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Identification and Characterization of Genetic Aberrations in Acute Childhood Leukemia

Identifikace a charakterizace genetických aberací dětských akutních leukémií

Doctoral thesis

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Prohlášení:

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Klíčová slova: TMD, trisomie 21, GATA1, JAK1, ETV6-ABL1, fúzní geny

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Abstract

Childhood acute leukemias are genetically complex disorders, with recurrent or random aberrations found in most patients. Their proper functional characterization is crucial for understanding the role they play in the process of leukemogenesis. We aimed to identify and characterize the genetic background of two leukemic entities.

The transient myeloproliferative disorder (TMD) is a preleukemic condition that occurs in 10% of newborns with Down syndrome. Trisomy 21 together with in-utero gained mutations in the GATA1 gene are essential in TMD and represent an ideal "multi-hit" model to study leukemogenesis. We investigated an alternative pathogenic mechanism enabling TMD development in a confirmed absence of trisomy 21. Novel deletions in the GATA1 and JAK1 genes were described as potential drivers of this TMD. The deletion D65_C228 in GATA1 results in the expression of an aberrant isoform, which is predicted to lose transactivation potential and, more importantly, to partially lose the ability of recognizing physiological DNA binding sites, possibly triggering TMD alone. Our thorough characterization of JAK1 F636del questions its role in TMD development. Analysis of JAK/STAT signaling suggested decrease of kinase activity upon F636 loss. Cells harboring the aberrant JAK1 did not obtain cytokine-independent growth when assessed in the Ba/F3 assay. Moreover, JAK1 F636del had no impact on cell proliferation and maturation when studied in a "prenatal" environment represented by fetal hematopoietic stem and progenitor cells expressing mutated GATA1. Combined, we described the molecular events in the first case of trisomy 21-independent GATA1-mutated TMD.

The ETV6-ABL1 fusion gene represents a rare recurrent event in acute lymphoblastic leukemia (ALL). We characterized a single chromosomal rearrangement leading to the formation of ETV6-ABL1 together with two novel fusion genes: ABL1-AIF1L and AIF1L-ETV6. The production of three in-frame fusion genes from a single rearrangement is a rare event. Moreover, we report, to the best of our knowledge, the first disruption of the AIF1L gene in leukemias. Chimeric protein analysis in HEK293T cells showed that AIF1L-ETV6 is expressed and localized in the nucleus, where it may bind to DNA via its ETV6 domain. We demonstrated the prenatal origin of the observed rearrangement by detecting the patient-specific ETV6-ABL1 fusion gene breakpoint sequence in the patient's Guthrie card by PCR, therefore

confirming that all three fusion genes are insufficient to cause overt leukemia. Additional "second" hit mutations were required, in this case probably represented by deletions in the IKZF1 and/or CDKN2A/B genes.

Our findings regarding trisomy 21-independent TMD shed new light on the pathogenesis of this intensely investigated leukemia-like condition. Furthermore, our thorough characterization of a unique chromosomal rearrangement resulting in the prenatal production of multiple in-frame fusion genes expands our knowledge regarding ETV6-ABL1-positive ALL.

Abstrakt

Dětské akutní leukémie jsou geneticky komplexní poruchy hematopoézy. U většiny pacientů se vyskytují rekurentní či náhodné aberace, jejichž pečlivá funkční charakterizace je zásadní pro pochopení role, kterou hrají v procesu leukemogeneze. Naším cílem byla identifikace a charakterizace genetického pozadí dvou leukemických entit.

Transientní myeloproliferativní porucha (TMD) je preleukémie, která postihuje 10% novorozenců s Downovým syndromem. Trisomie 21 chromozomu a in-utero získané mutace v genu GATA1 jsou kauzální pro vznik TMD a společně představují ideální "více-zásahový" model leukemogeneze. Zaměřili jsme se na zkoumání alternativního mechanismu vzniku TMD bez účasti trisomie 21. U jedinečného případu TMD jsme jako potenciálně spouštěcí aberace popsali nové delece v genech GATA1 a JAK1. Delece D65_C228 v GATA1 způsobuje tvorbu poškozené izoformy, u níž se předpokládá ztráta transaktivační schopnosti a rovněž částečná ztráta vazby ke specifickým vazebným místům v DNA, což může stačit ke spuštění TMD. Námi provedená podrobná charakterizace delece F636 v JAK1 zpochybňuje význam této aberace pro patogenezi TMD. Analýza signalizace JAK/STAT odhalila pokles kinázové aktivity po ztrátě F636. Růst buněk Ba/F3, které vytvářejí poškozený JAK1, je závislý na cytokinech, podobně jako je tomu u buněk produkujících JAK1 divokého typu. Dále jsme prokázali, že delece F636 v JAK1 neměla žádný dopad na růst a dozrávání buněk v "prenatálním" prostředí, reprezentovaném fetálními hematopoietickými kmenovými a progenitorovými buňkami vytvářejícími mutovaný GATA1. V předkládané práci popisujeme molekulární děje v prvním známém případu TMD s mutovaným genem GATA1 a zároveň nezávislém na trisomii 21 chromozomu.

Fúzní gen ETV6-ABL1 je vzácný, avšak opakovaně se vyskytující genetický jev při akutní lymfoblastické leukémii (ALL). Popsali jsme chromosomální přestavbu vedoucí ke vzniku ETV6-ABL1 a dvou dalších dosud nepopsaných fúzních genů ABL1-AIF1L a AIF1L-ETV6. Vznik tří fúzních genů, se zachovaným čtecím rámcem, z jediné přestavby je v hematologii ojedinělý jev. Dále zde popisujeme první známé poškození genu AIF1L u leukémií. Analýza chimérické bílkoviny v buňkách HEK293T ukázala, že AIF1L-ETV6 je tvořen a umístěn v jádře, kde se pomocí své ETV6 domény může vázat na DNA. Pomocí metody PCR se nám v pacientově Guthrieho kartě podařilo prokázat specifickou zlomovou sekvence fúzního genu

ETV6-ABL1 a tedy prenatální původ studované chromozomální přestavby. Díky tomu jsme následně potvrdili, že ETV6-ABL1 není dostatečný ke spuštění diagnostikovatelné leukémie a musí následovat další druhotné aberace, kterými jsou v tomto případě pravděpodobně delece v genech IKZF1 a/nebo CDKN2A/B.

Naše výsledky zabývající se TMD bez trisomie 21 chromozomu přinášejí nové informace ohledně patogeneze tohoto intenzivně studovaného onemocnění připomínajícího leukémii. Podrobná charakterizace jedinečné chromozomální přestavby, která vyústila v prenatální vznik několika in-frame fúzních genů, prohlubuje naše znalosti o ETV6-ABL1-pozitivních ALL.

My role in the described projects

I have been responsible for the description and functional characterization of novel mutations and fusion genes identified in both of the here described projects. I have actively participated in the planning and in the design of all experiments. Importantly, I have implemented all of the *in vitro* experiments in both cell lines (HEK293T, K562, TF1, NIH3T3, Ba/F3) and in isolated hematopoietic stem cells (adult peripheral blood, murine bone marrow, murine fetal liver) both at the home institute and abroad. I carried out the Western blot assays and all of the experiments regarding fusion gene breakpoint identification together with the subsequent backtracking analysis. Moreover, I wrote and successfully obtained both local (Charles University Grant Agency: 86218) and international (European Hematology Association Research Mobility Grant, European Cooperation In Science And Technology Grant) funding grants for the experiments conducted during my Ph.D. study.

Structure of the thesis

This thesis consists of the following sections: review of literature, aims, methods, results, discussion, conclusions, list of publications and presentations, list of abbreviations, references and the publications that I have contributed to during my Ph.D. study. Two full-text published first-author publications, together with one first-author manuscript under consideration and one co-author manuscript in revision are attached.

Structure of the review of literature

The opening section of the review of literature intends to comprehensively summarize important knowledge regarding childhood acute leukemia. An introduction to both pediatric acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) is presented. Emphasis is given to recurrent genetic alterations in pediatric AML and B-cell precursor ALL.

The transient myeloproliferative disorder is together with a subtype of myeloid leukemia present exclusively in children with Down syndrome. An introduction to these two entities, together with relevant information regarding the GATA and JAK genes families, which are involved in their development, is presented. Moreover, the role of trisomy 21 in the leukemogenic process is introduced in detail.

Review of literature

1. Childhood acute leukemia

Acute leukemia is caused by a series of events that prevent normal maturation of a hematopoietic precursor cell into their progeny. These mutational events on the other hand allow the precursors to uncontrollably proliferate, which finally results in observable clinical symptoms.

Leukemia is the most common type of cancer in children and teenagers and accounts for 30% of all pediatric cancers (Linabery and Ross, 2008; Siegel et al., 2018; Smith et al., 2010; Steliarova-Foucher et al., 2017). Its treatment has become a success story of modern medicine. Virtually an untreatable disease in the 1950s, it is now curable in around 85% of cases (Siegel et al., 2018). This has been attributed to the development of chemotherapeutic agents, proper dosing, precise diagnostics and last but not least to correct supportive care. Despite of these positive developments it still remains the principal cause of death from cancer before 20 years of age (Smith et al., 2010). Heterogeneity presents the main challenge in the assessment of childhood acute leukemias. Leukemia subtypes differ not only in morphology and clinical presentation, but also in response to treatment and prognosis. This is mainly due to a broad spectrum of primary and secondary genetic aberrations (Hunger and Mullighan, 2015b; Iacobucci and Mullighan, 2017). Acute leukemias can be divided into two main subgroups by lineage origin. Acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), with ALL being five times more abundant than AML in pediatric patients (Hunger and Mullighan, 2015a). The incidence of acute leukemias gradually switches during aging, with AML taking lead and finally dominating in older adults (Fig. 1) (Dores et al., 2012; Hein et al., 2020).

The etiology of leukemias remains largely unknown. The generally accepted theory suggests that leukemias originate from a combination of exposition to various risk factors, genetic predisposition and chance. A minority of cases have been linked to chemicals like for example benzene (Savitz and Andrews, 1997) and to exposure to radiation (Greaves, 2006; Preston et al., 1994). The main genetic factor associated with increased risk of leukemia is Down syndrome, followed by rare entities generally predisposing to various types of cancer, namely Ataxia telangiectasia, Fanconi anemia and Li Fraumeni (Stieglitz and Loh, 2013).

Moreover germline variants are believed to be involved in the etiology of about 5% of childhood leukemias (Zhang et al., 2015).

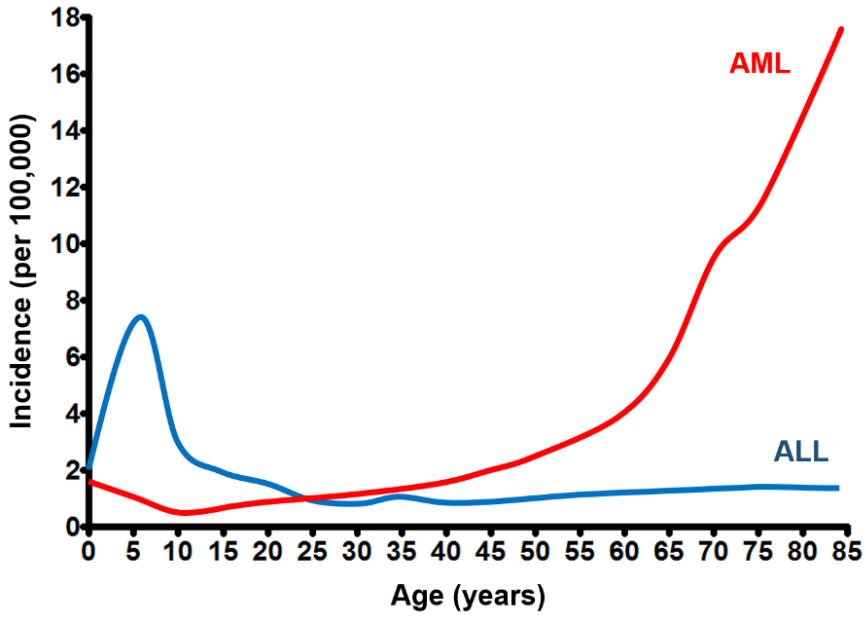


Figure 2: Age-specific incidence rates of AML and ALL (adapted from Hein et al., 2020)

1.1. Genetic aberrations in leukemia

Sequential accumulation of both genetic and epigenetic aberrations represent the main hallmark of cancer. These alterations can be either inherited (germline, constitutional) or acquired (somatic). During the process of leukemogenic transformation, cells may acquire a wide spectrum of mutations, including small insertions and deletions (indels), single nucleotide substitutions (point mutations), as well as a wide array of structural variations, like for example chromosomal translocations (Fig. 2) or more complex rearrangements. These changes of the genetic material of a cancer cell lead to gene expression profile alterations, subsequently affecting cell differentiation, growth and regulation of apoptosis (Bailey et al., 2018; Dawson and Kouzarides, 2012; Mitelman et al., 2007; Stratton et al., 2009). The advent of next generation sequencing technologies has allowed detailed investigation of these genetic events.

The main subtypes of both AML and ALL involve a broad spectrum of genetic alterations, including deletions and point mutations, however, they are mainly characterized

by chromosomal changes such as translocations or hyperdiploidy (Look, 1997; Raimondi et al., 1999). Chromosomal translocations (Fig. 2) involve gene recombination or juxtaposition, which can result in dysregulation of oncogene expression by an association with a constitutively active regulatory element, like for example MYC with the T-cell receptor (TCR) or immunoglobulin heavy chain (IGH) enhancer (Rabbitts, 1994). However, in leukemia, usually an in-frame fusion or chimeric gene is created, resulting in the production of a hybrid protein. This altered protein frequently results in aberrant kinase activity or transcriptional regulation (Look, 1997; Rowley, 1998). A striking feature of leukemic chromosomal translocations is their association with biologically distinct leukemic subtypes. In general, chromosomal translocations in leukemia tend to be balanced or reciprocal and stable. These chromosomal changes usually reflect a stable early occurring single "hit" (Rabbitts, 1994). This distinguishes them from other pediatric malignancies, like for example epithelial carcinomas, commonly harboring unbalanced translocations and numerous deletions (Lengauer, 2001). The principal types of structural chromosomal aberrations are shown in Figure 2.

For a given chromosomal translocation, the genomic regions in which recombination occurs are either clustered or specific and localized. When clustered, they are known as breakpoint cluster regions (BCR) which occur in introns and vary in size, therefore each patient harbors a unique breakpoint in the DNA of a particular leukemic clone (Reiter et al., 2003; Xiao et al., 2001). In lymphoid cancers DNA breaks may occur by aberrant processing of DNA by RAG (recombination activating gene) proteins which coordinate V(D)J recombination (Kuppers and Dalla-Favera, 2001). V(D)J recombination is frequently involved in translocations found in pediatric T-cell precursor ALL, which repeatedly involve the TCR loci (Brown et al., 1990). The more abundant B-cell precursor ALL (BCP-ALL) however only rarely involves V(D)J recombination. In this case, the more common chromosomal translocations harbor dispersed breakpoints, similarly to those found in myeloid leukemias (Reichel et al., 1998; Reiter et al., 2003; van der Reijden et al., 1999; Wiemels et al., 2000; Xiao et al., 2001). Another mechanism involved in translocation and DNA breakpoint formation has been linked with the dysfunction of topoisomerase II, which plays a crucial role during DNA replication (Felix, 1998; Pedersen-Bjergaard and Rowley, 1994; Rowley and Olney, 2002). Treatment-related leukemias have been associated with topoisomerase II dysfunction due to the use of topoisomerase II–targeting drugs (Cowell and Austin, 2012; Felix et al., 2006; Rowley and Olney, 2002).

Interestingly, similar chromosomal translocations as found in therapy-related leukemias are commonly seen in *de novo* infant acute leukemias, like for example those resulting in KMT2A fusions (De Braekeleer et al., 2005; Eguchi et al., 2003; Felix and Lange, 1999; Slater et al., 2002). The possibility of a prenatal origin of a leukemia associated chromosomal translocation originating already *in utero* was first indirectly suggested by a case of monozygotic twins harboring an identical leukemia genotype (Clarkson and Boyse, 1971; Greaves et al., 2003). International studies of concordant twin leukemia cases helped to investigate this hypothesis by studying genomic breakpoints of translocations for KMT2A fusions (Ford et al., 1993; Gill Super et al., 1994; Megonigal et al., 1998). Moreover, archived neonatal blood spots, also known as Guthrie cards, invented by Robert Guthrie in 1963 in order to screen newborns for phenylketonuria, provided valuable material to study non-twinned leukemia patients (Guthrie and Susi, 1963; Wiemels et al., 1999). Gale and colleagues gave the first evidence of the presence of leukemia fusion genes in archived neonatal blood spots (Gale et al., 1997). Subsequently prenatal origin was shown for the ETV6-RUNX1 translocation (Hjalgrim et al., 2002; McHale et al., 2003; Wiemels et al., 1999). Not only fusion genes, but also point mutations specific for leukemias were detected in Guthrie cards. Importantly, mutations in the GATA1 gene in Down syndrome acute megakaryoblastic leukemia (DS-AMKL), in transient myeloproliferative disease (TMD) patients (Ahmed et al., 2004) and in the NOTCH1 gene present in T-ALL patients (Eguchi-Ishimae et al., 2008). The backtracking studies revealed that preleukemic cells harboring early genetic aberrations can persist in the patient's bone marrow for years before acquiring additional necessary hits for leukemic transformation. Most pediatric leukemias therefore very probably originate prenatally through various chromosomal translocations (Bateman et al., 2010; Cazzaniga et al., 2011; Greaves, 1999; Greaves et al., 2003; Hong et al., 2008; Maia et al., 2004; Wiemels et al., 1999). Importantly, various studies indicate that a single chromosomal translocation, representing the so-called "first hit" which initiates a preleukemic state where the hematopoietic precursor is provided with possible proliferative or function advantage, is usually insufficient to cause overt leukemia and additional genetic alterations are required (Fig. 3) (Gonzalez-Herrero et al., 2018; Higuchi et al., 2002; Hong et al., 2008; Ma et al., 2013; Yuan et al., 2001). Twin studies confirmed that these additional alterations, including single nucleotide variants (SNV) and copy number aberrations (CAN), most likely occur postnatally (Bateman et al., 2010; Cazzaniga et al., 2011). Similarly, the vast majority of ongoing V(D)J rearrangements occurring in IGH are

subclonal and differ in twin pairs (Alpar et al., 2015). Common cooperating oncogenic lesions, which reprogram the cell into an autonomously proliferating blast with blocked differentiation and clonal expansion potential, are in ALL represented by alterations in the CDKN2A, CDKN2B, PAX5, RAG1, RAG2, IKZF1 genes (Iacobucci and Mullighan, 2017; Mullighan et al., 2007) and in AML by alterations in the JAK2, FLT3, KIT, NRAS, KRAS, DNMT3A genes (Beghini et al., 2000; Krauth et al., 2014; Schnittger et al., 2002; Shin et al., 2016).

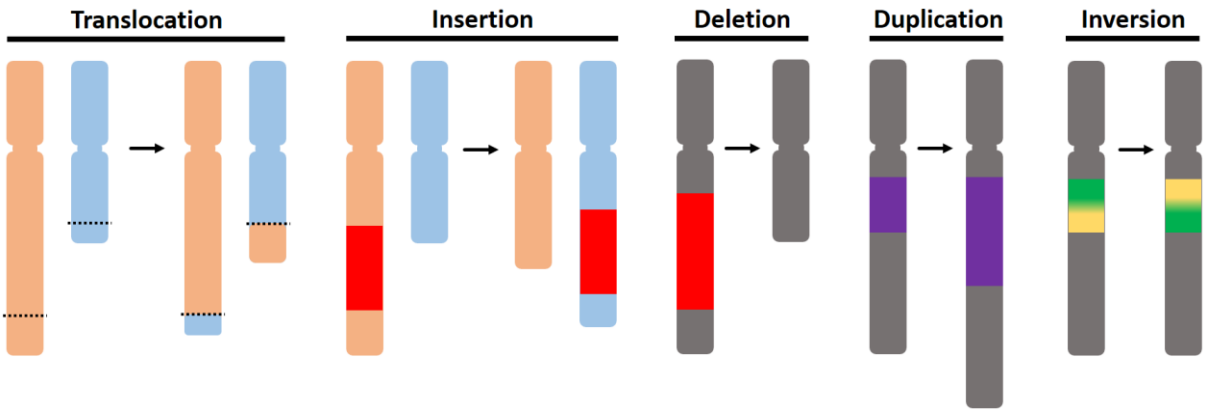


Figure 2: Principal types of structural chromosomal aberrations.

