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RESEARCH ARTICLE

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The impact of an additional copy of chromosome 21 in B-cell precursor acute lymphoblastic leukemia

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Abstract

A common finding in pediatric B-cell precursor acute lymphoblastic leukemia (BCPALL) is that chromosome 21 is never lost and an extra chromosome 21 is often gained. This implies an important role for chromosome 21 in the pathobiology of BCPALL, emphasized by the increased risk of BCPALL in children with Down syndrome. However, model systems of chromosome 21 gain are lacking. We therefore developed a BCPALL cell line (Nalm-6, *DUX4*-rearranged) with an additional chromosome 21 by means of microcell-mediated chromosome transfer. FISH, PCR, multiplex ligation-dependent probe amplification, and whole exome sequencing showed that an additional chromosome 21 was successfully transferred to the recipient cells. Transcription of some but not all genes on chromosome 21 was increased, indicating tight transcriptional regulation. Nalm-6 cells with an additional chromosome 21 proliferated slightly slower compared with parental Nalm-6 and sensitivity to induction chemotherapeutics was mildly increased. The extra copy of chromosome 21 did not confer sensitivity to targeted signaling inhibitors. In conclusion, a BCPALL cell line with an additional human chromosome 21 was developed, validated, and subjected to functional studies, which showed a minor but potentially relevant effect in vitro. This cell line offers the possibility to study further the role of chromosome 21 in ALL.

KEYWORDS
acute lymphoblastic leukemia, cell line, chromosome 21, IFNGR2, microcell-mediated chromosome transfer, Nalm-6

1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common oncologic childhood disease. The majority (85%) of ALL cases is B-cell precursor ALL (BCPALL).[1] Based on genetic rearrangements in the leukemic cells, BCPALL is further divided into subgroups that are used for risk stratification in treatment protocols.[2] Chromosome 21 is involved in multiple of these subgroups, such as intrachromosomal amplification of chromosome 21 (iAMP21) and t(12;21)(p13;q22) leading to the fusion gene *ETV6::RUNX1*. Additionally, chromosome 21 is—of all chromosomes—most frequently involved in numerical abnormalities, either in a near-diploid leukemia or in the context of high hyperdiploidy (≥51 chromosomes), involving one or more extra copies of chromosome 21 in leukemic cells.[3–5] In addition, in hypodiploid leukemic cells

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(≤45 chromosomes)—chromosome 21 is always retained.[6] In contrast, in solid tumors chromosome 21 is more frequently lost than gained.[7] Children with Down syndrome (DS) have a 20- to 40-fold increased risk of developing acute leukemia, while the incidence of solid tumors is lower than in non-trisomy 21 peers.[8–10] These findings indicate a role for an additional chromosome 21 in the development and/or establishment of leukemia. The underlying mechanism is yet to be elucidated, but research regarding this topic is challenging due to the lack of appropriate models. Primary patient material is less suitable for this purpose since leukemic cells do not proliferate or survive for extended periods of time ex vivo. In addition, patient cells often show secondary genetic differences that can hamper comparison of results obtained from experiments conducted with material from different patients.[11,12]

Several BCPALL cell lines are available, but none of these have an additional copy of chromosome 21. Microcell-mediated chromosome transfer (MMCT) is a method applied for transferring large amounts of DNA and has been proven successful in transferring fully intact chromosomes.[13] Recently, the human chromosome 21 has been successfully transferred by MMCT into human embryonic stem cells.[14] Development of a trisomy 21 BCPALL cell line would enable experimental research and would also prevent the bias introduced by germline and somatic genetic differences in primary patient materials. This study aimed to develop and validate a trisomy 21 BCPALL cell line and investigate whether the presence of an extra chromosome 21 influences cell proliferation and drug sensitivity. Ultimately, identification of the effects and traits of an additional chromosome 21 in BCPALL could aid in the development of new—targeted—treatments.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Nalm-6 (DSMZ) cells were cultured in RPMI 1640 medium + GlutaMAX™ Supplement, supplemented with 10% Fetal Calf Serum (FCS) and penicillin, streptomycin, fungizone (PSF). Cells were passaged twice a week at 0.2×10^6 cells/mL. STR profiling was routinely performed with the Nalm-6 cell line every 3–4 months to confirm cell line identity. A9 (21-16) cells were kindly provided by prof. Oshimura and prof. Kazuki[14] and cultured in Dulbecco's modified Eagle medium (DMEM) high glucose + GlutaMAX™ Supplement pyruvate, supplemented with 10% FCS and geneticin (G418 sulfate; 800 µg/mL). Cells were passaged twice a week at 70%–90% confluency. All culturing was performed in 37°C and 5% CO_2. All cell lines were routinely tested for mycoplasma every 6 weeks and remained negative.

2.2 | Microcell-mediated chromosome transfer

A detailed protocol is available in the Supporting Information. A previously published protocol[15] was adjusted for the current study (Figure 1). A9 (21-16) cells were grown in Nunc™ Cell Culture Tubes (flat bottom, growth surface 5.5 cm^2) to 70%–80% confluence. Subsequently, cells were exposed to colcemid (50 µg/L) in DMEM. After 48 h, colcemid was replaced by cytochalasin B (10 µg/mL) in DMEM and the culture tubes were centrifuged (1 h, 12 000 *g*, 34°C) in a fixed rotor (Beckman-Coulter BC avant J-E, rotor JA-10), ensuring the centrifugal force was directed away from the growth surface. Resulting pellets were resuspended in FCS-free DMEM, dissociated with an 18G needle, and purified through subsequently 8, 5, and 3 µm Whatman® Nucleopore track-etched filter membranes. The purified microcell suspension was centrifuged (500 *g*, 5 min). The pellet was resuspended in PHA-P solution (2 mL, 50 mg/L) in FCS-free DMEM, mixed with 1.0×10^6 Nalm-6 recipient cells (washed twice with FCS-free RPMI) and centrifuged (500 *g*, 5 min). The resulting pellet was resuspended for 1 min in Polyethylene Glycol 1000 (PEG-1000; 1 mL 47% [w/v]; 5 g PEG-1000 in 1 mL dimethyl sulfoxide [DMSO] and 6 mL RPMI), after which FCS-free medium (10 mL) was added. The suspension was centrifuged (500 *g*, 5 min), and the resulting pellet

