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# Abbreviations

|        |                                                           |
|--------|-----------------------------------------------------------|
| aa     | Amino Acids                                               |
| AC     | Adenocarcinoma                                            |
| AML    | Acute Myeloid Leukaemia                                   |
| att    | Attachment Sites                                          |
| BSA    | Bovine Serum Albumin                                      |
| Cas    | CRISPR-associated System Genes                            |
| CCD    | Coiled Coil Domain                                        |
| cDNA   | Complementary DNA                                         |
| CDS    | Coding Sequence                                           |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| crRNA  | CRISPR RNA array                                          |
| DBD    | DNA-binding Domain                                        |
| DMEM   | Dulbecco Modified Eagle Medium                            |
| DSB    | Double Strand Breaks                                      |
| EF1A   | Elongation Factor 1 $\alpha$ Promoter                     |
| EGF    | Epidermal Growth Factor                                   |
| FAB    | French-American-British                                   |
| FBS    | Fetal Bovine Serum                                        |
| G-CSF  | Granulocytic Colony-stimulating Factor                    |
| gDNA   | Genomic DNA                                               |
| GFP    | Green Fluorescent Protein                                 |
| HDR    | Homology Directed Repair                                  |
| IL     | Interleukin                                               |
| Indels | Insertions and Deletions                                  |
| JAK    | Janus Kinase                                              |
| KO     | Knock-out                                                 |
| LEDGF  | Lens Epithelium-derived Growth Factor                     |
| LSC    | Leukaemic Stem Cell                                       |
| LB     | Lysogeny Broth                                            |
| MMP    | Matrixmetalloprotease                                     |
| MPO    | Myeloperoxidase                                           |
| NaF    | Sodium Fluoride                                           |

|          |                                                  |
|----------|--------------------------------------------------|
| NHEJ     | Non-homologous End Joining                       |
| nt       | Nucleotides                                      |
| NTD      | N-terminal Domain                                |
| PAC      | Puromycin N-acetyl-transferase                   |
| PAM      | Proto-spacer Adjacent Motifs                     |
| PCAF     | p300/CBP-associated Factor                       |
| RPMI     | Roswell Park Memorial Institute                  |
| RT       | Room Temperature                                 |
| RT-qPCR  | Reverse Transcription Quantitative PCR           |
| sg       | Single Guide                                     |
| SH2      | Src-homology-2                                   |
| ssODN    | Single-stranded Oligo Dinucleotides              |
| STAT     | Signal Transducer and Activator of Transcription |
| STAT3C   | Constitutively active STAT3                      |
| STAT3-DN | Dominant-negative STAT3 mutant                   |
| TAD      | Transactivation Domain                           |
| TALEN    | Transcription Activator-like Effector Nuclease   |
| TIDE     | Tracking of Indels by Decomposition              |
| tracrRNA | Transactivating crRNA                            |
| TRE      | Tetracycline Response Element                    |
| WHO      | World Health Organisation                        |
| ZFN      | Zinc-finger Nuclease                             |

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## 1. INTRODUCTION

### 1.1 Acute myeloid leukaemia

Acute leukaemia is a monoclonal disease of the haematopoietic system which can be classified according to the lineage of occurring malignant cells into myeloid or lymphoid leukaemias. In comparison to chronic leukaemias, the acute forms are characterised by a rapid growth of malignant cells in the bone marrow, interfering with normal haematopoiesis and a sudden onset of symptoms indicating the reduction of normal blood cells, including fatigue, easy bruising and bleeding, and increased vulnerability to infections<sup>1</sup>.

One of the most common acute leukaemia in adults is acute myeloid leukaemia (AML), most frequently diagnosed in people aged 65 to 74 with a median age of 68 years at diagnosis<sup>2</sup>. According to the National Cancer Institute, roughly 1.3 % of all diagnosed cancer types in the USA are attributed to AML with 4.2 per 100,000 new cases per year. In general, AML is a rather rare disease compared to other cancer forms. Regarding disease outcome, the 5-year survival rate was at 26.9 % in the years from 2007 to 2013 and is highly dependent on disease and non-disease related factors such as cytogenetics of the malignant cells, age and general health condition of the patient<sup>2,3</sup>.

#### 1.1.1 Classification of the different AML types

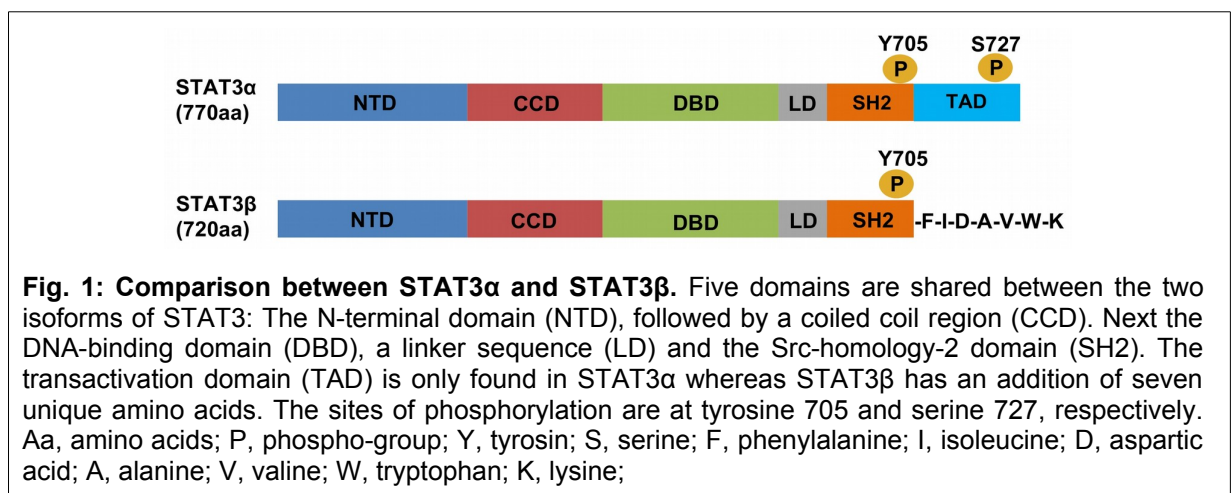
In 1976, Bennet et al proposed a classification scheme for acute leukaemias (French-American-British Co-operative group, FAB classification) based on morphology of bone marrow and peripheral blood smears upon Romanowsky staining, supported by additional cytochemical reactions<sup>4</sup>. Acute leukaemias were separated into myeloid and non-myeloid, also called lymphoblastic, leukaemias and dysmyelopoietic syndromes which are also associated with hypercellular bone marrows but not defined as full blown leukaemias. However, the latter is known to be capable of progressing into so-called secondary leukaemias<sup>4</sup>. In general, myeloid leukaemias were categorised into six groups (M1 to M6) defined by one or more predominant cell types found in the bone marrow and peripheral blood, as well as the degree of maturation of these cells. Group M1 to M3 are mainly derived from the granulocytic lineage with an increase in maturation from M1 to M3, additionally

characterised by an increase of azurophilic granules in the cytoplasm, which can aggregate, forming so-called needle shaped Auer bodies. M4 is characterised by a mixture of granulocytic, which resemble M2, and monocytic cells. M5 is defined by cells from the monocytic lineage, subdivided into M5a and M5b, with more blast-like cells in the first group and more differentiated ones in the latter. M6 predominantly shows erythroblastic cells, with abnormalities in the erythropoiesis in the bone marrow and erythroblasts appearing in the peripheral blood. To distinguish the different precursor cells from each other and between lineages, additional biochemical reactions are needed, such as the myeloperoxidase (MPO) reaction, for which all myeloid leukaemias are positive in contrast to the lymphoid, and the non-specific esterase reaction before and after exposure to sodium fluoride (NaF). The latter reaction is tested positively in the majority of monocytic and granulocytic cells with a stronger reaction in the monocytic compartment, namely monoblasts, promonocytes and monocytes. Additionally, in these cells the reaction can be completely inhibited by NaF whereas the reaction in granulocytes remains unaffected. In the following years, the classification was further extended, considering surface antigens for diagnosis and cytogenetics for prognosis, and additional categories were added, now including acute megakaryoblastic variants (M7)<sup>5</sup>. In 1999, the World Health Organisation (WHO) published their own recommendations for the diagnosis of neoplastic diseases in the haematopoietic system focusing on cytogenetic abnormalities<sup>6</sup>. The consortium attempted a more clinically relevant classification with prognostic information, taking into account that certain disease categories are associated with favourable abnormalities, such as t(8;21) or t(15;17) or unfavourable, like translocations or inversions on chromosome 11 (at the spot q23) or t(9;22)<sup>3</sup>. Since then, the WHO classification is extended every few years incorporating discoveries of new genetic arrangements and molecular targets available for advanced prognosis and therapies<sup>7,8</sup>. Currently, both classifications are used side by side in the clinics. The FAB classification is more stringent, requiring up to 30 % of occurring blasts in the bone marrow and peripheral blood whereas the WHO classification defines AML already with 20 % of blasts<sup>6,9</sup>.

## 1.2 The transcription factor STAT3

STAT3 is part of a family of seven members of transcription factors (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) named after their dual function, hence signal

transducer and activator of transcription (STAT)<sup>10</sup>. These proteins are usually found in an inactive state in the cytoplasm and are activated upon tyrosine phosphorylation by Janus kinases (JAKs)<sup>11</sup>. The JAKs are non-covalently bound to transmembrane receptors and are activated upon formation of ligand-receptor complexes, e.g. with cytokines or growth factors, leading to transphosphorylation of tyrosine residues on the intracellular domains of these receptors<sup>12</sup>. The new phosphate groups provide binding sites the STAT proteins can attach to via their Src-homology-2 (SH2) domain. The canonical view is, that the STATs are recruited to the activated JAKs and phosphorylated on a tyrosine around position 700 in their amino acid (aa) sequence. Phosphorylated STATs dimerise via their phosphorylated tyrosines, forming homo- or heterodimers and are imported into the nucleus where they directly bind to promoters of target genes, influencing their gene expression. Newer studies suggest, that STATs can occur as homo- or heterodimers already in the unphosphorylated state and bind to DNA in the nucleus, though the biological function of the unphosphorylated complexes is yet not fully understood<sup>13</sup>. Other classes of non-receptor protein tyrosine kinases can additionally facilitate STAT phosphorylation, including the Src family kinases (Src, Lck, Lyn, Fyn)<sup>14</sup>.



STAT3 exists in two isoforms and two additional proteolytically generated forms, transcribed from a single gene located on chromosome 17. The most abundant form is the full-length transcript STAT3α consisting of six protein domains, shown in figure 1<sup>15</sup>. The N-terminal domain (NTD) is followed by a helical coiled coil region (CCD) allowing protein-protein interactions, directly next to the DNA-binding domain (DBD) built from several β-sheets. Associated to it is a highly conserved linker domain of unknown function, directly followed by

the SH2 domain containing the tyrosine position (Y705) which is phosphorylated upon JAK activation. The transactivation domain (TAD) is found at the carboxy terminus, responsible for cofactor recruitment (e.g. CBP/p300) and harbours an additional phosphorylation site at serine 727 which is needed for maximal transcriptional activity of STAT3<sup>16,17</sup>. STAT3 $\beta$  is an alternatively spliced version of the *STAT3* gene, lacking the TAD and therefore also the phosphorylation site on serine 727. The isoform is generated by utilisation of an alternative splice acceptor site in exon 23 of the *STAT3* gene, resulting in a deletion of 50 nucleotides (nt) and a +2 frameshift. The latter leads to an addition of seven amino acids in the STAT3 $\beta$  protein<sup>18</sup>. Both isoforms are expressed ubiquitously, whereas STAT3 $\alpha$  usually is the predominant form with alpha to beta ratios of 3:1 to 10:1 on the mRNA level and 1:3 to 10:1 on the protein level, depending on the origin and activation status of the cells<sup>18,19</sup>. Notably during myeloid differentiation, overall levels of STAT3 decrease with a shift in expression from STAT3 $\alpha$  to mainly STAT3 $\beta$ . This results suggest that STAT3 $\alpha$  is important for proliferation, since bone marrow granulocytes have overall higher STAT3 $\alpha$  levels, whereas mature granulocytes start to mainly express STAT3 $\beta$ <sup>20</sup>. STAT3 $\gamma$  and STAT3 $\delta$  are the result of limited proteolytic cleavage of STAT3 $\alpha$ , primarily found in mature neutrophils. Their physiological relevance remains unclear and they are probably a by-product of the shift from high STAT3 $\alpha$  to high STAT3 $\beta$  levels in the process of granulocytic maturation<sup>21</sup>.

### 1.2.1 STAT3 orchestrates a variety of cellular processes

Due to its biological role as a transcription factor, STAT3 is widely involved in cellular functions, such as survival, proliferation, migration, differentiation and apoptosis further differing among tissues. STAT3 is activated by many different stimuli including cytokines (e.g. Interleukin (IL)-6, IL-10, IL-12), growth factors such as the granulocytic colony-stimulating factor (G-CSF) or epidermal growth factor (EGF), hormones (e.g. prolactin) and oncogenes (e.g. v-Src) resulting in activation of distinct gene sets depending on the biological context<sup>19</sup>. The role of STAT3 was first described for its ability of binding to acute-phase response elements in hepatocytes stimulated with IL-6, activating acute-phase genes<sup>22,23</sup>. In the following years, tissue-specific functions were discovered among others by deletion of *Stat3* in mice. General ablation of STAT3 leads to embryonic lethality indicating that it plays a major role during early development that cannot be compensated by the other STAT family members<sup>24</sup>. Surprisingly, looking at different adult tissues and biological

settings, knock-out (KO) of STAT3 revealed contrasting roles during inflammation, regeneration, proliferation and homeostasis. The heterogeneity of STAT3 responses is most likely linked to the different isoforms as well as their ability to form heterodimers with other transcription factors, such as the c-Jun-STAT3 $\beta$  dimer<sup>25</sup>. STAT3 $\beta$  is lacking the TAD domain necessary for co-factor recruitment and DNA-binding, which led to the suggestion that it would act in a dominant-negative fashion and has an impaired transcriptional activity<sup>26</sup>. However, recent studies demonstrated that STAT3 $\beta$  has its own unique gene sets distinct from STAT3 $\alpha$ , among others by redirecting the splicing mechanism from predominantly STAT3 $\alpha$  to STAT3 $\beta$ . The change in gene expression included down-regulation of lens epithelium-derived growth factor (LEDGF), p300/CBP-associated factor (PCAF) and CyclinC<sup>27,28</sup>.

#### 1.2.2 STAT3 plays a dual role as oncogene and tumour-suppressor

In cancer, STAT3 is considered to act as an oncogene when constitutively activated by phosphorylation, whereas when inactivated, cells are impeded in their growth resulting in the end in cell death. Common targets of activated STAT3 in cancer are CyclinD, c-Myc and Pim members inducing proliferation together with members of the Bcl-2 family and Survivin which are providing resistance to apoptosis. Additionally, E-Cadherin, integrins and matrix-metalloproteases (MMPs) facilitate invasion and metastasis, altogether fulfilling the criteria for the hallmarks of cancer<sup>29</sup>. The oncogenic potential of constitutive active STAT3 was first analysed using a mutant protein (STAT3C) which was able to effectively induce malignant transformation by activation of the aforementioned genes<sup>30</sup>. In line, a dominant-negative STAT3 mutant (STAT3-DN), which cannot be phosphorylated, was able to mediate cell-cycle arrest together with induction of apoptosis<sup>31</sup>. Contrasting these data, some studies demonstrated a tumour suppressive function for STAT3. A simultaneous deletion of PTEN and STAT3 increased the proliferative capacity of astrocytes and hence led to tumour formation whereas siRNA-mediated knock-down of PTEN, with normal STAT3 expression, resulted in a reduced tumorigenicity<sup>32</sup>. A second study investigated lung tissue-specific inactivation of *Stat3* in mice, observing increased KRAS driven adenocarcinoma (AC) initiation and malignant progression, supporting the role of STAT3 as a tumour suppressor in this context. Consistent with this data, reduced *STAT3* expression was also observed in high-risk lung AC patients with poor survival<sup>33</sup>. However, findings of the last few years point

to the fact that the two STAT3 isoforms need to be considered separately to identify in which context they play a role in tumour formation or suppression. In oesophageal squamous cell carcinoma, high STAT3 $\beta$  expression correlated with favourable prognosis indicated by longer overall and relapse-free survival. Additionally, cancer stem cell populations were reduced here and cancer cells showed increased sensitivity to treatment with chemotherapeutics<sup>34</sup>. Furthermore, two-stage skin carcinogenesis and colitis-associated cancer induced in mice lacking the STAT3 $\beta$  isoform showed aggravated disease onset accompanied by enhanced inflammation<sup>35</sup>. Interestingly, focusing on leukaemia, treatment of AML cells with G-CSF and thus activation of STAT3 sensitises them towards successful chemotherapy. In AML, leukaemic stem cells (LSCs) often prove to be a problem since they are highly resistant to conventional therapies and reside in the bone marrow niche which provides a protective environment. LSCs start to react to chemotherapies when additionally treated with G-CSF in a stroma-dependent manner<sup>36</sup>. G-CSF is able to activate STAT3, which in turn appears to play a major role in granulocytic differentiation<sup>20</sup>. As described in previous chapters, differentiation of the granulocytic lineage is accompanied by a shift of STAT3 isoforms from a dominant STAT3 $\alpha$  expression to predominantly STAT3 $\beta$ , thus suggesting that the LSCs started to differentiate which was accompanied by a switch in STAT3 isoforms<sup>37</sup>.

### 1.3 Design of the study

So far we have limited knowledge of which role the distinct STAT3 isoforms play in the setting of leukaemia development. Preliminary data from our research group suggests that STAT3 $\beta$  has a tumour-suppressive role in the context of AML. So far, only experimental data from mice and patient-derived samples are available, both indicating that a higher STAT3 $\beta$  expression is beneficial regarding disease progression and survival data. However, the underlying mechanism for this phenomenon is not yet fully understood.

The aim of this master project was to establish two *in vitro* models based on human AML cell lines. With the first one we attempted a total STAT3 knock-out mediated by CRISPR/Cas9 and with the second model overexpression of one of the two STAT3 isoforms, either STAT3 $\alpha$  or STAT3 $\beta$ . These models should in the end help to unravel the role of STAT3 in the context of AML, allowing to examine which impact the change of STAT3 expression has on AML cell line behaviour, such as migration, differentiation and proliferative capacity compared to the

parental cell lines. Six cell lines with distinct features were selected. Two of these cell lines would have been classified as favourable in the clinical context (HL-60, NB-4), three cell lines are harbouring the MLL-AF9 fusion which is described as unfavourable when detected in patients (MONO-MAC-6, MOLM-13 and NOMO-1) and one cell line (HEL) is already expressing by itself high levels of both *STAT3* isoforms. Prior to modifying the cell lines, their basic levels of *STAT3* expression were analysed on the transcriptional as well as the protein level. Additionally, the growth rates of each cell line were evaluated.