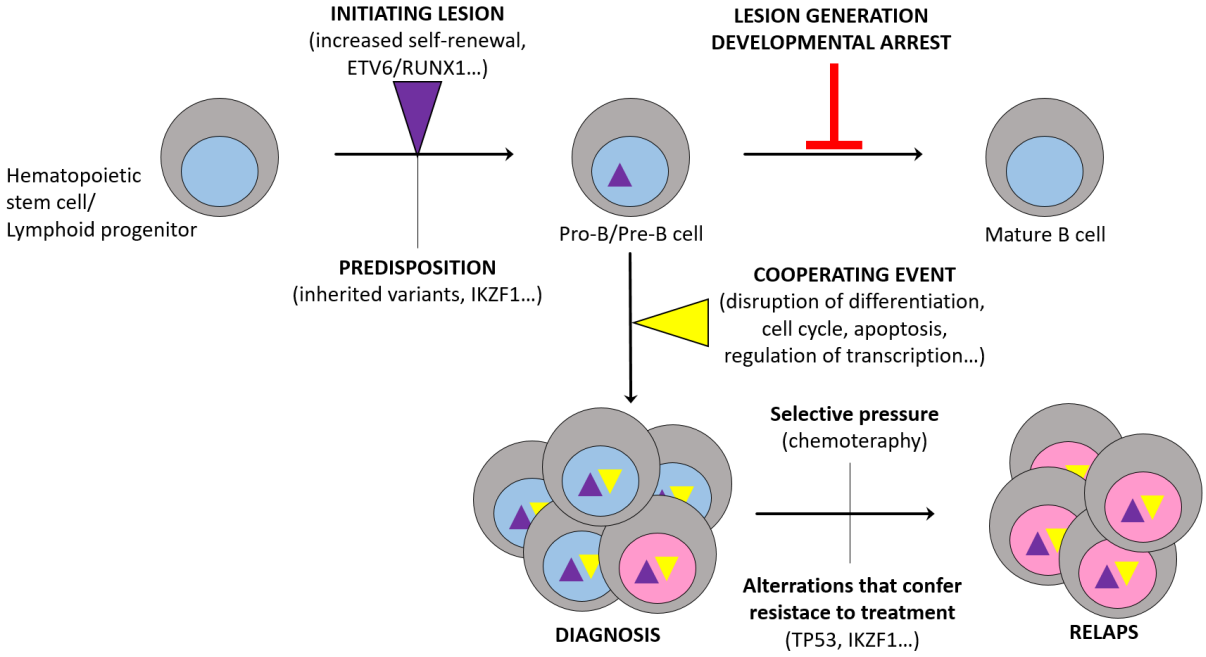


Figure 3: Multi-step model of leukemogenesis. Genetic alterations leading to B lymphoblastic leukemia development are depicted (adapted from Inaba et al., 2013)

1.2. Acute myeloid leukemia

Acute myeloid leukemia accounts for 15-20% of childhood leukemias. The highest incidence of AML in pediatric patients is within the first year of life (Fig. 1) (Dores et al., 2012). Although major improvements in the survival rates for pediatric AML patients have been achieved in the past decade, with a 5-year overall survival of around 70% (Creutzig et al., 2012), they are still considerably lower than for children with ALL (Siegel et al., 2018; Ward et al., 2014).

AML is a very heterogeneous disease, both from a clinical, molecular and pathophysiological view point. Various myeloid hematopoietic progenitors, encompassing the erythroid, granulocyte, monocyte and megakaryocyte lineages can be affected giving rise to a myriad of presentations. Originally myeloid malignancies have been divided and characterized by the French-American-Berlin (FAB) classification mainly by morphology and immunophenotyping (Bennett et al., 1976). However, with gradual emergence of genetic approaches, which enabled a more complex characterization of the blast population on molecular level, and their incorporation into routine diagnostics required a revision of the classification, which was introduced by the World Health Organization (WHO) in 2002 (Vardiman et al., 2002) and was then revised in 2008 (Tefferi and Vardiman, 2008; Vardiman et al., 2009). Mainly, due to numerous advances associated with next-generation sequencing methods and gene expression analysis, an even more precise classification of AML and related neoplasms was presented in 2016 (Arber et al., 2016).

Leukemic blast infiltration into numerous tissues and organs results in a plethora of clinical symptoms in AML. The alteration of bone marrow microenvironment leads to anemia, thrombocytopenia and neutropenia. Extramedullary lesions include the spleen (resulting in hepatosplenomegaly), skin, lymph nodes and the central nervous system (CNS).

Morphology, immunophenotyping, fluorescent in-situ hybridization (FISH), karyotyping and molecular genetic analysis of the bone marrow, eventually of the peripheral blood, are the basic diagnostic requirements in pediatric AML. Moreover, CNS involvement is investigated at diagnosis by examining the cerebrospinal fluid (Creutzig et al., 2012).

Assessing proper treatment intensity in AML patients follows risk stratification, which is based on the underlying leukemia biology, molecular genetics and response to therapy. Intensive induction therapy is initiated shortly after diagnosis of AML.

1.2.1. Genetic aberrations in AML

A number of aberrantly expressed genes and gene mutations have been described in childhood AML (Fig. 4). These genetic events are generally divided into two classes (Renneville et al., 2008). Type I class includes mutations that present the progenitor cell with a proliferative advantage without fully transforming it into a leukemic cell, like for example mutation in the JAK2, FLT3, KIT, CBL genes and mutations in the RAS-RAF-ERK signal transduction pathway, NRAS, KRAS and PTPN11 (Zuna and Zaliova, 2015). On the other hand genetic mutations of the type II class mostly affect the differentiation potential and subsequently apoptosis of the leukemic cell. Fusion genes, like for example PML-RARA, RUNX1-RUNX1T1 and CBFB-MYH11, that result from chromosomal translocations, are the main representatives of the second class aberrations (Dash and Gilliland, 2001; Kelly and Gilliland, 2002; Renneville et al., 2008). With respect to the two-hit theory, AML results from a cooperation of at least two mutations from the above described classes (Kelly and Gilliland, 2002). The acquisition of genetic aberration in AML occurs in a stepwise manner. Both the order and the type of the acquired mutations affects the hematopoietic stem cell (HSC). It makes it either more likely or less likely to evolve into full-blown leukemia (Grove and Vassiliou, 2014).

Alterations that are associated with a favourable prognosis in pediatric AML include the fusion genes RUNX1-RUNX1T1, CBFB-MYH11, PML-RARA and mutations in genes NPM1 and CEBPA (Harrison et al., 2010; Rubnitz and Inaba, 2012; Rubnitz et al., 2010; von Neuhoff et al., 2010).

Monosomy 7, monosomy 5 and del(5q) have been associated with poor prognosis (Hasle et al., 2007). Fortunately, these chromosomal abnormalities present only 2-4% of AML cases. On the other hand, activating mutations in the form of internal tandem duplications of the FLT3 gene (FLT3/ITD mutations) represent approximately 10% of AML cases and are associated with poor prognosis when exhibiting a high ratio of mutant to normal alleles (Levis and Small, 2003; Meshinchi et al., 2006; Staffas et al., 2011).

The most common recurrent karyotypic abnormalities in pediatric AML are discussed below. The list is not exhaustive.

KMT2A-rearranged AML

KMT2A gene (previously known as the MLL gene) fusions resulting from chromosome 11q23 rearrangements are common in pediatric AML, comprising 10 to 20% of cases overall. They are especially prevalent in infants (Balgobind et al., 2009; Creutzig et al., 2012; Forestier et al., 2003; Masetti et al., 2015). The KMT2A gene is fused to more than 20 gene partners, however the MLLT3 gene (AF9 gene) located on chromosome 22 is the most common translocation partner (Meyer et al., 2006; Shih et al., 2006). Acute myeloid leukemia with the KMT2A-MLLT3 fusion gene is considered as a distinct subtype of AML by the WHO classification and is associated with an intermediate prognosis. However, other translocation partners of KMT2A in children demonstrated a wide variability in survival (Coenen et al., 2011).

t(8;21)/RUNX1-RUNX1T1 AML

The t(8;21)(q22;q22) chromosomal translocation is found in 8-13% of pediatric AML cases and results in the production of the fusion gene RUNX1-RUNX1T1 (Forestier et al., 2003; Rubnitz et al., 2002). Secondary cooperating mutations in NRAS, KRAS or KIT are frequently present in AML patients harboring this particular translocation (Goemans et al., 2005; Krauth et al., 2014). Interestingly the RUNX1-RUNX1T1 fusion gene was one of the first to be used for the monitoring of minimal residual disease (MRD) (Yin et al., 2012). The RUNX1-RUNX1T1 chimeric protein causes a disruption of the normal function of the transcription factor complex CBF which regulates normal hematopoiesis (Downing, 1999; Licht, 2001).

inv(16)/t(16;16)/CBFB-MYH11 AML

The inversion inv(16)(p13.1;q22) or translocation t(16;16)(p13.1;q22) of chromosome 16 is present in 5-10% of childhood AML cases (Creutzig et al., 2012). Both of these cytogenetic abnormalities give rise to a fusion of the CBFB gene with the MYH11 gene on chromosome 16 (Sinha et al., 2015; Speck and Gilliland, 2002). The CBFB-MYH11 chimeric fusion protein deregulates transcriptional activity effecting cell differentiation, regulation of apoptosis and proliferation, similarly to RUNX1-RUNX1T1 (Shigesada et al., 2004; Steffen et al., 2005). This subtype of pediatric AML usually presents with a myelomonocytic morphology. Frequent

eosinophils are present in the bone marrow and have characteristic abnormal immature basophilic granules (Larson et al., 1986).

t(15;17)/PML-RARA AML

The PML-RARA fusion gene results from the translocation t(15;17)(q22;q12) and is associated with acute promyelocytic leukemia (APL) in which it represents the most common genetic aberration (Warrell, 1993). The fusion gene is present in 6-10% of all pediatric AML patients (Creutzig et al., 2012). The RARA gene encodes the retinoic acid receptor alfa protein which serves as a nuclear receptor. It is fused to the PML gene, which encodes for a myeloid transcription factor. The chimeric fusion protein results in a permanent repression of genomic expression finally leading to dysregulation of cell differentiation, self-renewal and apoptosis (Steffen et al., 2005). The repressive function of PML-RARA can be inhibited by high doses of the all-trans retinoid acid (ATRA), representing the first successful targeted molecular therapy applied in the treatment of leukemia which changed the prognosis of APL from dismal to excellent (Huang et al., 1988; Wang and Chen, 2008).

1.2.2. Minimal residual disease monitoring in AML

The persistence of leukemic cells after therapy at levels undetectable by morphology is termed minimal residual disease (MRD). Its assessment in routine clinical practice has become indispensable in both pediatric and adult ALL (Pui et al., 2017; van Dongen et al., 2015). On the other hand, its applicability in pediatric AML still remains largely problematic. Several platforms are currently available for MRD monitoring in AML, including multiparametric flow cytometry (MFC), real-time quantitative polymerase chain reaction (RT-qPCR) and next-generation sequencing (NGS), each suitable for distinct leukemia subtypes (Schuurhuis et al., 2018; Voso et al., 2019). RT-qPCR assesses MRD by amplifying genetic abnormalities associated with leukemia, mainly RNA transcripts of fusion genes. The currently validated molecular targets for MRD monitoring by RT-qPCR, in which it plays a superior role over MFC, include the PML-RARA translocation, the core-binding factor (CBF) translocations RUNX1-RUNX1T1 and CBFB-MYH11, and mutations in the NPM1 gene (Gabert et al., 2003; Inaba et al., 2012; Kronke et al., 2011; Schuurhuis et al., 2018; Yin and Frost, 2003).

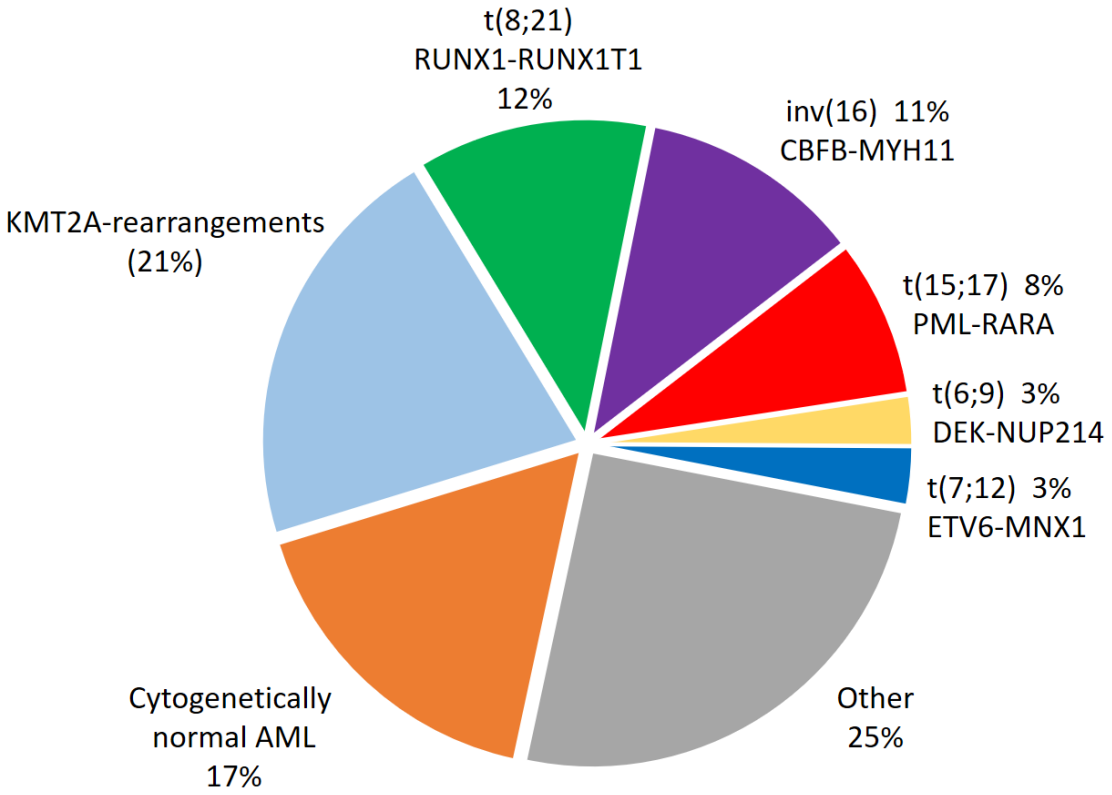


Figure 4: Recurrent cytogenetic aberrations in childhood AML, t (translocation), inv (inversion) (adapted from Creutzig et al., 2012)

1.3. Acute lymphoblastic leukemia

Treatment of pediatric ALL has advanced tremendously in the last few decades, with current event free survival rate of approximately 90% (Hunger et al., 2012). Nevertheless cure rates range from as low as 20% to as high as 95% depending on the ALL subgroup. ALL accounts for 25% of all childhood cancers, making it the most common pediatric malignancy, with a peak incidence between 2 to 5 years, being more prevalent in boys (Fig. 1) (Hunger and Mullighan, 2015a; Pui et al., 2004). A number of genetic factors were linked with an increased risk of pediatric ALL, most notably DS (Buitenkamp et al., 2014). Moreover, several polymorphic variants identified by genome-wide association studies, including IKZF1, GATA3, ARID5B, CDKN2A and CEBPE, were associated with increased ALL risk (Papaemmanuil et al., 2009; Sherborne et al., 2010; Trevino et al., 2009).

Clinical presentation usually reflects the extent of bone marrow infiltration with leukemic blasts and extramedullary involvement. Typical symptoms include spleno/hepatosplenomegaly, lymphadenopathy, fever and signs of bleeding. Laboratory

findings usually demonstrate anemia, neutropenia and thrombocytopenia, reflecting a disorder of hematopoiesis. The presence of leukemia in the CNS usually results from the spread of leukemic cells. Fortunately, it is found in only 5% of B-ALL patients at the time of diagnosis (Laningham et al., 2007).

A bone marrow aspirate is ordinarily necessary for establishing definite diagnosis of leukemia, by identifying the morphology of lymphoblasts via microscopical analysis together with defining cell lineage and developmental stage by flow cytometry (Pui et al., 2008).

Proper risk stratification of ALL patients has immensely improved the therapy of this disease. Risk of relapse is the main factor driving therapy intensity. Treatment usually lasts for approximately 2 to 3 years. The first phase of treatment, the induction phase, is initiated right after diagnosis and is meant to achieve disease remission and the re-establishment of normal hematopoiesis in the bone marrow. Consolidation phase, together with intensification play a major role in the eradication of persisting leukemic cells that are below the morphological detection levels and in the achievement of long lasting remission.

The majority (80-85%) of pediatric ALL cases are represented by BCP-ALL. These cases originated from an early B-cell progenitor. Approximately 15% of pediatric ALL, originated from an early T-cell progenitor and therefore represent T-lymphoblastic leukemia (T-ALL).

1.3.1 Genetic aberrations in ALL

Pediatric ALL comprises of multiple subtypes with various chromosomal rearrangements, gains/deletions of DNA and mutations of common cellular pathways (Fig. 5). Precise identification of these genetic alterations is important for diagnosis, prognostic risk stratification and subsequent adjustments of therapy. The detection of genetic abnormalities is done by conventional genetic methods like fluorescence in-situ hybridization (FISH), karyotyping and polymerase chain reaction (PCR) together with the measurement of DNA ploidy by flow cytometry (Pui et al., 2004).

The introduction of genome-wide approaches, namely whole-genome sequencing, whole-exome sequencing (WES), whole-transcriptome sequencing (RNA-seq), single nucleotide polymorphism arrays (SNP array) together with genome wide gene expression

profiling has enabled us to distinguish novel subtypes of ALL (Gu et al., 2019; Iacobucci and Mullighan, 2017; Lilljebjorn and Fioretos, 2017).

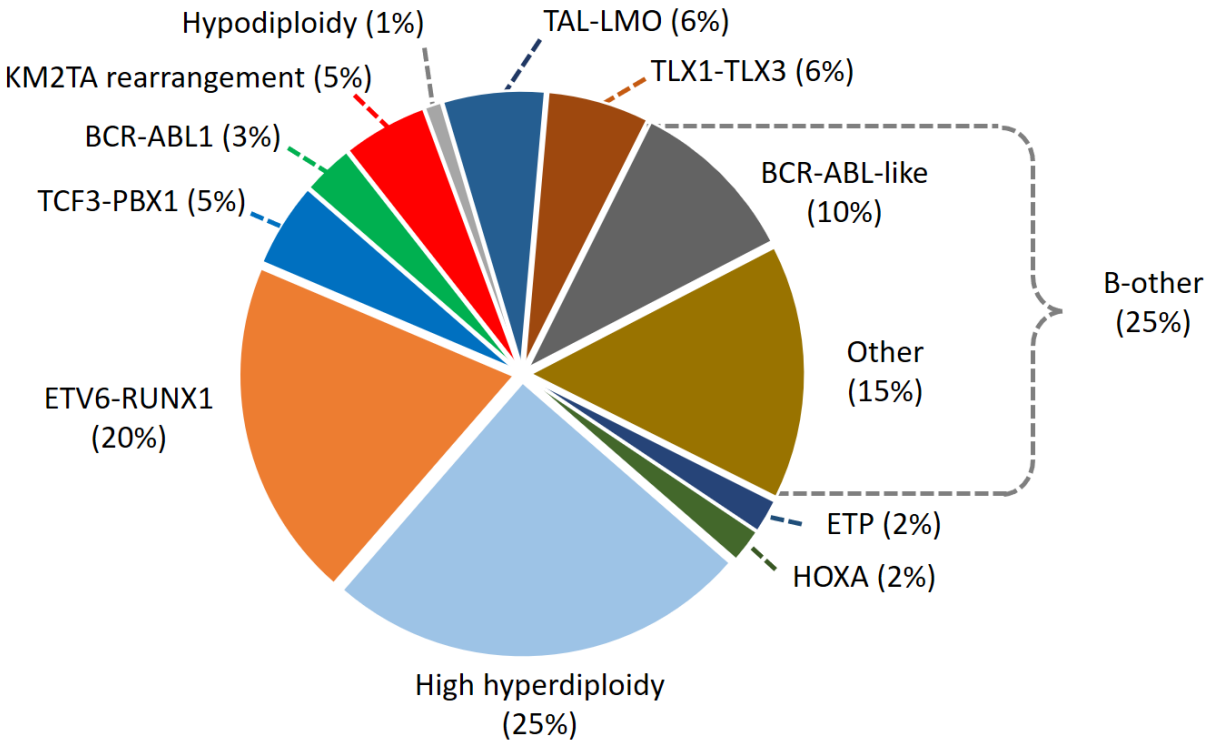


Figure 5: Prevalence of the most common genetic aberrations in pediatric ALL (adapted from (Lilljebjorn and Fioretos, 2017; Mullighan, 2012a; Mullighan, 2012b; Schwab and Harrison, 2018)

1.3.1.1. BCP-ALL

Selected BCP-ALL subtypes are introduced below. The list is not exhaustive and doesn't include all known and described BCP-ALL subtypes.