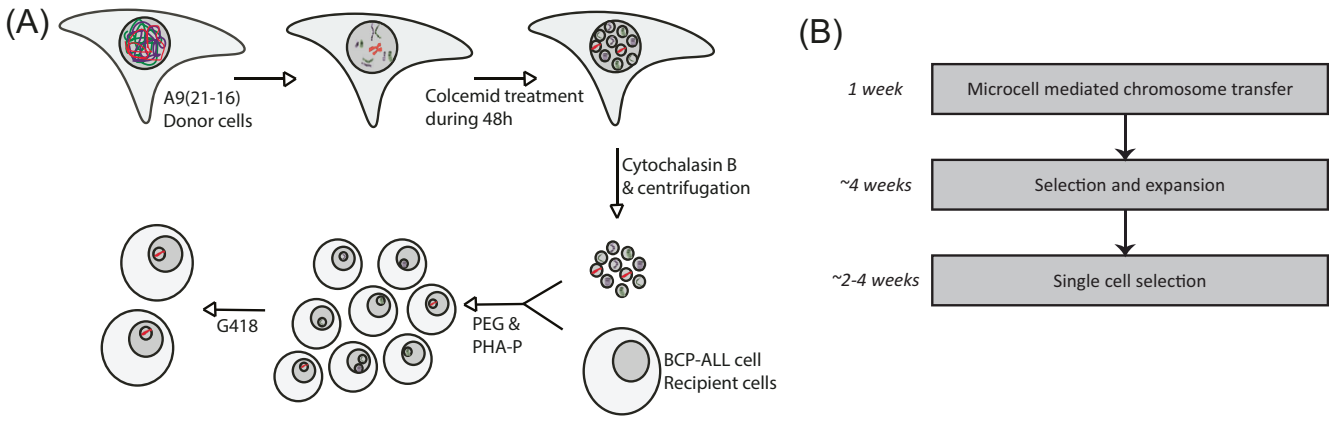

FIGURE 1 Schematic overview of the microcell-mediated chromosome transfer (MMCT) protocol. (A) Schematic diagram of the MMCT protocol. Chromosome of interest—human chromosome 21 with the neo/G418 resistance gene as selection marker—is displayed in red. (B) Overview and timeline of the complete process.

resuspended in nonselective culture medium (5 mL) and put in a 6-well plate. After 3 days, nonselective culture medium was replaced with selection medium (500 µg/mL G418). Cells were cultured on selection medium until exponential growth and limiting dilution was started (5.5 weeks for batch 1, N6-T21-B1).

2.3 | Limiting dilution

Based on a previously published protocol,[16] a cell suspension of 1.0×10^6 cells/mL was generated in nonselective culture medium. Twice, the solution was diluted 1:100, followed by an additional dilution of 1:20, resulting in a cell suspension with an estimated concentration of 5 cells/mL. One hundred microliters of the resulting cell suspension was put in each well of a 96-well round bottom plate. Twice a week, half of the total volume was replaced with fresh medium until cell pellets were visible. Subsequently, cells were passaged at regular culturing density (0.2×10^6 cells/mL).

2.4 | Genomic polymerase chain reaction

Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Venlo, The Netherlands) according to manufacturers' protocol. Polymerase chain reaction (PCR) was performed using AmpliTaq Gold™ DNA Polymerase with Buffer II and $MgCl_2$ (Life Technologies, Carlsbad, CA). Primers were designed for the neomycin resistance gene (*pSTneo*): forward primer 5′-TATGTCCTGATAGCGGTCCG-3′; reverse primer: 5′-GTTGTCACTGAAGCGGGAAG-3′. DNA quality was assessed using a FLT3 PCR primer set.[17] For gel-electrophoresis, PCR products were stained with TriTrack loading dye (Thermo Fisher Scientific, Waltham, MA) and loaded onto an agarose gel (2% [w/v]) containing MIDORI^{GREEN} (NIPPON Genetics). A GeneRuler (100 bp) Plus DNA Ladder (0.1 µg/µL) was used. PCR for the *pSTneo* gene was routinely performed to confirm the continued presence or absence of the additional copy of chromosome 21 in the selected cell lines.

2.5 | Drug sensitivity assay

Cells were exposed to six concentrations of prednisolone (0.0076–250 µg/mL), daunorubicin (0.0020–2 µg/mL), asparaginase (0.0032–10 IE), gilteritinib (0.0032–10 µM; Selleckchem, Houston, TX), ruxolitinib (0.3125–10 µM; Selleckchem), and trametinib (0.00064–5 µM; Selleckchem) in duplicate for 4 days at normal culturing density. Gilteritinib, ruxolitinib, and trametinib were tested in the presence of 32 ng/mL FLT3 ligand (Thermo Fisher Scientific), 20 ng/mL TSLP (R&D Systems, Minneapolis, MN), and 8 ng/mL IL7 (Miltenyi Biotec, Leiden, The Netherlands) after 30 min of prestimulation with a 1.25× concentration. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL in PBS) was added and incubated at 37°C and 5% CO_2 for 2 h and dissolved with acidified isopropanol (0.04 N HCl). After 5 min of incubation, the absorbance was read on a spectrophotometer at wavelengths of 562 and 720 nm with Softmax pro 7.0.3 software.

2.6 | Multiplex ligation-dependent probe amplification

The following kits were used for multiplex ligation-dependent probe amplification (MLPA): SALSA MLPA P327 iAMP21-ERG probemix and SALSA MLPA EK1 reagent kit (FAM) (MRC Holland, Amsterdam, the Netherlands). MLPA was conducted according to manufacturers' protocol. Amplified fragments were quantified by an ABI3500 genetic analyzer (Applied Biosystems, Carlsbad, CA). Software used for data analysis was Coffalyser.Net (v.140721.1958) (MRC Holland). Cutoffs for heterozygous deletion and heterozygous duplication were <0.65 and >1.30, respectively.

2.7 | RNA sequencing

Total RNA was isolated using TRIzol. RNA concentration was determined using the DeNovix (Life Science Research, Wilmington, DE) and Qubit with the Qubit RNA broad range kit (Thermo Fischer Scientific). RNA integrity was analyzed using Bioanalyzer and Agilent RNA 6000 nano kit (Agilent Technologies, Santa Clara, CA). Subsequently, library preparation and RNA sequencing was performed by Novogene (Hong Kong, China). Library preparation was done with the TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Human/Mouse/Rat kit. Paired-end sequencing, of 150 base pair long reads, was done on the NovaSeq6000 (Illumina, San Diego, CA), generating at least 50 million raw reads per sample. The aligning of fastq files to the reference genome (GENCODE v29 GRCh38) and read counting were done using STAR version 2.6.0c. Fusion detection was done using FusionCatcher and STAR-fusion. RNA sequencing data were only used for fusion detection and expression levels, not for mutation analysis.

2.8 | Whole exome sequencing

Genomic DNA was isolated the Qiagen DNeasy Blood & Tissue Kit (Qiagen). DNA concentration was determined using the DeNovix (Life Science Research) and the Qubit and corresponding Qubit dsDNA broad range kit (Thermo Fischer Scientific). Subsequently, library preparation and DNA sequencing was performed by Novogene (Hong Kong, China). In short, sequencing libraries were generated using Agilent SureSelect Human All ExonV6 kit (Agilent Technologies). Paired-end sequencing, of 150 base pair long reads, was done on the NovaSeq6000 (Illumina), generating at least 60 million raw reads per sample. The fastq files were aligned to the reference genome (GENCODE v29 GRCh38) using Minimap2 version 2.12. Variant calling was done using Mutect version 2.2 and copy number estimations were done using GATK version 4.1.9.0.

2.9 | Fluorescent in situ hybridization

Cytospins of the donor cell line A9 (21-16), the parental Nalm-6, and MMCT-generated trisomy 21 clones N6-T21-C5 and C7 were hybridized using the 21q22/*RUNX1* break-apart probeset (Cytocell, Cambridge, UK). For each fluorescent in situ hybridization (FISH), 100 interphase nuclei were counted by two independent analysts. No metaphases were detected.