### 1.3.1 Utilisation of the CRISPR/Cas9 system for genomic mutagenesis in mammalian cells

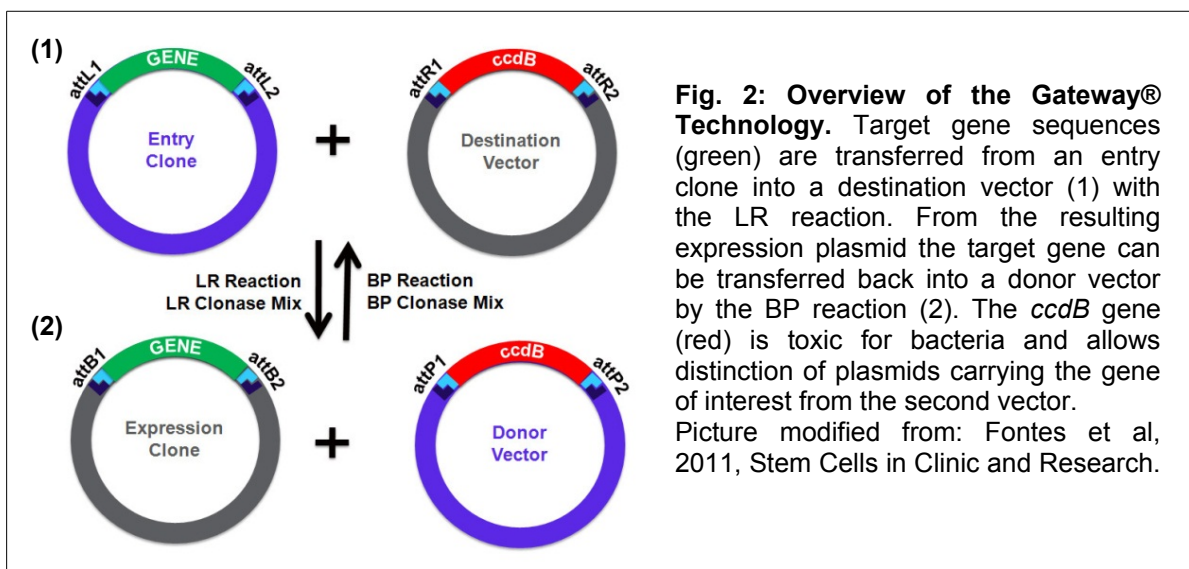
The CRISPR/Cas system originated in prokaryotic and archaea cells, resembling an acquired immune system and conferring resistance against foreign genetic elements which are incorporated as small snippets in a CRISPR array in the host genome<sup>38</sup>. The abbreviation CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats and describes how the short foreign DNA elements, stemming from invading organisms like phages or plasmids, are stored in the prokaryotic genome<sup>39</sup>. Palindromic repeats are alternated by short interspacing DNA sequences (e.g. alien plasmid sequences) which are later processed into short guide RNAs, followed by small clusters of CRISPR-associated system genes (Cas)<sup>39</sup>. So far, three types of CRISPR systems (I-III) are described, with type II being the best characterised one. The type II system consists of a single large nuclease (Cas9), the CRISPR RNA array (crRNA), as well as an additional transactivating crRNA (called tracrRNA) which allows processing of the crRNA array into separate guide RNA segments of 20 nucleotides in length by Cas9<sup>40</sup>. Which genetic elements from the invading organisms are incorporated into the CRISPR array, and later on can again be cut out to produce guide RNA segments, is dependent on so-called proto-spacer adjacent motifs (PAMs) which are specific for each Cas nuclease and the host organism<sup>41</sup>.

In recent years, the CRISPR/Cas9 system was utilised to facilitate precise genome editing through the process of Watson-Crick pairing of small RNAs to guide the Cas9 to its position, which is easier to design, highly specific and efficient when compared to DNA-binding protein technologies such as zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs)<sup>42</sup>. The editing process itself is stimulated by DNA double strand breaks (DSB) at the site of guide RNA binding and either repaired by high-fidelity homology directed repair (HDR), provided a repair template is present, or the highly error-prone non-

homologous end joining (NHEJ)<sup>43</sup>. The NHEJ repair mechanism often results in random insertion or deletion of basepairs leading to frameshift mutations and premature stop codons which in the end effectively disrupts normal gene function and can be exploited for gene knock-out.

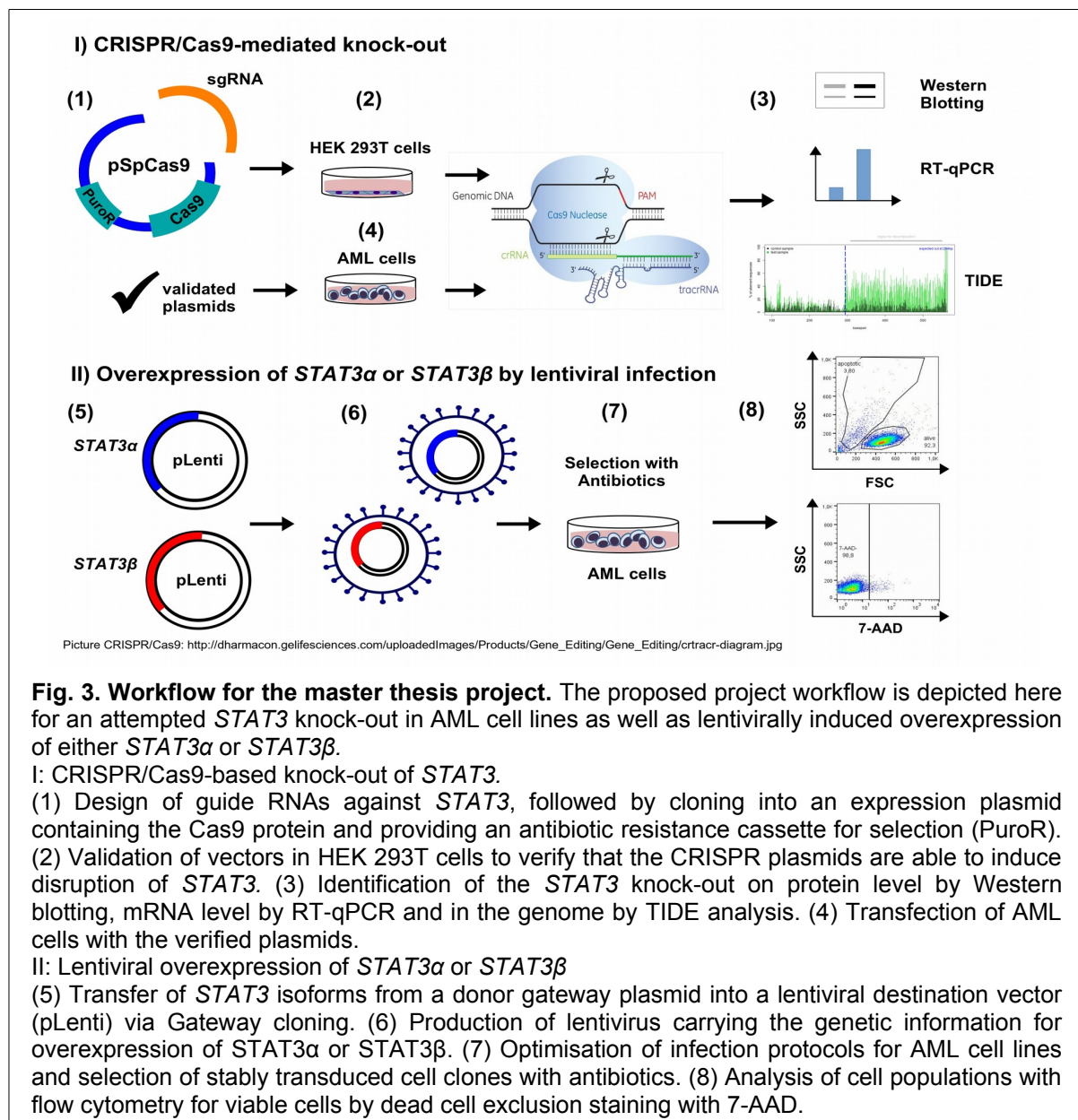
### 1.3.2 Preparation of lentiviral plasmids via Gateway cloning

The Gateway® Cloning Technology is a proprietary system developed by ThermoFisher based on the site-specific recombination events of the lambda bacteriophage described 1989 by Landy<sup>44</sup>. This process allows the phage to integrate into the chromosome of *E. coli* as well as remove itself again from it, thereby switching from a lysogenic to a lytic pathway<sup>45</sup>. The recombination process is dependent on DNA recombination sequences (*att*, short for attachment sites) and proteins that mediate the actual reaction. The *E. coli* genome provides one part of the recognition sites (*attB*) whereas the Lambda chromosome carries the corresponding *attP* site. During recombination, new sequences are created, termed *attL* and *attR* sites, and the genes flanked by the attachment sites are exchanged (see figure 2). The gene of interest in the entry vector is changing places with the *ccdB* gene sitting in the destination vector. Subsequently, bacteria transformed with the plasmid carrying the *ccdB* gene are inhibited in their growth, due to the fact that this gene encodes for a protein able to inhibit prokaryotic DNA-gyrases<sup>46</sup>. Therefore, colonies growing after transformation are selected for plasmids carrying the target gene. With this technology, genes of interest can be easily transferred to different expression plasmids.



### 1.3.3 Aim of the study and workflow

For generation of the AML cell lines, we proposed the following workflow, depicted in figure 3. In summary, the aim of this project is to establish mutant cell lines which allow investigation of the role of *STAT3* and the specific isoforms in AML. In further experiments, the oncogenic capacity of these cell lines will be analysed, as well as their potential for migration and differentiation, especially in cells overexpressing one of the two isoforms.



**Fig. 3. Workflow for the master thesis project.** The proposed project workflow is depicted here for an attempted *STAT3* knock-out in AML cell lines as well as lentivirally induced overexpression of either *STAT3α* or *STAT3β*.

I: CRISPR/Cas9-based knock-out of *STAT3*.

(1) Design of guide RNAs against *STAT3*, followed by cloning into an expression plasmid containing the Cas9 protein and providing an antibiotic resistance cassette for selection (PuroR). (2) Validation of vectors in HEK 293T cells to verify that the CRISPR plasmids are able to induce disruption of *STAT3*. (3) Identification of the *STAT3* knock-out on protein level by Western blotting, mRNA level by RT-qPCR and in the genome by TIDE analysis. (4) Transfection of AML cells with the verified plasmids.

II: Lentiviral overexpression of *STAT3α* or *STAT3β*

(5) Transfer of *STAT3* isoforms from a donor gateway plasmid into a lentiviral destination vector (pLenti) via Gateway cloning. (6) Production of lentivirus carrying the genetic information for overexpression of *STAT3α* or *STAT3β*. (7) Optimisation of infection protocols for AML cell lines and selection of stably transduced cell clones with antibiotics. (8) Analysis of cell populations with flow cytometry for viable cells by dead cell exclusion staining with 7-AAD.

**Table 1: Overview of selected AML cell lines.**

| Name              | Patient          |        | FAB classification           | Translocation             | Fusion protein | Additional gene alterations                                                                                                           | Prognosis      |
|-------------------|------------------|--------|------------------------------|---------------------------|----------------|---------------------------------------------------------------------------------------------------------------------------------------|----------------|
|                   | Age at diagnosis | Gender |                              |                           |                |                                                                                                                                       |                |
| <b>MONO-MAC-6</b> | 64               | M      | Myeloid metaplasia, later M5 | t(9;11)(p22;q23)          | MLL-AF9        |                                                                                                                                       | non-favourable |
| <b>MOLM-13</b>    | 20               | M      | MDS (RAEB), later M5a        | ins(11;9)<br>(q23;p22p23) | MLL-AF9        | <i>FLT3-ITD</i> , <i>p15<sup>INK4B</sup></i> deletion, <i>p16<sup>INK4A</sup></i> deletion                                            | non-favourable |
| <b>NOMO-1</b>     | 39               | F      | M5a                          | t(9;11)(p22;q23)          | MLL-AF9        |                                                                                                                                       | non-favourable |
| <b>HEL</b>        | 30               | M      | Hodgkin's disease, M6        |                           |                | <i>p15<sup>INK4B</sup></i> deletion, <i>p16<sup>INK4A</sup></i> deletion, <i>JAK2 V617F</i> mutation                                  |                |
| <b>HL-60</b>      | 35               | F      | initially M3, later M2       | t(9;14)                   |                | altered amplification of <i>MYC</i> , <i>NEU</i> , <i>NRAS</i> ; <i>p15<sup>INK4B</sup></i> , <i>p16<sup>INK4A</sup></i> , <i>p53</i> | favourable     |
| <b>NB-4</b>       | 23               | F      | M3                           | t(15;17)(q22;q11)         | PML-RARA       | <i>p15<sup>INK4B</sup></i> deletion, <i>p16<sup>INK4A</sup></i> deletion                                                              | favourable     |

M: male, F: female, FAB: French-American-British, MDS: myelodysplastic syndrome, RAEB: refractory anaemia with excess blasts, ITD: internal tandem duplication.

## 2. MATERIAL AND METHODS

### 2.1 Basic characterisation of six selected AML cell lines

In this study, six human cell lines were chosen for further experiments according to their cytogenetics and AML cell type assigned by the FAB classification. An overview of these cell lines and their characteristics is given in Tab. 1. Suspension cells were grown in RPMI medium supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, US-MA) and split every three to four days at a 1:6 ratio.

#### 2.1.1 Growth curve analysis

For each cell line,  $1.5 \cdot 10^5$  cells were seeded as quadruplicates in 1 ml supplemented RPMI medium in a 24-well plate. Cell counts were measured for four consecutive days with the CASY® Cell Counter (OLS Omni Life Science, Bremen, Germany), using 50 µl of cell suspension in 5 ml CASYton (OLS Omni Life Science).

#### 2.1.2 Measurement of basal *STAT3* levels by RT-qPCR

Relative gene expression of *STAT3α* or *STAT3β* isoforms, total *STAT3* and *β-actin* levels was analysed by reverse transcription quantitative PCR (RT-qPCR). RNA from separate cell lines were extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany), followed by cDNA synthesis and RT-qPCR with primers detecting the different *STAT3* isoforms, total *STAT3* or *β-actin* (Tab. 2.). In brief, one dense 6-well from each cell line was harvested and washed once with 1x PBS, followed by centrifugation at 300 g for 5 minutes. Subsequently, cell pellets were lysed in 350 µl RLT buffer and supplemented with 350 µl 70 % ethanol (Australco, Spillern, Austria). The resulting 700 µl of this solution was transferred to a RNA binding column and centrifuged at 15,000 g for 20 seconds. The flow-through was discarded and the columns were washed once with 700 µl RW1 buffer and twice with 500 µl RPE buffer, each washing step followed by centrifugation at 15,000 g for 20 seconds for the first two wash steps and an elongated step of 1 minute for the last one. Next, RNA binding columns were placed in 1.5 ml tubes and eluted with 30 µl RNase-free water by

centrifugation at 15,000 g for 1 minute. RNA concentrations and 260/280 ratios were measured in duplicates with the Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland).

For cDNA synthesis, 1 µg RNA each was used as template and added to a mastermix prepared according to manufacturer's protocol, containing 5x reaction buffer, dNTP mix, random hexamer primes, RevertAid reverse transcriptase and RiboLock RNase Inhibitor in a final volume of 20 µl (RevertAid First Stand cDNA Synthesis Kit, ThermoFisher Scientific). Random hexamer primed synthesis was performed in a thermocycler (Eppendorf, Hamburg, Germany) with a starting temperature of 25 °C for 5 minutes followed by 60 minutes at 40 °C and a final incubation at 70 °C for 5 minutes to terminate the reaction. The final cDNA was diluted 1:5 with ddH<sub>2</sub>O and 5 µl of this was used as a template for the RT-qPCR reaction. The mastermix for the RT-qPCR consisted of 2x GoTaq qPCR mix (GoTaq® qPCR Master Mix, Promega, US-WI) with reverse and forward primers in a final concentration of 0.2 µM, filled up to 20 µl with ddH<sub>2</sub>O. The reaction was run in triplicates in a CFX Connect™ Real-Time PCR Detection System (Biorad, Berkeley, US-CA) with an initial heating step of 95 °C for 2 minutes, followed by 40 cycles of denaturing at 95 °C for 15 seconds and annealing at 60 °C for 60 seconds. The PCR cycles were followed by a melting curve analysis with a gradient from 60 °C to 95 °C run over 20 minutes. The mean for each triplicate sample was calculated and normalised to the corresponding *β-actin* levels with the  $2^{-\Delta\Delta CT}$  method to determine either the relative gene expression levels or the fold change compared to a control sample.

**Table 2: RT-qPCR primer sets**

| PCR for            | Primer | Orientation | Primer sequence (5' → 3') |
|--------------------|--------|-------------|---------------------------|
| <i>STAT3α</i>      | DS514  | Fw          | TGCAGCAATACCATTGACCT      |
|                    | DS515  | Rv          | AGATTGCTCAAAGATAGCAGAAGT  |
| <i>STAT3β</i>      | DS518  | Fw          | GTTTATCTGTGTGACACCATTTCAT |
|                    | DS515  | Rv          | AGATTGCTCAAAGATAGCAGAAGT  |
| total <i>STAT3</i> | DS557  | Fw          | TGACATTCCCAAGGAGGAGGC     |
|                    | DS558  | Rv          | TGCAGCTTCCGTTCTCAGCTCC    |
| <i>β-actin</i>     | DS500  | Fw          | GCTCATAGCTCTTCTCCAGGG     |
|                    | DS501  | Rv          | CCTGAACCCTAAGGCCAACCG     |

Fw: forward, Rv: reverse.

### 2.1.3 Western blotting of STAT3 isoforms

Protein levels of STAT3 and phosphorylated STAT3 (pSTAT3) were detected by Western blotting using corresponding antibodies and HSC-70 as loading control. AML cells were either stimulated with 100 ng human IL-6 (Peprotech, Rocky Hill, US-NJ) for 40 minutes at 37 °C or left unstimulated. Cell pellets were harvested and washed once with 1x PBS prior to lysis in 1x RIPA buffer (NEB, Ipswich, US-MA) completed with protease and phosphatase inhibitors (1x complete protease inhibitor, 1 mM PMSF, 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> (Sigma-Aldrich, St. Louis, MO-USA)), using 1 µl of buffer per 10<sup>4</sup> cells. Cells were incubated on ice for at least 20 minutes, sonicated for two cycles with 10 seconds on and 5 seconds off in between (Bioruptor® Plus Diagenode, Seraign, Belgium), and afterwards centrifuged at 20,000 g for 30 minutes at 4 °C. The supernatant was collected and the protein concentration was determined with the Coomassie Plus (Bradford) Assay Kit (ThermoFisher Scientific) in samples, diluted 1:10 in RIPA buffer. SDS gel electrophoresis was performed with a 8 % resolving polyacrylamide gel topped with a 5 % stacking polyacrylamide gel. For each sample, 50 µg of protein was loaded, completed with 1x SDS loading buffer containing 50 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 1 % β-mercaptoethanol, 12.5 mM EDTA and 0.02 % bromophenol blue. Gels were initially run at 90 V for 30 minutes, followed by 1.5 to 2 hours at 140 V. After electrophoresis, samples were transferred to nitrocellulose membranes at 80 mA per membrane for 1.5 hours with a subsequent blocking step in TBST with 5 % skim milk for up to 2 hours at room temperature (RT). Primary antibodies were diluted 1:1,000 and secondary antibodies 1:10,000 or 1:50,000 in TBST with 5 % bovine serum albumin (BSA). Signals were detected with the Super Signal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) on CL-Xposure films (ThermoFisher Scientific) in a x-ray film processor (AGFA, Mortsel, Belgium). Stripping was performed using a medium stripping buffer, consisting of 62.5 mM TRIS-HCl (pH 6.8), 2 % SDS and 100 mM 2-Mercaptoethanol, for 5 minutes at 50 °C, followed by 15 minutes at RT with agitation.