High hyperdiploid ALL

Leukemic blasts harboring 51-67 chromosomes are defined as high hyperdiploid. This most common cytogenetic abnormality in pediatric BCP-ALL is associated with a favorable prognosis. The higher the chromosome number, the better the prognosis (Dastugue et al., 2013). The gain of chromosomes in high hyperdiploid ALL is nonrandom and is featured by

chromosomes 21, X, 6, 4, 10, 17, 14 and 18 (Paulsson et al., 2010; Paulsson and Johansson, 2009). High hyperdiploidy is considered an early event in leukemogenesis as was confirmed by backtracking studies (Gruhn et al., 2008; Taub et al., 2002). It has been suggested that the gain of extra chromosomes occurs simultaneously in a single cell division (Paulsson and Johansson, 2009).

Hypodiploid ALL

Hypodiploid ALL with less than 44 chromosomes has an extremely poor outcome. Patients with hypodiploid blast cells are further stratified by chromosome number into three subgroups. The near haploid group (24-31) has an event free survival (EFS) as low as 30%. Slightly better are the EFS for the two remaining subgroups, low hypodiploid (32-39) and high hypodiploid (40-43) (Holmfeldt et al., 2013; Nachman et al., 2007).

t(12;21)/ETV6-RUNX1 ALL

The most common chromosomal translocation in pediatric BCP-ALL, t(12;21)(p13;q22), results in the production of the fusion gene ETV6-RUNX1 (TEL-AML1) (Romana et al., 1995b). It is very common in pediatric patients and almost absent in adults. It has been shown that this aberration frequently originates *in utero* (Wiemels et al., 1999). Usually cryptic, the translocation is detectable only by FISH analysis and not by conventional karyotyping. The oncogenic fusion protein promotes self-renewal and differentiation of B-progenitor cells. It seems to be necessary in leukemogenesis, but is alone insufficient to cause overt leukemia (Morrow et al., 2004). Patients with this ALL subtype have a very favorable prognosis. Occasionally relapses do occur, but tend to respond well to chemotherapy (Bhojwani et al., 2012; Loh et al., 2006; Zuna et al., 1999).

KMT2A-rearranged ALL

Rearrangements of the KMT2A gene occur in both ALL and AML. They are specifically related to infant leukemia in patients younger than 1 year (Muntean and Hess, 2012; Slany, 2009). Nearly all KMT2A abnormalities are KMT2A N-terminus in-frame fusions with fusion partners, which create a novel oncogene. The KMT2A gene is considered quite promiscuous, with more than 120 gene fusion partners described (Meyer et al., 2013; Meyer et al., 2009). Nevertheless, nine partner genes represent almost 90% of all rearrangements (Meyer et al.,

2013; Muntean and Hess, 2012). KMT2A most commonly fuses with AFF1 (AF4), MLLT1 (ENL) and MLLT3 (AF9). Evidence points to the *in utero* origin of KMT2A-rearranged leukemias. Moreover, they harbor fewer cooperating mutations, when compared with other B-ALL leukemias, indicating the strength of the KMT2A oncogene. Patients with the KMT2A-rearrangement have a significantly poorer prognosis and are treated with intensified therapy in most protocols (Meyer et al., 2013; Meyer et al., 2009).

t(9;22)/BCR-ABL1 ALL

The BCR-ABL1 fusion oncogene, located on the Philadelphia (Ph) chromosome, results from a balanced translocation of chromosome 9 and 22. It was the first cytogenetic abnormality to be associated with a particular malignity (Nowell and Hungerford, 1960; Rowley, 1973). A portion of the ABL1 tyrosine kinase, residing on chromosome 9, is fused with the BCR gene on chromosome 22. The resulting BCR-ABL1 fusion gene generates a constitutively active tyrosine kinase, which subsequently deregulates and aberrantly activates a number of crucial signal transduction pathways (Lugo et al., 1990). The fusion gene is a typical feature of adult chronic myeloid leukemia (CML) and also ALL. It is less common in pediatric ALL were it is presents in about 3% of cases. There are two main forms of the fusion gene depending on the BCR break point. The Major BCR-ABL1 variant, resulting from breakpoints in the major breakpoint cluster region, is typically present in CML patients and gives rises to the 210kD protein p210. In ALL patients we tend to find the minor BCR-ABL1 fusion gene variant which results in the production of protein p190 (Melo, 1996). Until recently, the BCR-ABL1 fusion gene was associated with an inferior prognosis (Arico et al., 2000). The introduction of a specific tyrosine kinase inhibitor, imatinib mesylate, which is active against the fusion protein has significantly improved survival (Biondi et al., 2012; Druker et al., 2001; Jeha et al., 2014; Schultz et al., 2009).

B-other ALL

An important subgroup of precursor B-ALL leukemias are the so-called “B-others”, which are represented by leukemias without the above described genetic aberrations and account for approximately 25% of all B-ALL cases (Fig. 5) (Inaba et al., 2013; Zaliova et al., 2019). The availability of modern genome-wide approaches enabled investigation of the genetic background of B-other leukemias. A number of novel subtypes have been described

within B-other ALL based on unique gene expression signature profiles and recurrent genetic aberrations (Gu et al., 2019; Hirabayashi et al., 2017; Lilljebjorn et al., 2016; Rand et al., 2011; Zaliova et al., 2019).

In addition to the subtype defining aberrations, additional genetic abnormalities have been identified, with some proven to be therapeutically relevant, like for example the IKZF1 deletion (IKZF1del) which has been associated with increased risk of relapse (Clappier et al., 2015; Dorge et al., 2013; Mullighan et al., 2009b). Moreover a group of aberrations in genes encoding cytokine receptors, kinases and regulators of intracellular signaling which eventually result in the activation of kinases seems to be therapeutically relevant, namely aberrations in the CRLF2, KRAS, NRAS, ZEB2 and fusions involving JAK2, ABL1, EPOR (Mullighan et al., 2009a; Perentesis et al., 2004; Roberts et al., 2012; Zhang et al., 2011).

BCR-ABL1-like/Ph-like ALL

The BCR-ABL1-like ALL is a new entity, which was recognized by the 2016 WHO classification, mainly due to its responsiveness to tyrosine kinase inhibitors (TKI) and clinical relevance (Arber et al., 2016). This subgroup lacks the BCR-ABL1 fusion gene, but its expression profile resembles BCR-ABL1-positive ALLs (Den Boer et al., 2009; Mullighan et al., 2009b). The incidence of BCR-ABL1-like ALL increases with age (Roberts et al., 2017; Roberts et al., 2014a). It is associated with poor response to therapy and overall poor prognosis (Roberts et al., 2014a). Recurrent genetic aberrations in BCR-ABL1-like ALLs are represented by IKZF1 deletions, mutations and rearrangements of the CRLF2 gene, rearrangement of JAK2 and ABL-class genes and mutations activating the RAS and JAK/STAT signaling pathways (Roberts et al., 2017; Roberts et al., 2014a).

In BCR-ABL1-like ALLs CRLF2 is either translocated into the heavy chain locus of the immunoglobulin gene (IGH) or a deletion upstream of the CRLF2 gene causes the production of a P2RY8-CRLF2 fusion (Yoda et al., 2010). CRLF2-rearranged ALLs usually harbor additional aberrations activating signaling pathways (Mullighan et al., 2009c; Roberts et al., 2017). Poor prognosis has led to the investigation of specific kinase inhibitors which would target the aberrant signaling in these patients (Maude et al., 2012; Waibel et al., 2013).

ETV6-RUNX1-like ALL

The ETV6-RUNX1-like leukemias display the same gene expression pattern as ETV6-RUNX1-positive ALL cases, albeit not having the fusion gene (Lilljebjorn et al., 2016; Zaliova et al., 2017). This subgroup harbors deletions or various structural aberrations of the ETV6 gene together with alterations of IKZF1. The expression of surface markers CD27 and CD44 is also similar as in ETV6-RUNX1-positive ALL cases (Zaliova et al., 2017), supporting the biological proximity of these two ALL subgroups.

DUX4-rearranged ALL

The deregulation of the transcription factor DUX4 (double homeobox 4 gene) in childhood ALL has been linked with a distinct gene expression profile and immunophenotype (Lilljebjorn et al., 2016; Liu et al., 2016; Yasuda et al., 2016; Zhang et al., 2016). DUX4 is located on chromosome 4, in the subtelomeric D4Z4 repeat region. The translocation of DUX4 to IGH causes the expression of its truncated isoform in B lymphocytes (Lilljebjorn et al., 2016; Liu et al., 2016; Yasuda et al., 2016; Zhang et al., 2016). Rarely, DUX4 can be inserted into the ERG gene (Lilljebjorn et al., 2016). The short DUX4 protein binds to the intragenic region of the gene encoding ERG and leads to the expression of an aberrant ERG protein, which inhibits ERG wild type transcriptional activity (Zhang et al., 2016).

ZNF384-rearranged ALL

The zinc finger encoding gene ZNF384 plays an important role in the process of matrix metalloprotease regulation. The rearrangements of ZNF384 involves a fusion partner gene, usually a chromatin modifier or transcriptional regulator (for example: CREBBP, EP300, TAF15, ARID1B, TCF3) (Hirabayashi et al., 2017; Liu et al., 2016; Shago et al., 2016; Yasuda et al., 2016). The B precursor leukemias harboring the ZNF384 rearrangement have intermediate prognosis.

MEF2D-rearranged ALL

Rearrangements of the myocyte enhancer factor 2D (MEF2D) are present in approximately 3% of children with BCP-ALL and 6% of adults (Liu et al., 2016; Zaliova et al., 2019). MEF2D is most commonly rearranged to BCL9 (Gu et al., 2016). The resulting fusion proteins are more active and show transforming potential. Increased transcriptional activity

in the leukemic cells leads to enhanced expression of HDAC9 (histone deacetylase 9), making them particularly sensitive to histone deacetylase inhibitors *in vivo* (Gu et al., 2016).

1.3.1.2. ETV6-ABL1-positive ALL

The ETV6-ABL1 fusion is a rare event in ALL were it represent 0,2% of cases in children and 0,4% of cases in adults. Moreover it is also recurrently found in patients with CML without BCR-ABL1 and in AML (Zaliova et al., 2016; Zuna et al., 2010). Similarly to the BCR-ABL1 fusion gene, which is far more frequent, it is a kinase activating lesion leading to the deregulation of cellular survival and growth and subsequently transforming the affected cell into a leukemic blast (Hannemann et al., 1998; Million et al., 2004; Okuda et al., 1996; Papadopoulos et al., 1995; Pendergast et al., 1993). Interestingly ETV6-ABL1 does not induce leukemic transformation in mice, unlike BCR-ABL1. On the other hand, it causes a chronic myeloproliferation, that is similar to the one induced by BCR-ABL1 in CML (Million et al., 2002). Its genomic profile closely resembles the BCR-ABL1-positive and BCR-ABL1-like ALLs. Most patients harbor deletions in the CDKN2A/B and IKZF1 genes (Zaliova et al., 2019). The expression profile analysis clusters patients positive for ETV6-ABL1 parallel to the BCR-ABL1 cases, therefore usually classifying them into the BCR-ABL1-like ALL subgroup. The ETV6-ABL1 fusion gene is associated with poor prognosis in acute leukemias (Zuna et al., 2010). It has been shown *in vitro* that the aberrant kinase can be inhibited by imatinib, a TKI used for treatment of BCR-ABL1-positive leukemias, therefore making it a potential treatment option also in leukemias harboring the ETV6-ABL1 fusion (Carroll et al., 1997; Zaliova et al., 2016).

1.3.1.3. T-ALL

More than half of T-ALL patients harbor chromosomal translocations. These translocations usually involve the T-cell receptor alfa (TRA), T-cell receptor delta (TRD) loci (14q11) and the T-cell receptor beta (TRB) region (7q34). The chromosomal rearrangements juxtapose the T-cell receptor genes to genes encoding for transcription factors, such as LYL1, TAL1, TAL2, LMO1, LMO2, TLX1, TLX3, MYC, MYB and HOXA genes. Moreover, T-ALLs can contain cryptic rearrangements of ABL1, like ETV6-ABL1, EML1-ABL1 and NUP214-ABL1 (Durinck et al., 2015; Liu et al., 2017).

Activating mutations in NOTCH1 are present in approximately 60% of T-ALLs (Weng et al., 2004). They are together with CDKN2A and CDKN2B deletions the most prevalent T-ALL alterations (Girardi et al., 2017). Other molecular alterations include JAK1 and JAK3 mutations resulting in constitutive activation of JAK/STAT signaling (Liu et al., 2017; Van Vlierberghe and Ferrando, 2012).

2. Myeloid leukemogenesis in Down syndrome

Down syndrome (DS) was described clinically in 1866 by Langdon Down (Down, 1866) and was associated with trisomy of the 21 chromosome a century later (Lejeune et al., 1959). Prevalence of the disorder correlates with maternal age and is approximately 1/700 (Mai et al., 2019). In 95% of cases, trisomy 21 in Down syndrome patients is due to chromosomal non-disjunction in meiosis. Three percent of patients harbor translocations that involve the additional 21 chromosome or its part. A small group of patients, approximately 2%, are affected by mosaic Down syndrome, where a portion of the patient's cells have trisomy 21, while the rest are disomic. The range of the affected cells may vary significantly and can be organ or tissue specific. The clinical presentation of DS is associated with typical facial features (almond shaped eyes, flattened face, macroglossia, short neck), weak muscle tone, developmental delay, congenital heart disease, Alzheimer's and importantly leukemia (Roizen and Patterson, 2003). Interestingly, DS is associated with a lower incidence of solid tumours (Hasle et al., 2000).

A broad spectrum of hematological malignancies has been described in patients with DS, ranging from benign to malignant conditions (David et al., 1996; de Hingh et al., 2005; Henry et al., 2007; Kivivuori et al., 1996; Starc, 1992; Watts et al., 1999; Webb et al., 2007). The incidence of both AML and ALL is increased in DS patients (Table 1) (Hasle et al., 2000). Acute myeloid leukemia in DS occurs at a younger age. The most striking difference from the general population is the risk of acute megakaryoblastic leukemia (AMKL), which is 500 times higher in patients with DS, than in healthy age-matched individuals. AMKL is a subtype of AML and it may be preceded, in DS patients, by a preleukemic phase termed transient myeloproliferative disorder (TMD).

Children with DS are more sensitive to chemotherapy and therefore intensity reduced regiments in the treatment of AML are recommended, usually without the need of

hematopoietic stem cell transplantation (HSCT) (Creutzig et al., 2012). This approach results in survival rates of more than 85% (Creutzig et al., 2005; Kudo et al., 2010; Kudo et al., 2007). On the other hand, survival rates of children with ALL in DS are poorer than in non-DS ALL patients, mainly due to higher relapse occurrence (Buitenkamp et al., 2014).

Type of leukemia	Frequency in non-DS	Frequency in DS
Acute lymphoblastic leukemia (ALL)	80%	60%
Acute myeloid leukemia (AML)	20%	40%
Acute megakaryoblastic leukemia (AMKL)	6% of all AML cases	62% of all AML cases
Transient myeloproliferative disorder (TMD)	Not applicable	10%
Myelodysplastic syndrome (MDS)	8% of all AML cases	20-62% of all AMKL cases

Table 1: Acute leukemia in children with and without Down syndrome (adapted from Hitzler and Zipursky, 2005).

Trisomy 21 is believed to play an essential role in the pathogenesis of TMD and DS-AMKL. This is exemplified by the fact that both TMD and DS-AMKL require trisomy 21 in the blast cells.

Despite, that the long arm of chromosome 21 (HSA21) is the most studied chromosome (Antonarakis, 2017) its biological role in leukemogenesis remains elusive. Trisomy 21 has been shown to increase fetal hematopoietic stem cell self-renewal. It accelerates the expansion of early hematopoietic progenitor cells, namely the erythro-megakaryocytic progenitor compartment (Chou et al., 2008). Moreover, it has been postulated by Banno and colleagues, that gene dosage alterations of ERG, RUNX1 and ETS2, which are located in a 4-Mb region on HSA21, is critical for the deregulating effects on hematopoiesis (Banno et al., 2016).

To study the contribution of trisomy 21, induced pluripotent stem (iPS) cells originating from DS patients have been established and analyzed for hematopoietic differentiation (Chou et al., 2012; Li et al., 2012; Maclean et al., 2012). When cultured under conditions supporting primitive hematopoiesis, erythropoiesis was enhanced, myelopoiesis was reduced and megakaryocytes were normally produced (Chou et al., 2012). Interestingly, when the iPs cells were cultured in conditions supporting differentiation into fetal liver-derived definite hematopoietic cells, the trisomic iPs cells showed an increase in multi-lineage colony forming potential (Maclean et al., 2012). There was no difference between trisomic iPS cells and disomic iPs cells when assessed in conditions appropriate for generating erythroblast co-expressing fetal and embryonic globin genes (Li et al., 2012). These studies on iPS cells suggest that the influence of the additional chromosome 21 on hematopoiesis is dependent on the hematopoietic microenvironment.

Multiple DS mouse models were used to examine the hematopoietic phenotype induced by trisomy 21. The Tc1 mice are a transchromosomic line which carries a freely segregating copy of human chromosome 21 (Wiseman et al., 2009). These mice present with macrocytic anemia together with an increased number of megakaryocytes and in the elderly with signs of extramedullary hematopoiesis. Interestingly, major changes in frequencies of erythroid progenitors, myeloid progenitors and megakaryocytes were not seen in the fetal liver (Alford et al., 2010). Ts16 mice are trisomic for mouse chromosome 16, which is synthetic of human chromosome 21 (Epstein et al., 1985; Gropp et al., 1974; Vacano et al., 2012). The Ts16 mouse line showed reduced myelopoiesis and increased erythropoiesis during the embryonic period (Gjertson et al., 1999). These mice do not survive postnatally, therefore their defects in hematopoiesis during this period are uncertain. Ts1Rh, Ts1Cje and Ts65Dn mice are lines of euploid DS model mice that bear a region of mouse chromosome 16 which contains 33, 81 and 104 genes, respectively. The Ts1Rh mice showed thrombocytosis and anemia in adulthood. The number of B-cell progenitors was reduced in Ts1Rh mice and their bone marrow cells differentiated preferentially toward granulocytes and monocytes (Lane et al., 2014; Malinge et al., 2012). Except for an increase in the HSC population, no hematological abnormalities were observed in the embryonic stage of Ts1Rh mice. Disturbed erythropoiesis was found in Ts1Cje mice. However, they never developed myeloproliferative diseases or thrombocytosis (Carmichael et al., 2009). In contrast, progressive myeloproliferative diseases,

defects of stem cell function and macrocytic anemia were seen in the Ts65Dn mice (Kirsammer et al., 2008). The hematological phenotypes of these studied DS model mice lines show partially overlapping features with those seen in DS patients, however none of the studied mice acquire Gata1 mutations or develop leukemia (Shimizu and Yamamoto, 2015).

2.1. Transient myeloproliferative disorder

Transient myeloproliferative disorder (TMD), or transient abnormal myelopoiesis (TAM), is a unique and complex preleukemic condition with a specific genetic background and a perturbance of fetal hematopoiesis, which affects 10% of neonates with DS. TMD usually presents right after birth (Klusmann et al., 2008; Massey et al., 2006; Muramatsu et al., 2008) and in most cases resolves spontaneously without intervention in the first few months of life, hence the description transient (Klusmann et al., 2008). Nevertheless, approximately 20% of TMD patients progress to AMKL in the first 4 years of life, therefore these patients have to be closely monitored in this given timeframe (Gamis et al., 2011; Klusmann et al., 2008; Lange et al., 1998; Massey et al., 2006).

The majority of patients present with clinical symptoms and approximately 10% are asymptomatic (Klusmann et al., 2008). Hepatomegaly and splenomegaly are common features of this disorder, due to the fact, that blasts in TMD likely originate in the fetal liver (Klusmann et al., 2008). Rarely, liver fibrosis can occur with life-threatening consequences (Al-Kasim et al., 2002). Skin infiltrations in the form of a rash are another common presentation. Less frequent symptoms include pericardial effusions, pulmonary edema, ascites and hydrops fetalis (Al-Kasim et al., 2002; Zipursky, 2003). Morphological examination of the peripheral blood and the bone marrow usually reveals a myeloid-appearing blast population that can be quite heterogeneous and vary in number. Megakaryoblasts are commonly present, some with characteristic protruding cytoplasmic blebs, together with features of dyserythropoiesis. Various alterations in the level of white blood cells, thrombocytes and hemoglobin may be observed (Roy et al., 2009).