2.10 | Proliferation assay

Cells were stained with CarboxyFluorescein Succinimidyl Ester (CFSE) (Abcam, Waltham, MA) according to the manufacturers' protocol and seeded at 0.1×10^6 cells/mL. After 24, 48, 72, or 96 h of culture cells were stained with 1:1000 Sytox Red (Thermo Fisher Scientific, S34859) in FACS buffer (2% FCS in PBS) and incubated at 4°C for 30 min. Subsequently, fluorescence was measured with Cytoflex S (Beckman Coulter, Brea, CA) and analyzed using FlowJo (version 10.7.1).

2.11 | IFNGR2 protein expression

Cells were incubated with human IFN-gamma R2 APC-conjugated antibody (R&D systems; FAB773A) and isotype control antibody goat IgG APC-conjugated antibody (R&D systems; IC108A) in 1:50 FACS buffer at 4°C for 30 min. Subsequently, cells were exposed to 1:1000 Sytox Green (Thermo Fischer Scientific, S7020) in FACS buffer and incubated at 4°C for 30 min. Fluorescence was measured using Cytoflex S and analyzed using FlowJo.

2.12 | Statistical analyses

Differential gene expression was determined using the R package EdgeR (version 3.32.1), with an FDR correction for multiple testing. Statistical analysis of functional experiments was performed in Prism. A one-way ANOVA was used to determine differences in all functional experiments, with a Dunnett post-hoc test, using the parental Nalm-6 as the control sample. Data were considered significant if $p < 0.05$. All functional experiments were repeated at least three times, unless otherwise specified. Data are presented as mean ± SEM, unless otherwise specified.

3 | RESULTS

3.1 | Characterization of recipient cell line

As recipient cell line for the MMCT procedure, Nalm-6 was selected. This is a widely used BCPALL cell line characterized by *DUX4* rearrangement to the *IGH* locus,[18] resulting in high expression of *DUX4* (Figure S1A). *DUX4* rearrangement can co-occur with an additional copy of chromosome 21 in ALL patients with a frequency of around 20% (10/49 *DUX4*-rearranged samples with informative karyotype.[19] It was confirmed that Nalm-6 does not contain an additional chromosome 21, but an intragenic *ERG* deletion (exon 5 to 11, NM_001243428.1) on chromosome 21 was found (Figure S1B). The latter is in line with the description of *ERG* deletions in *DUX4*-rearranged BCPALL.[18,20,21] In addition, Nalm-6 has copy number variations on several chromosomes (Table S1), including a deletion on chromosome 5 that leads to a monoallelic loss of *PDGFRB* (Figure S1C). Although it has been suggested that Nalm-6 has an *ETV6::PDGFRB* fusion,[22] no evidence supporting the presence of this fusion was found (Figure S2). A drug sensitivity assay showed that Nalm-6 is sensitive to the selection drug G418 with an inhibitory concentration of 50% (IC50) at approximately 500 µg/mL (Figure S1D). Thus, Nalm-6 was selected as recipient cell line.

3.2 | Microcell-mediated chromosome transfer

Using MMCT with Nalm-6 (designated N6-WT) as recipient cells, we generated two batches of Nalm-6 trisomy 21 cells (N6-T21), designated as N6-T21-B1 and N6-T21-B2 (B1 and B2 indicating batch 1 and batch 2, respectively). After chromosome transfer, it took approximately 3 weeks for the resulting cell population to start growing steadily under G418 selective pressure at 500 µg/mL (Figure 2A). Selection and subsequent drug sensitivity assay showed increased resistance of N6-T21 cells to the G418 as compared with N6-WT cells (Figure 2B). Both batches tested positive for the neomycin resistance gene on genomic PCR (Figure 2C). By selection with the IC50, it is possible that this resulting population has a heterogeneous chromosome 21 copy number. Therefore, subsequently limiting dilution experiments were performed using the N6-T21-B1 cells to obtain clonal cell populations. Based on the protocol by Ryan,[16] a limiting dilution experiment was conducted from which initially 16 clones were derived, named N6-T21-C[*clone_number*]. Clones 2 and 13 died during expansion. The remaining 14 clones tested positive for the neomycin resistance gene (Figure 2D), indicating that the clones harbored the additional copy of chromosome 21.

3.3 | Validation of the additional copy of chromosome 21

Using MPLA, we confirmed that the N6-T21 clones gained a copy of chromosome 21 (Figure 3A; Figure S3). N6-T21-C9 showed inconclusive MLPA results and was excluded from subsequent experiments. The intragenic *ERG* deletion originally present in N6-WT was present in all N6-T21 clones. We performed whole exome sequencing (WES) on N6-WT and four randomly selected clones (N6-T21-C5, C7, C11, and C15), confirming the gain of chromosome 21 (Figure S4). Both MLPA and WES indicated a deletion from 18 134 084 to 23 079 642 bp on chromosome 21 (human genome build GR38), with the only protein-coding genes being *TMPRSS15* and *NCAM2*. Since this region was diploid in N6-WT (Figure S1B), the deletion was likely