### 2.1.4 Antibiotic kill curves for AML cell lines

Dose-response curves with increasing concentrations of puromycin or blasticidin were performed to determine the optimal concentration for selecting stable cell clones. For this, 1 · 10<sup>4</sup> cells of each cell line were seeded in a 96-well plate and 200 µl supplemented RPMI

with increasing concentrations of antibiotics were added. The concentrations tested ranged from 0.25 to 10 µg/ml for puromycin (Sigma-Aldrich) and from 2 to 100 µg/ml for blasticidin (Carl Roth, Karlsruhe, Germany). Cell survival was measured after 96 hours with a resazurin viability assay by adding 40 µl of 700 µM resazurin solution (Sigma-Aldrich) to each well. One well containing only medium and resazurin was used as blank. The plate was incubated for 4 hours at 37 °C. Reduction of resazurin to resorufin induces a shift in absorbance from 600 nm to 570 nm which can be measured with a plate reader (Infinite 200 PRO plate reader, Tecan). Measured absorbance was normalised to the absorbance of medium containing only resazurin and the 570/600 nm ratio was calculated for all samples.

## 2.2 Process of a *STAT3* knock-out based on CRISPR/Cas9

The CRISPR/Cas9 systems allows irreversible genome editing mediated by guide RNAs resulting in a disruption of normal gene function and eventually gene knock-out. With this approach a stable *STAT3* KO in AML cell lines was attempted.

### 2.2.1 Selection of target sequences for *STAT3* specific single guide RNAs

Single guide (sg) RNAs were designed with the online CRISPR Design Tool (<http://tools.genome-engineering.org/>) from the input sequences of exon 7, 8 and 9 of *STAT3*, taken from Ensemble from the references sequence NM\_003150.3 (Ensemble release 87 – December 2016)<sup>47</sup>. Sequences were selected according to the following requirements: (1) starting with a guanine, (2) 20-nt guide sequence was immediately followed by a PAM sequence (NGG) in the genome, (3) highest Zhang scores indicating low off-target activity (Tab. 3.). Additional overhangs were added to the sequences for the top and bottom sgRNA (top: CACC, bottom AAAC) to allow ligation of the paired sgRNA strands into a pSpCas9(BB)-2a-Puro vector cut with Bpil (ThermoFisher Scientific) in a predetermined orientation. Sequences were ordered from MWG Eurofins (Ebersberg, Germany) and single-stranded oligo dinucleotides (ssODN) were diluted to a final concentration of 100 µM.

**Table 3: ssODN used for CRISPR/Cas9 mediated *STAT3* knock-out in AML cell lines.**

| Name      | Zhang score | Off-targets                      | Sequence 5'-3'            | Genomic location   |
|-----------|-------------|----------------------------------|---------------------------|--------------------|
| R1-top    | 78          | 144 (33 in genes, all have >3MM) | CACCGGGAACAGATGCTCACTGCGC | Exon7 <i>STAT3</i> |
| R1-bottom |             |                                  | AAACGCGCAGTGAGCATCTGTTCCC |                    |
| R2-top    | 92          | 84 (12 in genes, all have >4MM)  | CACCGGCAGAAACTCTCACGGACG  | Exon8 <i>STAT3</i> |
| R2-bottom |             |                                  | AAACCGTCCGTGAGAGTTTTCTGCC |                    |
| R3-top    | 90          | 70 (18 in genes, all have >3MM)  | CACCGGGCGGGGCTTTTGTCAGCGA | Exon8 <i>STAT3</i> |
| R3-bottom |             |                                  | AAACTCGCTGACAAAAGCCCCGCCC |                    |
| R4-top    | 94          | 42 (7 in genes, all have >4MM)   | CACCGTGTACAGCACCGGCCGATGC | Exon9 <i>STAT3</i> |
| R4-bottom |             |                                  | AAACGCATCGGCCGGTGCTGTACAC |                    |
| R5-top    | -           | -                                | CACCGGATGATAACTGGTCCGCAG  | Renilla control    |
| R5-bottom |             |                                  | AAACCTGCGGACCAGTTATCATCC  |                    |

ssODN: single-stranded oligo dinucleotides, MM: mismatches. In bold is indicated the leading guanine.

### 2.2.2 Cloning of vectors co-expressing sgRNA and Cas9

Oligo inserts were prepared by phosphorylating the top and bottom ssODN, followed by combining the two strands in order to create dimers. For this, 100  $\mu$ M of each matching ssODN was mixed with 10x T4 ligation buffer, 10 U T4 polynucleotide kinase (ThermoFisher Scientific) and added up to a final volume of 10  $\mu$ l with ddH<sub>2</sub>O. The reaction was carried out in a thermocycler at 37 °C for 30 minutes, followed by a heating step at 95 °C for 5 minutes and a ramp down of 5 °C per minute to a final temperature of 25 °C.

The paired oligos were diluted 1:200 in ddH<sub>2</sub>O and 2  $\mu$ l of this was mixed with the cloning reaction, consisting of 100 ng pSpCas9(BB)-2a-Puro vector (#48139, Addgene), 10 U BpiI (NEB), 10x T4 ligase buffer and 10 U T4 ligase (ThermoFisher Scientific), added up to 20  $\mu$ l with ddH<sub>2</sub>O. As a negative control, a no-insert control was set up with only pSpCas9(BB)-2a-Puro. The reactions were incubated for 1 hour, alternating between 37 °C and 21 °C every 5 minutes. Of each ligated vector, 2  $\mu$ l were transformed into chemically competent Stbl3 bacteria with an incubation of 10 minutes on ice and a subsequent heat-shock for 45 seconds at 42 °C. The cell suspensions were put back on ice for 10 minutes and a 1:10 dilution was prepared for each sample. Lysogeny broth (LB) medium was added to all tubes, plated onto LB agar plates supplemented with 100  $\mu$ g/ml ampicillin (Sigma-Aldrich) and incubated overnight at 37 °C. After overnight incubation, five clones were picked from each plate and subcultured in 5 ml LB medium with 100  $\mu$ g/ml ampicillin at 37 °C on a shaker.

Plasmids from resulting overnight cultures were extracted with the PureYield™ Plasmid Miniprep System (Promega, Fitchburg, US-WI) and sent for sequencing with the U6 primer (5'-GGG CAG GAA GAG GGC CTA T-3') to the IMP sequencing facility (VBC, Vienna, Austria).

### 2.2.3 Validation of sgRNA in HEK 293T cells

Sequence verified pSpCas9(BB)-2a-sgRNA-Puro vectors were validated in HEK 293T cells by confirming the KO with RT-qPCR, Western blotting and TIDE analysis after genomic DNA (gDNA) extraction.

#### 2.2.3.1 Transfection of HEK 293T cells with CRISPR plasmids

HEK 293T cells were cultured in DMEM, supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cells were calcium-phosphate transfected with 10 µg of plasmid in 500 µl ddH<sub>2</sub>O with 0.25 M CaCl<sub>2</sub>. The DNA-calcium complexes were mixed with 500 µl 2x HBS (pH 7.0) buffer, incubated for 15 minutes at RT and added dropwise to ~60 % confluent HEK 293 T cells. Control transfections were included with pSpCas9(BB)-2a-Puro cloned with a sgRNA for Renilla and a no-DNA transfection control. After 48 hours at 5 % CO<sub>2</sub> and 37 °C, cells were harvested for Western blotting from 10 cm dishes, according to the protocol described in section 2.1.3.

For RNA and DNA extraction, HEK 293T cells were transfected with 6 µg of plasmid DNA and 0.25 M CaCl<sub>2</sub> in 6-well plates and harvested after 48 hours. Afterwards, cells were washed once with ice-cold PBS, detached with 0.25 % Trypsin-EDTA (Gibco) and collected after inhibition of the enzymatic reaction in 1.5 ml tubes. RNA extraction, cDNA synthesis and RT-qPCR were performed as described in section 2.1.2. DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. Concentrations of extracted gDNA and 260/280 ratios were measured in duplicates with the Infinite 200 PRO plate reader (Tecan).

Duplicate reactions for each transfection were prepared according to the above described protocols, with the exception that 2 µg/ml of puromycin were added 24 hours after introduction of the CRISPR plasmids into the cells. Cells were harvested as described after three days of selection.

### 2.2.3.2 Tracking of Indels by Decomposition (TIDE) analysis

While the cleaving of the genomic sites by the CRISPR/Cas9 in combination with the distinct sgRNAs is a sequence specific process, the subsequent repair mechanism of these breaks by NHEJ is a random insertion or deletion of basepairs. TIDE analysis allows detection of the spectrum of the different mutations in a cell bulk culture. For this, PCR fragments were amplified with primers binding approximately 300 bp before and after the sgRNA binding site (Tab. 4). PCR reactions consisted of 1 µl of template DNA with 5x GoTaq buffer, 0.5 U GoTaq (Promega), 0.2 mM dNTPs (ThermoFisher Scientific) and 0.4 µM of each primer pair added up to a final volume of 25 µl with ddH<sub>2</sub>O. No template controls were included for each sgRNA-specific PCR and run with the following programme in a Eppendorf thermocycler: 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 1 minute, 50 °C for 30 seconds and 72 °C for 1 minute. The PCR run was concluded with 10 minutes at 72 °C and a cool-down to 4 °C. Samples were checked for products on a 1.5 % agarose gel and afterwards purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's protocol. Eluted PCR products were sent for sequencing with the corresponding forward and reverse primers (IMP sequencing facility). For TIDE analysis, .ab1 files of sequences were uploaded to the TIDE webtool (<https://tide-calculator.nki.nl/2>) and results were checked for certain quality criteria, such as overall sequence quality, breaksite location and size of decomposition window.

**Table 4: Primer sets for TIDE analysis**

| PCR for | Primer | Orientation | Primer sequence (5' → 3') |
|---------|--------|-------------|---------------------------|
| sgRNA#1 | DS716  | Fw          | GTGAGTTTGTGTTGTTGAGTTG    |
|         | DS717  | Rv          | GAGTTTCTGCACGTA CTCC      |
| sgRNA#2 | DS691  | Fw          | TGAATGGAAACAACCAGTCAG     |
| sgRNA#3 | DS692  | Rv          | ATTACAGGTGTGAGCCACAG      |
| sgRNA#4 | DS687  | Fw          | TTTTCAGCATCCACCCAAC       |
|         | DS688  | Rv          | CAACTCTACCCTCACCTAAC      |

Fw: forward, Rv: reverse.

#### 2.2.3.3 Measurement of cell cycle status in *STAT3* KO HEK 293T cells

After transfection of HEK 293T cells and subsequent selection with puromycin for three days (detailed description in section 2.2.3.1), cells were harvested for PI staining to evaluate the effect of *STAT3* KO on the cell cycle status. In brief, cells were vortexed while adding 3 ml 70 % Ethanol in PBS and frozen overnight at -20 °C. Following, cells were washed once in cold PBS and resuspended in 300 µl PI/RNase staining solution (ThermoFisher Scientific). After an incubation period of 30 minutes at RT in the dark, analysis was performed by flow cytometry with the FACSCanto II (BD, Franklin Lakes, US-NJ) and histograms were analysed with the FlowJo software (FlowJo LLC, Ashland, OR-USA).

#### 2.2.4 Nucleofection

Electroporation was used to transfer the prior cloned pSpCas9(BB)-2a-sgRNA-Puro or control plasmid (pSpCas9(BB)-2a-sgRenilla-Puro) into AML cell lines. For each cell line, different programme settings were tested first in duplicates with the pMAX-GFP plasmid (Lonza, Basel, Switzerland). The setup with the best viability and highest transfection efficiency was chosen for further experiments. In brief, 1.5 µg of plasmid DNA was added to electroporation cuvettes (VWR, Radnor, US-PA) and mixed with  $1 \cdot 10^6$  cells in 100 µl 1 M nucleofection buffer (5 mM KCl; 15 mM MgCl<sub>2</sub>; 120 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.2; 50 mM Mannitol). Afterwards, cells were electroporated with the corresponding programme of the Amaxa Nucleofector II (Lonza), washed out of the cuvettes with 500 µl pre-warmed supplemented RPMI medium and transferred to 12-well plates with 1.5 ml medium. After 48 hours at 37 °C and 5 % CO<sub>2</sub>, cells were harvested for viability staining with 0.1 µg 7-AAD per sample in 200 µl FACS buffer (1x PBS with 2 % FBS). Samples were analysed with the FACSCanto II (BD) for GFP and 7-AAD and evaluated with the FlowJo software (FlowJo LLC).

#### 2.3 Overexpression of *STAT3* isoforms by lentiviral infection

Lentiviral transduction was used to introduce exogenous *STAT3* isoforms into AML cell lines to induce stable overexpression. *STAT3α* and *STAT3β* sequences were cloned into a viral vector which was transfected into a packaging cell line, producing lentivirus and subsequently allowing infection of AML cell lines.

### 2.3.1 Ordering of *STAT3α* and *STAT3β* sequences

Human *STAT3α* and *STAT3β* sequences were ordered in a pENTR221 Gateway vector (#12536017, ThermoFisher Scientific) via the GeneArt Gene Synthesis service (ThermoFisher Scientific). Sequence information for *STAT3* was downloaded from the Ensemble website (Ensemble release 87 – December 2016)<sup>47</sup>. The coding sequences for *STAT3α* and *STAT3β* were retrieved from the transcripts ENST00000264657.9 (encoding a 770aa protein) and ENST00000585517.5 (coding for a protein of 722aa), respectively. Restriction sites for EcoRI, XhoI, BamHI, Eco147I (StuI) and BglII were removed from both coding sequences (CDS) with the GeneDesign online tool (<http://54.235.254.95/gd/index.html>)<sup>49</sup>. Additionally, the Kozak consensus sequence (GCC ACC) was added to the 5' end of both sequences.

### 2.3.2 Cloning of lentiviral vectors via Gateway cloning

LR Gateway cloning was performed in order to transfer the *STAT3α* and *STAT3β* sequences from the donor vector into a destination vector suitable for lentiviral infection. For the gateway reaction, 5x LR Clonase Reaction buffer (Gateway™ LR Clonase™ II Enzyme mix, ThermoFisher Scientific) was mixed with 1 µl of prior diluted plasmids and filled up to a final volume of 4 µl. In brief, one reaction for each entry vector, either pENTR221-h*STAT3α* or pENTR221-h*STAT3β*, was prepared with 25 ng donor vector mixed with 50 ng of the destination vector pLenti6/V5-DEST (#V49610, ThermoFisher Scientific). Two control reactions were set up in parallel, one containing only the destination vector (insert control) and one with 50 ng of a gateway control plasmid pENTR-gus (positive control), provided with the reaction kit. The reactions were incubated for 1 hour at 25 °C. Afterwards, 2 µl of Proteinase K were added and incubated for 10 minutes at 37 °C to halt the reaction. DH10B bacteria were transformed with 2 µl of the resulting gateway reactions and left on ice for 30 minutes. Bacteria were heat-shocked at 42 °C for 30 seconds and immediately transferred back on ice. Plating of bacteria, subculturing and plasmid extraction was performed as described in section 2.2.2. Plasmids were digested with BsrGI to verify clones presumably containing the correct insert. Restriction digestion was set up with 0.5 µg plasmid DNA, 10x NEB buffer 2.1 and 5 U BsrGI (NEB), filled up to 25 µl with ddH<sub>2</sub>O. Reactions were incubated for 1 h at 37 °C and inactivated subsequently at 80 °C for 20 minutes.

Digests were next mixed with 6x loading dye and run on a 1 % agarose gel for 1 h at 140 V. Additionally, primers capable of distinguishing between the two different isoforms were used for sequencing (IMP sequencing facility).

### 2.3.3 Induction of overexpression by lentiviral infection

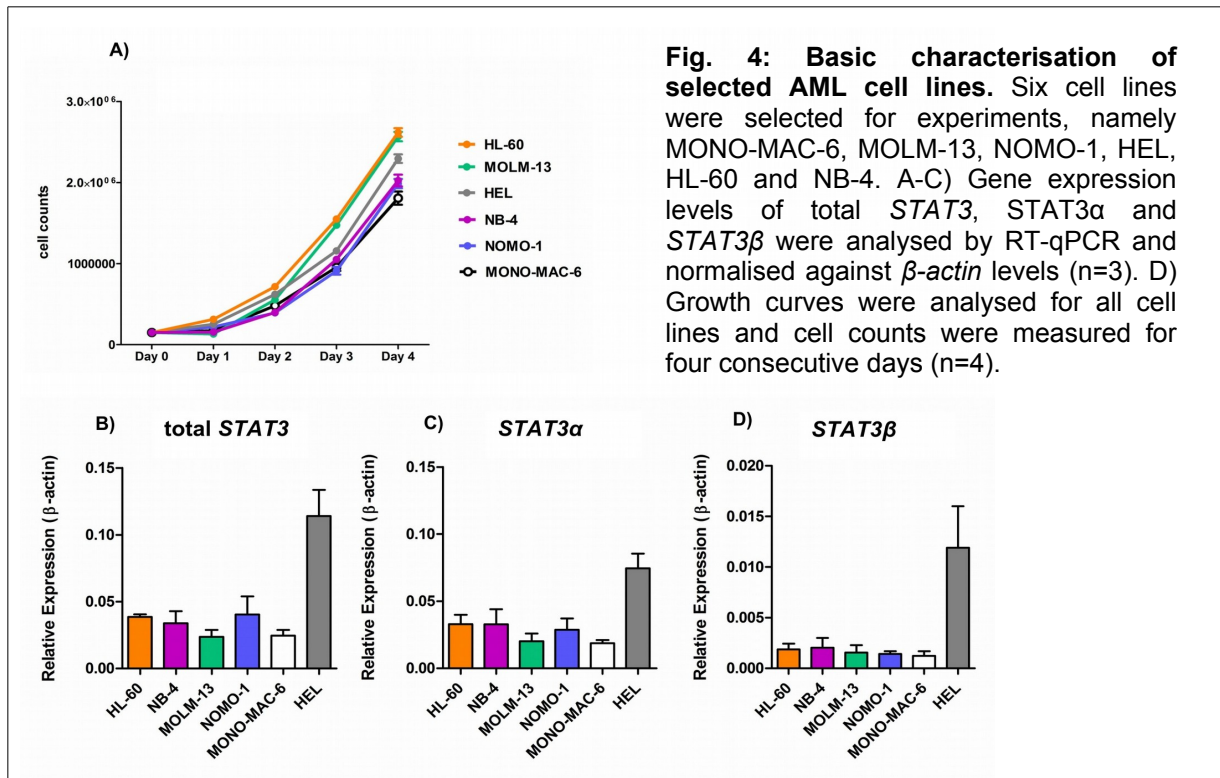
Overexpression of one of the two isoforms of *STAT3* was achieved by lentiviral transduction of human AML cell lines. For this, Lenti-X™ 293T cells were calcium-phosphate transfected with transfer vectors for either the pLenti6-h*STAT3*α-V5-Blasticidin or pLenti6-h*STAT3*β-V5-Blasticidin. Control transductions were performed with the pLentiGuide-sgRenilla-Puro (#52963, Addgene) or the parental pLenti6/V5-DEST (#V49610, ThermoFisher Scientific). Additionally, two lentiviral envelope and packaging plasmids were co-transfected, pMD2.G (#12259, Addgene) and psPAX2 (#12260, Addgene), coding for the viral proteins gag, pol, rev and tat, as well as the vesicular stomatitis virus envelope glycoprotein G (VSV-G), respectively.

In total 25 µg of DNA were used per reaction, made up of 12.5 µg transfer vector, 7.81 µg of psPax and 4.69 µg pMD2G and prepared in 500 µl ddH<sub>2</sub>O with a final concentration of 0.25 M CaCl<sub>2</sub>. The transfection mix was prepared as described in section 2.2.3 and added dropwise to ~60 % confluent Lenti-X™ cells, cultured in supplemented DMEM with 10 % FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, US-MA). Cells were incubated for 24 hours at 37 °C and 5 % CO<sub>2</sub>, followed by a medium change to supplemented DMEM medium with 30 % FBS. Virus was harvested at 48 and 72 h after transfection by removing the supernatant of Lenti-X™ cells and filtering through a 0.45 µm filter (TPP, Trasadingen, Switzerland) to remove any cell debris. For lentiviral transduction, 3 · 10<sup>6</sup> target AML cells were resuspended in 4 ml pooled virus suspension with 10 µg polybrene (Merck), followed by spinoculation for 90 minutes at 1000 g and 37 °C. Virus containing media was removed from target cells after 18 hours of incubation and infected cells were resuspended in supplemented RPMI containing the appropriate amount of selection antibiotics for the chosen plasmid. Cell viability and transduction efficiency was monitored by flow cytometry, measuring 7-AAD and GFP every 24 h, 48 h and 5 days after viral infection.

