instrument with a magnification scale: 200 μm.



Supplementary Fig. 5. ATRA induces expression of leukocyte β2 integrin receptors MAC-1 and p150,95, and their high-affinity state on the cell surface of NB4 cell lines.
FACS analysis of cell surface expression of L-selectin, differentiation marker CD11b/activated form and CD11c on 1 μM ATRA or 1 μM ATRA plus 30 μM NC9 -treated NB4 N, NB4 C, NB4 TG2-KO, NB4 TG2-ha and NB4 TG2-KO cells at the indicated days. Measurements were conducted in triplicates; values were validated by Flowing software. Graphs show the average of the percentage of the labelled positive cells ± SD.



Supplementary Fig. 6. Expression of inflammatory cytokines in Normal and TG2-deficient NB4 cell lines in response to ATRA and the TG2 inhibitor NC9.
In the cell culture supernatants, secreted chemokines and inflammatory cytokines were quantified by ELISA based fluorescent detection (Raybiotech). Figures show secreted protein levels from 3 independent experiments measured in triplicates. Statistical significance was determined by using the ANOVA (Bonferroni Test) $P < 0.05$.

Extended (supplementary) materials and methods

Cell culturing

APL cell line NB4 was cultured in RPMI 1640 HEPES-containing medium, supplemented with 10% (vol/vol) foetal bovine serum (Gibco, Paisley, Scotland), 2 mM L-glutamine and 1% (vol/vol) 100 U/mL penicillin-streptomycin solution (Sigma-Aldrich). Cultures were maintained at 37°C with 5% CO_2.

Preparation of total cell lysate

A total of 1–2x 10^6 cells were collected and centrifuged at 12,000 rpm at 4ºC for 10 min, and the resulting pellet was washed with pre-cooled 1x phosphate buffered saline (PBS). The pellet was then lysed in lysis buffer (50 mM Tris, 1mM EDTA, MEA, 0,5% Triton X 100, 1 mM PMSF) containing a protease inhibitor cocktail (Sigma-Aldrich) with a 1:100 dilution ratio, and homogenised with 5 to 7 strokes in a sonicator at the intensity of a 40% cycle (Branson Sonifer® 450). After sonication, the lysed samples were centrifuged at max rpm at 4ºC for 15 min. Finally, the supernatant was collected for measurement of the protein concentration with a Bradford assay (BIO-RAD).

Preparation of SDS-PAGE samples

The collected clear supernatant protein concentration was measured using 96-well plates (Wallac 1420 VICTOR2™ Multilabel counter) with a Bradford assay (BIO-RAD) at a wavelength of 595 nm. Every sample was measured three times in parallel, using BSA standard calibration (stock concentration 0.5 mg/ml). The samples were diluted up to a 2 mg/ml concentration, mixed with equal volumes of SDS denaturation buffer (0.125 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, 10% MEA, and 0.02% bromophenol blue) and incubated at 100°C for 10 min.

Preparation of cytosolic and nuclear fractions

A total of 4-5x 10^6 cells were collected and centrifuged at 12,000 rpm at 4ºC for 10 min, and the resulting pellet was washed with pre-cooled 1x PBS. Pellets were re-suspended in pre-cooled Nuclei isolation buffer (Nuclei Isolation Kit - Sigma-Aldrich NUC101-1KT) supplemented with protease inhibitor cocktail (Sigma-Aldrich), PMSF (Sigma-Aldrich) with a 1:100 dilution ratio and with phosphatase inhibitor cocktail (Sigma-Aldrich) with a 1:50 dilution ratio. After the resuspension, the lysed samples were centrifuged at 500 rcf at 4ºC for 5 min followed by the collection of the supernatant, was considered as the cytosolic fraction. The remaining pellets were washed two more times with pre-cooled Nuclei isolation buffer.

After washing the supernatant was removed, pellet was lysed in lysis buffer (50 mM Tris, 1mM EDTA, MEA, 0,5% Triton X 100, 1 mM PMSF) containing the supplements mentioned previously, and then homogenised with 7-10 strokes in a sonicator at the intensity of a 40% cycle (Branson Sonifer® 450). Nuclear and cytosolic fractions were centrifuged at max rpm at 4ºC for 20 min. Clear supernatants were collected for protein concentration measurement.