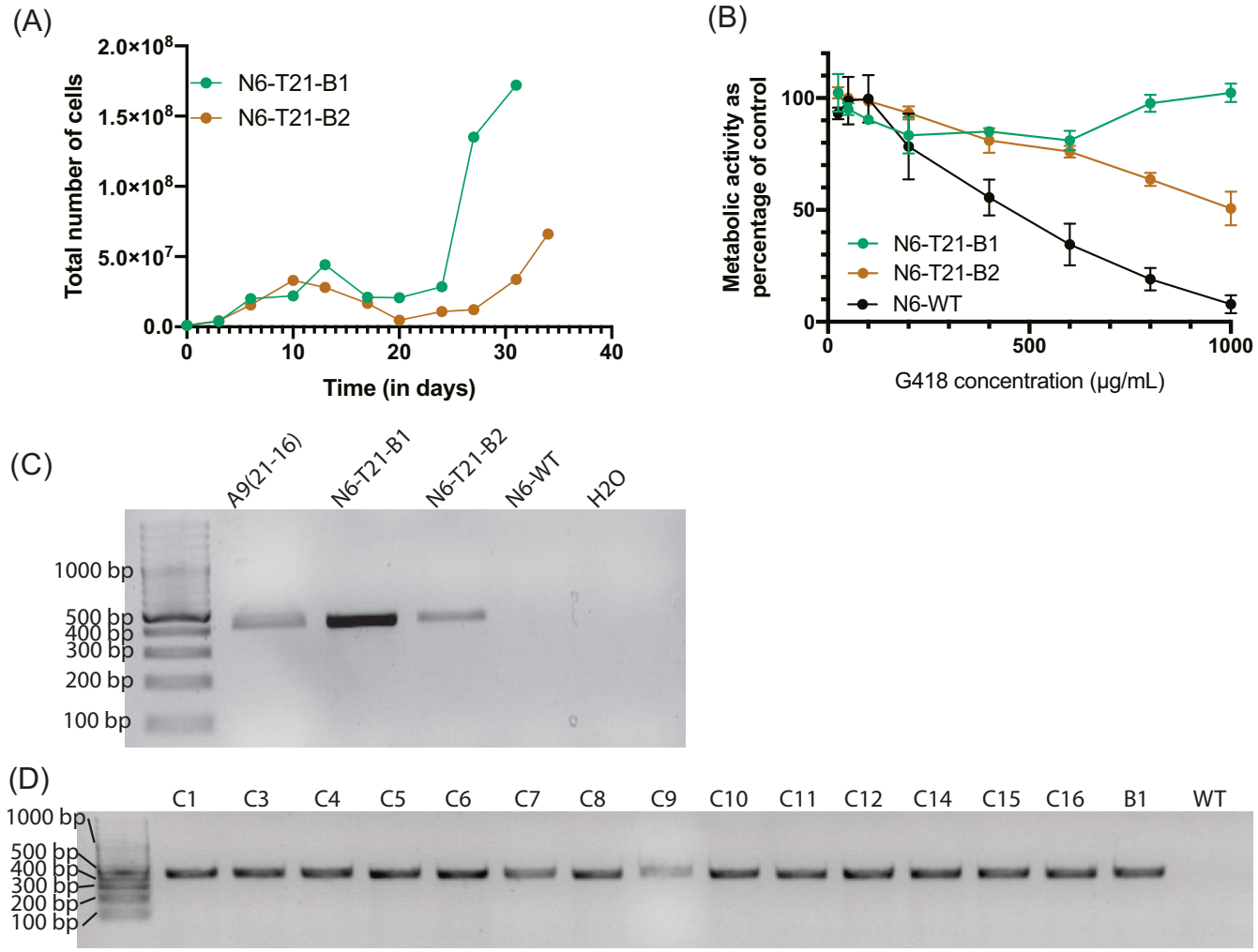

FIGURE 2 Generation of N6-WT clones with additional human chromosome 21. (A) Growth curves of the pool of recipient cells in the first weeks of selection with 500 µg/mL G418. Manual cell count is shown on the y-axis, time since MMCT procedure is shown on the x-axis. N6-T21-B1, Nalm-6 after MMCT for chromosome 21, batch 1; N6-T21-B2, idem, batch 2. (B) Metabolic activity of the wildtype, parental Nalm-6 (N6-WT) and pools of N6-T21 cells for batch 1 (N6-T21-B1) and batch 2 (N6-T21-B2) as percentage of no G418 control upon exposure to increasing concentrations of G418. The mean ± SD of 3–6 technical replicates is shown for each batch. (C) Gel electrophoresis of PCR product between 400 and 500 bp for neo/G418 resistance gene to confirm the presence of the selection gene, encoded from the donor chromosome 21, in recipient cells. (D) Gel electrophoresis of PCR product for neo/G418 resistance gene on single cell-derived clones, to confirm the presence of an additional chromosome 21 from donor cells. Sample names are shortened to C for single clone and B for batch.

present on the additional copy of chromosome 21 from the donor cell line. Both genes were not expressed in the N6-WT or N6-T21 clones. The N6-T21 clones shared 188 single nucleotide variants (SNVs) with a variant allele frequency (VAF) >10%. Of these, 48 were located on chromosome 21 and likely to be single nucleotide polymorphisms on the additional chromosome 21 as they were not detected in the N6-WT cells and had an average VAF of 31%. The shared variations, including copy number variants present in the N6-WT cells (Figure S4), suggest that the studied subclones were derived from a common ancestor after MMCT and G418 selection. In addition, the clones also showed between 19 and 102 unique SNVs (Figure S5; Table S2). Total RNA sequencing, using the same selected N6-T21 clones, the N6-WT and an independent Nalm-6 sample, showed that N6-T21 clones exhibit higher expression of genes on chromosome 21 compared with N6-WT cells, especially in genes expressed above approximately 30 FPKM (Figure 3B). Thus, RNA sequencing confirmed that the extra chromosome in N6-T21 cells is actively transcribed, with a median 1.3-fold increased expression of 198 expressed genes (Table S3). Using FISH, the presence of trisomy 21 was confirmed in N6-T21 clones (Figure S6).

3.4 | Expression of chromosome 21-associated leukemia genes

Several genes on chromosome 21 are implicated in leukemia, for example, *RUNX1* is important for hematopoiesis and fusions with this gene are often found in leukemia.[23] Furthermore, *ERG* is implicated in DS-related acute megakaryoblastic leukemia.[24] In addition, *HMGN1*

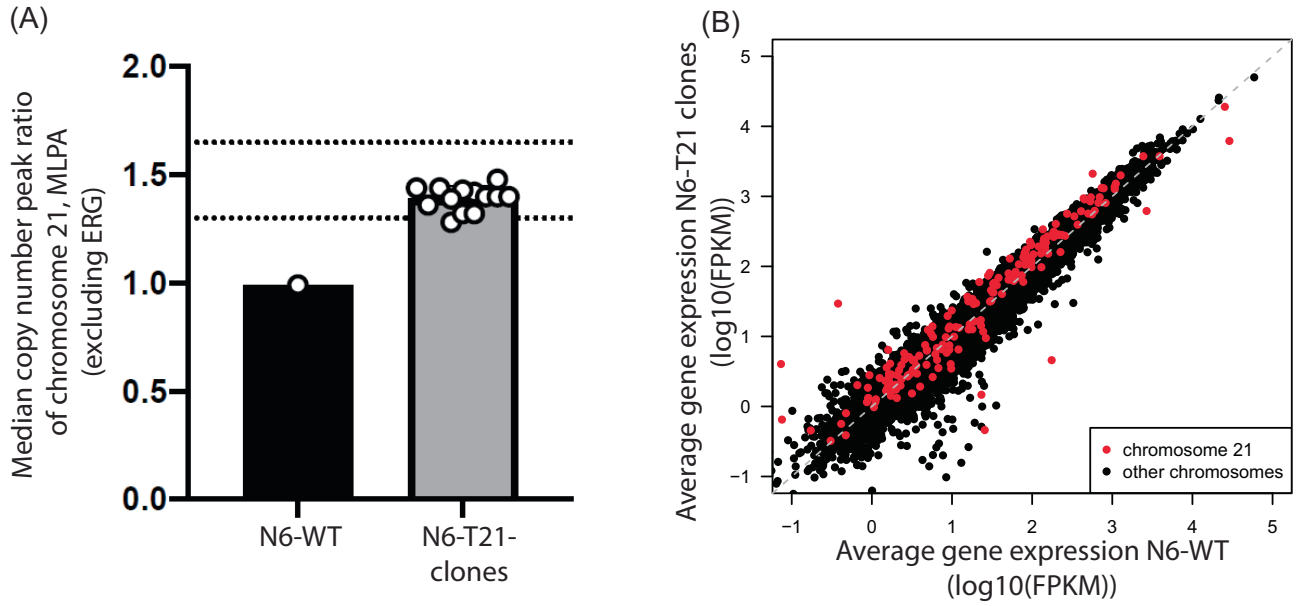

FIGURE 3 Validation of additional chromosome 21 in N6-T21 clones. (A) Median peak ratio of all MLPA probes (excluding the ERG probes; y-axis) located on chromosome 21 in the N6-WT compared with the N6-T21 clones. A peak ratio between 1.3 and 1.65 (dotted lines) indicates heterozygous duplication. (B) Expression of genes in N6-T21 clones (y-axis) versus expression in N6-WT cells (x-axis) as determined by RNA sequencing. Expression of the 4 clones is averaged, as well as expression of the N6-WT and the independent Nalm-6. Dashed line indicates equal expression between the N6-WT and the N6-T21 clones, red dots indicate genes located on chromosome 21, black dots indicate genes located on other chromosomes. Increased expression of chromosome 21 genes can be seen as red dots above the dashed line. Only genes that are expressed (counts per million > 1 in at least two samples) are shown. For some genes, there was (almost) no expression in the N6-WT samples, while there was expression in the N6-T21 clones, leading to extreme fold changes (LogFC > 6).