Most TMD patients do not require chemotherapy. Nonetheless, symptomatic neonates with liver dysfunction or a high percentage of blasts in the peripheral blood, may profit from brief treatment with low doses of cytosine arabinoside (Klusmann et al., 2008;

Massey et al., 2006). The mortality rate of TMD is about 20% (Klusmann et al., 2008; Massey et al., 2006; Muramatsu et al., 2008; Zipursky, 2003).

The clonal expansion of hematopoietic progenitors, resulting in TMD is exclusively associated with two molecular factors – the extra copy of chromosome 21 and mutations in the megakaryocyte-erythroid transcription factor gene GATA1. This unique leukemic predisposition, presented only in neonates with DS, or in phenotypically normal neonates with trisomy 21 mosaicism, or very rarely in patients with somatic trisomy 21 solely in the blast population, underlines the important role that trisomy 21 is believed to play in the pathogenesis of this condition. Moreover, somatic mutations in GATA1 result in the sole expression of a shorter isoform of GATA1, the so called GATA1s protein, while eliminating the expression of full-length GATA1. Additional genetic aberrations may be present, as was recently exemplified by the largest sequencing study of TMD and AMKL patients so far by Labuhn and colleagues (Labuhn et al., 2019).

Due to the omnipresence of GATA1 mutations and trisomy 21 in TMD, both should be investigated and validated to achieve proper diagnosis. Mutations in GATA1 may also serve as useful markers to monitor MRD and TMD progression in individual patients. In the recent recommendations of the British Society for Haematology, DS-TMD was defined as the presence of >10% of blasts in the peripheral blood, together with a GATA1 mutation and/or clinical features of DS-TMD in a child with DS or mosaic trisomy 21 (Tunstall et al., 2018).

The multi-step process of TMD progression into AMKL has provided an essential *in vivo* model to study myeloid leukemogenesis. Trisomy 21 together with acquired somatic mutations of the GATA1 gene in stem cells or hematopoietic progenitors initiate the process of transformation during prenatal hematopoiesis (Fig. 6). At birth, multiple clones harboring GATA1 mutations may be present. However, in most cases of overt TMD, one clone is predominant (Hitzler and Zipursky, 2005). TMD manifests itself before, or usually right after birth as a preleukemia, in most cases resolves spontaneously and may later on progress to full-blown leukemia. In the majority of cases, trisomy 21 together with GATA1s is sufficient to initiate TMD, were exonic variants are relatively rare (Labuhn et al., 2019). The progression into leukemia on the other hand occurs, when the GATA1-mutated cells acquire additional somatic aberrations. The secondary transforming events most frequently occur in genes

encoding signaling molecules (JAK/STAT pathway, RAS-RAF-ERK pathway, RUNX1, TP53…), epigenetic regulators (KANSL1, SUZ12, EZH2…) and members of the cohesin protein family (STAG2, RAD21, SMC1A, CTCF…) (Labuhn et al., 2019; Nikolaev et al., 2013; Walters et al., 2006; Yoshida et al., 2013).

2.2. Acute megakaryoblastic leukemia of Down syndrome

A characteristic molecular signature of trisomy 21, GATA1 mutations and additional somatic aberrations, characterizes DS-AMKL (Bourquin et al., 2006; Gruber and Downing, 2015). Together with better outcome, it distinguishes itself from non-DS AMKL which frequently harbors chimeric oncogenes of hematopoietic gene origin and in which outcome tends to be poor (Gruber and Downing, 2015). Virtually all cases of DS-AMKL occur in the first 5 years after birth (Hasle et al., 2008) and about 20-30% of them are preceded by TMD (Fig. 6).

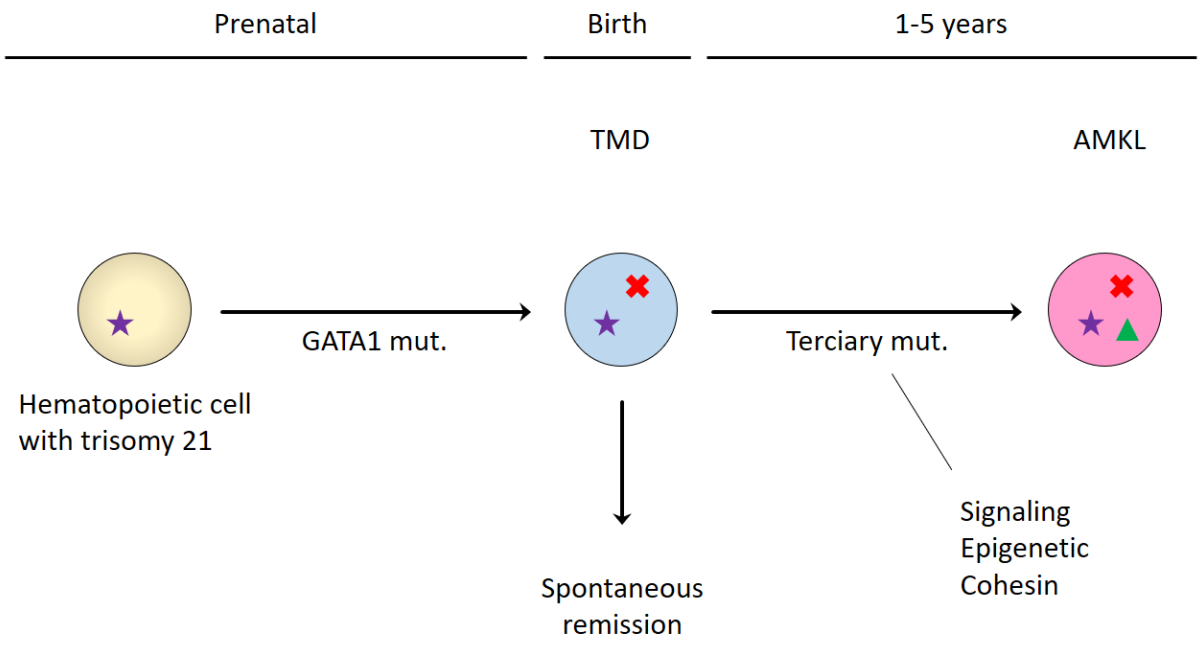


Figure 6: Multi-step model of leukemogenesis in TMD and AMKL (adapted from Crispino and Horwitz, 2017; Gruber and Downing, 2015; Hitzler and Zipursky, 2005)

3. GATA transcription factor family

The GATA gene family consists of six members (GATA1-6) that are structurally related and function as master regulators of transcription in a tissue-specific manner (Bresnick et al., 2012; Chlon and Crispino, 2012). Each transcription factor contains a C-terminal and an N-terminal Cys4-type zinc finger. GATA1 plays together with GATA2 a major role in normal hematopoiesis. As mentioned above, when mutated in the cell context of trisomy 21, GATA1 mutations lead to TMD and/or AMKL. When mutated in germline, GATA1 aberrations result in a wide range of hereditary sex-linked forms of anemias and thrombocytopenias (Crispino and Weiss, 2014; Millikan et al., 2011; Nichols et al., 2000). Somatic mutations of GATA2 can be associated with myelodysplastic syndrome or AML, germline mutations on the other hand lead to the GATA2 deficiency syndrome (Collin et al., 2015; Shiba et al., 2014). GATA1 and GATA2 regulate each other's expression. First, GATA2 initiates the expression of GATA1 in early megakaryocyte-erythroid progenitor cells. In comparison, GATA2 gene expression is down regulated by GATA1, which additionally activates its own expression. This phenomenon is known as GATA factor switching (Kaneko et al., 2010). Balance between these two transcription factors is crucial for proper hematopoiesis. GATA3 is widely expressed. In the immune system it controls T-cell proliferation (Wang et al., 2013). Interestingly, common GATA3 variants have been linked with Ph-like ALL in children and with the risk of relapse (Perez-Andreu et al., 2013). The remaining members GATA4, GATA5 and GATA6 play a role in the cardiovascular system, mainly during heart formation and are recurrently mutated in patients with congenital heart disease (Peterkin et al., 2005; Wei et al., 2013).

3.1. GATA1 in hematopoiesis

The GATA1 gene is located on the short arm of chromosome X, consists of 6 exons and encodes the GATA-binding factor 1 protein, which has a transactivation domain (TAD) and two centrally located zinc-finger domains, the N-terminal zinc-finger (NZF) and the C-terminal zinc-finger domain (CZF) (Fig. 7). It is expressed in megakaryocytes, erythroid cells, mast cells, basophils and also in Sertoli cells (Onodera et al., 1997a; Onodera et al., 1997b; Yamamoto et al., 1997). The NZF plays a role in stabilizing GATA1 during its binding to DNA and in the specificity of the binding (Fig. 8). It enables the binding of GATA1 to a number of binding sites. These binding sites contain a palindromic recognition sequence (Trainor et al., 1996).

Moreover, NZF recruits and interacts with FOG1, a key cofactor of GATA1 (Tsang et al., 1997) (Fig. 7, 8). The CZF is necessary for GATA consensus sequence (A/T)GATA(A/G) recognition and for proper DNA binding activity (Evans et al., 1988; Martin et al., 1989; Tsang et al., 1997; Wall et al., 1988; Yang and Evans, 1992). Moreover, it plays an important role in the interaction of GATA1 with transcription factors like PU.1 and Sp1 (Merika and Orkin, 1995; Rekhtman et al., 1999). The N-terminally located 83 residues of TAD have a strong transactivation potential on reporter genes that contain the GATA binding consensus sequence (Martin and Orkin, 1990). The necessity of GATA1 in erythropoiesis was first demonstrated with Gata1-null mouse embryos, which died from anemia at E10.5-E11.5 (Fujiwara et al., 1996). Remarkably, a different effect of GATA1 loss has been observed in megakaryocytes, which proliferate extensively, but fail to differentiate (Shivdasani et al., 1997). Megakaryocytes lacking GATA1 possess various abnormalities (Vyas et al., 1999). Moreover, GATA1 has been associated with the development of basophils (Nei et al., 2013), mast cells (Migliaccio et al., 2003), eosinophils (Hirasawa et al., 2002; Yu et al., 2002) and dendritic cells (Kozma et al., 2010).

Friend of GATA 1 (FOG1), an important cofactor of GATA1 as its name suggests, is a zinc finger protein that plays an essential role in hematopoiesis by binding and interacting with GATA1, through the NZF domain (Fig. 8). This interaction seems to be indispensable for FOG1 in order to fulfil its role in megakaryopoiesis (Chang et al., 2002). Interestingly, absence of Fog1 in mice causes embryonic lethality due to severe anemia (Tsang et al., 1998). A number of point mutations in GATA1 that result in benign hematological disorders have been identified. Some, most notably mutations in V205 (Nichols et al., 2000), D218 (Freson et al., 2001; Freson et al., 2002) and G208 (Mehaffey et al., 2001), which lead to various forms of anemias and thrombocytopenias, affect the affinity of GATA1 for FOG1, without influencing binding to DNA (Fig. 8). Aberrations of the NZF, like for example R216W effect GATA1 binding to DNA and GATA1 target gene expression (Fig. 8) (Phillips et al., 2007).

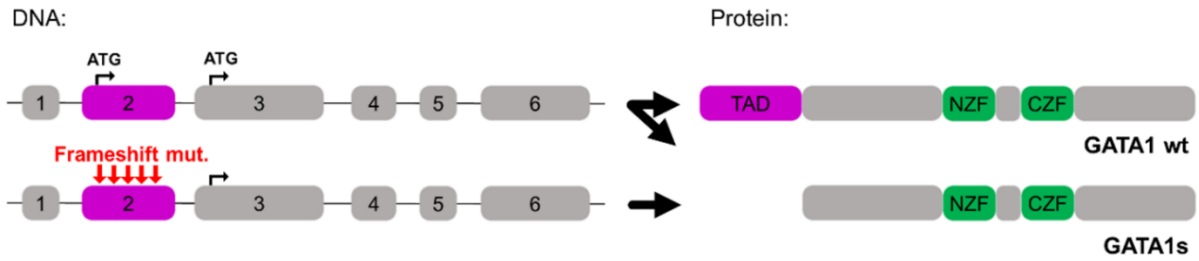


Figure 7: Schematic representation of the GATA1 gene. Both GATA1 wild type (wt) and GATA1s (GATA1 short) are expressed in healthy hematopoietic cells. Frameshift mutations in the second exon lead to the sole expression of GATA1s, with the loss of expression of the longer wt isoform. GATA1s lacks the transactivation domain (TAD). N-terminal zinc finger (NZF), C-terminal zinc finger (CZF) (Lukes et al., 2020).

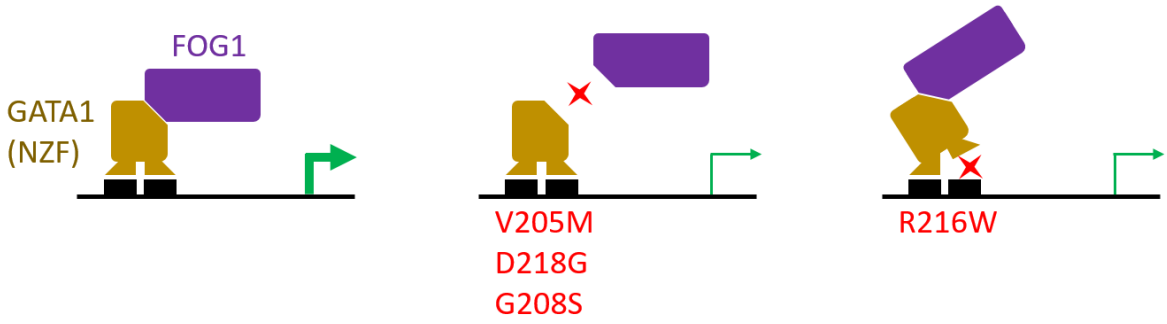


Figure 8: Benign hematological disorders frequently harbor mutations affecting the N-terminal zinc finger (NZF) of GATA1 (adapted from Crispino and Horwitz, 2017)

3.2. GATA1 mutations in TMD and AMKL

Healthy human hematopoietic cells express two types of the GATA1 protein. The full-length GATA1 and an alternative splicing variant, which originates from skipping of the second exon and is identical to GATA1s found in TMD and lacks the 83 N-terminal amino acids, which encode for TAD (Shimizu and Yamamoto, 2015; Wechsler et al., 2002). It utilizes an alternative translation initiation codon (Met84) in the third exon, instead of the translation initiation codon used by GATA1 full-length in exon 2 (Fig. 7)

In TMD and DS-AMKL somatic mutations in GATA1 result in the exclusive production of GATA1s, nullifying the expression of GATA1 full-length (Fig. 7). Mutations, usually in the form of frameshift and nonsense mutations in the second exon, lead to an introduction of a

premature stop codon or possibly to the loss of the adjoining splice site (Alford et al., 2011). On a large cohort of DS patients using targeted NGS, Roberts and colleagues showed that 30% of patients with DS harbor GATA1 mutations. Interestingly, only one-third of them had clinical and hematological findings, which correlates with the previously published studies that suggest a 10% occurrence of TMD in DS (Roberts et al., 2013).

Transgenic mice expressing GATA1s have been established in the past to investigate the function of GATA1s *in vivo*. Transgenic expression of GATA1s rescued males deficient for GATA1 from embryonic lethality (Shimizu et al., 2001). Interestingly, an accumulation of immature megakaryocytes was observed in fetal liver of the rescued mice (Shimizu et al., 2009). However, this phenotype disappeared after birth, pointing to the fact that in mice GATA1s can provoke a TMD-like condition regardless of trisomy of chromosome 16, which serves as an equivalent to the human chromosome 21 (Shimizu et al., 2009). Transgenic mouse lines expressing low or high levels of GATA1s have been assessed for rescue analysis. Interestingly, low levels of GATA1s predisposed the mice to developing leukemia, while high levels of GATA1s never led to leukemia development in the studied mice (Shimizu and Yamamoto, 2015). Gene targeting has led to the establishment of mouse lines expressing GATA1 which lacked the 63 N-terminal amino acids (Li et al., 2005). This mouse model showed, that GATA1s leads to a hyperproliferation of a unique population of fetal liver progenitors and doesn't affect the adult hematopoiesis (Li et al., 2005). Another mouse model was created by deleting the second exon of GATA1 ($Gata1^{\Delta e2}$), leading to exclusive production of GATA1s in the $Gata1^{\Delta e2}$ mice, also causing a transient hyperproliferative phenotype of the early embryonic megakaryocytes (Li et al., 2005). The here described models support the postulation that GATA1s alone causes the hypeproliferation of fetal liver megakaryocytic progenitors in mice (Li et al., 2005; Shimizu et al., 2009; Shimizu and Yamamoto, 2015). This observation was later validated in a CRISPR/Cas9 model of TMD in human fetal HSPCs (Gialesaki et al., 2018). Altogether, evidence clearly suggests that TMD initiates during fetal hematopoiesis and that GATA1s plays a major role in its pathogenesis.

4. JAK/STAT signalling pathway

The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway plays a key role in hematopoiesis, growth, cell differentiation and immunity by mediating signals of more than fifty cytokines, hormones and growth factors (Constantinescu et al., 2013; Ihle, 1995; Rawlings et al., 2004; Schindler et al., 2007; Schindler, 2002; Villarino et al., 2015). There are four members in the JAK family: JAK1, JAK2, JAK3 and Tyk2. These intracellularly located, receptor associated, non-receptor protein tyrosine kinases are activated by receptor multimerization after ligand-binding (Haan et al., 2006; Ihle and Kerr, 1995). Activated JAKs phosphorylate their constitutively associated receptors and most importantly the STAT molecules, which consequentially dimerize and are trafficked into the nucleus, where they bind to specific DNA sequences and regulate target gene transcription (Becker et al., 1998; Chen et al., 1998; Darnell, 1997; Horvath and Darnell, 1997) (Fig. 9).

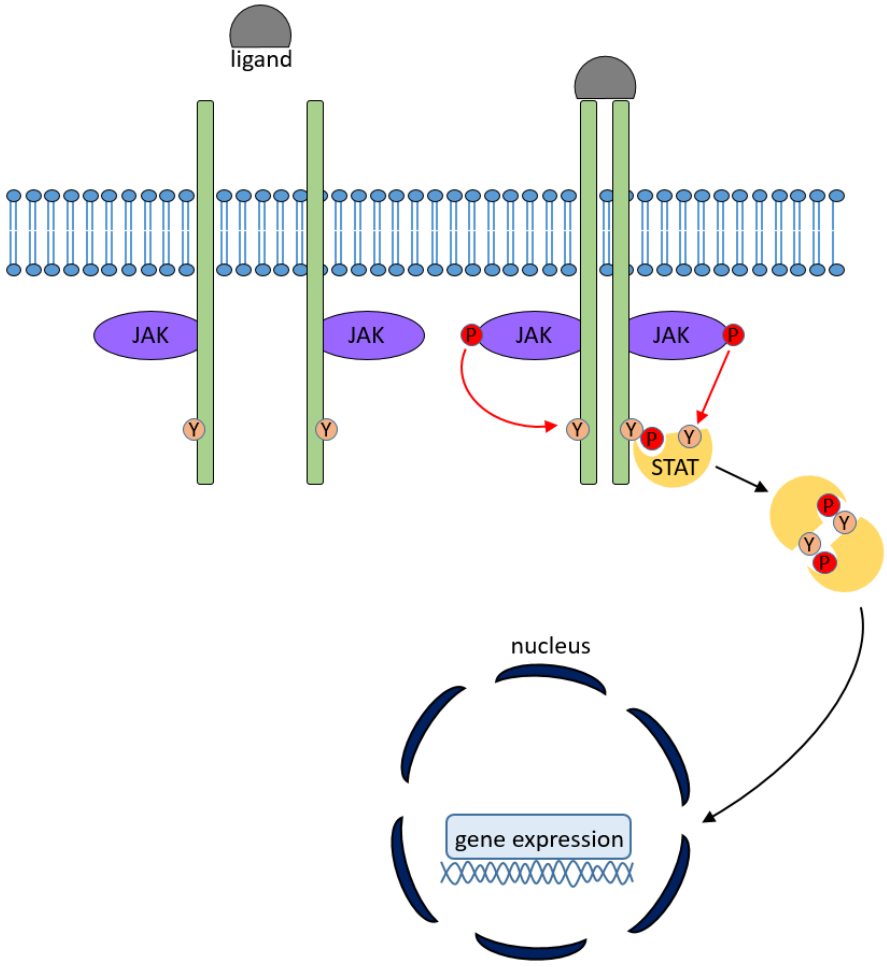


Figure 9: JAK/STAT signalling pathway (adapted from Heinrich et al., 2003 and Haan et al., 2006). Y (Tyrosine residues), P (phosphorylation).