Western blot analysis

A total of 10–25 μg protein was electrophoresed on 10% SDS-polyacrylamide gels and blotted onto a polyvinylidene difluoride membrane (Millipore/Advansta) using a semi-dry blotting method. The membranes were blocked with 5% milk powder in 1x Tris-buffered saline and Tween 20 (TTBS) for 1 hour at room temperature. Monoclonal CUB7402-TG2 (Neomarkers, Fremont, CA) and monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich) were added as primary antibodies, diluted in 0.5% milk powder in 1x TTBS, with a dilution ratio of 1:5.000–1:7.000, and incubated overnight at 4°C, or for 2 hours at RT. The membranes were washed three times with 1x TTBS for 15 min at RT and then incubated with horseradish peroxidase-labelled, affinity-purified goat anti-mouse IgG secondary antibody (Advansta) at a 1:10.000 dilution ratio for 1 hour at RT. The TG band was visualised using the ECL Kit chemiluminescence-based method (Advansta). Images were taken using a ProteinSimple AlphaImager® HP instrument.

Nitroblue-tetrazolium (NBT) test

NB4 cell line differentiation was assessed using NITROBLUE-TETRAZOLIUM (NBT) (Sigma Aldrich) reduction assay following the manufacturer's instructions. All the photographs and documentation were obtained using the FLoid® Cell Imaging Station (Life Technologies) instrument.

Measurement of differentiation cell surface marker of CD11c/CD11b and Selectin-L

Approximately 4x 10^5 cells were plated, treated, collected and washed with pre-cooled 1x PBS, followed by centrifugation at 3000 rpm for 3 min. All the following steps were performed at 4°C. Pellets were washed and incubated with 2% BSA containing 1x PBS for 15 minutes and then centrifuged and washed and incubated at dark for 2 hrs with the PE labelled CD11c/CD11b, and FITC labelled Selectin-L human antibody in 1:25 dilution ratio (R&D Systems). For each treatment and sample, appropriate isotype control was prepared respectively. Incubation followed by repeated washing steps and the samples were measured

by FACS (BD FACScalibur instrument). The geometric mean fluorescence of the cells was used to calculate the CD11c/CD11b/Selectin-L surface expression. Data validated by WINMDI 2.9/Flowing software, normalised and corrected to the isotype controls.

Determination of superoxide anion production by luminol-amplified based chemiluminescence

Differentiated NB4 cells reactive O_2^- production was measured by a chemiluminescence assay using L-012 dye. The reaction volume of 100 µl containing 1 × 105 cells and 5.0 µl L-012 (100 µM). 2 µl PMA (100 nM) was added and incubated for 5 minutes, and then the chemiluminescence was measured Synergy Multi-Mode Microplate Reader (BioTek Instruments, Inc) at intervals of 10 seconds. Production of generated light by the reaction was recorded in relative luminometer units (RLUs) and corrected with the protein concentration level of the samples.

Transduction of NB4 cells with Luciferase lentiviral reporter

To evaluate the NF-kB pathway in NB4 cells, Ready-to-transduce transcription factor-responsive lentiviral reporter system (CLS-013-L8 -QIAGEN) was used in transient and in permanent transfections as well. The method was performed according to the protocol of the company.

Selection of the cells

The transfected cells were selected using puromycin solution (Sigma-Aldrich) dissolved in cell culture medium (RPMI 1640 HEPES using in 10 µg/ml final concentration.

Luciferase activity measurement

The Luciferase activity measurement was performed using Bright-GloTM Luciferase Assay system (Promega). Before measurement, the cell lines were each cultured in RPMI-1640 medium supplemented with 2% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, and 4 mmol/L L-glutamine and treated as described above. The analysis was performed according to the manufacturer's manual. The data was validated by Graphpad Prism software, using a parallel normalising method based on cell numbers and protein concentration. The latter was performed as previously described in the "Preparation of total cell lysate" section.

Cell cultures for the knock out experiments

As a basic cell line for the knock out experiments: APL wild-type NB4 (NB4 N) were cultured in RPMI 1640 HEPES-containing medium, supplemented with 10% (vol/vol) foetal bovine serum (Gibco; Paisley, Scotland), 2 mM L-glutamine and 1% (vol/vol) 100 U/mL penicillin-streptomycin solution (Sigma-Aldrich). For the functional analysis of TG2: NB4 wild-type and its sub-cell-lines, NB4 C (virus control) and NB4 TG2 KD cell lines (shRNA based KD targeting TG2; Csomós et al. 2010) were used and cultured under similar circumstances. All the cultures were maintained at 37°C with 5% CO_2.

TALENs plasmids

Talen Library Resource (Seoul National University) designed the plasmids for the human tissue transglutaminase 2 gene. To validate the effectiveness of transfection and FokI nuclease action, we used a GFP reporter-containing plasmid supplied by LAMOBICS company (GeneCopoeia, Labomics, Nivelles, Belgium): 3-pRG2S fluorescence surrogate reporter (custom target sequence) PRG2S-CS.