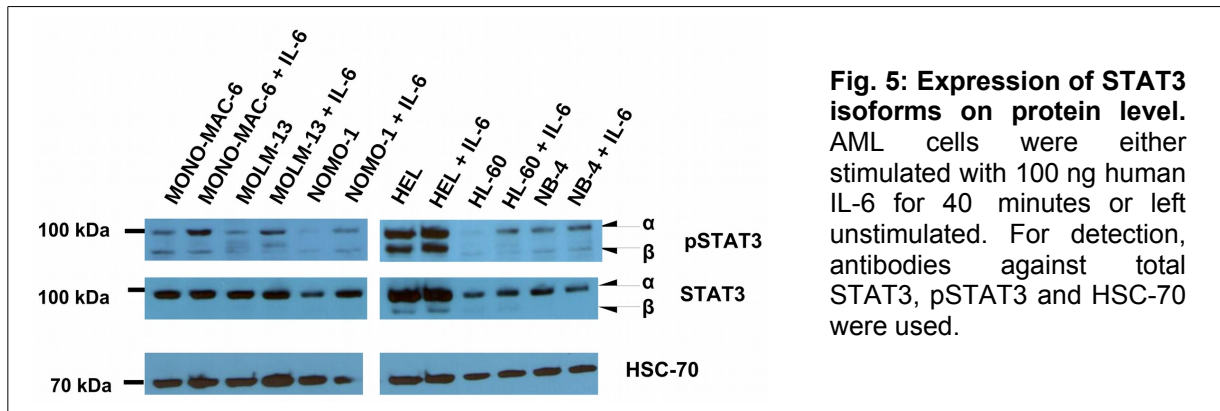
### 3. RESULTS

#### 3.1 Characterisation of *STAT3* expression and growth behaviour in AML cell lines

Six selected AML cell lines were characterised by assessing their growth behaviour by growth curve analysis. All human AML cell lines demonstrated a similar exponential growth, with HL-60 showing the fastest and MONO-MAC-6 the slowest growth rate compared to the others (Fig. 4A). Additionally, mRNA and proteins levels of *STAT3* isoforms were analysed by RT-qPCR and Western blotting, respectively. Relative expression of *STAT3* mRNA was assayed from all AML cell lines and normalised to  $\beta$ -actin levels. With the exception of HEL, which showed significantly higher expression of both *STAT3* isoforms and total *STAT3* levels, all AML cells exhibited similar levels for total *STAT3*, *STAT3* $\alpha$  and *STAT3* $\beta$ , respectively (Fig. 4B, 4C, 4D). In general, the *STAT3* $\beta$  isoform was expressed at far lower levels than *STAT3* $\alpha$  in all cell lines.



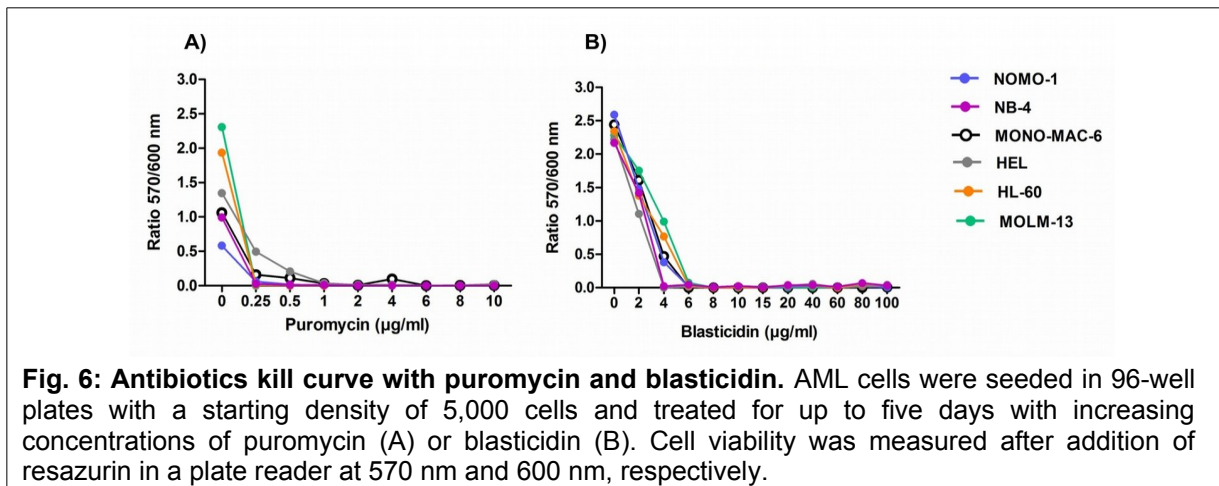
Analysis of protein levels was performed by Western blotting with antibodies against total STAT3, phosphorylated STAT3 (Y705) and HSC-70, serving as a loading control (Fig. 5). Cells were either stimulated with human IL-6 or left untreated. Detected bands for HSC-70, with a size of 70 kDa, were similar in all samples, whereas blots for STAT3 and pSTAT3 showed higher levels in HEL, corresponding to the RT-qPCR results (Fig. 4). When the STAT3 antibody was used, a single band for STAT3 $\alpha$  at approximately 93 kDa was detectable on the blot and a prominent one for STAT3 $\beta$  (84 kDa) in HEL samples. The other samples exhibited a band for STAT3 $\beta$  only with longer exposure times (data not shown). The antibody for pSTAT3 was able to detect both isoforms, with faint STAT3 $\beta$  bands for all cell lines, with the exemption of HEL depicting an especially strong signal. The IL-6-stimulated cells showed a clear increase in phosphorylated isoforms in all samples whereas total levels of STAT3 remained similar to the unstimulated cells. Phosphorylated STAT3 isoforms were especially low in NOMO-1 and HL-60 when compared to the other cell lines. Additionally, detection of phosphorylated STAT3 exhibited a third, unspecific band between STAT3 $\alpha$  and STAT3 $\beta$  in MOLM-13 cells in both stimulated and unstimulated cells. In summary, IL-6-stimulated AML cells demonstrated an increase of active, phosphorylated STAT3.



### 3.1.1 Determining concentrations for antibiotic selection of cell clones

In later experiments, transduced AML cell clones were planned to be selected with either puromycin or blasticidin. For determination of a suitable concentration for antibiotic selection, cells were seeded in 96-well plates and treated with increasing concentrations of antibiotics for up to five days, followed by analysis of cell viability with resazurin. For puromycin, concentrations up to 10  $\mu$ g/ml were tested. Cells started to die after three days in medium

containing 0.25 µg/ml puromycin and higher. Cells were dead in all cell lines in wells containing 1 µg/ml puromycin after five days of incubation, with most cells already dead at concentrations of 0.25 µg/ml. One exception was the cell line HEL which was more resistant and required up to 1 µg/ml to show significantly reduced viability (Fig. 6A). Blasticidin was tested in concentrations from 0 to 100 µg/ml for all selected AML cell lines. Cells took up to five days to die and viability was significantly reduced in the medium containing 4 µg/ml blasticidin for NB-4 and HEL, whereas the other cell lines required concentrations of up to 6 µg/ml blasticidin to exhibit significantly reduced viability (Fig. 6B). As a result, for subsequent experiments concentrations of 2 µg/ml were chosen for selection with puromycin and 8 µg/ml for blasticidin.

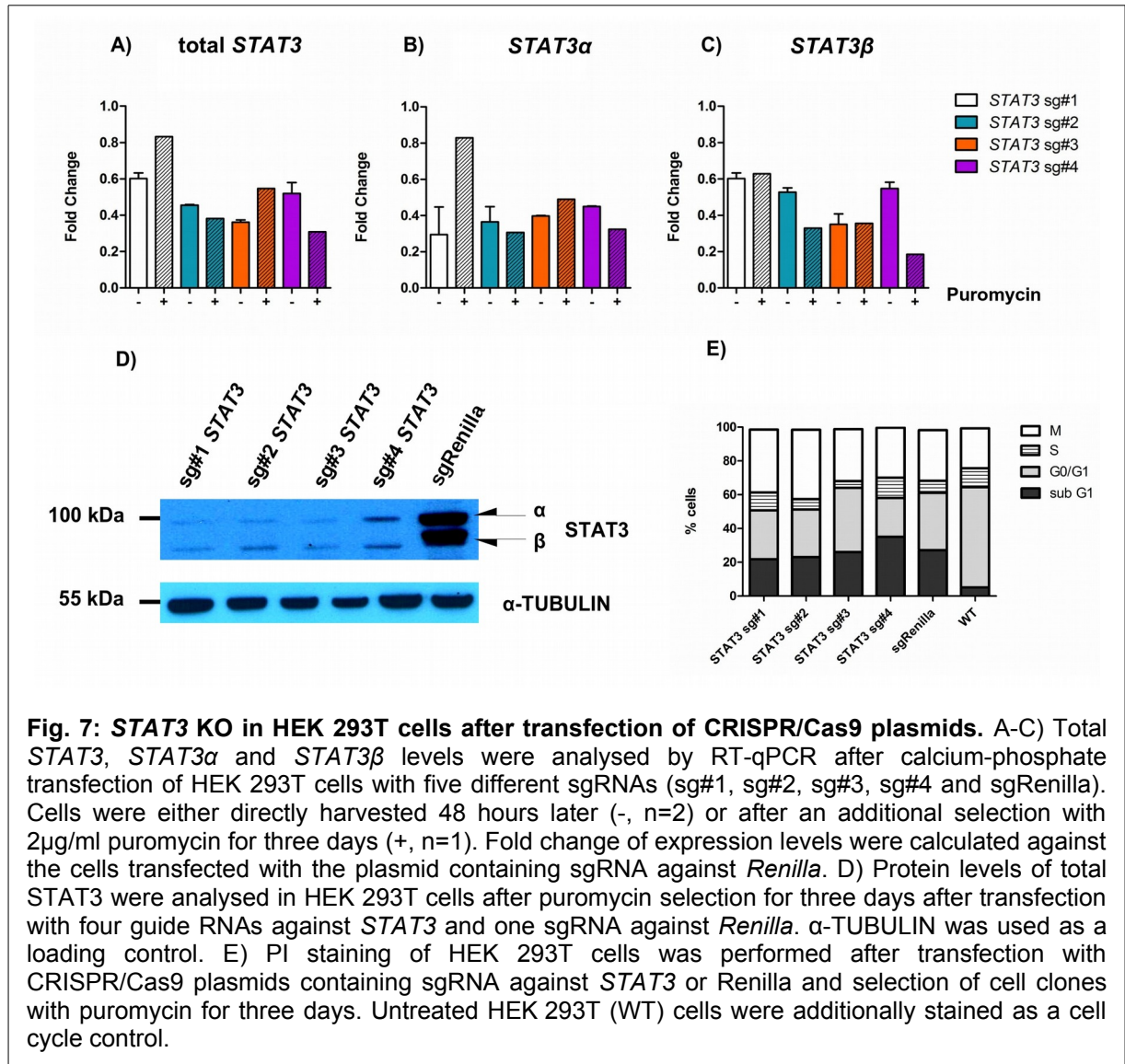


### 3.2 Knock-out of total *STAT3* by CRISPR/Cas9

STAT3 KO was aimed with a CRISPR/Cas9-based approach and validated in HEK 293T cells. Disruption of the STAT3 gene was verified on genomic and transcriptional level by TIDE analysis and RT-qPCR, respectively. Reduction of protein levels was evaluated by Western blotting after selection via puromycin. Flow cytometry of PI stained cells allowed analysis of cell cycle status of transduced HEK 293T cell clones. Thereafter, selected AML cell lines were transfected with the validated CRISPR plasmids.

### 3.2.1 Validation of *STAT3* KO in HEK 293T cells after transfection with CRISPR plasmids

Guide RNAs specifically targeting genomic h*STAT3* were cloned in plasmids containing the information for the Cas9 protein as well as providing resistance against puromycin. Validation of these plasmids was performed in HEK 293T cells (Fig. 7).



**Fig. 7: *STAT3* KO in HEK 293T cells after transfection of CRISPR/Cas9 plasmids.** A-C) Total *STAT3*, *STAT3α* and *STAT3β* levels were analysed by RT-qPCR after calcium-phosphate transfection of HEK 293T cells with five different sgRNAs (sg#1, sg#2, sg#3, sg#4 and sgRenilla). Cells were either directly harvested 48 hours later (-, n=2) or after an additional selection with 2μg/ml puromycin for three days (+, n=1). Fold change of expression levels were calculated against the cells transfected with the plasmid containing sgRNA against *Renilla*. D) Protein levels of total *STAT3* were analysed in HEK 293T cells after puromycin selection for three days after transfection with four guide RNAs against *STAT3* and one sgRNA against *Renilla*. α-TUBULIN was used as a loading control. E) PI staining of HEK 293T cells was performed after transfection with CRISPR/Cas9 plasmids containing sgRNA against *STAT3* or *Renilla* and selection of cell clones with puromycin for three days. Untreated HEK 293T (WT) cells were additionally stained as a cell cycle control.

Relative expression of *STAT3* mRNA was measured by RT-qPCR before and after puromycin selection. Fold changes in relation to cells transduced with a guide RNA against *Renilla* were calculated for each experimental sgRNA with the  $-2^{\Delta\Delta CT}$  method, resulting in a difference in expression of the *STAT3* isoforms from the control plasmid. Values above one

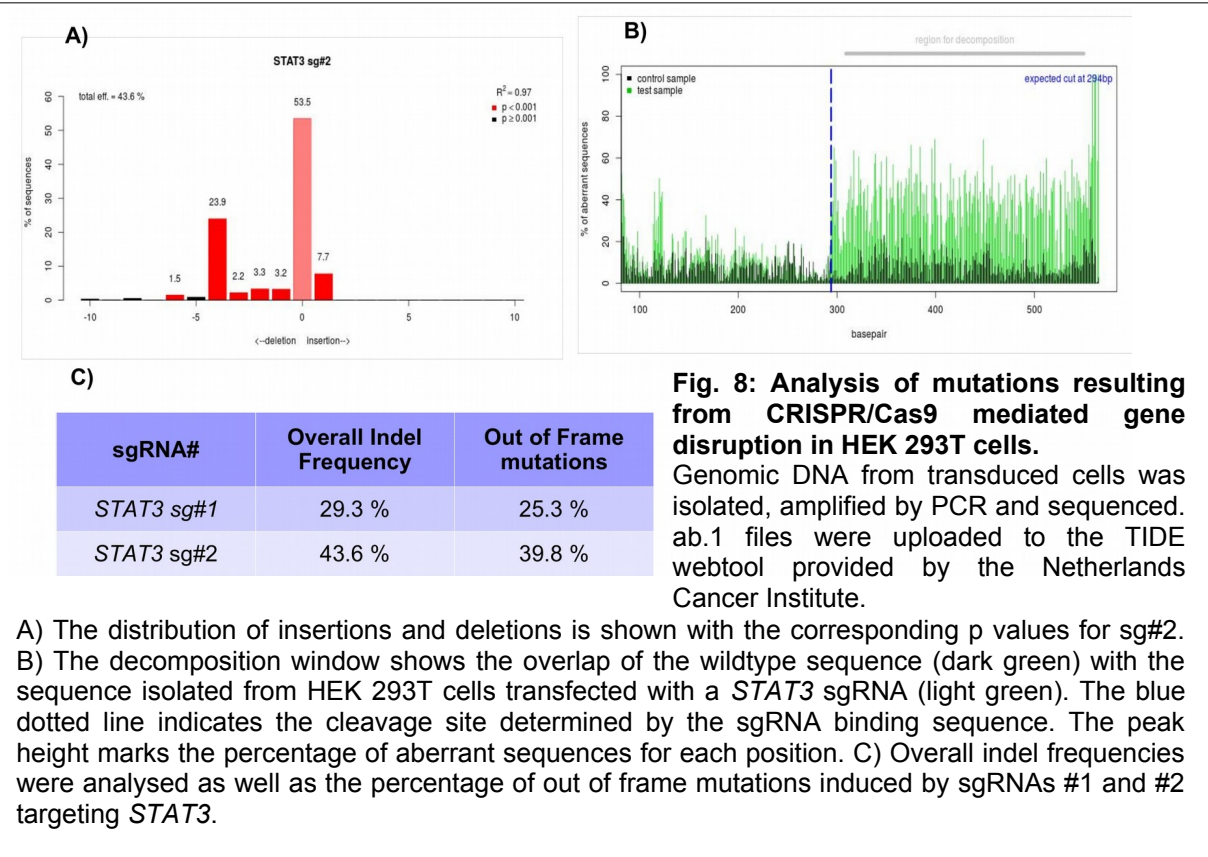
indicate an upregulation and values below one a downregulation of *STAT3* normalised to the guide RNA against *Renilla*. All sgRNAs against *STAT3* showed a clear reduction in fold change after 48 h of transfection varying from 40 % for sg#1 for total *STAT3* and *STAT3* $\beta$  to around 60 % in *STAT3* $\alpha$  (Fig. 7A, 7B, 7C). Overall, levels of *STAT3* $\alpha$  were reduced to a greater extent for all sgRNAs when compared to *STAT3* $\beta$ . Notably, expression of *STAT3* $\beta$  was further decreased in case of *STAT3* sg#2 and sg#4 after puromycin selection, from around 40 % reduction to 70 % and even lower in case of sg#4 with only 20 % expressed *STAT3* $\beta$  left. However, *STAT3* levels for *STAT3* sg#3 were slightly elevated compared to the 48 h time point. In addition, sg#1 against *STAT3* showed the highest increase in fold change of total *STAT3* and *STAT3* $\alpha$  after puromycin treatment, changing from 40 % to only 20 % knockdown in total *STAT3*. *STAT3* $\alpha$  levels were initially reduced by 70 % and gained after selection again 80 % of mRNA expression, whereas *STAT3* $\beta$  expression remained stable before and after selection. In addition, *STAT3* protein levels were analysed after puromycin treatment of HEK 293T cells with Western blotting and antibodies against total *STAT3* and  $\alpha$ -TUBULIN (Fig. 7D). For loading control, bands for  $\alpha$ -TUBULIN were detected at 53 kDa in equal signal strength for all samples. *STAT3* blots showed two bands for all samples for *STAT3* $\alpha$  at the height of 93 kDa and for *STAT3* $\beta$  at approximately 84 kDa (Fig. 7D). HEK 293T cells transduced with a sgRNA against *Renilla* showed strong signals for both isoforms whereas all guide RNAs for *STAT3* knock-out exhibited significant reduction of both isoforms. In summary, all four sgRNAs resulted in substantial decrease of *STAT3* expression, shown on RNA and protein expression level, with sg#2 and sg#4 being the most potent after puromycin selection.

PI staining and subsequent flow cytometry were performed with HEK 293T cells after puromycin treatment to assess the cell cycle state of *STAT3* KO cells. Cells in sub G1 represent apoptotic and necrotic cells, whereas resting and non-dividing cells are found in G0/G1 phase. Actively dividing cells are visible in S and M phase. In wild-type HEK 293T, most cells were in G0/G1 phase, with 34.8 % of cells in M and S phase, and 5.16 % of cells in sub G1. In contrast, the amount of transfected cells in sub G1 phase was elevated, ranging from 21.8 % to 35.7 %. In addition, less cells were in G0/G1 phase, accounting for only 23.5 % to 38.1 % of cells. The percentage of M and S phase cells was comparable to the wild-type situation with the exception of *STAT3* sg#3, which showed a decreased number

of cells in S phase (Fig. 7E). Summing this up, deletion of *STAT3* in HEK 293T cells did not alter the cell cycle status of these cells in comparison to wild-type cells.