JAKs consist of four domains, the N-terminally located FERM-domain, SH2-like domain, pseudokinase domain and a C-terminally located signalling protein kinase domain, that is catalytically active (Fig. 10). The two-faced god Janus gave JAKs their name, due to its resemblance with the two kinase domains that are present in JAKs. The JAK-receptor interaction is mediated by the FERM domain and the SH2-like domain (Radtke et al., 2005; Zhao et al., 2009), which also seem to play a role in JAK kinase activity regulation (Zhao et al., 2010). The pseudokinase domain, shares a similarity with other kinase domains, like with the adjacent tyrosine kinase domain, but lacks crucial residues and therefore remains catalytically inactive. Nevertheless, it is believed to play a critical role in regulating the JAK kinase domain (Saharinen et al., 2000; Toms et al., 2013).

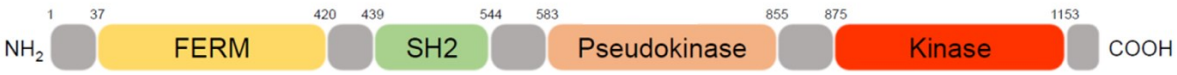


Figure 10: Domain organization of the JAK1 protein (Lukes et al, 2020)

4.1. JAK mutations in hematology

Aberrant activation of JAK/STAT signalling plays a major role in hematological malignancies. Mutations in the JAK family are usually point mutations and are often associated with inferior prognosis (Flex et al., 2008). Interestingly, somatic gain of function mutations are most frequently located in the pseudokinase domain (Haan et al., 2010).

Somatic JAK1 mutations occur recurrently in both childhood and adult acute leukemias (Jeong et al., 2008; Zhang et al., 2012). In adults they are present in about 20% of T-cell precursor ALL and have been linked to poor prognosis and response to therapy (Flex et al., 2008). JAK1 mutations are less common, but still recurrent, in B-cell ALL (Mullighan et al., 2009c), T-cell prolymphocytic leukemia (Bellanger et al., 2014) and in AML (Xiang et al., 2008). The aberrations are usually located in the pseudokinase domain, as for example the V658F mutant (Flex et al., 2008; Jeong et al., 2008), that has been proven as activating (Staerk et al., 2005) and is an equivalent to the frequent and well-studied JAK2 V617F. The crystal structure of the JAK1 pseudokinase mutant V658F has served as a model for deciphering the role of the

pseudokinase in JAK activation. It has been postulated that three residues, Val658, Phe575 and Phe636, termed as the F-F-V triad, play an important role in this process (Toms et al., 2013). The triad is highly conserved and seems to be evolutionarily coupled. If Val658 is mutated into Phe658, as in the JAK1 V658F mutant, upon activation the Phe658 displaces Phe575, causing a switch resulting in the rearrangement of the SH2-PK linker (Fig. 11).

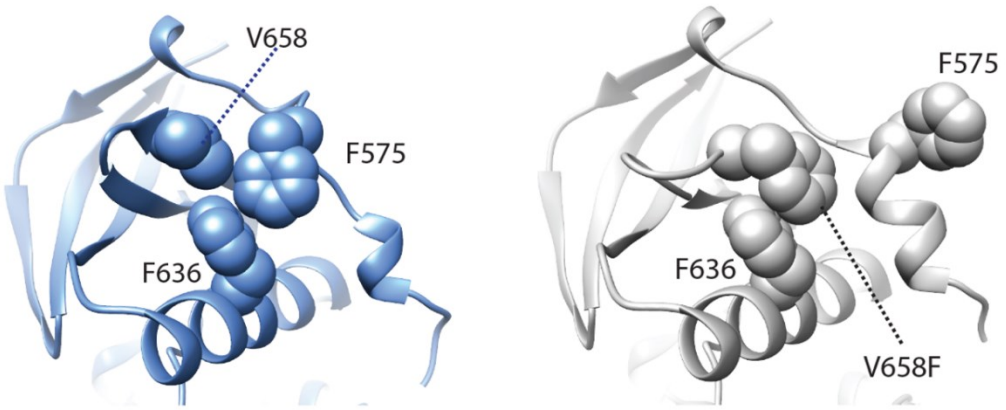


Figure 11: The pseudokinase domain in JAK1 wild type (blue) and in the JAK1 V658F mutant (grey). A conformation switch can be observed in JAK1 V658F, with the displaced F575 residue.

JAK2 mutations, which are associated with rearrangements of CRLF2 (Mullighan et al., 2009a), are present in 20% of B-ALL patients with DS (Gaikwad et al., 2009; Kearney et al., 2009) and in a lesser extent also in non-DS patients (Mullighan et al., 2009c). Importantly, JAK2 mutations have been tightly linked with myeloproliferative neoplasms (Baxter et al., 2005). The substitution of valine 617 to phenylalanine (JAK2 V617F) is present in more than 90% of patients with polycythemia vera (James et al., 2005; Kralovics et al., 2005) and in 50% of patients with essential thrombocythemia and primary myelofibrosis (Levine et al., 2005). It is a gain of function mutation that is thought to activate JAK2 by the same conformation switch as described above on the JAK1 V658F mutant (Fig. 11). In this case the three crucial residues are represented by V617, F537 and F595 (Bandaranayake et al., 2012; Leroy et al., 2016; Toms et al., 2013). *In vitro*, JAK2 V617F induced cytokine independent growth (James et al., 2005) and a myeloproliferative-like disorder *in vivo* in mouse models (Lacout et al., 2006).

Mutations in JAK3 have been associated with T-ALL (Zhang et al., 2012), adult T-cell leukemia and lymphoma (Elliott et al., 2011; Kameda et al., 2010) and also with TMD and AMKL (De Vita et al., 2007; Kiyoi et al., 2007; Malinge et al., 2008; Riera et al., 2011; Walters et al., 2006).

Aims

The aim of this Ph.D. study was to identify and subsequently characterize novel genetic aberrations in childhood acute leukemias.

1. To elucidate the alternative pathogenic mechanism of TMD development in the absence of trisomy 21.

a. perform comprehensive genomic and transcriptomic profiling of a non-Down syndrome TMD.

b. characterize the novel GATA1 D65_C228del mutation

c. characterize the novel JAK1 F636del mutation

d. study the impact of JAK1 F636del on GATA1s induced deregulation of erythroid and megakaryocytic lineage development in a murine TMD model

2. To characterize two novel fusion gene, AIF1L-ETV6 and ABL1-AIF1L, in an ETV6-ABL1-positive pediatric ALL and to describe the chromosomal rearrangement(s) that led to their formation.

a. thoroughly analyze the genomic profile of an exceptional ETV6-ABL1-positive ALL

b. identify genomic fusion sites of the AIF1L-ETV6, ABL1-AIF1L and ETV6-ABL1 fusion genes

c. characterize the chromosomal rearrangement(s) that led to the production of the fusion genes

d. perform backtracking analysis in order to investigate the potential prenatal origin of the observed rearrangement(s).

e. analyze potential AIF1L chimeric proteins

3. To investigate the feasibility of genomic fusion identification and subsequent fusion-gene based MRD monitoring in patients harboring PML-RARA, CBFB-MYH11 or RUNX1-RUNX1T1.

- results not discussed in the thesis; manuscript under consideration (attached)

Methods

Biological samples

Total DNA and RNA was isolated from mononuclear cells that were separated by density centrifugation of patient's diagnostic and remission bone marrow aspirates and peripheral blood samples as part of the routine sample processing procedure. The archived Guthrie card containing the patient's neonatal blood was obtained from the national central repository. The study was approved by the Institutional Review Board of the University Hospital Motol and informed consent was obtained from patients' parents in accordance with the Declaration of Helsinki.

Single-nucleotide polymorphism assay (SNPa)

Copy number aberrations (CNA) and regions of uniparental disomy (UPD) were analyzed using CytoScan HD array (Affymetrix, Santa Clara, CA, USA). The Chromosome Analysis Suite software (Affymetrix) was used for genotype calling, quality control, CNV/UPD identification and data visualization. Results were manually curated, deletions corresponding to somatic rearrangements of the immunoglobulin and T-cell receptor gene loci and common population variations were excluded. In case of the TMD patient a sample of peripheral blood containing 56% of blasts (as assessed by flow cytometry) was utilized for the analysis. For the patient with pre-BCP ALL, the SNPa analysis was performed as a service in the Laboratory for Molecular Biology and Tumor Cytogenetics at the Department of Internal Medicine of Hospital Barmherzige Schwestern (Linz, Austria).

Analysis of acquired mutations and fusion transcripts by whole exome and whole transcriptome sequencing

DNA and total RNA was used for sequencing libraries preparation using Agilent SureSelectXT HumanAllExon V5 and Agilent SureSelect mRNA Strand Specific kits, respectively, according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Read pairs were aligned to the human genome reference (hg19) using BWA (Li and

Durbin, 2010) (WES) and STAR (Dobin et al., 2013) (RNAseq) aligners and further processed by Picard tools (http://broadinstitute.github.io/picard/). VarScan (Koboldt et al., 2012) (WES) and Samtools (http://samtools.sourceforge.net/; RNAseq) were used for variant calling. Variants detected in remission samples were excluded from the analysis. TopHat (Kim and Salzberg, 2011) and deFuse (McPherson et al., 2011) algorithms were used for the analysis of presence of fusion transcripts in RNAseq data.

In the case of the trisomy 21-independent TMD patient the peripheral blood sample containing 56% of blasts was utilized for RNA isolation and subsequent RNAseq. DNA for WES was isolated from blasts that were sorted based on their immunophenotype by a fluorescence assisted cell sorter (FACS). WES was also performed on DNA isolated from non-tumor cells (FACS-sorted peripheral blood B and T lymphocytes) to facilitate the identification of somatic tumor-specific SNV and indels. Identified mutations were confirmed by Sanger sequencing and were annotated using the following NCBI transcript reference sequences: GATA1 – NM_002049.4, JAK1 – NM_002227.2, FN1 – NM_212482.1, SPIRE2 – NM_032451.1.

Fusion gene screening

The BCR-ABL1, ETV6-RUNX1, KMT2A-AFF1 and TCF3-PBX1 fusions were screened by in-house developed multiplex reverse transcription PCR (RT-PCR). This was done as part of the routine molecular genetic diagnostics. A single PCR reaction, which combined primers for all four fusion, enabled the amplification of the ETV6-ABL1 fusion.

Identification of genomic fusion sites

Long distance PCR was performed using AccuPrime™ Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. A series of primers annealing to various regions of the respective introns of ETV6, ABL1 and AIF1L were used. PCR products were analyzed by Sanger sequencing.

Backtracking of the ETV6-ABL1 fusion in archived neonatal blood (Guthrie card)

The specific detection of the ETV6-ABL1 genomic fusion was performed with the following PCR primers:

Primers used for ETV6-ABL1 detection (5' to 3')
forward: GGAAGGAGAGGGAACTATACTTGG
reverse: CCAGGCCCAATACAATGTAAAATAAAC

The DNA from the patient's diagnostic bone marrow sample was serially diluted into control healthy DNA and was used for the optimization of PCR conditions and to assess proper sensitivity. This initial optimization allowed us to achieve a sensitivity of 0.001%. The final PCR reaction included 12.5 µl of 2x SureDirect Blood PCR Master Mix (Agilent, Santa Clara, CA, USA), 1 µl of each primer (10 µM) and 2.5 µl $MgCl_2$ (25 µM). The final reaction volume was 25 µl. The cycling conditions were as follows: 5 min at 90^{o}C; 14 cycles at 95^{o}C for 30 sec, 65^{o}C for 30 sec (-0.5^{o}C each cycle), and 72^{o}C for 1 min; 40 cycles at 95^{o}C for 30 sec, 58^{o}C for 30 sec, and 72^{o}C for 1 min; 72^{o}C for 5 min. Guthrie card segments (1/12 of blood spot) with the archived patient's neonatal blood were directly added to the PCR tube. Guthrie card segments of the corresponding size, but without any blood, were added into the PCR tubes with positive control. The positive control tubes contained diluted patients diagnostic DNA. This was done in order to demonstrate the actual sensitivity of this assay which could potentially be compromised by Guthrie card material presence. The PCR products were finally analyzed by Sanger sequencing.

Cloning

Cloned AMV First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used for RNA transcription into cDNA. PCR-amplified whole coding sequences of GATA1 wt, GATA1 D65_C228del and JAK1 wt were cloned into a pWCC19 vector. InFusion HD Cloning Kit (Clontech, Takara Bio, Japan) was used for cloning. Sanger sequencing was used for the analysis of inserted coding sequences. pWCC19 vector-based constructs of JAK1 V658I, JAK1 K908G, JAK1 F636del, JAK1 F636del+K908G and GATA1 M1V (resulting in GATA1s) were generated from respective wild type constructs using QuikChange Lightning Site-Directed

Mutagenesis kit (Agilent, Santa Clara, CA, USA). Primers used for the PCR reactions are listed below.

Primers used for amplification from cDNA and for cloning (5' to 3')
JAK1 forward: GTCGACCTCGAATCGGATCCGAACACTGGACAGCTGAATAAATGC
JAK1 reverse: AGATTCCTGCAGCCCGGGCAGGAGAAGGACTTGATAATCTGTGG
GATA1 forward: GTCGACCTCGAATCGGATCCAGGTTAATCCCCAGAGGCTCC
GATA1 reverse: AGATTCCTGCAGCCCGGGCATGCTCTGTGCCCTCATGAG

Primers used for mutagenesis (5' to 3')
JAK1 K908G forward: GGAGCAGGTGGCTGTTGGATCTCTGAAGCCTG
JAK1 K908G reverse: CAGGCTTCAGAGATCCAACAGCCACCTGCTCC
JAK1 F636del forward: GGATATTTCCCTGGCCTTCGAGGCAGCCAGCATGATGAG
JAK1 F636del reverse: CTCATCATGCTGGCTGCCTCGAAGGCCAGGGAAATATCC
JAK1 V658I forward: CCTCTATGGCGTCTGTATCCGCGACGTGG
JAK1 V658I reverse: CCACGTCGCGGATACAGACGCCATAGAGG
GATA1 M1V forward: CCCAGAGGCTCCGTGGAGTTCCCTGGCCTGG
GATA1 M1V reverse: CCAGGCCAGGGAACTCCACGGAGCCTCTGGG

ABL1-AIF1L, AIF1L-ETV6 and wild type AIF1L constructs were produced from total RNA isolated from the patient's diagnostic bone marrow sample and from HEK293T cells. Cloned AMV First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used for reverse transcription of RNA. HEK293T cDNA was used for the amplification of full-length coding sequence of AIF1L wild type. The patient's cDNA was used for the amplification of coding sequences of fusion transcripts ABL1-AIF1L and AIF1L-ETV6. InFusion HD Cloning Kit (Clontech) was used for cloning of the PCR products into the pIRES2-EGFP vector. Inserted sequences were confirmed by Sanger sequencing.

Primers used for amplification from cDNA and for cloning (5' to 3')
AIF1L forward: CTCGCCATGTCGGGCG
AIF1L reverse: CGGGGTCCTCAGGGCAG
ETV6 reverse: GGTGGACTGTTGGTTCCTTCAGC
ABL1 forward: CCCTCTTCTGGAAAGGGGTACC

Analysis of the AIF1L-ETV6 and ABL1-AIF1L fusion transcripts

RT-PCR and subsequent Sanger sequencing verified the AIF1L-ETV6 and ABL1-AIF1L fusion transcripts identified by RNAseq. Fusion sequences from RNAseq served as bases for RT-PCR primer design:

Primers used for RT-PCR (5' to 3')
AIF1L-ETV6 forward: GCAGCTACAGAGGATTTCATGTTCC
AIF1L-ETV6 reverse: CATAGGTCATGTTTGTTCTGTTCTTATGG
ABL1-AIF1L forward: CTCTACGCTCGCTGACCGTTC
ABL1-AIF1L reverse: TGAGGACAGCCGACCGTTTC

The same PCR systems were used for the analysis of the presence of these particular fusion transcripts in 10 ETV6-ABL1-positive leukemia patients collected during our previous study (Zaliova et al., 2016).

Cultivation and transient transfection of HEK293T cells and NIH cells

The HEK293T (human embryonic kidney carcinoma) and NIH3T3 (murine fibroblast) cell lines were kindly provided by the Tenen lab (Harvard Medical School). The cell lines were cultivated in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Biosera, Nuaille, France) and Antibiotic-Antimycotic (Thermo Fisher Scientific). Lipofectamine 2000 (Thermo Fisher Scientific) was used for the transfection of plasmid constructs according to manufacturer's instructions.

Western Blot

RIPA buffer was used to prepare whole cell extracts. NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) supplemented with Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) were used to extract nuclear and cytoplasmic protein lysates. Proteins were resolved by the Bolt 4–12% Bis-Tris Plus protein gels (Thermo Fisher Scientific). The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Non-specific binding was blocked with phosphate-buffered

saline containing 0.1% Tween 20 (Bio-Rad) and 5% dry milk for 1 hour. The following primary antibodies were used for immunoblotting: GATA1 (ab11852, 1:500, Abcam, Cambridge, UK), Jak1 (sc-376996, 1:500, Santa Cruz, Dallas, TX, USA), p-Jak1 (Tyr1034/1035, 74129, 1:1000, Cell Signaling, Danvers, MA, USA), TBP (ab63766, 1:1000, Abcam), GAPDH (G8795, 1:10000, Sigma-Aldrich), Stat3 (sc-8019, 1:500, Santa Cruz), p-Stat3 (Tyr705, 9145, 1:1000, Cell Signaling). Primary antibodies against the N-terminus of AIF1L (HPA056852; 1:500, Sigma-Aldrich) and the C-terminus of AIF1L (HPA020522; 1:250, Sigma-Aldrich). Incubation with the primary antibody was done overnight. The primary protein bound antibodies were detected with an appropriate secondary antibody, which was conjugated with horseradish peroxidase and Super Signal West Pico Chemiluminescent Substrate kits (Thermo Fisher Scientific). Visualization was performed using an ECL system (Bio-Rad).

Lentivirus production

HEK293T cells were used for lentivirus production. pWCC19 vectors together with packaging plasmids (p-gag-pol, p-VSV-G) were co-transfected into the cells using Xfect Transfection Reagent (Clontech). Centricon Plus-70 Centrifugal Filter Devices (Merck Millipore, Burlington, MA, USA) were used for the concentration of the collected supernatants. Virus titer was assessed in the NIH3T3 cell line.

Ba/F3 cell proliferation

Ba/F3 cells were purchased from DSMZ (ACC 300). They were cultured in RPMI (Thermo Fisher Scientific) with 10% FBS, 10µg/ml interleukin 3 (IL3; Sigma-Aldrich, St. Louis, MO, USA) and Antibiotic-Antimycotic. Transduced Ba/F3 cells positive for GFP were sorted. For the IL3 withdrawal experiments Ba/F3 cells transduced by JAK1 F636del/F636del+K908G/V658I/JAK1 wild type or the empty vector were washed with PBS 3 times. Then they were cultured in the absence of IL3 for 10 days. Cells were counted every other day using trypan blue to assess proliferation.

TF1 cell proliferation

TF1 (human erythroleukemia) cells were transduced with JAK1 F636del/JAK1 wild type/GATA1 D65_C228del/GATA1 M1V/GATA1 wild type or empty vector. The target transduction efficiency was 10%. After 72 hours cells were split into two populations. The control population was cultured in the presence of 5ng/ml of human recombinant granulocyte macrophage-colony stimulation factor (GM-CSF). The second cell population was washed 3 times with PBS and cultured in the absence of GM-CSF. Cell proliferation was monitored by assessing GFP positivity.

K562 cell assay

The K562 (chronic myeloid leukemia in blast crisis) cell line was transduced with GATA1 D65_C228del/GATA1 M1V/GATA1 wild type or empty vector. Transduction efficiency was assessed by GFP-positivity. The cells were stimulated with 10ng/ml Phorbol-12-myristate-13-acetate (PMA) to induce differentiation. Dimethylsulfoxid (DMSO) served as control. Flow cytometry analysis was conducted 1, 2 and 3 days after stimulation.

Human adult CD34-positive HSPC assay

Human CD34-positive adult hematopoietic stem and progenitor cells (HSPCs) were isolated from peripheral blood samples of healthy donors. The cells were transduced with GATA1 D65_C228del/GATA1 M1V/GATA1 wild type or empty vector. Differentiation was induced by change of media two days after transduction. The differentiation media included 5ng/ml stem cell factor (SCF), 5ng/ml GM-CSF, 10ng/ml granulocyte-colony stimulating factor (G-CSF) and 5ng/ml IL3. Differentiation was evaluated by flow cytometry 5 and 9 days after stimulation.