overexpression was identified to promote BCPALL *in vivo*.[25,26] Finally, RNA sequencing using iAMP21 samples identified potential candidates such as *CHAF1B*, *DYRK1A*, and *SON*.[27] We tested whether these genes-of-interest were overexpressed in the N6-T21 clones. Interestingly, *RUNX1*, *DYRK1A*, and *ERG* were not upregulated (Figure 4A), suggesting tight transcriptional regulation. *HMGN1* (LogFC = 0.84), *SON* (LogFC = 0.61), and *CHAF1B* (LogFC = 0.55) were upregulated in the trisomy 21 clones (Figure 4B; FDR < 0.05). In total, 1919 genes were significantly differentially expressed (FDR < 0.05; Table S4) in the N6-T21 samples (of which 754 up and 1165 down), of which 66 (3.4%) were located on chromosome 21 (56 up and 10 down). The trisomy 21-related upregulated genes in Nalm-6 overlapped with differentially expressed genes between BCPALL patients without a sentinel subtype (B-other, negative for *BCR*::*ABL1*, *ETV6*::*RUNX1*, *KMT2A* rearrangement, *TCF3*::*PBX1*, high hyperdiploidy) with somatic gain of chromosome 21 (27 genes) or DS ALL (33 genes) compared with B-other BCPALL without chromosome 21 aberration. Among the overlapping upregulated genes were *HMGN1*, *SON*, and *CHAF1B* (Table S3; Figure S7). *RUNX1* and *ERG* were not upregulated in these subtypes, similarly to the N6-T21 clones, while *DYRK1A* was upregulated in these subtypes, in contrast to the N6-T21 clones. Interestingly, *DUX4* expression was downregulated in the N6-T21 clones (LogFC = −1.3; Figure 4C). We compared *DUX4* gene expression in *DUX4*-rearranged BCPALL patients with disomy and trisomy of chromosome 21 and found that *DUX4* expression was similar, but with a trend to higher expression (p-value = 0.15), in these patients who also have +21 (Figure S8). We confirmed that the increase of *IFNGR2* expression on RNA level (LogFC = 6.2; Figure 4D) led to increased surface protein expression in N6-T21 clones (Figure 4E). There was a trend toward increased IFNGR2 expression in BCPALL patient samples with a chromosome 21 aberration and in DS BCPALL patients, although this did not reach statistical significance (chromosome 21 aberration, LogFC = 0.49, p-value = 0.037, FDR = 0.27; DS BCPALL, LogFC = 0.34, p-value = 0.024, FDR = 0.13; Table S3). Although the N6-T21 clones had an additional copy of the Y chromosome, most genes on this chromosome were not expressed (Table S4). David functional annotation[28] using all significant genes (FDR < 0.05) indicated no differentially expressed pathways but indicated enrichment of several chromosomes and cytogenetic locations (Table S5). Interestingly, chromosome 19 was even more enriched than chromosome 21 and about three quarters of chromosome 19 located genes were downregulated in the N6-T21 samples.

3.5 | An additional chromosome 21 resulted in slower proliferation

Since BCPALL is characterized by rapid expansion of developing B-cells, we determined the effect of an extra chromosome 21 on proliferation. Using CFSE, a dye that dilutes out over the two daughter cells with every cell division, a higher proliferation rate can be observed by a faster loss of the dye. All cells within one sample grew at the same speed, showing no slow dividing or non-dividing subpopulations of cells. Interestingly, an additional chromosome 21 resulted in