### 3.2.2 Tracking of Indels by Decomposition (TIDE) in HEK 293T cells

Frequencies of insertions and deletions (indels) after CRISPR/Cas9 were evaluated by TIDE analysis. Genomic DNA was extracted from HEK 293T cells transfected with sgRNA against *STAT3* or *Renilla*, followed by selection with puromycin. PCR fragments resulting from amplifying approximately 300 bp before and after the sgRNA cleavage sites were sequenced and analysed with an online tool provided by the Netherlands Cancer Institute (<https://tide-calculator.nki.nl/><sup>48</sup>).



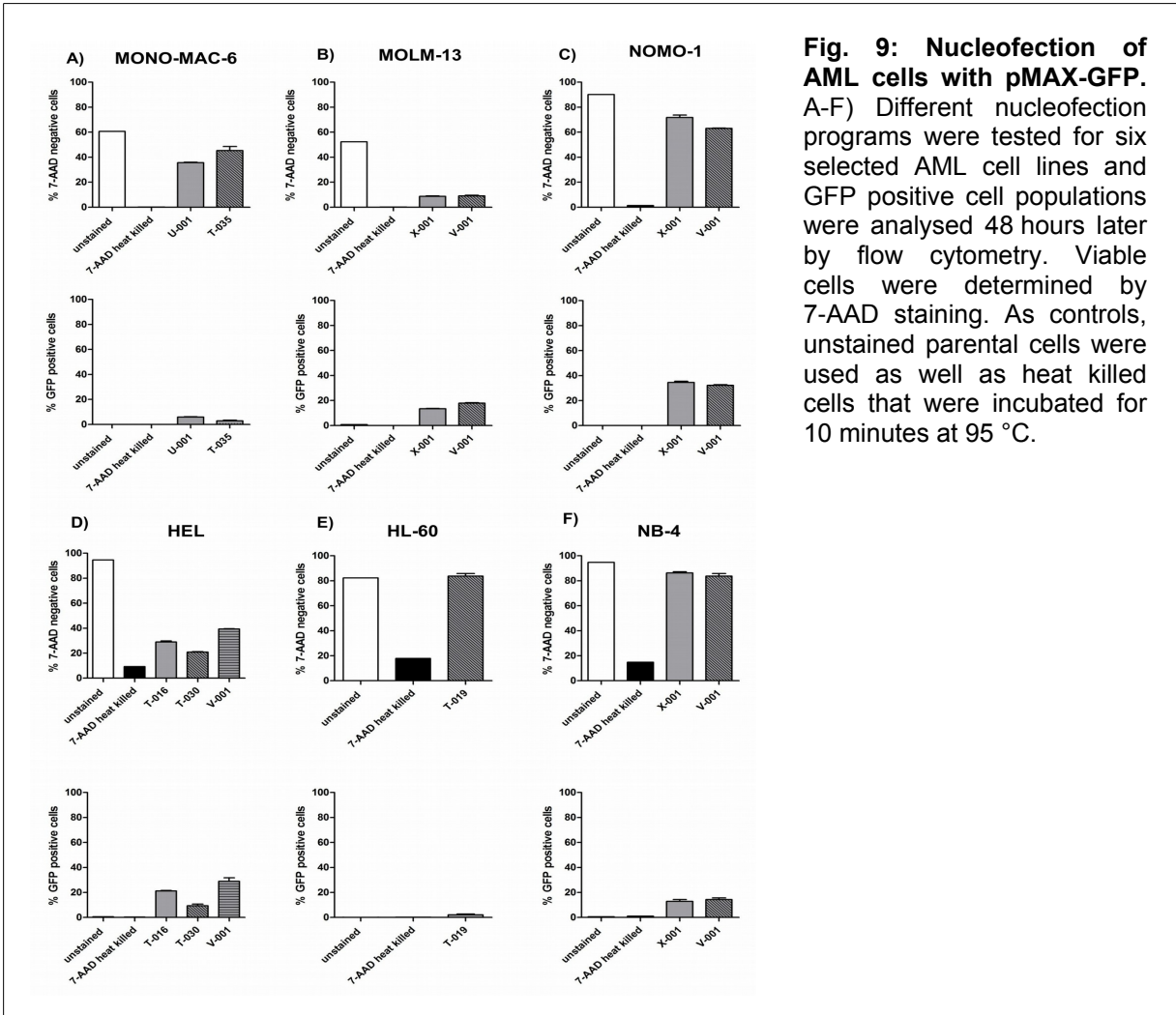
Results are shown exemplarily for the *STAT3* sg#2 to demonstrate the results from the previous mentioned webtool. The software displays two diagrams after sequence upload. One is displaying the percentage of all sequences harbouring indels when compared to a control sequence of untreated cells (Fig. 8A). The second diagram indicates how many

different sequences show more than one basepair per position (aberrant sequences, Fig. 8B). The  $R^2$  coefficient of this experiment was 0.97 and red bars indicate indels detected with a p-value  $\leq 0.001$  whereas black bars have a p-value  $\geq 0.001$ . In this case 53.5 % of sequences showed no deletion or insertion events whereas the second highest bar indicated a -4 deletion detected in 23.9 % of all sequences in one sample (Fig. 8A). Only one insertion of +1 was found in 7.7 % of all sequences. The decomposition window displayed the break site at around 294 bp, indicated by a blue dotted line and overlapping with the sgRNA binding site (Fig. 8B). Percentages of aberrant sequences were low before the breakpoint in control and samples transduced with the CRISPR plasmids. The amount of differences detected per basepair position in the experimental samples increased significantly at the site of cleavage whereas the percentages in the control cells remained low. The overall sum of indel frequencies for sg#1 and sg#2 are shown in Fig. 8C. Additionally, the percentage of out of frame mutations was indicated separately, taking into account only positions that were not divisible by three. The distinct sgRNA exhibited differences in the percentage of induced mutation frequencies, when comparing sg#1 and sg#2. The guide RNA sg#2 led to 43.6 % targeted sequences, from which 39.8 % were out of frame mutations. In contrast, *STAT3* sg#1 showed lower frequencies of 29.3 % targeted sequences but also with a high number of out of frame mutations (25.3 %). In conclusion, the induction of a *STAT3* knock-out could additionally be shown on the genomic level with TIDE analysis for sgRNA#1 and #2.

### 3.2.3 Establishment of an optimised nucleofection protocol for human AML cell lines

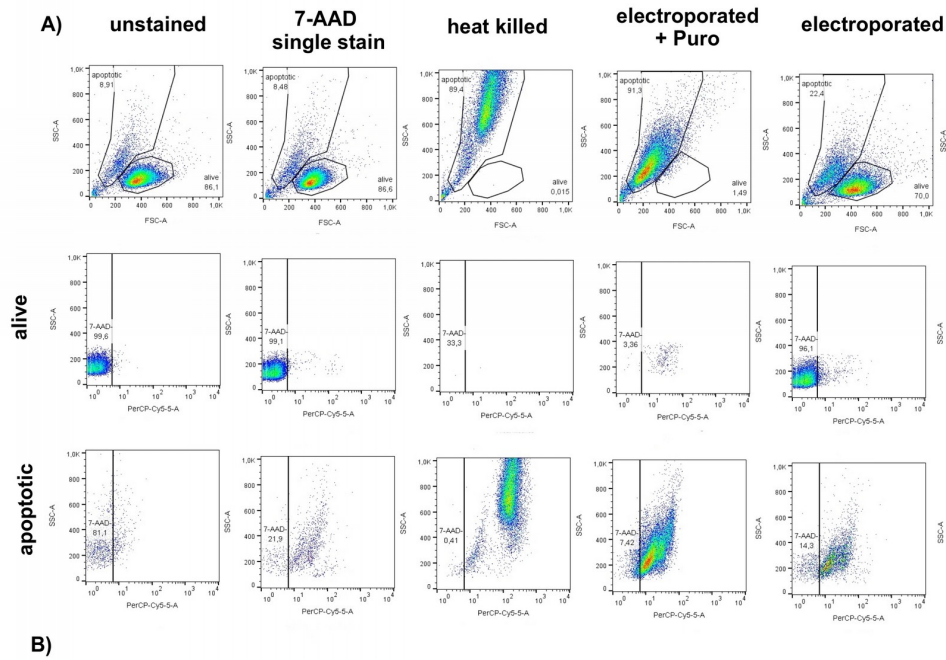
AML cells are difficult to manipulate, therefore cell lines were nucleofected with a control plasmid, the pMAX-GFP plasmid, to test which nucleofection programme resulted in the best combination of viability after electroporation and highest percentage of GFP positive cells. Viability was analysed with 7-AAD staining and subsequent flow cytometry as positive staining will only occur in cells with compromised cell membranes. Overall viability was dramatically reduced after electroporation of MOLM-13 and HEL cells (Fig. 9B, 9D), whereas the other cell lines only showed a slight decrease in viability compared to the untreated parental cell lines (Fig. 9A, 9C, 9E, 9F). Overall, AML cell lines displayed high variance in the percentage of viable GFP positive cells, ranging from 2 % to 35 %. The highest values were found in NOMO-1 cells in the two programmes tested, with 32.2 % and 34.6 % GFP positive cells, respectively (Fig. 9C). MOLM-13 and NB-4 cells exhibited GFP positive populations of

approximately 15 % in both settings (Fig. 9B, 9F). Transfection was most efficient in HEL cells with the programmes T-016 and V-001, resulting in 21.3 % and 29 % GFP positive cells, whereas the third programme tested only achieved transfection of 9 % of cells (Fig. 9D). In general, MONO-MAC-6 and HL-60 had the lowest transfection efficiencies with values from 2 % to 6 % GFP positive cells (Fig. 9A, 9E). Taking these results together, cell stress upon nucleofection differed, with MOLM-13 tolerating the procedure the least. Transfection efficiencies also varied between the different cell lines showing that NOMO-1, HEL and NB-4 cell lines are best suited for further experiments.



### 3.2.4 Transfection of AML cells with CRISPR/Cas9 plasmids mediating *STAT3* KO

AML cell lines as well as their corresponding nucleofection programmes, which showed the best viability and highest transfection efficiency, were selected for further experiments. NOMO-1, HEL and NB-4 cells were transfected with CRISPR/Cas9 plasmids carrying either sgRNAs against *STAT3* or a control sgRNA against *Renilla*. To select for positive clones, puromycin was added 24 hours after nucleofection and cells were analysed by flow cytometry for 7-AAD after three days of selection. Cells were grouped for analysis into two populations: the first group consisted of larger and less granular cells (alive) and a second group made up of smaller and highly granular cells (apoptotic). In a second step, cells were further separated into 7-AAD negative and positive cells, the latter corresponding to apoptotic and necrotic cells. The gating strategy and results are exemplarily shown only for NB-4 cells as results for NOMO-1 and HEL cells were comparable (data not shown). For controls, untreated NB-4 cells were used and distributed into three setups: unstained, 7-AAD stained and heat killed followed by 7-AAD staining. Electroporated cells were additionally included with and without puromycin treatment. In unstained and 7-AAD stained cells the living cell population accounted for approximately 99 % of total cells (Fig. 10A). The cell population labelled as apoptotic was in 80 % of cells positive for 7-AAD. The heat killed cells only had cells in the gate for apoptotic cells though the 7-AAD staining indicated mainly necrotic cells in the upper right area. Cells only electroporated showed similar distribution of cells in the two gates though the number of apoptotic cells was slightly increased to 22.4 % when compared to the 7-AAD stained controls. Additionally, cells electroporated without DNA and afterwards treated with puromycin showed only apoptotic cells shifting from early to late apoptosis indicated by 7-AAD. In cells transfected with the pSpCas9-sgRNA-Puro plasmids, the predominant cell population found was smaller and highly granular, accounting for about 90 % of all measured cells. Viable cells were 2 % and lower for all *STAT3* guide RNAs as well as the *Renilla* control (Fig. 10B). The amount of larger and more granular cells in the gate for apoptotic cells still showed around 3 % of viable cells whereas the percentages of 7-AAD negative cells were negligible in the alive gate. Due to the low number of viable cells, selection of stable *STAT3* KO cell clones was not possible and cells did not grow out even one week after nucleofection.



| sample         | % alive | % apoptotic | % alive: 7-AAD neg. | % apoptotic: 7-AAD neg. |
|----------------|---------|-------------|---------------------|-------------------------|
| NB-4 sgRenilla | 1.29    | 90.1        | 0                   | 2.88                    |
| NB-4 sg#1      | 1.21    | 91.6        | 0.83                | 3.42                    |
| NB-4 sg#2      | 1.52    | 91.2        | 0.66                | 2.87                    |
| NB-4sg#3       | 2.05    | 89.8        | 0.73                | 3.06                    |
| NB-4 sg#4      | 1.50    | 90.2        | 1.33                | 3.94                    |

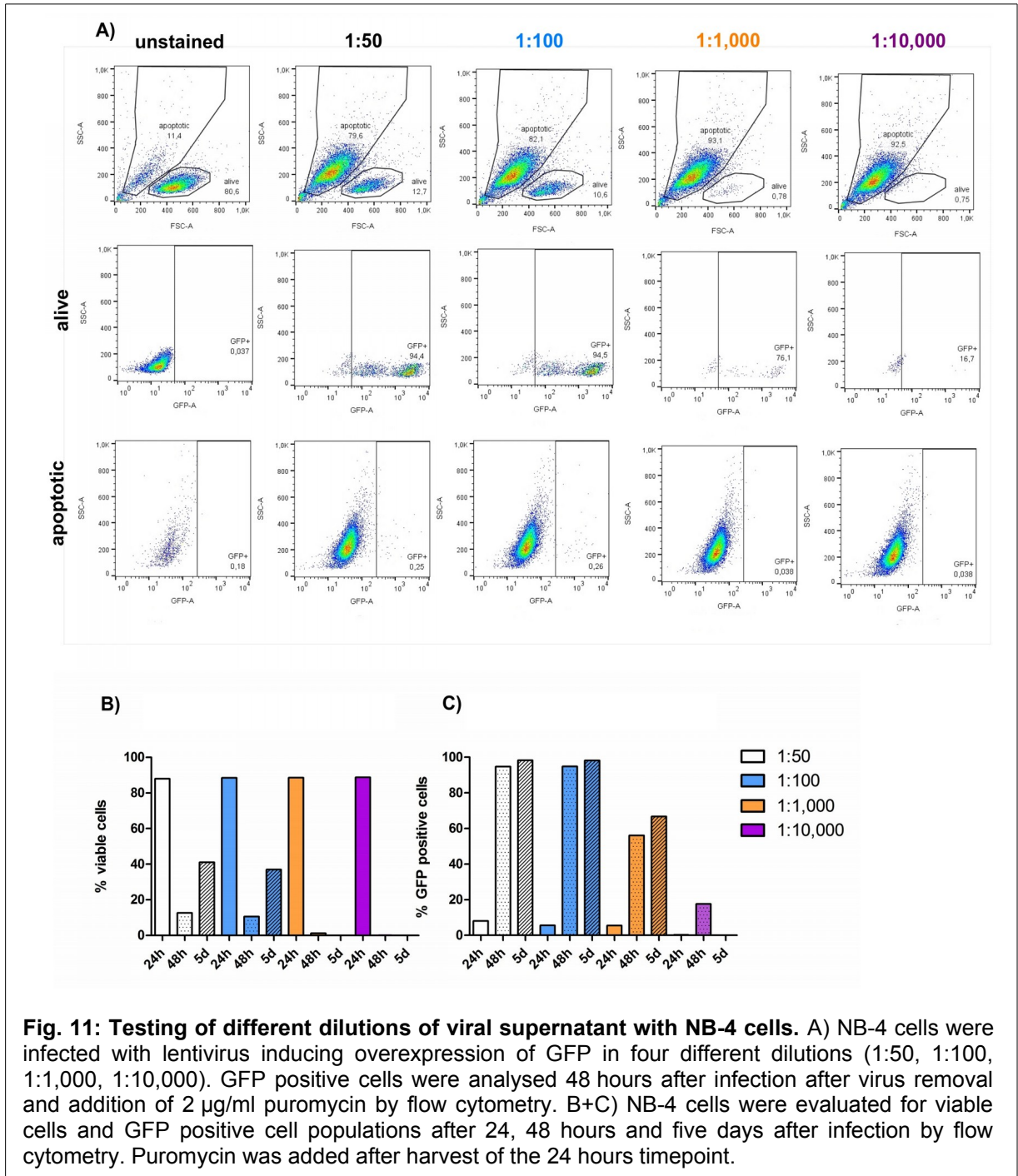
**Fig. 10: Nucleofection of AML cells with pSpCas9-sgRNA-Puro.** A) NB-4 cells were transfected with CRISPR/Cas9-sgRNA plasmids and treated 24 hours later with 2µg/ml puromycin for three days. Viability was analysed subsequently by 7-AAD staining. For controls, untreated parental cell lines were either left unstained, stained with 7-AAD or heat killed (10 minutes, 95 °C) prior to staining. Additionally, electroporated cells with (electroporated+Puro) or without puromycin treatment (electroporated) were included. B) Percentages of gated populations are shown for each sgRNA. Population “alive” refers to larger and less granular cells, population “apoptotic” in contrast indicates smaller and highly granular cells. For each population, the percentage of 7-AAD negative cells is indicated. Neg, negative.

### 3.3 STAT3 overexpression with lentiviral vectors

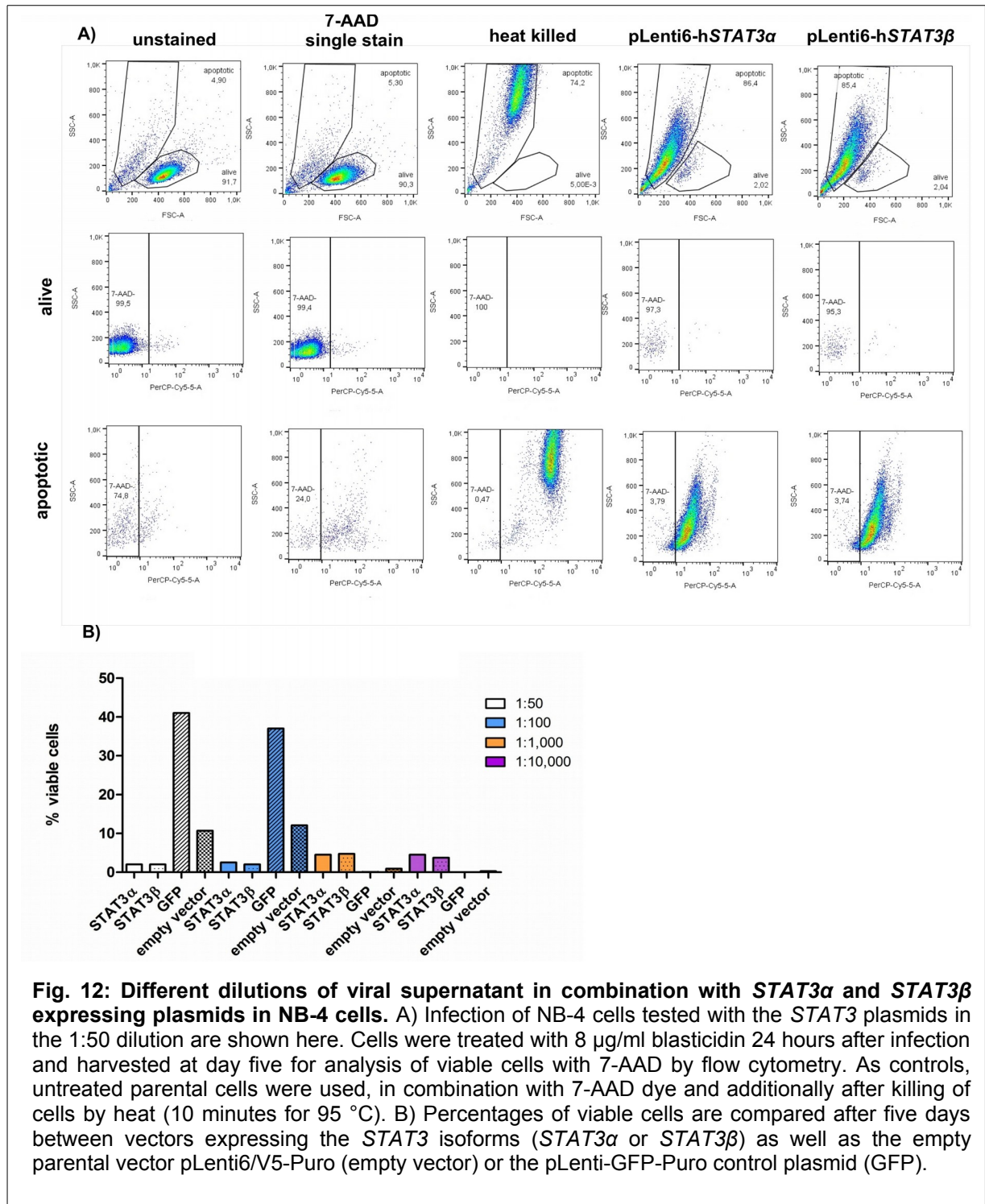
*STAT3α* and *STAT3β* CDS were obtained and transferred in the pLenti6/V5-DEST vector suitable for lentiviral overexpression. First, different dilutions of viral supernatant, with a control vector inducing overexpression of GFP, were representatively tested in NB-4 cells.