Colony-forming assays

Murine bone marrow was extracted from long bones and hips of C57BL/6J mice by crunching. Murine fetal liver cells were isolated from E13.5 mouse embryos. Erythrocytes were lysed using ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid). Biotinylated anti-CD117 antibody was used for c-kit+ cells staining. Cells were separated from the bone marrow using Anti-Biotin MicroBeads UltraPure (Miltenyi Biotec, Bergisch Gladnach, Germany) by MACS. The c-kit+ cells were lentivirally transduced with JAK1 F636del/JAK1 wild type or empty vector. Transduced cells were expanded for 48 hours in IMDM with 15% FBS, mSCF, mIL3, mIL6 (Peprotech, Rocky Hill, NJ, USA). Transduced c-kit+ GFP expressing cells were sorted and seeded in MethoCult GF M3434 medium (Stemcell Technologies, Vancouver, BC, Canada). Microscopy was used for counting and classification of the cell colonies.

Competitive growth assay of Gata1s-positive mouse fetal liver cells.

Ter119 depleted fetal liver cells were isolated from E13.5 Cas9 knock-in mouse embryos. Cells were transduced with a Gata1-sgRNA expression vector. Transduced cells were cultured under low cytokine conditions for the duration of 3 weeks. A population of transduced Gata1s cells was obtained by this selection as previously described (Labuhn et al., 2019). Gata1s expression in these cells was confirmed by Western blot analysis. The Gata1s-positive cells were transduced with JAK1 F636del/JAK1 wild type or empty vector. In one experimental setting the cells were simultaneously transduced with Gata1-sgRNA and JAK1 F636del/JAK1 wild type. Double positive cells were measured every other day by flow cytometric analysis to assess their percentage. Cells were cultured either in a fully cytokine-supplemented growth-supportive media (mSCF, mTPO) or in a cytokine-depleted growth-restrictive (only mSCF/only mTPO) media or in a media containing 0ng/ml / 0,1ng/ml / 1ng/ml or 10ng/ml of IL6.

Homology modelling

JAK1 F636del models were generated by Modeller (Webb and Sali, 2016) using the structure of JAK1 wild type as template (PDB entry 4L00) (Toms et al., 2013).

Results

Project 1. The alternative pathogenesis of TMD development in the absence of trisomy 21

1. The identification of a trisomy 21-independent TMD questions the indispensability of trisomy 21 in the pathogenesis of this preleukemic condition.

We have identified a unique TMD with a typical clinical and morphological manifestation (Table 1, Fig. 1-2) in a newborn without the features of Down syndrome. Immunophenotypic analysis of the peripheral blood, performed by flow cytometry, revealed 56% of atypical cells (Fig. 3-4). We compared the expression levels of selected antigens on the gated blast cells population from the non-DS-TMD with expression levels in DS-TMDs and with AMKL cases diagnosed and treated at our department. The non-DS-TMD exhibited an immunophenotype that differed from the DS-TMDs, particularly by a high expression of the CD61 and CD41 antigens and a weak expression of the CD33 antigen, resembling more an immunophenotype of AMKL (Fig. 3-4). Due to critical clinical manifestation, advanced symptomatic treatment was initially required. However, during the intensive 2-week treatment period after diagnosis (after birth) the clinical picture gradually improved and no chemotherapeutics were therefore administered. Due to the spontaneous remission of the blast population, which occurred at 2 months of age, the final diagnosis of TMD was confirmed (Fig. 5).

Clinical feature	DS-TMD (% of cases *)	Presented TMD
Hepatomegaly	40	Yes
Splenomegaly	30	Yes
Rash	11	Yes
Thrombocytopenia	50	Yes
Anemia	5-10	Yes (mild)
Circulating blast cells >10%	100	Yes

Table 1 (previous page): Typical clinical and hematological features of DS-TMD (based on data from Klusmann et al., 2008; Roberts et al., 2013; Tunstall et al., 2018) were also found in the here presented non-DS-TMD, exemplifying the clinical and hematological similarity of both entities.*

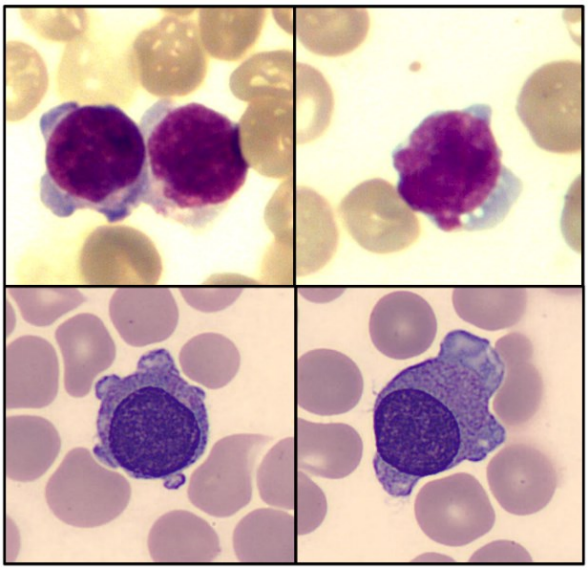


Figure 1: The examination of a bone marrow (top) and peripheral blood (bottom) smears identified blasts with prominent nucleoli, cytoplasmic blebs and a basophilic cytoplasm morphologically resembling megakaryoblasts. This finding is indicative of a TMD/AMKL.

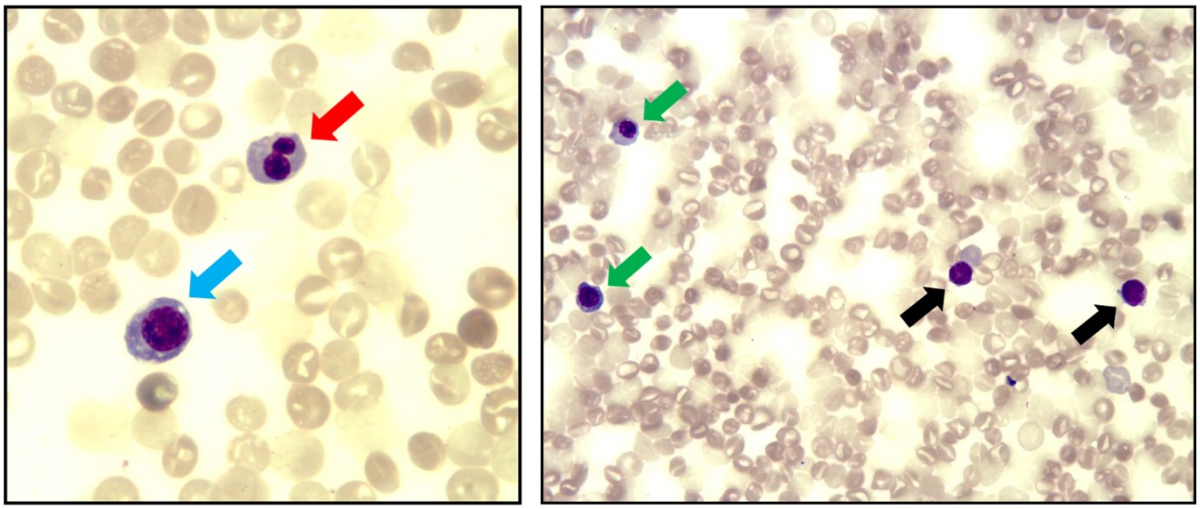


Figure 2. Signs of dyserythropoiesis in the bone marrow aspirate. The blue arrow shows one polychromatophilic erythroblast. The red arrow point an oxyphilic erythroblast. Its nucleus is atypically shaped with constrictions. The green arrows points to erythroblasts, whereas the black arrows point to atypical megakaryoblasts.

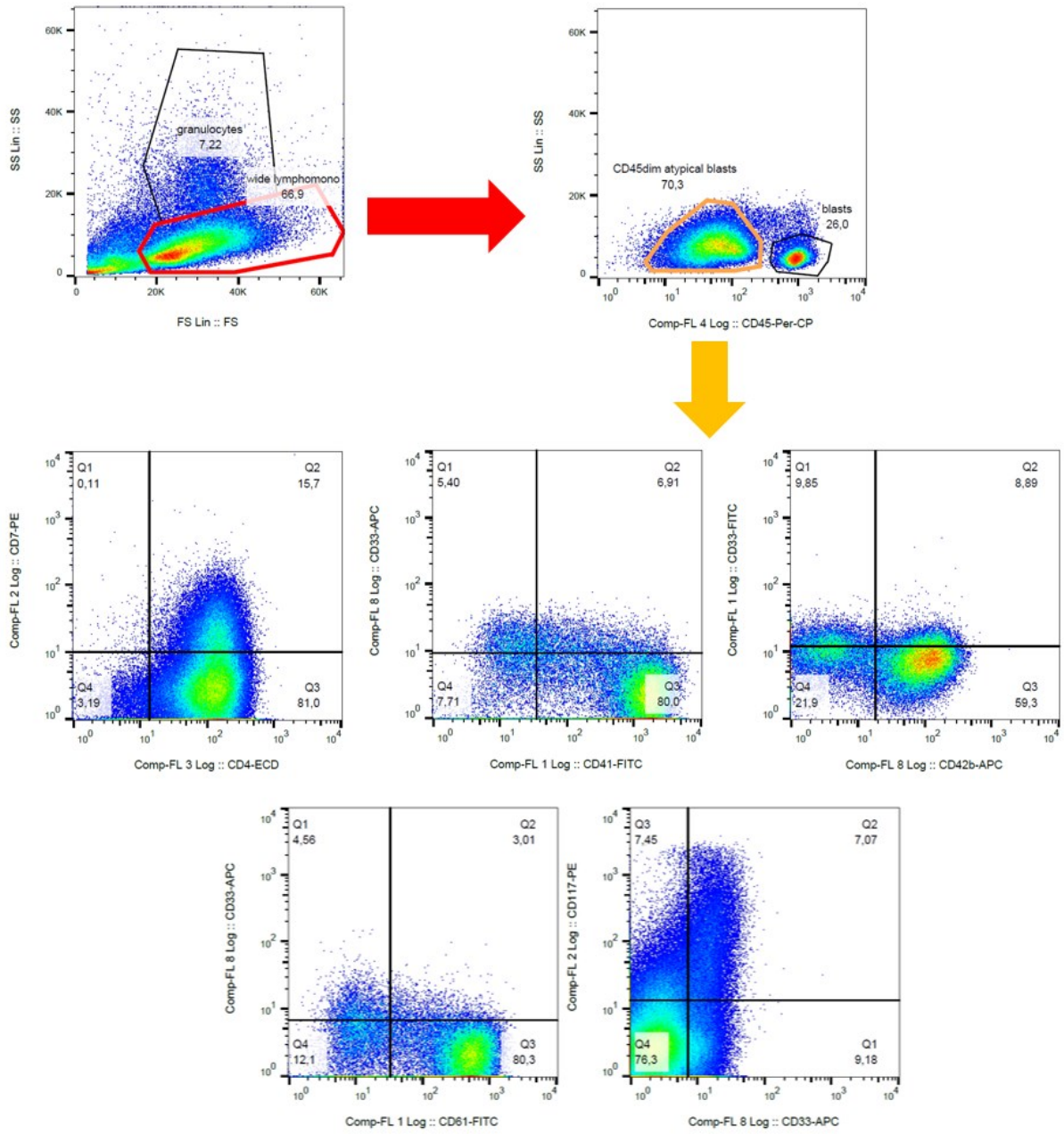


Figure 3: The diagnostic cell immunophenotyping analysis revealed atypical blasts that were gated as a CD45dim subpopulation from the lymphomonocytic compartment ("wide lymphomono"). A strong expression of CD41 (91%), CD61 (86%), CD42 (67%), CD4 (87%) and a weak expression of CD38 (26%), CD7 (14%), CD33 (12%), CD71 (42%) and CD117 (17%) was recorded. Dot plots show expression of selected antigens on the gated blasts.

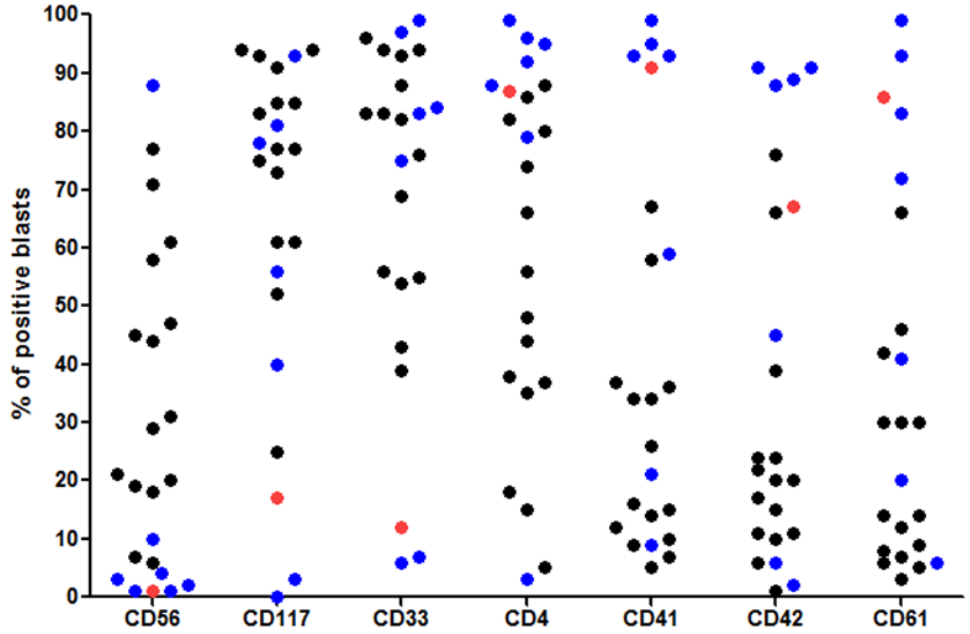


Figure 4: The comparison of immunophenotypes in AMKL patients (n=7, blue circles) and DS-TMD patients (n=15, black circles) treated at the University Hospital Motol (Department of Paediatric Haematology and Oncology) with the immunophenotype of the non-DS-TMD (red circles).

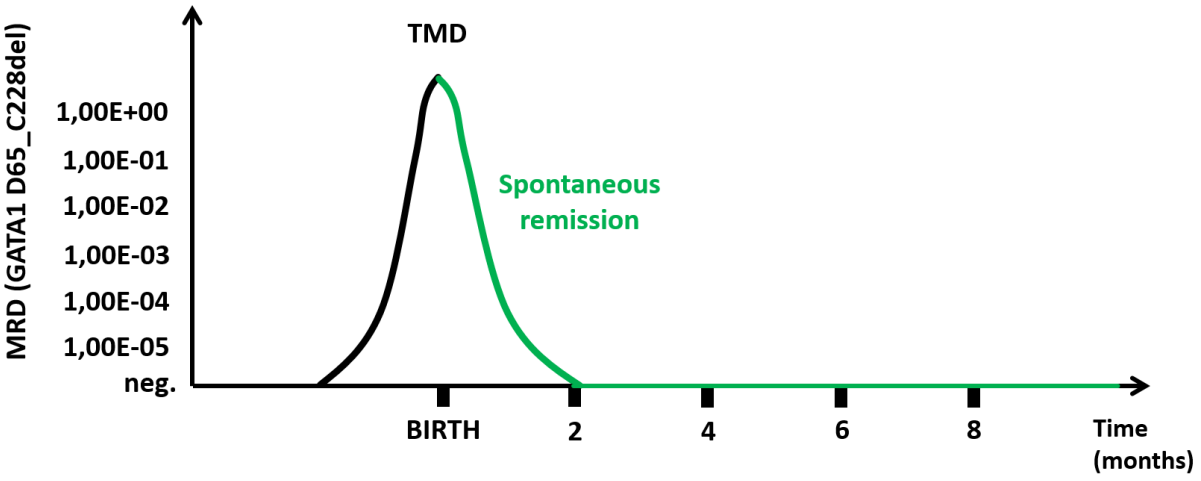


Figure 5: MRD monitoring of the patients' disease progression by quantifying GATA1 D65_C228del in peripheral blood samples. Spontaneous remission occurred at 2 months of age.

2. Cytogenetic and SNP array analysis confirms absence of trisomy 21 and genomic profiling identifies novel potential drivers of the trisomy 21-independent TMD development.

To exclude trisomy 21 involvement of the hematopoietic lineage, cytogenetic examination of the blast population was conducted. The FISH analysis of chromosome 21 was repeated twice and in both cases showed absence of trisomy 21. The examined blast cells had a normal male karyotype.

High-density SNP array analysis was performed to detect copy number changes (Fig. 6). Peripheral blood was used with a proportion of 56% of blasts (as assessed by flow cytometry). The analysis reliably excluded presence of whole-chromosome or partial trisomy 21 in the blasts.

We performed a comprehensive review of literature to establish if other trisomy 21-independent cases have been identified. All of the non-Down syndrome TMD cases that were described so far, harbored trisomy 21 in the blast population (Table 3). To the best of our knowledge the presented non-DS-TMD is the first described case of trisomy 21-independent GATA1-mutated TMD.

To elucidate the alternative pathogenesis of this exceptional TMD without trisomy 21 involvement we performed whole exome and whole transcriptome sequencing. We found in-frame deletions in the JAK1 and GATA1 genes and missense mutations in the SPIRE2 and FN1 genes (Table 2). All genetic aberrations were confirmed by Sanger sequencing.

JAK1 mutations are implicated in a number of hematological malignancies including TMD and AMKL. On the other hand SPIRE2 and FN1 are genes that do not have an established role in hematopoietic disorders.

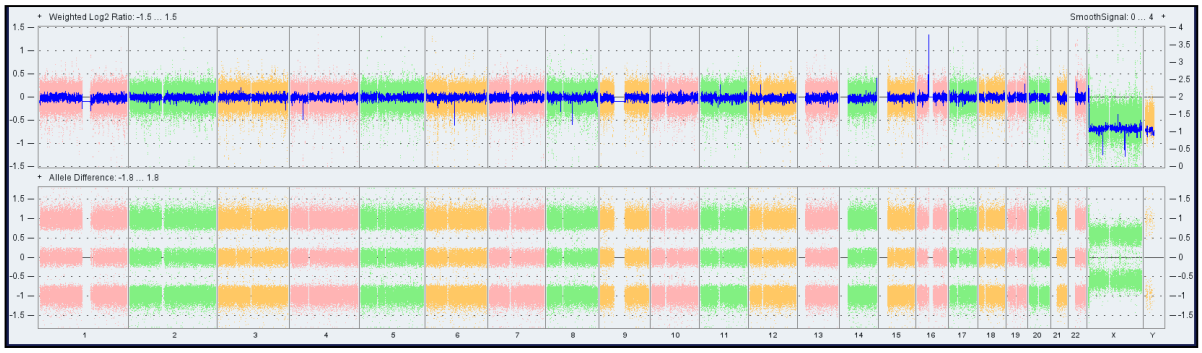


Figure 6: CNA analysis by SNP array. No aberrations were observed by CNA on any chromosome. No gain of chromosome 21 or its part was detected. Some regions with changed copy number are noticeable, mainly in the upper graph. These correspond to array specific artefacts and common population polymorphisms.

chr.	gene	mutation	VAF
X	GATA1	D65_C228del	100%
1	JAK1	F636del	35%
16	SPIRE2	R471W	52%
2	FN1	R2420C	48%

Table 2: Novel genetic aberrations discovered by WES which was performed using DNA from sorted blasts. Chr (chromosome), VAF (variant allele frequency).

Table 3 (next page): Literature review of non-Down syndrome TMD patients. M (male), F (female), CCR (complete clinical remission), AML (acute myeloid leukemia), AMKL (acute megakaryoblastic leukemia).