The sequences of the TALEN constructs containing the second exon of TG2 are as follows:

Human-H27143 Target site 1

TGGTCTTAGAGAGGTGTGAT ctggagctggag ACCAATGGCCGAGACCACCA

Left TALEN recognition site: TGGTCTTAGAGAGGTGTGAT
spacer: ctggagctggag
Right TALEN recognition site: ACCAATGGCCGAGACCACCA

Oligonucleotide primer for PCR and sequencing

The TALEN: Human H27143 plasmids of 6,763 bp sequences in length with cytomegalovirus (CMV) promoter. We designed a primer pair (Sigma-Aldrich) for later analysis.

The sequences of the designed primers were as follows:

Forward: (61.0 ºC) ACCTAGCCCCCGTGTGAC
Reverse: (60.6 °C) AGTGGGGTTGCAGGTACTCA

The length of the sequence spanned by the primers was 369 bp:

chr20: 36223215-36223583

5’ACCTAGCCCCCGTGTGACCAGGGCTGGGGAGACAGGTCAGTCGAAACAATGGC
TGTGCTTTGTGGGGAGTAAGAGGGCACAAGGCCCCCATGTCTAGGGAGGGCAGG
TGCATCACTGGAGGTCTGAAGGATGCCCCCGAGGCTCATGCGTCTCCTTCTGTTTC
CATAGAGCTGGTCTTAGAGAGGTGTGATCTGGAGCTGGAGACCAATGGCCGAGA
CCACCACACGGCCGACCTGTGCCGGGAGAAGCTGGTGGTGCGACGGGGCCAGCC
CTTCTGGCTGACCCTGCACTTTGAGGGCCGCAACTACGAGGCCAGTGTAGACAGT
CTCACCTTCAGTGTCGTGACCGGTGAGTACCTGCAACCCCACT 3’

3-pRG2S Fluorescence surrogate reporter (custom target sequence) PRG2S-CS

The surrogate reporter plasmid contained GFP and RFP genes for detection and selection of mutated cells. The total length of the plasmid was 6,122 bp.

Transformation of competent *E. coli* cells

Competent DH5α *E.coli* cells, or NEB® 5-alpha Competent *E. coli* (High Efficiency), with regard to the surrogate reporter plasmid, were separately transformed by the TALEN and surrogate reporter plasmids using the following steps (stock: TALEN LEFT 182 ng/µl; TALEN RIGHT 183 ng/µl; SURROGATE REPORTER 160 ng/µl):

1. 0.5 µl (TL: 91 ng; TR: 91.5 ng) plasmid DNA was added to 100 µl DH5α cells
2. 1.7 µl (90 ng) plasmid DNA was added to 100 µl NEB® 2529 cells
3. 60 min incubation on ice
4. Heat-shock for 90 min at 42°C
5. 2 min incubation on ice
6. Addition of 500 µl SOC (LB-Glucose)
7. Incubation with constant shaking at 37°C for 1–1.5 h
8. Plating the bacteria onto antibiotics-containing plates
9. Overnight incubation without shaking at 37°C
10. Collection of grown colonies (MIDI PREP/Restriction digestion)
11. Plasmid DNA multiplication and isolation
12. Restriction digestion

Plasmid DNA preparation

Plasmid DNA preparation was carried out using a NucleoSpin® Plasmid isolation kit (Macherey-Nagel, Düren, Germany) of high copy plasmid DNA from E.coli, following the manufacturer's instructions.

Restriction digestion

We carried out restriction analysis with both plasmids, and used the following restriction analysis mixture (an in silico test had previously been conducted using Serial Cloner software, involving XbaI and BamHI restriction enzymes) to analyse the digestion fragments by size and to visualise and purify them for further analysis [stock: TALEN LEFT (820,7 ng/µl); TALEN RIGHT (862,6 ng/µl); SURROGATE REPORTER (832,6 ng/µl)].

Restriction reaction mixture:

10x Tango buffer	2.0 µl
BamHI	1.0 µl
XbaI	1.0 µl
DNA plasmid	500.0 ng
NFW	15.5 µl
The total volume	20 µl.

Transfection of NB4 cells

NB4 cells were transfected using Amaxa® Cell Line Nucleofector® V Kit following the manufacturer's instructions. During transfection, the plasmid DNA ratio was 1:1:1. The maximum plasmid DNA amount should not exceed 2 µg. The transfection efficiency was measured by assessing the degree of GFP/RFP positivity in transfected cells after Days 0, 1 and 3.