### 3.3.1 Optimisation of viral infections in NB-4 cells with a control GFP-plasmid

NB-4 cell lines were infected with four different dilutions (1:50; 1:100; 1:1,000; 1:10,000) of lentivirus containing supernatant, carrying a plasmid coding for GFP, to test which virus dilution is best suited for the following experiments.



Cells were harvested 24, 48 hours and five days after infection and GFP positive populations were determined. After 24 hours, puromycin was added to cells to start selection for transfected cell clones. For flow cytometry analysis, the same gating strategy as described in 3.2.4 was used. Cells were divided into two populations, alive and apoptotic, and their corresponding GFP values were investigated. In general, the amount of apoptotic cells, by comparison to the viable population, increased with the magnitude of viral dilution. In dilutions as high as 1:1,000 and 1:10,000 most cells were dead after 48 hours (Fig. 11A). In the 1:50 and 1:100 dilutions, 94.4 % and 94.5 % of cells, respectively, were shown to be viable and GFP positive, whereas the apoptotic cell populations showed no GFP positive cells. Viable cells in the 1:1,000 populations exhibited also a high level of cells positive for GFP (76.1 %) though the overall viable population was vanishingly small, accounting for only 0.76 % of total cells. In the 1:10,000 dilution, cells in general were mostly negative for GFP in both cell populations. Cells were monitored over a period of five days after transduction, noting viable cells and corresponding GFP values (Fig. 11B, 11C). Cells in all dilutions indicated a high viability after 24 hours of transduction with approximately 80 % viable cells. At 48 hours, cell viability dropped under 20 % after addition of puromycin in the dilutions for 1:50 and 1:100, with an additional decrease in viability to levels below detection limit in dilutions as high as 1:1,000 and 1:10,000. Cell numbers were increasing to around 40 % after five days of incubation in dilution setup 1:50 and 1:100 whereas cell numbers in 1:1,000 and 1:10,000 stayed below detectable levels (Fig. 11B). The corresponding GFP levels showed only minor GFP positive populations after 24 hours of transduction which were increased after 48 hours and even went up further after five days in the dilutions of 1:50, 1:100 and 1:1,000 (Fig. 11C). GFP levels in the 1:10,000 dilution were the lowest from all setups and absent after five days of incubation. Those experiments show that viral dilutions of up to 1:100 still give satisfying infection efficiencies while higher dilutions failed to do so.



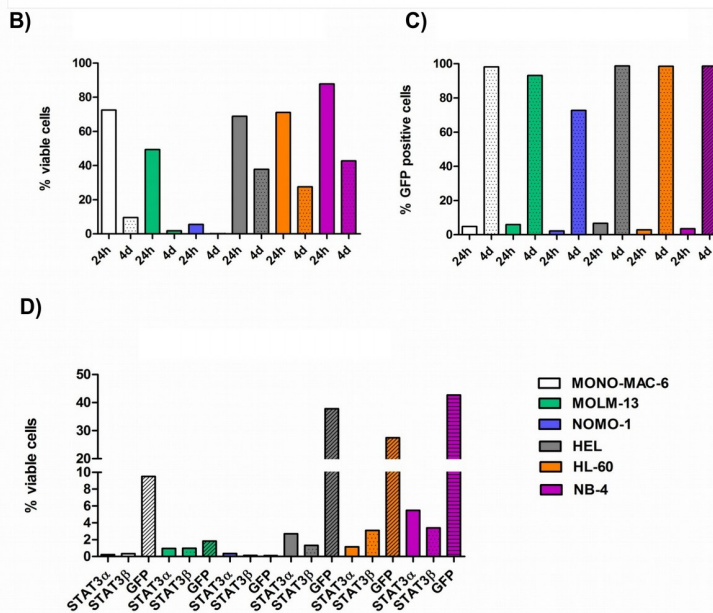
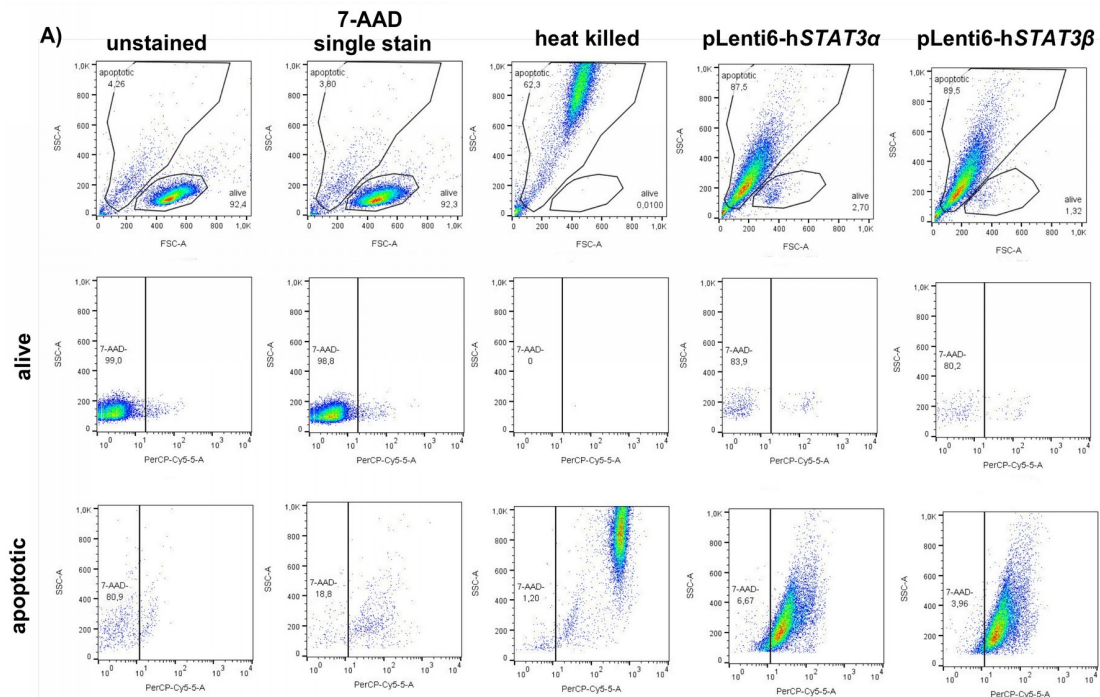
### 3.3.2 Infection of NB-4 cells with lentivirus overexpression *STAT3* isoforms

After testing different viral dilutions stemming from the pLenti-GFP-Puro plasmid, these defined dilutions were additionally used for infection with lentiviral plasmids to induce overexpression of either the *STAT3 $\alpha$*  or the *STAT3 $\beta$*  isoform in NB-4 cells. After transduction, blasticidin was added 24 hours later and cells were harvested at day five to determine the percentage of viable cells transduced with the pLenti6-h*STAT3 $\alpha$* -Puro or pLenti6-h*STAT3 $\beta$* -Puro plasmids via 7-AAD staining. As an example, the scatter plots for the 1:50 dilution are described here as the results for the other dilutions resulted in comparable percentages for each population (Fig. 12A). In comparison to the 7-AAD stained untreated parental cells, those transfected with the *STAT3* plasmids showed a significant decrease in viable cell populations from 99.4 % to around 2 % in both *STAT3* plasmids. In line, cells detected there were mainly 7-AAD negative. The apoptotic cell populations increased from 5.3 % to 86.4 % and 85.4 % in cells transduced with the plasmid for *STAT3 $\alpha$*  or *STAT3 $\beta$* , respectively, demonstrating a shift towards late apoptotic cells in the 7-AAD staining. Additionally, cell viability of the different dilutions in combination with the different *STAT3* plasmids were compared with the pLenti-GFP plasmid as well as the empty parental backbone from the pLenti6/V5-Puro five days after infection (Fig. 12B). Overall, the plasmids for the *STAT3* isoforms showed the lowest levels of viable cells in all dilutions of around 2 and 4 %. However, the empty parental backbone of these vectors also showed a decreased viability of around 10 % when compared to the GFP plasmid in the dilutions 1:50 and 1:100 which exhibited around 40 % viable cells. Neither the GFP nor the empty vector showed any viable cells left in the higher dilutions of 1:1,000 and 1:10,000. In conclusion, viral dilutions of 1:1,000 and higher turned out insufficient for infection with all plasmids. Although viability of the parental vector and the control plasmid were slightly decreased, the constructs for *STAT3 $\alpha$*  or *STAT3 $\beta$*  failed to give rise to stable infected clones after antibiotics selection.

### 3.3.3 Transduction of pLenti-h*STAT3 $\alpha$* and pLenti-h*STAT3 $\beta$* in AML cell lines

After testing of different viral dilutions and plasmids with NB-4 cells, the remaining human AML cell lines were infected with 1:50 dilutions of viral supernatant of *STAT3 $\alpha$* , *STAT3 $\beta$*  and GFP plasmids. Cell populations were again distributed into two groups as described in section 3.2.4 and the viability of the overexpression plasmids were compared four days after

infection with unstained, untreated but 7-AAD stained cells and 7-AAD stained, heat killed parental cells. Scatter plots for all AML cell lines looked comparable, therefore the gating strategy is only shown exemplarily for HEL cells (Fig. 13A). Populations of cells infected with plasmids coding for STAT3 isoforms were shifted towards the apoptotic compartment when compared to the 7-AAD stained parental cell line from 3.8 % to 87.5 % and 89.5 %, respectively. Cell lines transduced with the GFP plasmid showed variable levels of viability at 24 and 48 hours post infection (Fig. 13B). Especially, NOMO-1 showed a severe drop in viable cells already at 24 hours after infection and prior to the addition of puromycin. Selection antibiotics were added after 24 hours and cell viability was again assayed three days later (four day time point). Three out of six cell lines showed increased death whereas HEL, HL-60 and NB-4 displayed between 20 and 40 % viable cells. Corresponding GFP positive cells were similar in all cell lines with a low percentage after 24 hours which increased to over 90 % GFP positive cells four days after infection, with the exception of NOMO-1 which showed a smaller fraction of green cells (Fig. 13C). In addition, viability at the four day time point was compared between distinct AML cell lines and the different lentiviral plasmids pLenti-hSTAT3 $\alpha$ -Blasticidin, pLenti-hSTAT3 $\beta$ -Blasticidin and the pLenti-GFP-Puro vector. Overall, MONO-MAC-6, MOLM-13 and NOMO-1 showed a decrease in viable cells for the STAT3 plasmids, in the case of MOLM-13 and NOMO-1 also in the setup with the pLenti-GFP plasmid (Fig. 13D). Higher rates of viable cells of approximately 2 to 6 % were observed in HEL, HL-60 and NB-4 cells, where in addition the percentages of viable cells infected with the GFP vector were significantly higher when compared to the other AML cell lines. However, cells started to die at day seven in all setups infected with the plasmids encoding for the STAT3 isoforms. Concluding, though transduction with the GFP plasmid showed infection efficiencies of around 40 % in HEL, HL-60 and NB-4 cells, the plasmids for STAT3 failed to allow establishment of stable cell clones.



**Fig. 13: Transduction of AML cell lines with plasmids expressing STAT3 $\alpha$  and STAT3 $\beta$ .** AML cell lines were infected with a 1:50 dilution of viral supernatant containing either pLenti-hSTAT3 $\alpha$ -Puro, pLenti-hSTAT3 $\beta$ -Puro or pLenti-GFP-Puro plasmids. Cells were treated with puromycin after 24 hours of infection and harvested for viability staining with 7-AAD at 24 hours or four days later. A) HEL cells are exemplarily shown for flow cytometry analysis of 7-AAD. Untreated parental cells, single stains with 7-AAD and 7-AAD stained cells after killing with heat (10 minutes, 95 °C) were used as controls.

B+C) AML cells were transfected with the pLenti-GFP-Puro plasmid and cell viability and percentage of GFP positive cells are shown for 24 hours and four days after infection. D) Percentage of viable cells (7-AAD negative cells) is shown for all AML cells and in comparison to the three different lentiviral plasmids for STAT3 $\alpha$ , STAT3 $\beta$  or GFP.

## 4. DISCUSSION

At the beginning of this project, the role of the *STAT3* isoforms in acute myeloid leukaemia was incompletely understood. The focus of this thesis project was to establish *in vitro* models for studying the effect of *STAT3* knock-out as well as overexpression of *STAT3α* and *STAT3β* in AML cell lines. A genomic KO in selected cell lines was attempted with the CRISPR/Cas9 technology, first validated in HEK 293T cells and then applied to AML cell lines after nucleofection. For overexpression, several optimisation strategies for the viral transduction were tested in NB-4 cells before employing these protocols on the remaining AML cells.

### 4.1 AML cell lines show comparable gene and protein expressions of *STAT3* isoforms with the exception of HEL

Evaluating the expression of *STAT3* in AML cell lines allowed the selection of six cell lines that represented three categories. Regarding to cytogenetics of the human cell lines, two could be classified as favourable in clinical context (HL-60, NB-4) and three (MONO-MAC-6, MOLM-13 and NOMO-1) as non-favourable since they are harbouring the MLL-AF9 fusion which is correlated with an overall worse survival time and increased relapse in patients<sup>50</sup>. The last cell line represents a peculiarity as such that HEL is not of myelomonocytic origin like the other cell lines, but is derived from the erythropoietic lineage. However, this cell line was shown to respond to phorbol diester (TPA) treatment with a macrophage-like phenotype, indicated by a change in morphology as well as enhancement of phagocytic activity<sup>51</sup>. Additionally, we chose this cell line for further experiments because it showed high expression of *STAT3* isoforms on mRNA as well as protein level in contrast to the other cell lines, which all had similar expression levels. The growth behaviour was comparable in all six cell lines indicating that differences in expression levels are attributed to the distinct cell lines rather than the cells growth rates. For protein analysis by Western blotting, cells were either stimulated with IL-6 or left untreated to explore if all AML cell lines respond to stimulation since IL-6 is one of the main activators of *STAT3*<sup>52</sup>. As expected, levels of p*STAT3* increased in all AML cell lines upon stimulation with IL-6 whereas total *STAT3* levels remained unchanged. Interestingly, MOLM-13 exhibited for both conditions a third band on the blot

located between STAT3 $\alpha$  and STAT3 $\beta$ . Tumour cells are reported to receive post-translational modifications of the STAT3 protein such as acetylation of lysines or methylation of other residues<sup>53,54</sup>. These modifications can lead to an increased molecular weight when compared to the unmodified protein, indicated by a shift in band size<sup>55</sup>. Nevertheless, the occurrence of an unspecific band cannot be excluded so far and the blots should be repeated in addition with lysates from the original non-passaged cells.

## 4.2 Validation of a *STAT3* KO in HEK 293T cells

To understand the role of STAT3 isoforms in AML cell lines we aimed at using an approach based on CRISPR/Cas9. Inducing a KO of *STAT3* in human AML cell lines could help with investigation of the role of STAT3 in a cell type specific manner. The CRISPR technology itself is described as an efficient process, due to the Watson-Crick base pairing between the guide RNA and the target DNA, and a cost-effective method for gene disruption when compared to other genome editing mechanism such as ZFN or TALENs<sup>42</sup>. Prior to performing a *STAT3* KO in AML cells, the cloned CRISPR plasmids were first validated in HEK 293T cells to see if the selected guide RNAs in combination with the chosen backbone were able to induce a KO on genomic, transcriptional and translational level. We selected four guide RNAs which had the highest Zhang score, reflecting the off-target activity of the proposed guide RNAs, and were located in the first exons of *STAT3*, namely exon 7, 8 and 9. Therefore, disruption of the gene was supposed to lead to an early stop codon and impede translation of STAT3. After transfection of HEK 293T cells with CRISPR/Cas9-sgRNA-plasmids, cell pellets were harvested either directly for RT-qPCR analysis or after an additional round of selection with puromycin to compare resulting *STAT3* expression levels. Overall, levels of both isoforms were reduced with all sgRNAs, suggesting that the KO was induced with all plasmids. However, efficiency in downregulation varied between the guide RNAs. Sg#2 and sg#4 led to the highest reduction in *STAT3* $\alpha$  and *STAT3* $\beta$  when compared to the others. In contrast, sg#1 even showed an increase in *STAT3* levels after puromycin, indicating that cells were transfected with the plasmid which was providing resistance against the selection antibiotics but did not sufficiently induce KO. These cells could even have a growth advantage against other transduced *STAT3* KO clones in the same setup, which looked like a *STAT3* rescue when analysing the bulk culture. On protein level, sufficient

*STAT3* KO was visible with all guide RNAs in comparison to a guide RNA targeting *Renilla*. Bands of both isoforms detected with the antibody against total *STAT3* were faint when compared to the control, with equal band signal in the loading controls. This indicated, that the CRISPR plasmids were able to induce a *STAT3* KO. Further on, PI staining of the transduced HEK 293T cells was assayed after puromycin selection in an attempt to analyse if the *STAT3* KO has an effect on the cell cycle status. The cells treated with the CRISPR plasmids, whether carrying the guide RNAs targeting *STAT3* or *Renilla*, showed all similar distributions of cells. Most cells were detected in sub G1 or G0/G1 phase, which in sum represented similar values found in untreated (wild-type) HEK 293T cells. The high levels of sub G1 cells can be explained by the earlier selection with puromycin which causes cell death in those cells that do not have a resistance against the antibiotic. The amount of M and S phase cells was comparable between all setups, indicating that the *STAT3* KO does not alter the cell cycle status of these cells. For further information, also the growth behaviour could be assayed to observe possible changes in proliferation in *STAT3* KO cells.