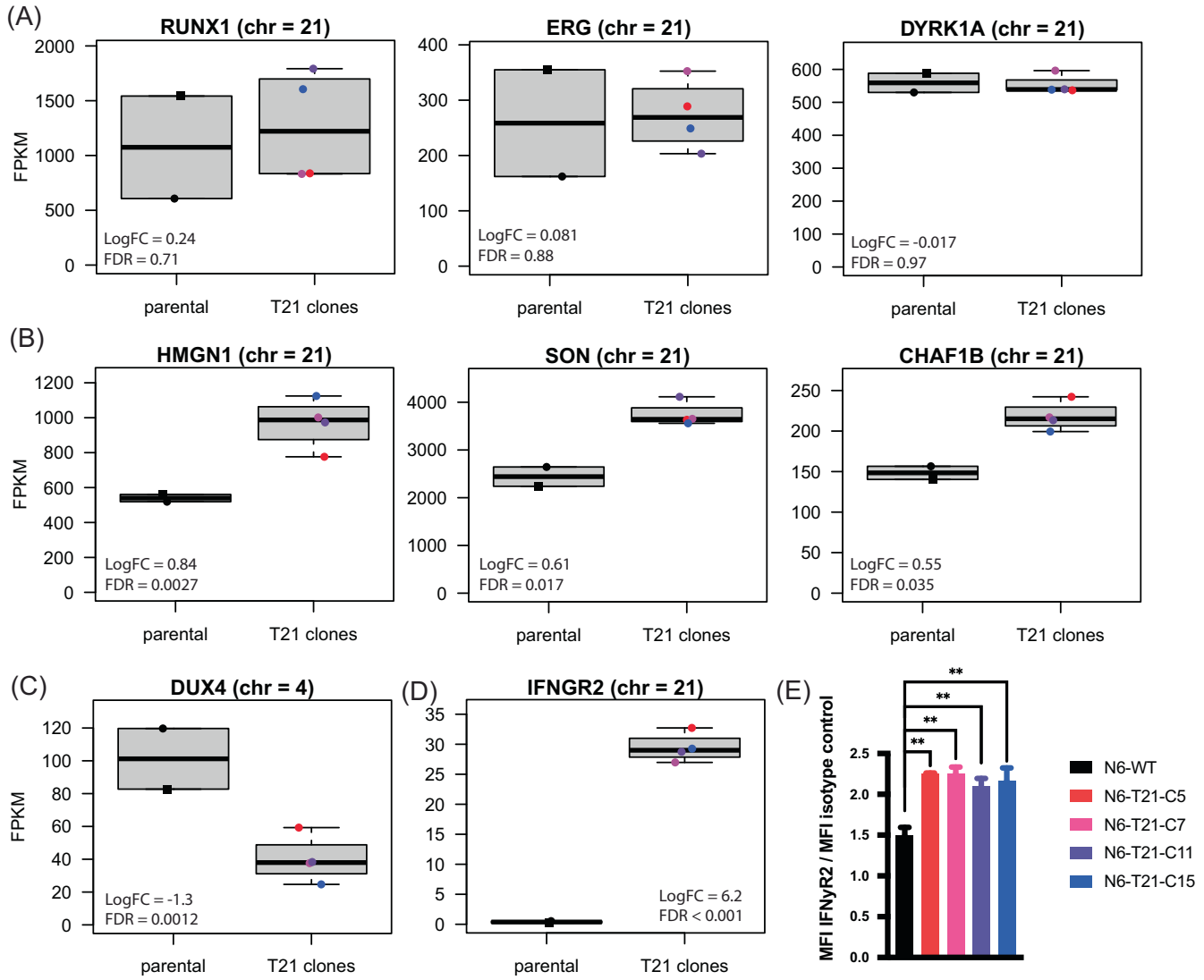

FIGURE 4 Expression of selected genes in the N6-T21 clones versus the N6-WT. (A–D) For each panel, expression is shown in fragments per kilobase per million (FPKM) on the y-axis. Each sample is shown with a symbol (black square = independent Nalm-6, black circle = N6-WT, red circle = N6-T21-C5, pink circle = N6-T21-C7, purple circle = N6-T21-C11, blue circle = N6-T21-C15), boxplots show median, interquartile range, and minimum and maximum. Gene symbol and chromosome are shown above each figure. Log2 fold change (LogFC) and FDR (determined with EdgeR) are written in each figure. (E) Surface expression of the IFNGR2 protein. Expression is shown as median fluorescence intensity (MFI) of IFNGR2 divided by MFI of isotype control. $^{*}p < 0.05$; $^{**}p < 0.01$, one-way ANOVA with Dunnett posthoc comparing the N6-WT to each of the N6-T21 clones individually. Bars indicate average ± SEM of three independent experiments.

slightly slower proliferation of each of the four analyzed N6-T21 clones compared with N6-WT cells, observed at three consecutive timepoints (48, 72, and 96 h) (Figure 5; Figure S9).

3.6 | N6-21 clones showed slightly increased sensitivity to prednisolone and asparaginase

We also investigated the role of an additional chromosome 21 in sensitivity to several drugs that play a key role in treatment of BCPALL.[29] This is of interest since it has been established that ex vivo drug sensitivity, as determined by MTT, is predictive of treatment response.[30] There was some heterogeneity in drug sensitivity among the N6-T21 clones, in particular N6-T21-C15 showed aberrant results. Averaging the remaining three clones, an additional chromosome 21 increased sensitivity to prednisolone by 4-fold (IC50 from 0.24 µg/mL to an average of 0.051 µg/mL, excluding N6-T21-C15) and asparaginase by 3-fold (IC50 from 1.2 Units/mL to an average of 0.43 Units/mL, excluding N6-T21-C15) (Figure 6A,B). There was no change in drug sensitivity to daunorubicin (Figure 6C).

3.7 | An additional chromosome 21 does not influence sensitivity to signaling inhibitors

Currently, there is no targeted therapy available for chromosome 21 gained leukemia. To determine whether an extra chromosome 21 makes the cells

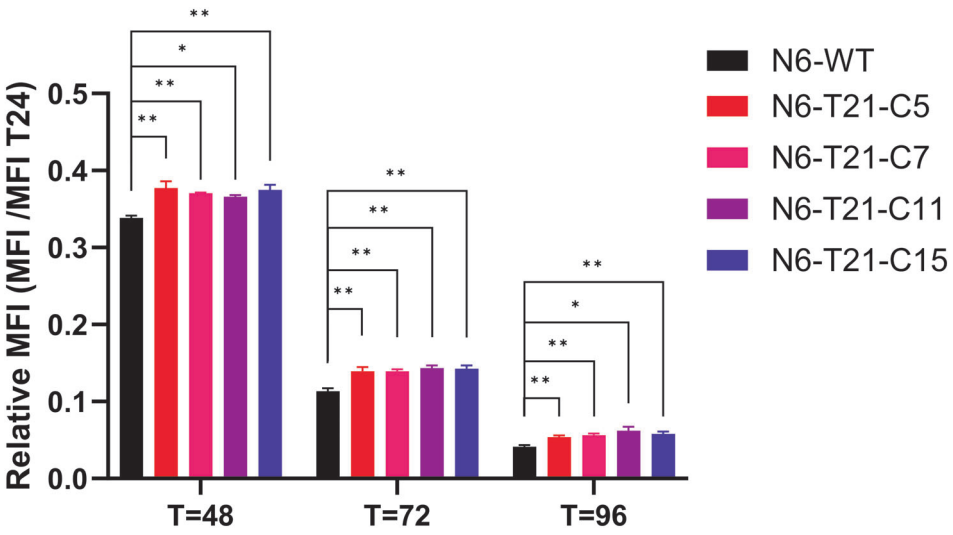

FIGURE 5 Slower proliferation of N6-T21 cells compared with N6-WT cells. Proliferation was assessed with CSFE. Median fluorescence intensity (MFI) of sytox red negative cells at timepoint 48, 72, and 96 h relative to MFI at 24 h after staining for N6-T21 clones versus N6-WT cells. Relative MFI for each N6-T21 clone was compared with relative MFI of N6-WT. *$p < 0.05$; **$p < 0.01$, one-way ANOVA with Dunnett posthoc comparing the N6-WT to each of the N6-T21 clones individually. Bars indicate average ± SEM of three independent experiments.

more sensitive to selected inhibitors of signal transduction pathways, we analyzed the sensitivity to gilteritinib (FLT3 inhibitor), ruxolitinib (JAK1/2 inhibitor), and trametinib (MEK1/2 inhibitor). N6-WT was not sensitive to these three targeted drugs, in line with absence of activating lesions. The presence of an extra chromosome 21 did not lead to increased sensitivity to these targeted drugs (Figure 7).

4 | DISCUSSION

We successfully generated the *DUX4*-rearranged BCPALL cell line Nalm-6 with an additional chromosome 21 from a donor cell line. The resulting N6-T21 clones were extensively validated to confirm that the extra chromosome 21 was present and that it was actively transcribed. Chromosome 21 genes were higher expressed, suggesting that our developed cell line could be a relevant model to study the consequences of chromosome 21 gain in leukemia. This is of particular importance since chromosome 21 is frequently gained in BCPALL yet elucidating the role of such an additional chromosome is hampered by the existence of additional genetic differences between patients, lack of primary patient material, and lack of proliferation in vitro. By comparing the N6-T21 clones to the N6-WT, the effect of additional genetic differences is reduced.