Flow cytometry and cell sorting

Cells were analysed and sorted on a BD FACSAria™ III flow cytometer (BD Biosciences, San Jose, CA). Dead cells were excluded from the analysis by forward scatter and side scatter gating, and 488 and 561nm lasers were used for excitation of enhanced GFP (EGFP) and monomeric RFP (mRFP). EGFP and mRFP fluorescence signals were detected using 530/30 and 582/15 band-pass filters, respectively. Triple transfected NB4 cells were sorted by EGFP and mRFP signals, and approximately 12,000 EGFP mRFP double-positive cells were sorted.

Limiting dilution and cloning

The sorted cells were cloned using the limited dilution method. A total of 1–5x 10^5 sorted cells were diluted and plated in the round (U) bottom 96-well plates (TPP) at a density of 1–2 cells/well. Clones had undergone clonal expansion at 37°C with 5% CO_2.

Determination of gene mutation in the TALENs KO cells

Genomic DNA isolation was performed using the TRIzol® (Invitrogen)-based and column-based methods. The latter method was performed following the manufacturer's protocol (UD GenoMed & Covalab tissue and blood DNA isolation kit) The PCR reaction with TALEN target site-specific primers are detailed in the supplemental materials and methods. The purified DNA samples were further analysed by sequencing, which was performed in the Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen, Hungary. The sequencing was carried out according to the Core Facility protocol. The results were analysed using Chromas LITE/Finch TV software, and TG2 KO sequences were aligned to reference sequences Genetic differences were evaluated using the National Center for Biotechnology Information basic local alignment search tool method. The result of the PCR-based genomic sequencing of the TALEN target site region in TG2 KO NB4 cell clones is demonstrated in Fig. 3-4, and detailed in Supplementary Fig. 2-3. Genomic DNA isolation and PCR reaction with TALENs-specific primers

Genomic DNA isolation

Genomic DNA isolation was performed using the TRIzol® (Invitrogen)-based and column-based methods respectively. The latter method was performed following the manufacturer's protocol (UD GenoMed & Covalab tissue and blood DNA isolation kit), while the former method was carried out using the following steps:

1. 1–2 x 10^6 cells were harvested and washed with 1x PBS
2. The centrifuged pellet was homogenised in TRIzol® reagent
3. 0.3 ml absolute ethanol per TRIzol® was added
4. The tube was gently inverted 2-3 times
5. The samples were maintained at RT for 2–5 min
6. Centrifugation at 2,000 g at 4°C for 5 min
7. The aqueous phase was removed
8. The DNA pellet was washed twice in 0.1 M tri-sodium citrate containing 10 % ethanol
9. The samples were incubated in wash solution at RT for 30 min with periodic mixing
10. Centrifugation at 2,000 g at 4°C for 5 min
11. The DNA was re-suspended in 70% ethanol
12. Incubation at RT for 30 min with periodic mixing
13. Centrifugation at 2,000 g at 4–25°C for 5 min

14. The ethanol phase was removed, and the pellet was briefly dried at RT for 2–5 minutes
15. The pellet was dissolved in 8 mM NaOH by passing it slowly through a pipette
16. DNA yield and quality were determined, and the A260 was measured.

PCR

The first PCR reaction was performed in 8-well strips in a volume of 50 µl per well using the following protocol: 95°C: 3 min, [95°C: 30 sec, 60°C: 30 sec, 72°C:1 min, 72°C: 5 min] x 30 cycle, 4°C

Reagents	µl
Taq polymerase buffer (10x)	5
dNTP mix (10 mM)	5
TALENs Forward primer (10 pMol)	1
TALENs Reverse primer (10 pMol)	1
$MgCl_2$ (25mM)	8
gDNA (50 ng/µl)	1
Taq polymerase	1
NFW	38
Total volume (µl)	50

For analysis, one-tenth of the product was loaded onto a 2% agarose gel, and the DNA fragments from this gel were isolated using a Macherey-Nagel gel extraction kit (Macherey-Nagel, Düren, Germany). The purified DNA samples were further analysed by sequencing, which was performed in the Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen, Hungary. The sequencing was carried out according to the Core Facility protocol. The results were analysed using Chromas LITE/Finch TV software, and TG2 KO sequences were aligned to reference sequences Genetic differences were evaluated using the National Center for Biotechnology Information basic local alignment search tool method.

Statistical analysis

All values on plots are expressed as the mean ± standard deviation of the indicated independent experiments. Statistical analysis was carried out using GraphPad Prism 7.04 version, by student T-test and Two-Way ANOVA (Bonferroni posthoc test; *P<0.05, **P<0.01 and ***P<0.001 ****P<0.001).