Verifying the KO also on the genomic level, the overall indel frequencies caused by the CRISPR-induced DSB was investigated by TIDE analysis which allowed tracking of single mutations in a bulk culture. Usually for detection of small sequence changes introduced either by programmable nucleases or CRISPR/Cas, enzymatic assays such as the Surveyor or T7 endonuclease I cleavage method are used. These methods are able to cut heteroduplex DNA resulting from sequence mismatches<sup>56</sup>. However, both techniques show a high signal-to-noise ratio and are not able to provide information about the type of mutations introduced. This information nonetheless is important, since only out of frame mutations sufficiently lead to gene disruption. In-frame mutations could lead to addition or removal of base triplets and there is a probability that instead of gene KO, a modified protein is produced. So far, only PCR fragments for sg#1 and sg#2 could be analysed since fragments for the other guide RNAs only showed wild-type sequences. Since all samples treated with the sgRNAs exhibited a reduction in *STAT3* on mRNA as well as protein level, it can be concluded that all sgRNA did induce a CRISPR-based gene disruption. Therefore, the occurrence of only wild-type sequences is most likely a result from either insufficient amplification or sequencing. Biased PCR amplification, in which low frequencies of mutated sequences in a wild-type pool are not expanded with the same efficiency as the remaining sequences, could result in a predominance of the wild-type sequence during sequencing<sup>57</sup>.

Since secondary structures and G-C contents of the templates are of importance here, additional primers with different annealing temperatures could be tested. Nevertheless, sg#1 and sg#2 both showed insertion and deletion of basepairs in the extracted genomic DNA. The sgRNA #2 even showed almost 50 % of targeted sequences in the bulk culture with mainly out of frame mutations. In contrast, the guide RNA #1 for *STAT3* resulted in lower efficiencies which is in accord with the results obtained from the RT-qPCR. In brief, comparing the results from all guide RNAs, sg#2 would be the best choice for *STAT3* knock-out mediated by CRISPR/Cas9 in cell lines.

#### 4.3 AML cells show high variance in transfection efficiency nucleofected with pMAX-GFP

After successful validation of the CRISPR-plasmids targeting *STAT3*, they were next introduced into AML target cell lines. AML cells are in general described as resistant to most non-viral transfection methods except for nucleofection, a technique based on electroporation<sup>58</sup>. For manipulation with the Amaxa Nucleofector™, several programmes are available depending on the distinct cell line. Unfortunately, since it is a proprietary system, the programmes are coded, which does not allow reconstruction of the used voltage and time applied for electroporation. Here, several programmes were tested, either published by different research groups or suggested by the company directly, in combination with the pMAX-GFP plasmid to identify the settings resulting in a high percentage of viable and GFP positive cells after nucleofection. The distinction between viable and apoptotic cells was on the one hand made by dividing cells according to their size and granularity and on the other hand with a viability exclusion staining based on 7-AAD, which can only intercalate with DNA when the cell membrane is permeable. Two cell lines were strongly impaired in viability after nucleofection, namely MOLM-13 and HEL, whereas the other cell lines seemed to tolerate the procedure. Nevertheless, GFP positive cell populations varied among all cell lines and tested programmes. The highest transfection efficiency was observed in NOMO-1 cells with 34.6 % GFP positive and around 70 % viable cells. For the following experiments, the three best cell lines were chosen with their respective programmes, namely NOMO-1, HEL and NB-4, since neither GFP values nor viability levels were sufficient for the other cell lines. Though the cell line HEL showed decreased viability after nucleofection, it was chosen for

testing because it still had a larger population of GFP positive cells compared to MONO-MAC-6, which showed more viable cells but only 5 % of these were transfected. One deviation from the protocol was the in-house prepared nucleofection buffer which was not purchased by the manufacturer, as recommended in their protocols, which could lead to low efficiencies as a result. Still, the transfection efficiencies achieved with the programmes used were comparable to reported efficiencies of not more than 30 to 50 % depending on the respective cell line<sup>58,59</sup>.

To finally introduce the CRISPR plasmids, cells were transfected with the same protocol as used for the pMAX-GFP setup with the exception that puromycin was added 24 hours after nucleofection to select for successfully transfected cells. For each cell line, samples were included that were only electroporated without addition of plasmid DNA to determine the level of cell damage caused by the method itself. The amount of apoptotic cells detected after nucleofection with the sgRNAs against *STAT3* were significantly higher than expected. However, the percentage of viable cells in the samples transduced with the sg*Renilla* plasmids were similarly low, suggesting that the nucleofection efficiencies with the pSpCas9-sgRNA-Puro backbone was too low rather than an effect induced by KO of *STAT3*. So far, we were not able to test if the poor viability rates were caused by poor transfection efficiencies or low levels of transcribed puromycin *N*-acetyl-transferase (PAC), which is conferring the resistance against puromycin. Currently, an improved version of the pSpCas9 vector is available which is described to provide a better resistance against puromycin (#62988, Addgene). In a subsequent experiment, the cells should be transduced without the addition of puromycin and KO of *STAT3* could be analysed in the same way as in the HEK 293T validation to determine whether an impaired puromycin resistance or the KO of *STAT3* causes cell death.

An additional approach to succeed in establishing stable KO cell lines, would be to electroporate ribonucleocomplexes composed of a recombinant Cas9 in combination with the desired sgRNA attached to a tracrRNA labelled with a fluorescent dye, to allow sorting of successfully transduced cells. This method has the advantage that a functional CRISPR/Cas9 complex is directly introduced into cells without prior transcriptional and translational steps and the transfection efficiency is higher when compared to nucleofection with plasmid DNA alone<sup>60</sup>.

#### 4.4 Lentiviral infection of NB-4 cells with a GFP plasmid results in viable cells but not with plasmids coding for *STAT3* isoforms

Transfection of AML cell lines can be more or less challenging depending on the available method, as described in the previous chapter. Lentiviruses can infect both dividing and non-dividing cells and additionally allow persistent gene expression by insertion of packaged plasmids into the host genome. Therefore, overexpression in AML cell lines was attempted with a lentiviral system that enable the generation of stable cell lines expressing a gene of interest. Plasmids for overexpression of *STAT3 $\alpha$*  or *STAT3 $\beta$*  were assembled by transferring the respective coding sequences in the pLenti6/V5-DEST vector which can be used for lentiviral transduction. First infections with undiluted virus resulted in high toxicity and cell death (data not shown). Thus, virus supernatant was used in four different dilutions (1:50, 1:100, 1:1,000, 1:10,000). Cells were monitored by flow cytometry for GFP and 7-AAD for five days after infection. Selection of transfected cells was started 24 hours after infection via the addition of puromycin. As expected, cells were highly viable after 24 hours of infection and these numbers significantly dropped upon the addition of puromycin. Cell numbers in the dilutions 1:50 and 1:100 recovered by day five and regained around 40 % viable cells. Higher dilutions of 1:1,000 and 1:10,000 did not allow the recovery of the cell population due to insufficient infection efficiencies. The surviving cells were all positive for GFP, indicating that the transduction with the control plasmid was highly efficient. In addition, cells detected in the gate assigned to the apoptotic cell compartment were all negative for GFP, demonstrating that these cells represented the not infected cell population which was targeted and eliminated by puromycin selection.

In a subsequent step, the proposed virus dilutions were tested in NB-4 cells for the experimental *STAT3* plasmids as well as the empty parental plasmid pLenti6/V5-DEST to determine any toxicity caused by *STAT3* overexpression. Cells were selected with the antibiotic, in this case with blasticidin, starting from 24 hours after infection and evaluated for viability with 7-AAD staining at day five, since blasticidin takes longer to induce cell death in not transfected cells. In comparison to the GFP control vector, cell viability after 24 hours was significantly reduced in cells infected with the pLenti6/V5 vectors, either the empty one or the ones containing the *STAT3* isoforms. Cells infected with plasmids encoding for *STAT3 $\alpha$*  or *STAT3 $\beta$*  failed to recover from selection even after removal of the antibiotic. In contrast, the empty parental vector was able to recover cell numbers after day seven (data

not shown). Still, from this data it cannot be concluded that overexpression of one of the two *STAT3* isoforms results in cell death. The gene of interest in this experiment is under the control of a strong CMV promoter and accumulation of overexpressed protein in the cells could be causative for apoptosis. Additionally, the cells could tolerate the GFP control plasmid better because GFP is a smaller protein of 238 aa when compared to the larger *STAT3* proteins, composed of up to 770 aa. Follow-up experiments should address this problem by examining a vector where *STAT3* expression is under control of a different promoter, e.g. the human elongation factor 1 $\alpha$  promoter (EF1A), which is used in the GFP plasmid. An additional consideration would be to use more concentrated virus supernatant for *STAT3* expression as the steep drop in viability after addition of the antibiotics could also indicate that the larger plasmids encoding for *STAT3* are less efficiently packed into viral particles.

#### 4.5 Viability is reduced in all AML cells transduced with *STAT3* plasmids

Previous experiments with NB-4 cells showed a distinct phenotype for cells infected with plasmids conferring overexpression of *STAT3* isoforms. Additional infection experiments were conducted to test if this is specific for NB-4 cells or visible in all AML cell lines. Cells were again infected with either pLenti-h*STAT3* $\alpha$ -Blasticidin, pLenti-h*STAT3* $\beta$ -Blasticidin or pLenti-GFP-Puro, selected for transduced clones with the corresponding antibiotics and evaluated for viable cells and GFP positive populations 24 hours and four days after infection. All six cell lines, except for NOMO-1 cells, showed high levels of viability after infection with the control plasmid coding for GFP which dropped as anticipated after addition of the selection antibiotics. Cells recovered until day four and started to increase in numbers when observed for one additional week (data not shown). Viable cells were all transduced, indicated by almost 100 % GFP positive cells. The NOMO-1 cell line did not tolerate the viral infection for what reason is not known so far. Repetition of this experiment is needed to analyse if this was a problem with the specific experiment or a general characteristic of this cell line. In addition, other virus dilutions could be tested for NOMO-1 to find the best ratio of virus particles and media for successful infection. Similar to what was observed before in the NB-4 cell line, the remaining AML cells transduced with plasmids encoding *STAT3* $\alpha$  or *STAT3* $\beta$  showed only a few viable cells at day four after infection which were all dead by day seven (data not shown). As discussed in the section above, the problem could have arisen

from the CMV promoter of the pLenti6/V5 vector. An additional method to rule out if the overexpression of STAT3 is causative for the apoptotic phenotype in the AML cells would be to try an inducible Tet-on system where the expression of *STAT3* isoforms is under control of a tetracycline response element (TRE). This would allow investigation of what induces toxicity in AML cell lines, the viral infection by itself or the overexpression of the *STAT3* isoforms. Furthermore, so far it could not be shown that an overexpression is induced with the *STAT3* plasmids as cells could not be stably selected. However, one could try to harvest the cell pellets for either Western blotting or RT-qPCR at different time points after infection when cells are still viable though the increase in *STAT3* isoforms could be only minor. Additionally, primers for RT-qPCR analysis need to distinguish between exogenous and endogenous mRNA for *STAT3* as the cells still have their genomic copy of *STAT3*. For this, the V5-tag encoded on the pLenti-*hSTAT3*-Blasticidin could be utilised which is not translated because of a stop codon in the exogenous *STAT3* sequence but should be transcribed since the termination sequence and poly-A-signals are located after the tag sequence.

#### 4.6 Conclusion and Outlook

In this thesis project, establishment of stable *STAT3* KO and overexpression of *STAT3* isoforms in human AML cell lines was attempted. So far, we were able to validate the efficient KO of *STAT3* in HEK 293T cells which did not alter the cell cycle status of the cells though a potential change in proliferative capacity still needs to be analysed. The transfection of AML cells with the CRISPR/Cas9 plasmids conveying the *STAT3* KO was so far not successful with nucleofection though it is not yet clear why and needs further investigation. Additionally, attempts to transduce AML cells lentivirally with plasmids encoding for *STAT3α* and *STAT3β* turned out to significantly reduce the viability of these cells when compared to plasmids encoding GFP or the parental pLenti6/V5-DEST vector. So far however, it cannot be excluded that the composition of the expression plasmids themselves used for overexpression of the *STAT3* isoforms is responsible for the drop in viable cells rather than an overexpression of the genes of interest since successful overexpression could not yet be demonstrated.

Further experiments should address and investigate the problem of reduced viability when AML cell lines are transduced with either the CRISPR or the lentiviral plasmids.

## 5. SUMMARIES

### 5.1 Summary

Signal Transducer and Activator of Transcription 3 (STAT3) is downstream of pathways activated by several different stimuli such as growth factors, cytokines such as IL-6, hormones or ligands binding to Toll-like receptors. In its role as transcription factor, STAT3 not only regulates target genes involved in development and differentiation of various tissues and cell types, but also plays a role during inflammation and disease progression. STAT3 occurs in two alternatively spliced variants, the full-length transcript STAT3 $\alpha$  (770 amino acids (aa)) and a truncated version of 720aa, STAT3 $\beta$ , which is missing the transactivation domain at the C-terminus and contains seven different amino acids instead. In the context of cancer, STAT3 was shown to be abnormally expressed and depending on the cellular context can play a role either as oncogene or tumour-suppressor. Preliminary data from our research group indicate that the STAT3 $\beta$  isoform has a tumour suppressive function in acute myeloid leukaemia (AML).

This master thesis project focused on the establishment of AML cell lines that either obtain a disruption in the *STAT3* gene by CRISPR/Cas9 or are overexpressing one of the two STAT3 isoforms, namely STAT3 $\alpha$  or STAT3 $\beta$ . Six AML cell lines were selected and analysed for their basic expression of the *STAT3* isoforms on transcriptional and translational level as well as their growth rates. Prior to the transfection of AML cell lines, the newly cloned CRISPR/Cas9-sgRNA plasmids were successfully validated for their efficiency for *STAT3* gene disruption in HEK 293T cells, demonstrating the KO on the mRNA, protein and genomic level. When AML cell lines were nucleofected with the verified plasmids they showed a rapid drop in viability under selection with antibiotics.

In a second approach, AML cell lines were infected with lentivirus containing plasmids carrying either *STAT3 $\alpha$*  or *STAT3 $\beta$*  to induce overexpression. AML cells transduced with a GFP control plasmid showed high efficiencies of lentiviral infection indicated by a predominant GFP positive cell population. In contrast, cells infected with the *STAT3* plasmids exhibited a drop in viability after addition of the selection medium containing antibiotics, suggesting that neither the infection protocol nor the plasmids supported efficient infection.

So far we were not able to create stable cell lines suitable for studying the role of STAT3 in acute myeloid leukaemia. The first steps for generating these cell lines have been done but additional experiments are necessary to address the problem of reduced viability when AML cells are nucleofected or lentivirally infected with the *STAT3* sgRNA or *STAT3* overexpression plasmids.

## 5.2 Zusammenfassung

„Signal Transducer and Activator of Transcription 3“, kurz STAT3, wird von verschiedensten Signalwegen mit unterschiedlichen Stimuli aktiviert, wie zum Beispiel Wachstumsfaktoren, Zytokinen wie IL-6, Hormonen oder Liganden die an Toll-like Rezeptoren binden. In seiner Rolle als Transkriptionsfaktor reguliert STAT3 nicht nur Zielgene die in der Entwicklung und Differenzierung verschiedenster Gewebe und Zelltypen eine Rolle spielen, er ist auch involviert in Entzündungsvorgänge und Krankheitsgeschehen. STAT3 kommt in zwei alternativen Zuständen vor, einmal in ganzer Transkriptlänge von 770 Aminosäuren (AS) und einer verkürzten Variante von 720 AS, STAT3 $\beta$ , welcher die Transaktivierungsdomäne am C-Terminus fehlt und statt dessen einen Anhang von sieben AS vorweist. Bezogen auf die Rolle die STAT3 in der Krebsentstehung spielt wurde gezeigt, dass es hierbei abnormal exprimiert wird und abhängig vom zellulären Kontext, entweder eine Rolle als Onkogen oder Tumorsuppressor einnimmt. Vorläufige Ergebnisse unserer Forschungsgruppe weisen darauf hin, dass die STAT3 $\beta$  Isoform eine Funktion als Tumorsuppressor in akuter myeloischer Leukämie (AML) hat.

Das vorliegende Masterarbeitsprojekt konzentriert sich auf die Etablierung von AML Zelllinien, die entweder eine Störung in *STAT3* vorweisen, verursacht durch CRISPR/Cas9, oder eine Überexprimierung einer der zwei *STAT3* Isoformen, STAT3 $\alpha$  oder STAT3 $\beta$ . Sechs ausgesuchte AML Zelllinien wurden auf ihre basale Expression der *STAT3* Isoformen auf transkriptioneller und translationeller Ebene hin untersucht, sowie ihres Wachstumsverhaltens. Vor Transfektion der AML Zelllinien wurden die neu geklonten CRISPR/Cas9-sgRNA Plasmide erfolgreich auf ihre Effizienz hin validiert, einen *STAT3* knock-out in HEK 293T Zellen zu induzieren. Der Knock-out wurde sowohl auf mRNA, Protein und genomischer Ebene gezeigt. Nukleofeziente AML Zellen mit den verifizierten

Plasmiden zeigten aber rapide Einbrüche im Überleben der Zellen sobald die Selektion mit Antibiotika begonnen wurde.

In einem zweiten Versuch wurden die AML Zelllinien mit Lentiviren infiziert die Plasmide enthielten, die entweder eine Überexpression von *STAT3α* oder *STAT3β* induzieren können. AML Zellen, die mit einem Kontrollplasmid für GFP transduziert wurden zeigten eine hohe Effizienz der lentiviralen Infektion, gezeigt durch eine vorherrschende GFP positive Zellpopulation. Im Gegensatz dazu zeigten Zellen, die mit den *STAT3* Plasmiden infiziert wurden eine Verminderung in lebenden Zellen nachdem das Selektionsmedium hinzu gegeben wurde. Dies weist daraufhin, dass weder das Protokoll für die Infektion noch die gewählten Plasmide effiziente Infektion unterstützen.

Zum momentanen Zeitpunkt war es nicht möglich stabile Zelllinien zu generieren, um die Rolle von STAT3 in akuter myeloischer Leukämie zu studieren. Die ersten Schritte für die Etablierung der Zelllinien sind gemacht, jedoch bedarf es zusätzlicher Experimente um abzuklären, warum sowohl mit sgRNAs gegen *STAT3* nukleofizierte als auch die lentivirale Infektion mit *STAT3* überexprimierenden Plasmiden in AML Zellen zu vermindertem Überleben führt.