Our functional studies suggest a role of chromosome 21 in proliferation and drug sensitivity. Reduced proliferation is more commonly found in aneuploid cell lines.[31] Our data suggest that an additional chromosome 21 reduces the cell proliferation rate and slightly increases cellular sensitivity to prednisolone and asparaginase. The reduced proliferation rate would not explain the observed increased cellular sensitivity to these drugs. Previously, an ex vivo study showed increased sensitivity of hyperdiploid leukemia—frequently exhibiting one or more additional chromosomes 21[3]—to l-asparaginase.[32] However, children with DS-ALL show no significant differences in sensitivity to l-asparaginase compared with non-DS-ALL patients in an ex vivo study.[33] Since DS-ALL shows a different frequency of genetic lesions,[34] this could interfere with proper evaluation of the role of chromosome 21 in sensitivity to prednisolone and asparaginase. Chromosome 21 cooperates with RAS pathway aberrations[35] and increased incidence of *P2RY8*::*CRLF2* fusions, often combined with *JAK2* mutations, in DS-ALL patients.[36] Although the pathways targeted by these three inhibitors often crosstalk,[37,38] and mutations in these pathways are associated with certain chromosome 21 aberrations,[11,12,36] we showed that an extra copy of chromosome 21 in itself did not confer sensitivity to these inhibitors in the N6-T21 cells.

A number of BCPALL subtypes co-occurs with an additional chromosome 21, among others hyperdiploidy, *KMT2A*-rearranged, *BCR*::*ABL1*, *ETV6*::*RUNX1*, *TCF3*::*PBX1*, and *CRLF2*-rearrangement.[19] For most of these subtypes, a cell line is readily available. For two reasons it would be interesting to develop a chromosome 21 gained leukemia cell line panel, with cell lines such as: REH (*ETV6*::*RUNX1*), SupB15 (*BCR*::*ABL1*), Rch-Acv (*TCF3*::*PBX1*), MHH-CALL-4 (*CRLF2*-rearranged), and Rs4;11 (*KMT2A*-rearranged). First, by using a larger panel, the general role of an extra chromosome 21 might be elucidated, as it could reduce the noise coming from the specific genetic background. Second, both DS-ALL and non-DS chromosome 21 gained leukemia often present with other leukemia-associated lesions,[19,34] suggesting an extra copy of chromosome 21 alone is not enough to result in leukemia. Therefore, the combination of both an extra chromosome 21 and the genetic background should be considered. As indicated before, *HMGN1* is an interesting candidate for the role of chromosome 21 in leukemia.[25] Mowery et al.[26] showed that a gain of *HMGN1*, responsible for suppressing H3K27me3, results in global amplification of gene expression, indicating that the role of chromosome 21 might be to increase gene expression of readily present expression programs. Their hypothesis might be tested using cell lines with an additional chromosome 21, which would give valuable insight in the biology of chromosome 21 related leukemia.

Additional SNVs were induced in the leukemic cells during the MMCT process. N6-T21-C15 gained the most unique variations which might explain why it was the most divergent in our drug sensitivity studies. Furthermore, the clones gained a copy of the Y chromosome, and the mono-allelic deletion on chromosome 10 became

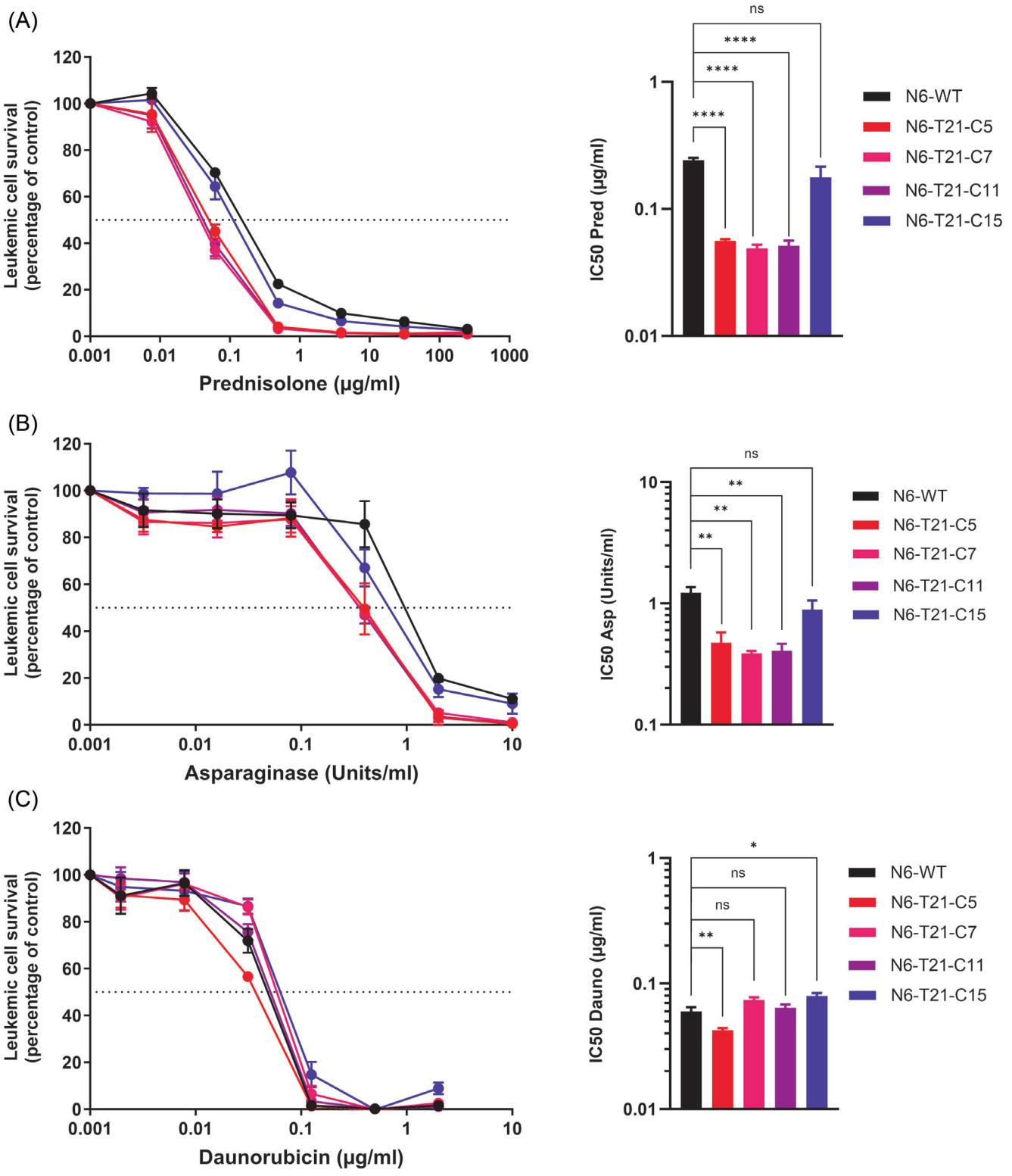

FIGURE 6 The effect of an additional chromosome 21 on sensitivity to induction drugs. N6-T21 clones were more sensitive to (A) prednisolone and (B) asparaginase than the N6-WT, while there was no difference in sensitivity to (C) daunorubicin. Left panel shows the sensitivity to the different drugs tested. Cell viability was determined using MTT, with cell survival (OD as percentage of untreated control) on the y-axis. Right panel shows the IC50 as determined using the data of the left panel. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, one-way ANOVA with Dunnett posthoc comparing the N6-WT to each of the N6-T21 clones individually. IC50 values were statistically tested (log10 transformed), dose response curve data were not statistically tested. One-way ANOVA p-values of IC50: (A) $p < 0.001$; (B) $p < 0.001$; (C) $p < 0.001$. Data are shown as mean ± SEM of three independent experiments.