Reference	Sex	Presentation	Karyotype	Trisomy 21 in blasts	Follow-up	Outcome
Van den Berghe et al., 1983	M		46 XY	Pentasomy 21		CCR
Hanna et al., 1985	F	Facial swelling (left side)	46 XX	Yes	2,3 years	CCR
Jones et al., 1987	F	Fetal distress, cardiac murmur, hepatosplenomegaly	46 XX	Yes	3,5 years	CCR
	M	Petechiae	46 XY	Yes	30 months	CCR
Kalousek et al., 1987	M	Respiratory distress, hepatomegaly, cyanosis	46 XY	Yes		
Faed et al., 1990	F	Tachypnea, hepatosplenomegaly	46 XX	Yes	21 months	CCR
Ridgway et al., 1990	M	Respiratory distress, hepatosplenomegaly	46 XY	Yes	12 months	CCR
	F	Mild respiratory distress, hepatosplenomegaly, maculopapular rash	46 XX	Yes	5,5 years	CCR
Jiang et al., 1991	M	Hepatosplenomegaly	46 XY	Yes	15 months	AML
Brissette et al., 1994	M	Petechiae, hepatosplenomegaly	46 XY	Yes	12 months	AMKL
Kempski et al., 1998	F	Rash, hepatosplenomegaly	46 XX	Yes	42 months	CCR
Richards et al., 1998	F	Macular-papular rash, swelling of extremities	46 XX	Yes	24 months	AMKL
	M	Moderate respiratory distress, hepatomegaly, splenomegaly, conjunctivitis and an erythematous maculopapular facial rash	46 XY	Yes	2 months	CCR
Polski et al., 2002	M	Petechiae, hematuria	46 XY	Yes	20 months	AMKL
Wolfe et al., 2003	F	Hepatomegaly, vesiculo-pustular rash	46 XX	Yes	28 months	CCR
Magalhaes et al., 2005	F	Respiratory distress, hepatosplenomegaly	46 XX	Yes	5 years	CCR
Sandoval et al., 2005	F	Pericardial effusion, hepatosplenomegaly, rash	46 XX	Yes	30 months	CCR
Cushing et al., 2006	M	Respiratory distress	46 XY	Yes	24 months	CCR
Appollonsky et al., 2008	M	Hepatosplenomegaly, respiratory distress	46 XY	Yes	5 months	AML
	M	Petechiae	46 XY	Yes	66 months	CCR
Yanase et al., 2010	M	Severe fetal distress, severe cardiorespiratory distress, hepatosplenomegaly, severe renal failure, hemorrhagic diathesis	46 XY	Yes	12 months	CCR
Inaba et al., 2011	F	Hepatosplenomegaly, petechiae, bloody stool, epistaxis	46 XX	Yes		CCR
Tsai et al., 2011	F	Respiratory distress, hepatosplenomegaly, skin rash, hypoxic encephalopathy, intracranial hemorrhage	46 XX	Yes	26 months	MDS
	M	Intraabdominal mass since 32 week of gestation, huge hepatomegaly	46 XY	Yes	16 months	CCR
Rozen et al., 2013	M	Transient tachypnoe	46 XY	Yes		CCR
Ono et al., 2015	M	Purpura, petechia	46 XY	Yes	6 years	CCR
Salvatori et al., 2017	F	hypotonia., hepatomegaly	46 XX	Yes		CCR

3. The novel GATA1 deletion, GATA1 D65_C228del, results in the expression of an internally truncated protein lacking the entire N-terminal zinc finger domain.

All TMD patients harbor mutations in the GATA1 gene usually represented by small insertions, duplications and deletions which cluster in the second exon. These aberrations lead to the introduction of a premature stop codon or to the loss of an adjoining splice site and finally result in the exclusive production of a shorter version of GATA1, the GATA1s protein, which lacks the transactivation domain (Fig. 7-8). The novel GATA1 aberration, GATA1 D65_C228del, described in the trisomy 21-independent TMD presented here, causes a deletion of 1106 base pairs (bp) and an insertion of 9 bp spanning between the second and forth exon. The deletion is in-frame and results in the expression of an internally truncated protein. This protein lacks part of the TAD and more importantly also the whole N-terminal zinc finger (Fig. 7-8).

To elucidate if the aberrant protein is expressed, we cloned the full-length cDNA of GATA1 wt, GATA1s and GATA1 D65_C228del into lentiviral vectors. The GATA1s expressing construct was prepared by introducing a M1V mutation in the canonical start codon of GATA1. We studied the expression of GATA1 D65_C228del chimeric protein in HEK293T cells. The presence of GATA1 proteins in transiently transfected HEK293T cells was analyzed using a polyclonal anti-GATA1 antibody. We confirmed the expression of the GATA1 D65_C228del protein and showed that it is trafficked into the nucleus, similarly to the physiological isoforms (Fig. 8).

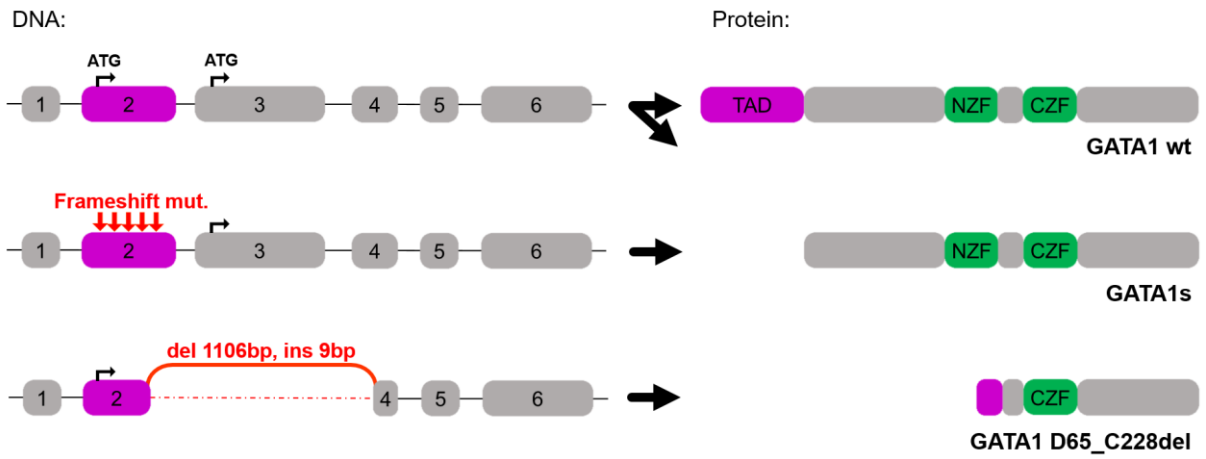


Figure 7: Schematic representation of the GATA1 gene. The upper model shows physiological production of GATA1 in a healthy individual where both GATA1 wt and GATA1s are expressed. The model in the middle represents the situation in trisomy 21-positive TMD patients were due to mutations in the second exon, exclusively GATA1s is expressed. The bottom diagram shows the novel mutation described in the presented patient, resulting in the production of an even shorter GATA1 variant. bp (base pair), del (deletion), ins (insertion), TAD (transactivation domain), NZF (N-terminal zinc finger), CZF (C-terminal zinc finger).

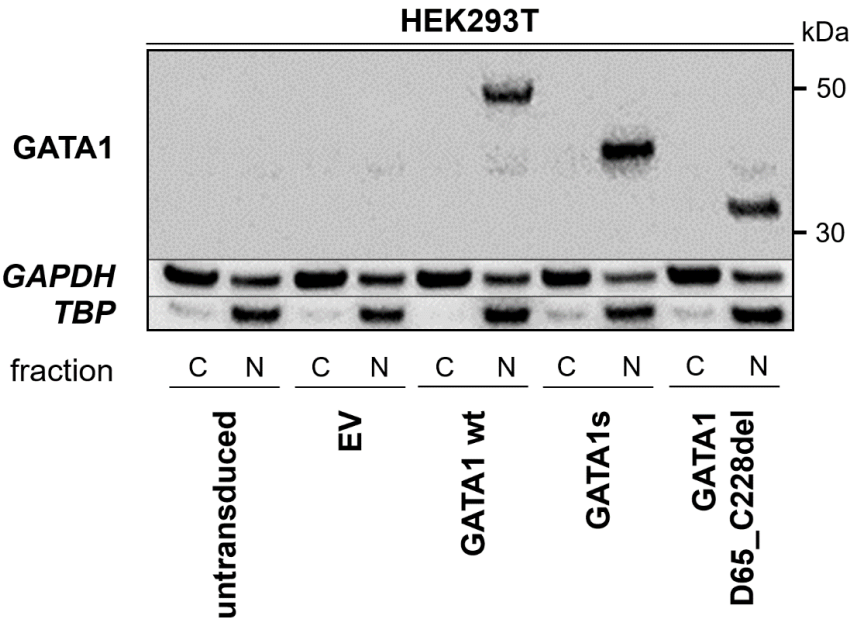


Figure 8: Western blot analysis of GATA1 wt, GATA1s and GATA1 D65_C228del expressing HEK293T cells. Different protein sizes are nicely visible. Protein separation into the cytoplasmic (C) and nuclear (N) fractions shows that all three transiently expressed proteins are trafficked into the nucleus. TBD and GAPDH serve as loading controls. EV (empty vector), kDa (kilodalton)

4. Searching for a model to study the effect of GATA1 D65_C228del on megakaryocytic and erythroid differentiation.

First, we decided to study the effect of GATA1 D65_C228del on megakaryocytic and erythroid differentiation in the human K562 erythroleukemia cell line. The K562 cell line has served as a valuable model for the study of mechanisms associated with the differentiation of leukemic cells (Jacquel et al., 2006; Lam et al., 2000; Racke et al., 2001; Rainis et al., 2005) and to study the role of GATA1 in hematopoiesis (Halsey et al., 2012; Huang et al., 2005; Matsumura et al., 2000). Most importantly, the K562 cells have been previously used to compare the effects of GATA1 wt and GATA1s (Halsey et al., 2012). The K652 cells express, in undifferentiated conditions, markers of both megakaryocytic and erythroid lineages. Depending on the stimulus, they can undergo further differentiation. Phorbol-12-myristate-13-acetate (PMA) stimulates K562 cells to undergo megakaryocytic differentiation (Dorsey et al., 2002; Huang et al., 2014; Kim et al., 2001; Pettiford and Herbst, 2003; Shelly et al., 1998). The PMA-induced megakaryocytic differentiation partially mimics the physiological processes that occur in the bone marrow (Long et al., 1990). The differentiation is accompanied by expression of specific megakaryocytic markers like CD61 and CD41, together with changes in morphology (Jacquel et al., 2006).

We introduced the empty vector (EV), GATA1 wt, GATA1s and GATA1 D65_C228del constructs into the K562 cells. A high (>90%) and similar transduction efficiency, measured by GFP positivity, was achieved with all four constructs (Fig. 9). Megakaryocytic and erythroid differentiation was induced two days after transduction by 10ng/ml of PMA. Dimethylsulfoxid (DMSO) served as control. Flow cytometry analysis was conducted one, two and three days after stimulation by measuring megakaryocytic (CD41, CD42b) and erythroid (CD235a, CD71) surface markers (Fig. 9). DAPI was used to distinguish viable from non-viable cells.

We hypothesised that introduction of GATA1 wt will allow the cells to differentiate into the megakaryocytic linage, whereas GATA1s introduction will decelerate or even arrest this differentiation as previously described (Halsey et al., 2012). Moreover we expected to see a similar or stronger effect induced by the GATA1 D65_C228del as compared to GATA1s. The PMA stimulation was successful. However, no difference between the GATA1 wt, GATA1s and GATA1 D65_C228del constructs in the expression of both erythroid and megakaryocytic

markers was visible (Fig. 10). We were not successful in optimizing the model. The lack of variation between GATA1 wt and GATA1s deemed this approach inappropriate for our usage.

Next, we conducted a similarly designed experiment on human CD34 positive adult hematopoietic stem and progenitor cells (HSPCs) isolated from peripheral blood samples. The cells were transduced with lentiviral empty vector, GATA1 wt, GATA1s and GATA1 D65_C228del constructs. Differentiation was induced by change of media two days after transduction. Myeloid (CD14, CD15), erythroid (CD71, CD235a) and megakaryocytic (CD41) differentiation was assessed by flow cytometry five and nine days after stimulation. Unfortunately, no difference was apparent between GATA1s and GATA1 D65_C228del, except for a slight difference in the expression of CD41, therefore making this model also unfit for assessing the impact of GATA1 D65_C228del (Fig. 11).

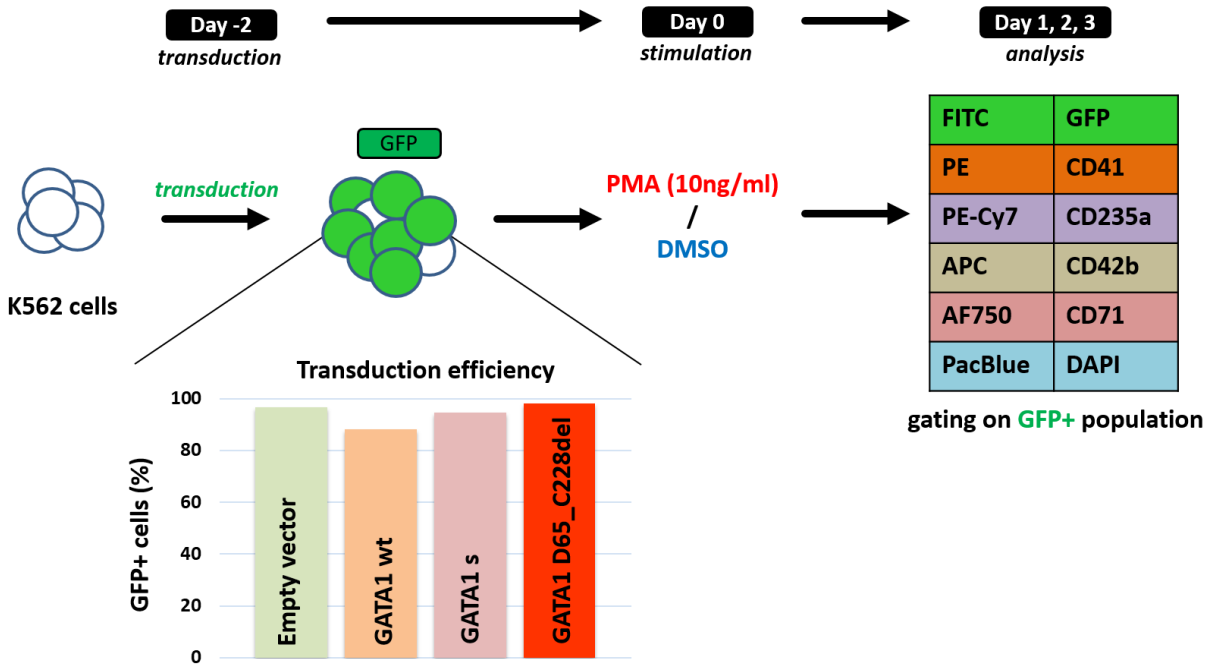


Figure 9: K562 experimental design and transduction efficiency

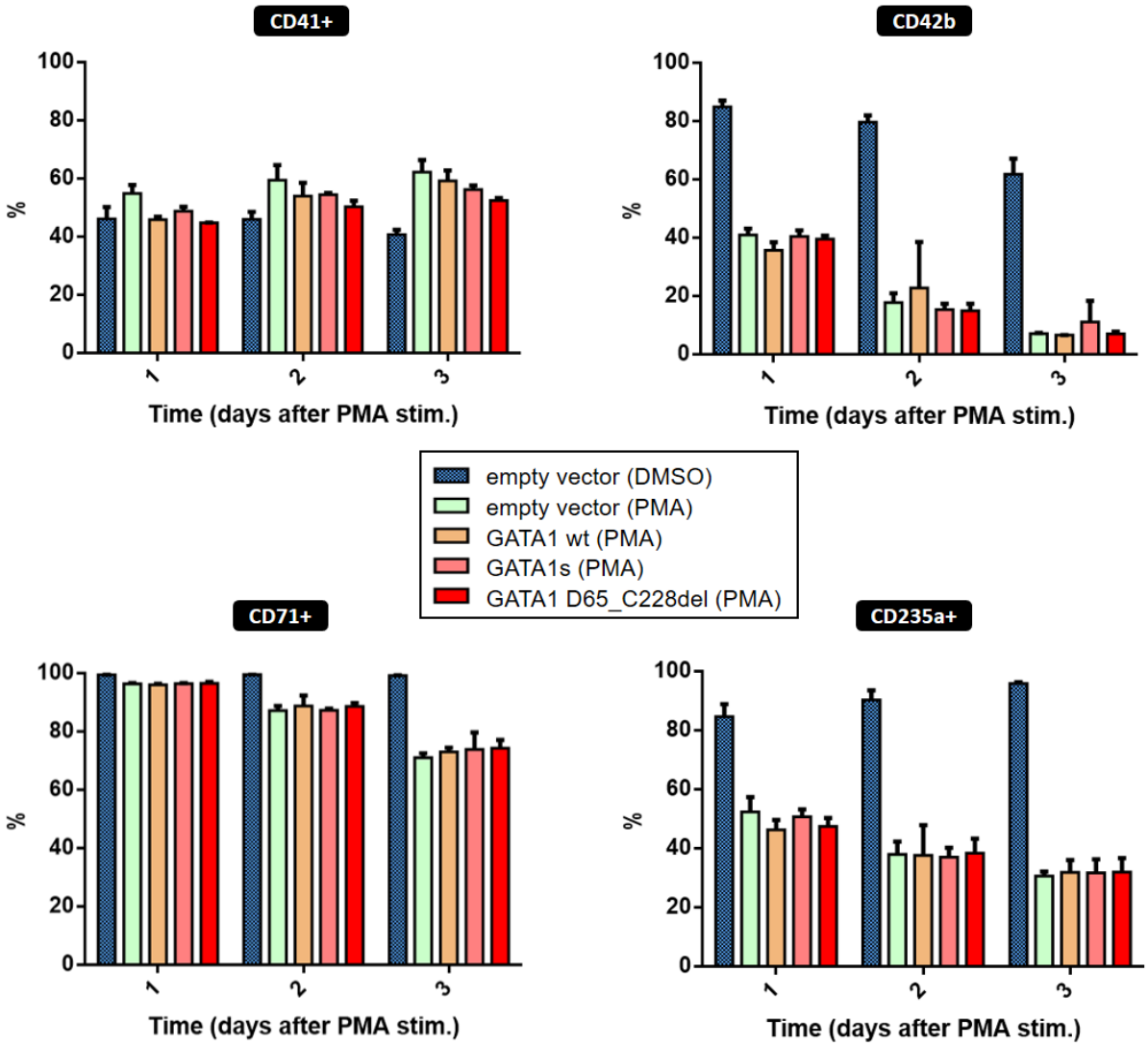


Figure 10: The expression of CD41, CD45b, CD71 and CD235a+ in lentivirally transduced K562 cells. Only GFP+ cells were used for the gating analysis.

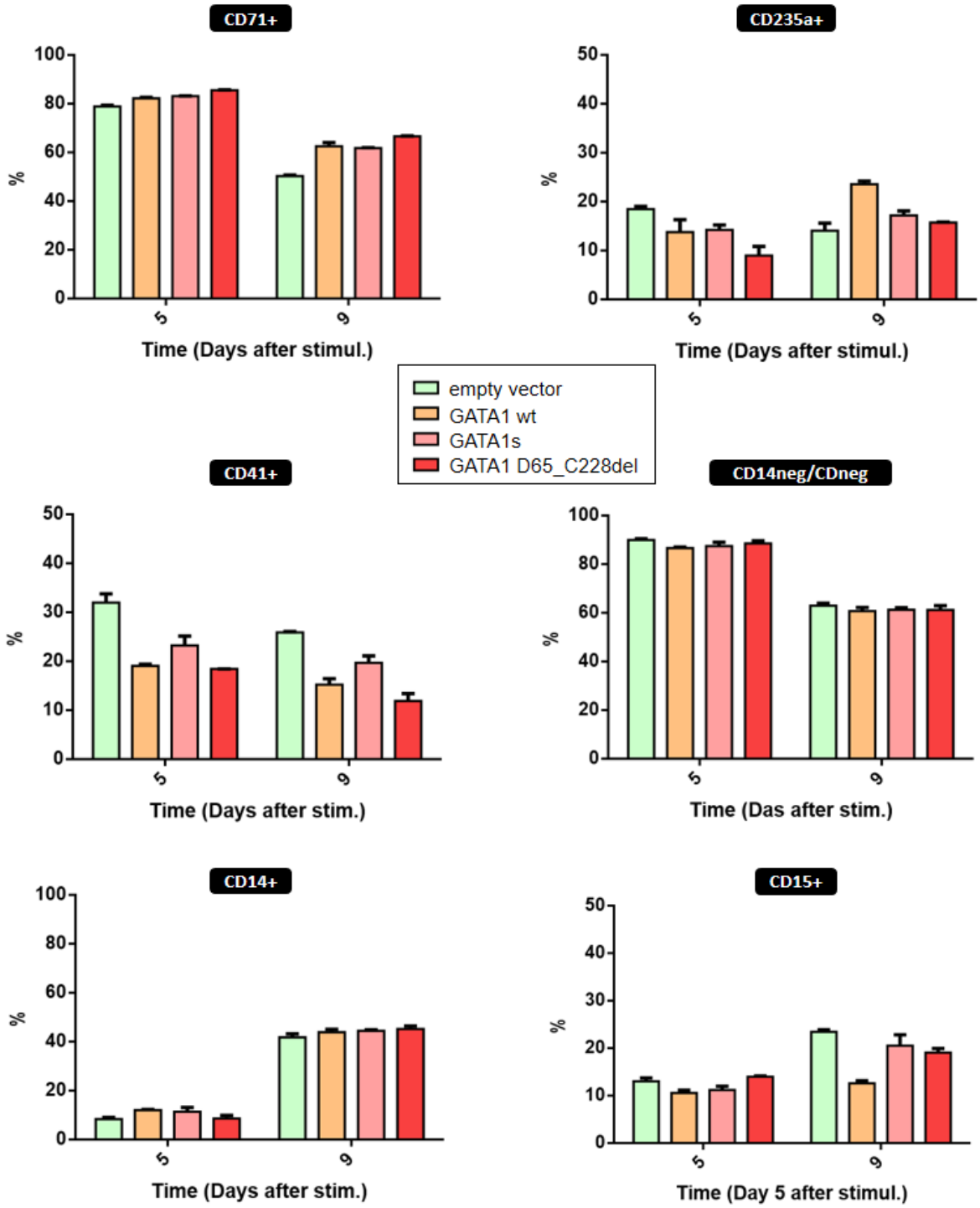


Figure 11: The expression of CD71, CD235a, CD41, CD14 and CD15 in lentivirally transduced human CD34+ HSPCs. Only GFP+ cells were used for the gating analysis.