## 6. LIST OF MATERIALS AND REAGENTS

**7-AAD Viability Staining Solution** (Biolegend), 420403

**Acrylamide/Bis-acrylamide, 30 % solution** (Sigma-Aldrich), A3574

**Agarose** (Sigma-Aldrich), A9539

**Ammonium persulfate, APS** (10 %, Sigma-Aldrich), A3678

**Ampicillin sodium salt** (Sigma-Aldrich), A9518

### **Antibodies for Western Blotting**

Anti-STAT3, D3Z2G Rabbit mAb (Cell Signaling Technology®), 12640

Anti-Phospho-STAT3, Tyr705 Rabbit mAb (Cell Signaling Technology®), 9131

Anti-HSC70, B-6 Mouse mAb (Santa Cruz Biotechnology), sc-7298

Anti-alpha-Tubulin, DM1A Mouse mAb (Abcam), ab7291

Anti-mouse IgG, HRP-linked (GE Healthcare Life Sciences), NA931V

Anti-rabbit IgG, HRP-linked (Cell Signaling Technology®), 7074

**Blasticidin S Hydrochlorid** (Carl-Roth), CP14.1

**Bovine Serum Albumin Fraction V, BSA pH 7.0** (Applichem), A1391

**Bpil** (10 U/μl, ThermoFisher Scientific), ER1011

**Bromphenol blue** (Sigma-Aldrich), B0126

**BsrGI** (10,000 U/ml, NEB), R0575S

NEB Buffer™ 2.1, 10x

Gel loading dye, 6x

**Calcium chloride dihydrate, CaCl<sub>2</sub>** (Sigma-Aldrich), C5080

**CASYton** (OLS Omni Life Science), 5651808

**cOmplete™ Protease Inhibitor Cocktail, 25x** (Sigma-Aldrich), 11697498001

**DNeasy Blood and Tissue Kit** (Qiagen), 69504

DNeasy Mini Spin Columns (colourless) in 2 ml Collection Tubes

Collection Tubes (2 ml)

Buffer ATL

Buffer AL

Buffer AW1 (concentrate)

Buffer AW2 (concentrate)

Buffer AE

Proteinase K

**dNTP Set** (100 mM, ThermoFisher Scientific), R0181

**Dulbecco's Modified Eagle Medium, DMEM** (Gibco®, ThermoFisher Scientific), 11960058

**Ethylenediaminetetraacetic acid, EDTA** (Applichem), A1104

**Ethanol Absolut 99.9 %** (AustrAlco), AX0X00

**Fetal Bovine Serum** (Gibco®, ThermoFisher Scientific), 10082139

**FxCycle™ PI/RNase Staining Solution** (ThermoFisher Scientific), F10797

**Gateway™ LR Clonase™ II Enzyme mix** (ThermoFisher Scientific), 11791100

Gateway™ LR Clonase™ II Enzyme Mix

Proteinase K Solution (2 µg/µL)

pENTR™-gus Positive Control (50 ng/µL)

**L-Glutamine** (200mM, Gibco®, ThermoFisher Scientific), 25030081

**Glycin** (Carl Roth), 3187.3

**Glycerol** (Sigma-Aldrich), G5516

**GoTaq® DNA Polymerase** (5 U/µl, Promega), M3005

5x Green GoTaq® Buffer

**GoTaq® qPCR Master Mix for Dye-based Detection** (Promega), A6001

GoTaq® qPCR Master Mix

Nuclease-Free Water

**4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES** (Sigma-Aldrich), H4034

**HEPES-buffered Saline, HBS 2x pH 7.08**

280 mM NaCl

50 mM HEPES

1.5 mM Na<sub>2</sub>HPO<sub>4</sub>

**Interleukine 6, recombinant human** (2 µg/ml, Peprotech), 200-06

**Lysogeny broth (LB)** (Sigma-Aldrich), L3022

**LB with agar** (Sigma-Aldrich), L7025

**Magnesium chloride, MgCl<sub>2</sub>** (Fluka), 63063

**D-Mannitol** (Sigma-Aldrich), M4125

**2-Mercaptoethanol** (Sigma-Aldrich), M7522

**Methanol** (Carl-Roth), 4627.2

**Nucleofection Buffer, 1M**

5 mM KCl

15 mM MgCl<sub>2</sub>

120 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.2

50 mM Mannitol

**peqGREEN** (Peqlab), 732-3196

**Penicillin-Streptomycin** (10,000 U/ml, Gibco®, ThermoFisher Scientific), 15140122

**Phenylmethylsulfonyl fluoride, PMSF** (Sigma-Aldrich), P7626

**Phosphate-buffered saline, PBS 10x** (pH 7.4, Gibco®, ThermoFisher Scientific), 10010023

**Pierce™ Coomassie Plus (Bradford) Assay Kit** (ThermoFisher Scientific), 23236

**Polybrene Transfection Reagent** (Merck), TR-1003-G

**Potassium chloride, KCl** (Sigma-Aldrich), 60128

**Primer** (Eurofins MWG Operon GmbH), custom made

stock solution: 100 µM (100 pmol/µl)

working solution: 10 µM

**Puromycin** (Sigma-Aldrich), P8833

**PureYield™ Plasmid Miniprep System** (Promega), A1221

Cell Lysis Buffer (CLC)

Neutralization Solution (NSC)

Endotoxin Removal Wash (ERB)

Column Wash Solution (CWC)

Elution Buffer (EBB)

PureYield™ Minicolumns

PureYield™ Collection Tubes

**Resazurin sodium salt** (Sigma-Aldrich), R7017

**RevertAid H Minus First Strand cDNA Synthesis Kit** (ThermoFisher Scientific), K1631

RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/μL)

RiboLock RNase Inhibitor (20 U/μL) 25 μL 120 μL

5x Reaction Buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl<sub>2</sub>, 50 mM DTT)

10mM dNTP Mix

Random Hexamer Primer (100 μM, 0.2 μg/μL (6 A<sub>260</sub> U/mL))

Water, nuclease-free

**RIPA Buffer, 10x** (NEB), 9806

**RNeasy Mini Kit** (Qiagen), 74104

RNeasy Mini Spin Columns (pink)

Collection Tubes (1.5 ml)

Collection Tubes (2 ml)

Buffer RLT

Buffer RW1

Buffer RPE (concentrate)

RNase-Free Water

**Roti®-Stock 20 % SDS** (Carl Roth), 1057.1

**RPMI Media 1640** (Gibco®, ThermoFisher Scientific), 11544446

**Skim milk powder** (Sigma-Aldrich), 1153630500

**Sodium chloride, NaCl** (Sigma-Aldrich), S9625

**Sodium dodecyl sulfate, SDS** (Sigma-Aldrich), L4509

**Sodium fluoride, NaF** (Sigma-Aldrich), 30105

**Sodium orthovanadate, Na<sub>3</sub>VO<sub>4</sub>** (Sigma-Aldrich), S6508

**Sodium phosphate dibasic dihydrate,  $\text{Na}_2\text{HPO}_4$**  (Sigma-Aldrich), 30435

**Sodium phosphate monobasic monohydrate,  $\text{NaH}_2\text{PO}_4$**  (Sigma-Aldrich), 71504

**Sodium pyruvate** (100 mM, Gibco®, ThermoFisher Scientific), 11360070

**SuperSignal™ West Femto Maximum Sensitivity Substrate** (ThermoFisher Scientific), 34095

**N,N,N',N'-Tetramethyl ethylenediamine, TEMED** (Merck), 110732

**T4 DNA Ligase** (5 U/μl, ThermoFisher Scientific), EL0011

**T4 DNA Ligase Buffer, 10x** (ThermoFisher Scientific), B69

**T4 Polynucleotide Kinase** (10 U/l, ThermoFisher Scientific), EK0031

**Tris-(hydroxymethyl)-aminomethan, TRIS** (Carl Roth), 5429

**Tris-buffered saline/Glycin, TBS/Glycin 10x**

25 mM TRIS

190 mM Glycin

**Tris-buffered saline/Glycin/SDS, TBS/Glycin/SDS 10x**

25 mM TRIS

190 mM Glycin

0.1 % SDS

**Tris/HCl**

1 M TRIS adjusted with HCl to pH 6.8 or 8.8

**Trypsin-EDTA** (0.25 %, Gibco®, ThermoFisher Scientific), 25200056

**TWEEN® 20** (Sigma-Aldrich), P9416

**Wizard® SV Gel and PCR Clean-Up System** (Promega), A9281

Wizard® SV Minicolumns

Collection Tubes

Membrane Wash Solution

Membrane Binding Solution

Nuclease-Free Water

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## 8. REFERENCES

1. Begemann M. *Praktische Hämatologie*. 11th ed. Georg Thieme Verlag; 1999.
2. Institute NC. Cancer Stat Facts: Acute Myeloid Leukemia (AML). <https://seer.cancer.gov/statfacts/html/amyl.html>. Accessed July 23, 2017.
3. Döhner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*. 2010;115(3):453-474. doi:10.1182/blood-2009-07-235358.
4. Bennett JM, Catovsky D, Daniel M-TT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976;33(4):451-458. doi:10.1111/j.1365-2141.1976.tb03563.x.
5. Hayhoe FG. Classification of acute leukaemias. *Blood Rev*. 1988;2(3):186-193. <http://www.ncbi.nlm.nih.gov/pubmed/23325837>.
6. Harris NL, Jaffe ES, Diebold J, et al. The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997. *J Clin Oncol*. 1999;17(12):3835-3849. <http://www.ncbi.nlm.nih.gov/pubmed/10643532>.
7. Arber DA. Realistic pathologic classification of acute myeloid leukemias. *Am J Clin Pathol*. 2001;115(4):552-560. doi:10.1309/K2PH-L2U7-722B-B1YR.
8. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391-2406. doi:10.1182/blood-2016-03-643544.The.
9. Amin HM, Yang Y, Shen Y, et al. Having a higher blast percentage in circulation than bone marrow: clinical implications in myelodysplastic syndrome and acute lymphoid and myeloid leukemias. *Leukemia*. 2005;19(9):1567-1572. doi:10.1038/sj.leu.2403876.
10. Levy DE, Darnell JE. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol*. 2002;3(9):651-662. doi:10.1038/nrm909.
11. Schindler C, Shuai K, Prezioso VR, Darnell JE. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science (80- )*. 1992;257(5071):809-813. <http://www.ncbi.nlm.nih.gov/pubmed/1496401>.
12. Mali SB. Review of STAT3 (Signal Transducers and Activators of Transcription) in head and neck cancer. *Oral Oncol*. 2015;51(6):565-569. doi:10.1016/j.oraloncology.2015.03.004.

13. Sgrignani J, Olsson S, Ekonomiuk D, et al. Molecular Determinants for Unphosphorylated STAT3 Dimerization Determined by Integrative Modeling. *Biochemistry*. 2015;54(35):5489-5501. doi:10.1021/bi501529x.
14. Silva CM. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. *Oncogene*. 2004;23(48):8017-8023. doi:10.1038/sj.onc.1208159.
15. Becker S, Groner B, Muller CW. Three-dimensional structure of the Stat3beta homodimer bound to DNA. *Nature*. 1998;394(6689):145-151. doi:10.1038/28101.
16. Decker T, Kovarik P. Serine phosphorylation of STATs. *Oncogene*. 2000;19(21):2628-2637. doi:10.1038/sj.onc.1203481.
17. Wen Z, Zhong Z, Darnell JE. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell*. 1995;82(2):241-250. doi:10.1016/0092-8674(95)90311-9.
18. Shao H, Quintero AJ, Tweardy DJ. Identification and characterization of cis elements in the STAT3 gene regulating STAT3 alpha and STAT3 beta messenger RNA splicing. *Blood*. 2001;98(13):3853-3856. <http://www.ncbi.nlm.nih.gov/pubmed/11739197>. Accessed July 27, 2017.
19. Dewilde S, Vercelli A, Chiarle R, Poli V. Of alphas and betas: distinct and overlapping functions of STAT3 isoforms. *Front Biosci*. 2008;13(6):6501-6514. <http://www.ncbi.nlm.nih.gov/pubmed/18508676>.
20. Hevehan DL, Miller WM, Papoutsakis ET. Differential expression and phosphorylation of distinct STAT3 proteins during granulocytic differentiation. *Blood*. 2002;99(5):1627-1637.
21. Chakraborty A, Tweardyabc DJ. Stat3 and G-CSF-Induced Myeloid Differentiation. *Leuk Lymphoma*. 1998;30(5-6):433-442. doi:10.3109/10428199809057555.
22. Raz R, Durbin JE, Levy DE. Acute phase response factor and additional members of the interferon-stimulated gene factor 3 family integrate diverse signals from cytokines, interferons, and growth factors. *J Biol Chem*. 1994;269(39):24391-24395. <http://www.ncbi.nlm.nih.gov/pubmed/7523373>.
23. Zhong Z, Wen Z, Darnell JE. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*. 1994;264(5155):95-98. <http://www.ncbi.nlm.nih.gov/pubmed/8140422>.
24. Takeda K, Noguchi K, Shi W, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *PNAS*. 1997;94(8):3801-3804. <http://www.ncbi.nlm.nih.gov/pubmed/9108058>.

25. Zhang X, Wrzeszczynska MH, Horvath CM, Darnell JE. Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation. *Mol Cell Biol*. 1999;19(10):7138-7146. doi:10.1128/MCB.19.10.7138.
26. Caldenhoven E, Dijk van TB, Solari R, et al. STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. *J Biol Chem*. 1996;271(21):13221-13227. <http://www.ncbi.nlm.nih.gov/pubmed/8675499>. Accessed July 27, 2017.
27. Ng IH, Ng DCH, Jans DA, Bogoyevitch MA. Selective STAT3- $\alpha$  or - $\beta$  expression reveals spliceform-specific phosphorylation kinetics, nuclear retention and distinct gene expression outcomes. *Biochem J*. 2012;447(1):125-136. doi:10.1042/BJ20120941.
28. Zammarchi F, de Stanchina E, Bournazou E, et al. Antitumorigenic potential of STAT3 alternative splicing modulation. *PNAS*. 2011;108(43):17779-17784. doi:10.1073/pnas.1108482108.
29. Siveen KS, Sikka S, Surana R, et al. Targeting the STAT3 signaling pathway in cancer: role of synthetic and natural inhibitors. *Biochim Biophys Acta*. 2014;1845(2):136-154. doi:10.1016/j.bbcan.2013.12.005.
30. Zhang HF, Lai R. STAT3 in cancer-friend or foe? *Cancers (Basel)*. 2014;6(3):1408-1440. doi:10.3390/cancers6031408.
31. Chen C-L, Cen L, Kohout J, et al. Signal transducer and activator of transcription 3 activation is associated with bladder cancer cell growth and survival. *Mol Cancer*. 2008;7(78):1-12. doi:10.1186/1476-4598-7-78.
32. De La Iglesia N, Konopka G, Puram SV, et al. Identification of a PTEN-regulated STAT3 brain tumor suppressor pathway. *Genes Dev*. 2008;22(4):449-462. doi:10.1101/gad.1606508.
33. Grabner B, Schramek D, Mueller KM, et al. Disruption of STAT3 signalling promotes KRAS-induced lung tumorigenesis. *Nat Commun*. 2015;6:6285. doi:10.1038/ncomms7285.
34. Zhang HF, Chen Y, Wu C, et al. The Opposing Function of STAT3 as an Oncoprotein and Tumor Suppressor Is Dictated by the Expression Status of STAT3 $\beta$  in Esophageal Squamous Cell Carcinoma. *Clin Cancer Res*. 2016;22(3):691-703. doi:10.1158/1078-0432.CCR-15-1253.
35. Marino F, Orecchia V, Regis G, et al. STAT3 $\beta$  controls inflammatory responses and early tumor onset in skin and colon experimental cancer models. *Am J Cancer Res*. 2014;4(5):484-494.

<http://www.ncbi.nlm.nih.gov/pubmed/25232490>%5Cn<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4163613>.

36. Nomdedeu M, Lara-Castillo MC, Etxabe A, et al. Treatment with G-CSF reduces acute myeloid leukemia blast viability in the presence of bone marrow stroma. *Cancer Cell Int*. 2015;15(1):122. doi:10.1186/s12935-015-0272-3.
37. Chakraborty A, White SM, Schaefer TS, Ball ED, Dyer KF, Tweardy DJ. Granulocyte colony-stimulating factor activation of Stat3 alpha and Stat3 beta in immature normal and leukemic human myeloid cells. *Blood*. 1996;88(7):2442-2449. <http://www.ncbi.nlm.nih.gov/pubmed/8839834>.
38. Horvath P, Barrangou R. CRISPR/Cas, the Immune System of Bacteria and Archaea. *Science* (80- ). 2010;327(5962):167-170. doi:10.1126/science.1179555.
39. Deveau H, Garneau JE, Moineau S. CRISPR/Cas System and Its Role in Phage-Bacteria Interactions. *Annu Rev Microbiol*. 2010;64(1):475-493. doi:10.1146/annurev.micro.112408.134123.
40. Makarova KS, Haft DH, Barrangou R, et al. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol*. 2011;9(6):467-477. doi:10.1038/nrmicro2577.
41. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Almendros C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*. 2009;155(3):733-740. doi:10.1099/mic.0.023960-0.
42. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013;8(11):2281-2308. doi:10.1038/nprot.2013.143.Genome.
43. Davis AJ, Chen DJ. DNA double strand break repair via non-homologous end-joining. *Transl Cancer Res*. 2013;2(3):130-143. doi:10.3978/j.issn.2218-676X.2013.04.02.
44. Landy A. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annu Rev Biochem*. 1989;58:913-949. doi:10.1146/annurev.bi.58.070189.004405.
45. Ptashne M. *A Genetic Switch: Phage [Lambda] and Higher Organisms*. 3rd ed. Cell Press; 1992. <https://books.google.at/books?id=baLqFvWJz30C>.
46. Bernard P, Gabant P, Bahassi EM, Couturier M. Positive-selection vectors using the F plasmid ccdB killer gene. *Gene*. 1994;148(1):71-74. <http://www.ncbi.nlm.nih.gov/pubmed/7926841>.
47. Aken BL, Ayling S, Barrell D, et al. The Ensembl gene annotation system. *Database*. 2016;2016:baw093. doi:10.1093/database/baw093.

48. Brinkman EK, Chen T, Amendola M, Steensel van B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* 2014;42(22):e168. doi:10.1093/nar/gku936.
49. Richardson SM, Wheelan SJ, Yarrington RM, Boeke JD. GeneDesign: rapid, automated design of multikilobase synthetic genes. *Genome Res.* 2006;16(4):550-556. doi:10.1101/gr.4431306.
50. Coenen EA, Raimondi SC, Harbott J, et al. Prognostic significance of additional cytogenetic aberrations in 733 de novo pediatric 11q23/MLL-rearranged AML patients: results of an international study. *Blood.* 2011;117(26):7102-7111. doi:10.1182/blood-2010-12-328302.
51. Papayannopoulou T, Nakamoto B, Yokochi T, Chait A, Kannagi R. Human erythroleukemia cell line (HEL) undergoes a drastic macrophage-like shift with TPA. *Blood.* 1983;62(4):832-845. <http://www.ncbi.nlm.nih.gov/pubmed/6309287>.
52. Wang Y, Boxel-Dezaire van AHH, Cheon HJ, Yang J, Stark GR. STAT3 activation in response to IL-6 is prolonged by the binding of IL-6 receptor to EGF receptor. *PNAS.* 2013;110(42):16975-16980. doi:10.1073/pnas.1315862110.
53. Lee H, Zhang P, Herrmann A, et al. Acetylated STAT3 is crucial for methylation of tumor-suppressor gene promoters and inhibition by resveratrol results in demethylation. *PNAS.* 2012;109(20):7765-7769. doi:10.1073/pnas.1205132109.
54. Kim E, Kim M, Woo D-H, et al. Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. *Cancer Cell.* 2013;23(6):839-852. doi:10.1016/j.ccr.2013.04.008.
55. Guan K-L, Yu W, Lin Y, Xiong Y, Zhao S. Generation of acetyllysine antibodies and affinity enrichment of acetylated peptides. *Nat Protoc.* 2010;5(9):1583-1595. doi:10.1038/nprot.2010.117.
56. Vouillot L, Th  lie A, Pollet N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *Genes, Genomes, Genet.* 2015;5(3):407-415. doi:10.1534/g3.114.015834.
57. Polz MF, Cavanaugh CM. Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol.* 1998;64(10):3724-3730. doi:10.1007/s00253-012-4244-4.
58. Schakowski F, Buttgereit P, Mazur M, et al. Novel non-viral method for transfection of primary leukemia cells and cell lines. *Genet Vaccines Ther.* 2004;2(1):1-11. doi:10.1186/1479-0556-2-1.
59. Larsen HO, Roug AS, Nielsen K, S  ndergaard CS, Hokland P. Nonviral transfection of leukemic primary cells and cell lines by siRNA-a direct comparison between

Nucleofection and Accell delivery. *Exp Hematol*. 2011;39(11):1081-1089.  
doi:10.1016/j.exphem.2011.08.003.

60. Mout R, Ray M, Yesilbag Tonga G, et al. Direct Cytosolic Delivery of CRISPR/Cas9-  
Ribonucleoprotein for Efficient Gene Editing. *ACS Nano*. 2017;11(3):2452-2458.  
doi:10.1021/acsnano.6b07600.