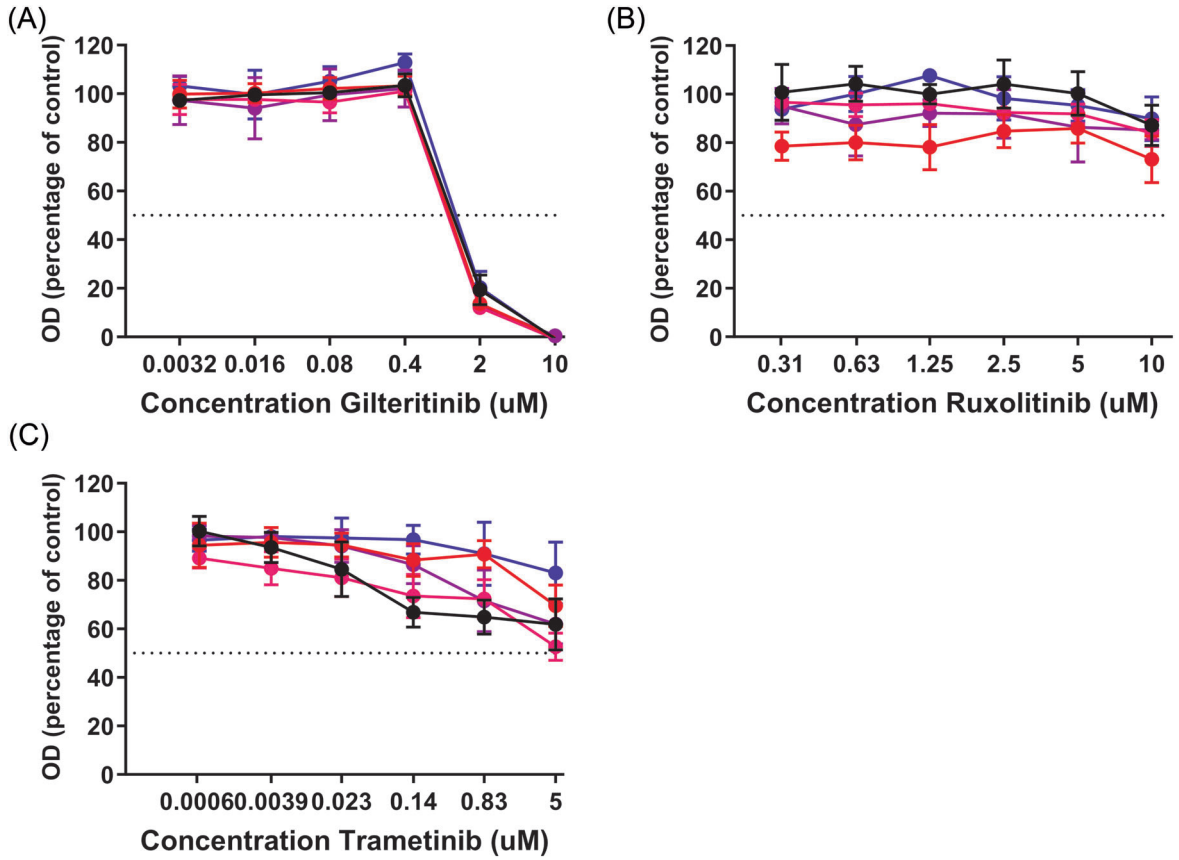

FIGURE 7 An additional chromosome 21 has no influence on the sensitivity to selected inhibitors. N6-T21 clones showed similar sensitivity to (A) gilteritinib, (B) ruxolitinib, and (C) trametinib. Cell viability was determined using MTT, with cell survival (OD as percentage of untreated control) on the y-axis. Data are shown as mean ± SEM of 3–4 independent experiments.

bi-allelic. It could be that the clones arose from a subclone from the N6-WT. It is difficult to predict the effect of the clones, but a panel with multiple cell lines, or clones from multiple MMCT processes, could eliminate the effect of the additional alterations.

Interestingly, two-third of the genes on chromosome 21 are not dose-dependently expressed. We noticed that *RUNX1*, *ERG* and *DYRK1A* were tightly regulated. Even though the copy number increased, this was not reflected in expression levels in N6-T21 clones. Similarly, DS induced pluripotent stem cells have reduced *ERG* expression and similar *RUNX1* expression (but increased expression of *DYRK1A*)[39] and dosage compensation is often seen in *RUNX1*.[27,40] This dosage compensation suggests that genes on chromosome 21 are tightly regulated. Since the genes in our cell lines are under regulation of their own promoters and enhancers, regulation might mimic the leukemia biology closely. An additional chromosome 21 might change gene expression locally on chromosome 21 (cis-effect), or it might change gene expression genome wide (trans-effect). As of the 1919 differentially expressed genes, only 66 (=3.4%) are located on chromosome 21; this strongly points to a potential trans-effect, where a slight change in gene expression on chromosome 21 can cause large gene expression changes genome wide. This trans-effect could possibly explain the altered gene expression of regions on other chromosomes, such as chromosome 19, and could also explain why it has been difficult to identify the gene or genes on chromosome 21 that are responsible for the association with leukemia.

We showed an increased expression of *IFNGR2* (type II IFN receptor), resulting from an extra chromosome 21. The IFN pathway is involved in mediating several processes, such as cell survival and stimulating antitumor activities.[41–43] The IFN pathway has a pro-tumorigenic role as well, mediated by IFN-related gene transcription via STAT1/2 signaling.[42,44] As the bone marrow niche is suggested to provide a beneficial microenvironment for leukemic cells, as well as protect leukemic cells from therapy,[45] the role of an extra chromosome 21 in leukemia might involve interactions with the niche.

In conclusion, we transfected the BCPALL cell line Nalm-6 with an additional chromosome 21 and validated that this chromosome is actively being transcribed similar to BCPALL samples with trisomy 21. With this model system, we showed that an additional chromosome 21 results in genome-wide gene expression changes, slower proliferation, and increased prednisolone and asparaginase sensitivity. This model system could be of interest in determining the role of chromosome 21 in leukemia and developing more targeted treatments for chromosome 21 altered leukemia.

AUTHOR CONTRIBUTIONS

The project was conceived by Femke M. Hormann, Judith M. Boer, and Monique L. den Boer. Experimental and computational analyses

were performed by Femke M. Hormann, Eva J. Mooij, Marieke van de Mheen, and H. Berna Beverloo. Data interpretation was performed by Femke M. Hormann, Eva J. Mooij, H. Berna Beverloo, Judith M. Boer, and Monique L. den Boer. The manuscript was drafted by Femke M. Hormann, Eva J. Mooij, and Judith M. Boer. All authors approved the manuscript. Both Femke M. Hormann and Eva J. Mooij contributed equally to the manuscript and reserve the right to list their name first in their CV and doctoral thesis.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article. Whole exome sequencing and total RNA sequencing datasets generated in the study are deposited in the Sequence Reads Archive under BioProject accession number PRJNA835628.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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