Finally, we attempted to study the differences between GATA1s and GATA1 D65_C228del in the human erythroleukemia cell line TF1, which is dependent on GM-CSF and was at our disposal. This model has been previously used to assess the functionality of the CSF2RB A455D variant (Labuhn et al., 2019). The TF1 cells were transduced with GATA1 wt, GATA1s and GATA1 D65_C228del constructs, aiming for 10% transduction efficiency. Cells were cultured either with or without human GM-CSF. Cells harboring either GATA1 wt, GATA1s or GATA1 D65_C228del ceased to proliferate in both cytokine rich and cytokine free conditions, demonstrating the inapplicability of this model to study differences between GATA1 variants (Fig. 12).

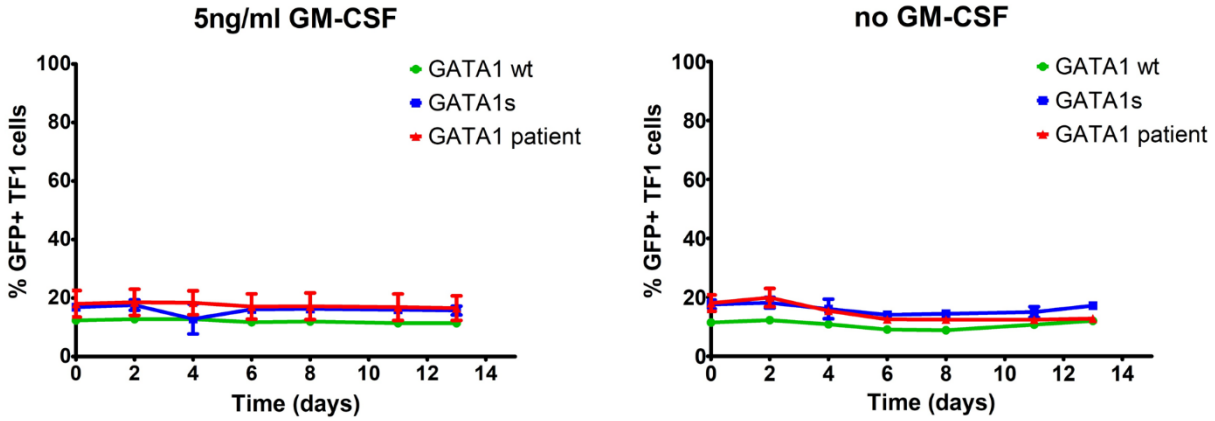


Figure 12: Transduced TF1 cells (with GATA1 wt, GATA1s or GATA1 D65_C228del*) cultured in the presence (left panel) or absence (right panel) of GM-CSF.*

5. The novel JAK1 mutation is located in a crucial part of the pseudokinase domain.

The identified novel mutation is located in the pseudokinase domain of JAK1, which is a hotspot region for activating mutations (Flex et al., 2008; Haan et al., 2010; Jeong et al., 2008) (Fig. 13). JAK mutations have been described previously as drivers in hematological malignancies, including AMKL (Chen et al., 2012; Labuhn et al., 2019). The deletion results in the loss of phenylalanine 636 (F636del) which is a highly conserved aminoacid among various species (Fig. 14). Moreover, it belongs to a highly conserved triad of aminoacids, namely V658, F636, F575, which is believed to control JAK1 catalytic activity by mediating a conformation switch between the active and inactive forms (Toms et al., 2013). Therefore JAK1 F636del

seemed as the likely candidate for a driver mutation that could cooperate with GATA1 D65_C228del on TMD induction without the involvement of trisomy 21.

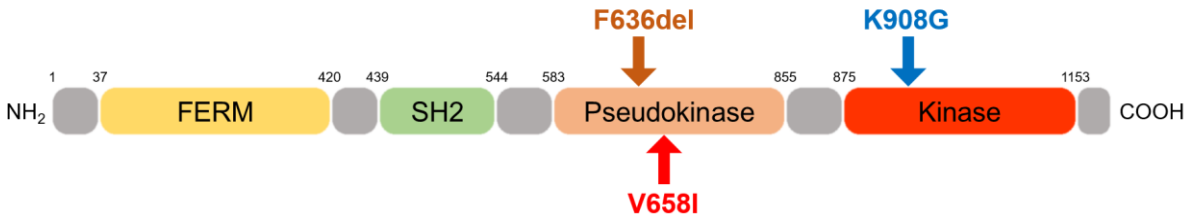


Figure 13: Schematic representation of the JAK1 protein and its domain organization. The brown arrow highlights the deletion of F636 which is located in the pseudokinase domain. The red arrow highlights a known activating mutation V658I, the blue arrow highlights an inactivating mutation in the kinase domain. V658I and K908G were used as controls.

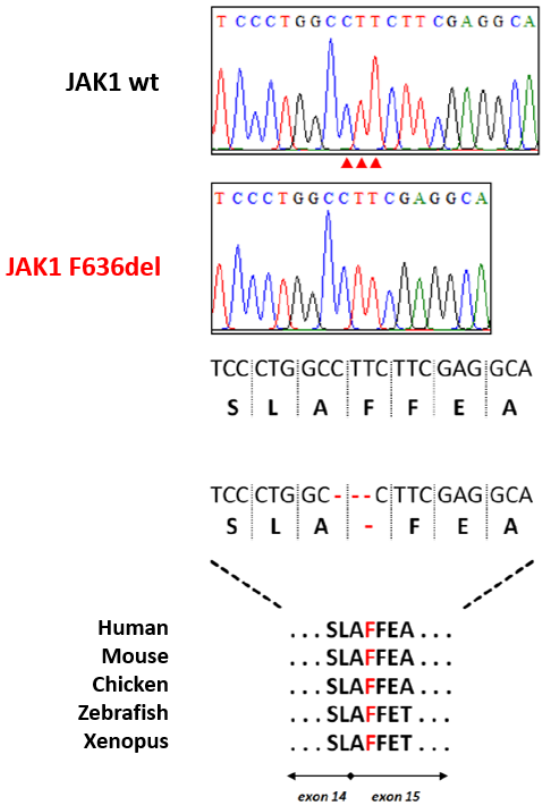


Figure 14: The comparison of the wild type and mutated allele F636del (shown in red) of the JAK1 gene. The nucleotides are denoted by capital letters and aminoacids in bolt, dashes indicate missing nucleotides/aminoacids. The comparison between various animal species points to a high conservation of the phenylalanine located on the 636 position in the JAK1 gene (shown in red).

We performed homology modeling of the mutated JAK1 pseudokinase domain which suggested that JAK1 F636del is compatible with both the active and the inactive conformation. Moreover, the mutated pseudokinase may adopt a third conformation. This alternative conformation, which was not achieved, or previously described, by the wild type JAK1 pseudokinase, may potentially mimic the “inactive” state (Fig. 15). We hypothesized that the neighboring phenylalanine F635 could replace the deleted phenylalanine F636 in its vacant position. The modeling suggested otherwise. F635 of the mutated JAK1 F636del, in both the active and inactive conformation, was modelled into a similar position as in JAK1 wild type (Fig. 16).

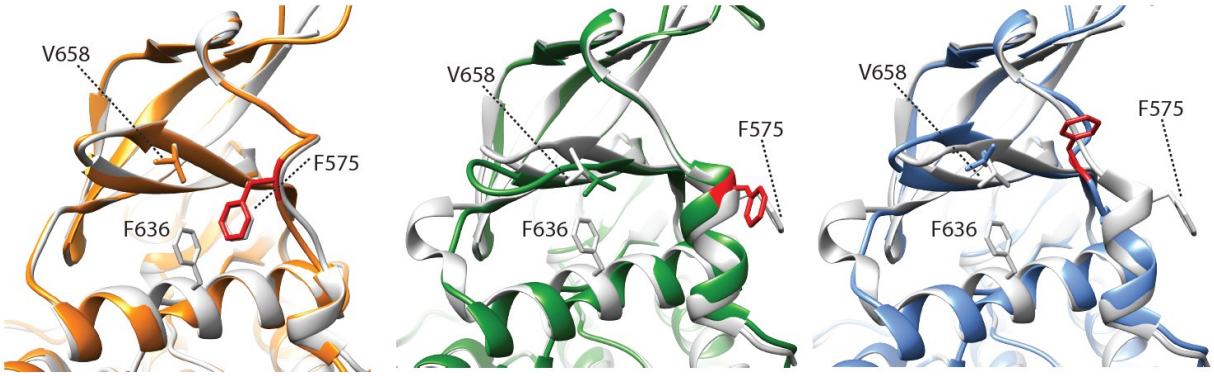


Figure 15: Homology modeling of the JAK1 pseudokinase domain. Three predicted conformations of the mutated pseudokinase domain (JAK1 F636del) were superposed with the JAK1 wild type structure (white; PDB entry 4L00; Toms et al, 2013) in the inactive (crystallographic molecule A; left panel) or active (crystallographic molecule A; middle and right panel) state. F575, F636, and V658 in JAK1 wild type are labelled. The modelling suggests that the SH2-PK linker (with F575 shown in red) of JAK1 F636del can adopt either an inactive (orange) or active (green), or an “alternative” (blue) conformation, the latter of which has not been experimentally documented for JAK1 wild type.

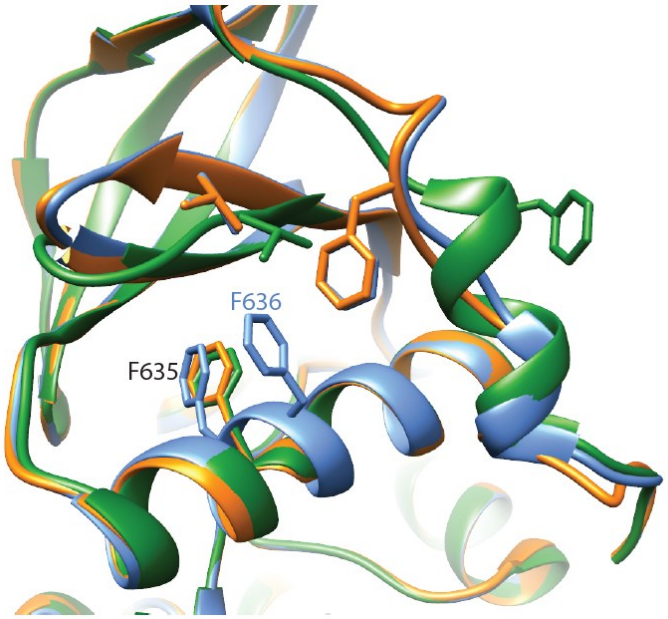


Figure 16: Homology modelling of JAK1 F636del in the active (orange) and inactive (green) conformation superimposed with JAK1 wild type (blue) showing the side chain of F635.

6. JAK1 F636del does not activate the JAK1 kinase but rather attenuates its function.

We assessed the kinase activity of JAK1 F636del. In order to distinguish between auto- and trans-phosphorylation, we utilized side-directed mutagenesis and created a JAK1 construct harboring an inactivating mutation of an ATP-binding site (K908G) and a construct harboring both the JAK1 F636del and K908G mutations together (Fig. 13). The JAK1 F636del, but not JAK1 F636del + K908G, was autophosphorylated on Y1034/Y1035 both under steady state conditions and after non-specific PMA stimulation (Fig. 17-19).

JAK1 F636del induced STATs phosphorylation both in non-stimulated and stimulated HEK293T cells. However, all phosphorylation levels, at all studied time points were lower when comparted to wild type JAK1, except immediately after PMA stimulation, when STAT3 phosphorylation was comparable between JAK1 F636del and JAK1 wt (Fig. 19) These data suggest the decrease of kinase activity upon F636 loss.

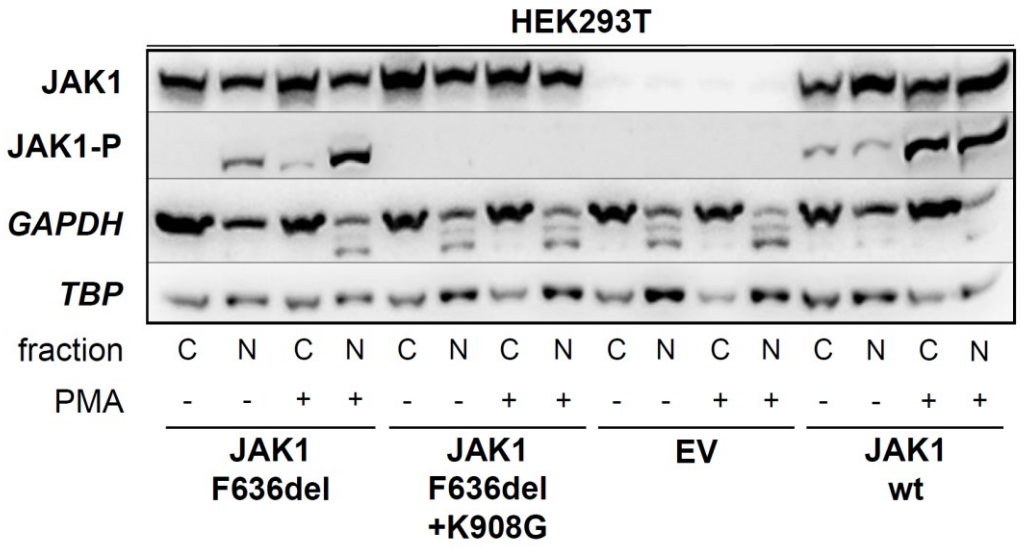


Figure 17: Western blot analysis of the kinase activity in HEK293T cells transiently transfected with empty vector (EV), JAK1 wt, JAK1 F636del or the catalytically deficient form (bearing the K908G mutation). Catalytic activity is preserved in JAK1 F636del which was autophosphorylated on Y1034/Y1035. GAPDH and TBP serve as loading controls. Cells were stimulated for 15 minutes with 1µg/ml PMA. Separated cytoplasmic (C) and nuclear (N) protein fractions were analyzed.

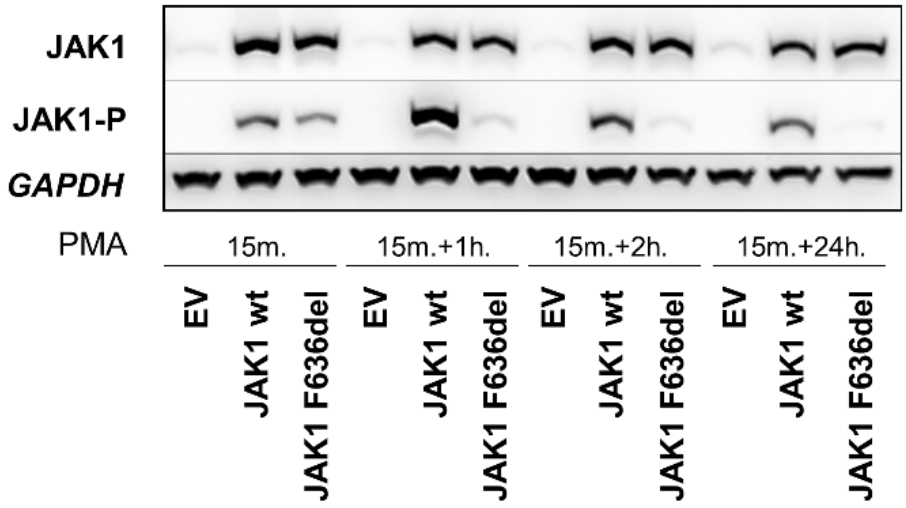


Figure 18: Western blot analysis of the kinase activity in HEK293T cells transiently transfected with empty vector (EV), JAK1 wt, JAK1 F636del. Stimulation was carried out for 15 minutes with 1µg/ml PMA. Cells were harvested and analyzed immediately after stimulation, 1 hour (h), 2h and 24h after stimulation. GAPDH served as loading control.

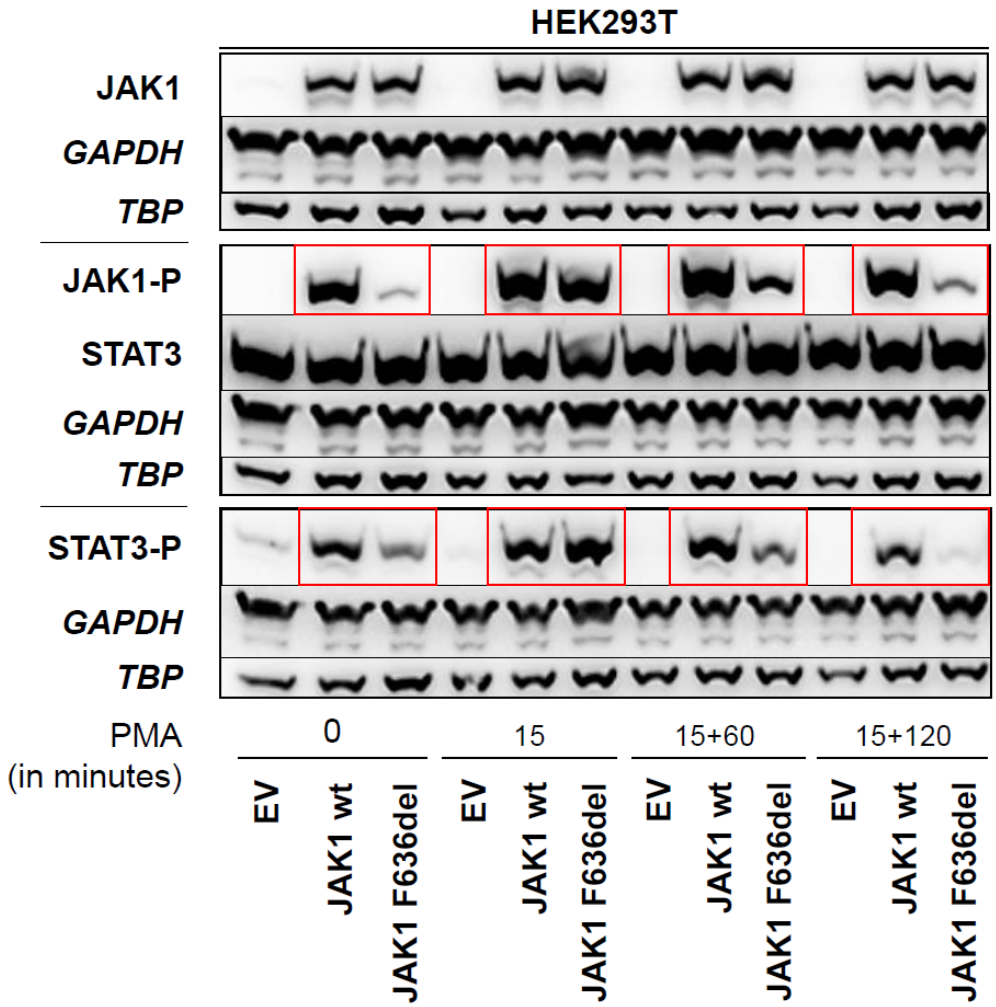


Figure 19: Western blot analysis of JAK/STAT signalling in transiently transfected HEK293T cells with empty vector (EV), JAK1 wt or JAK1 F636del. Lower levels of STAT3- and auto-phosphorylation when compared to JAK1 wild type suggest decrease in JAK1 F636del kinase activity.

To assess the activating potential of JAK1 F636del, we utilized the well-known murine Ba/F3 cell transformation assay (Lacronique et al., 1997; Palacios and Steinmetz, 1985; Warmuth et al., 2007). For example, the activating potential of the JAK2 V617F mutation that is present in almost 90% of polycythemia vera patients has been validated via this assay (James et al., 2005). Similarly several novel JAK1 mutations were shown activating in this particular assay (Arulogun et al., 2017; Li et al., 2017). We used the previously described activating mutation JAK1 V658I as a positive control (Fig. 13) (Arulogun et al., 2017).

Unlike V658I, JAK1 F636del did not induce IL3-independent growth in the Ba/F3 assay after 10 days of culture in a cytokine depleted environment (Fig. 20). Moreover, JAK1 F636del did not induce cytokine independent growth in the human TF1 cell line (Fig. 21). These data suggest, that F636del does not activate the JAK1 kinase but rather attenuates its function.

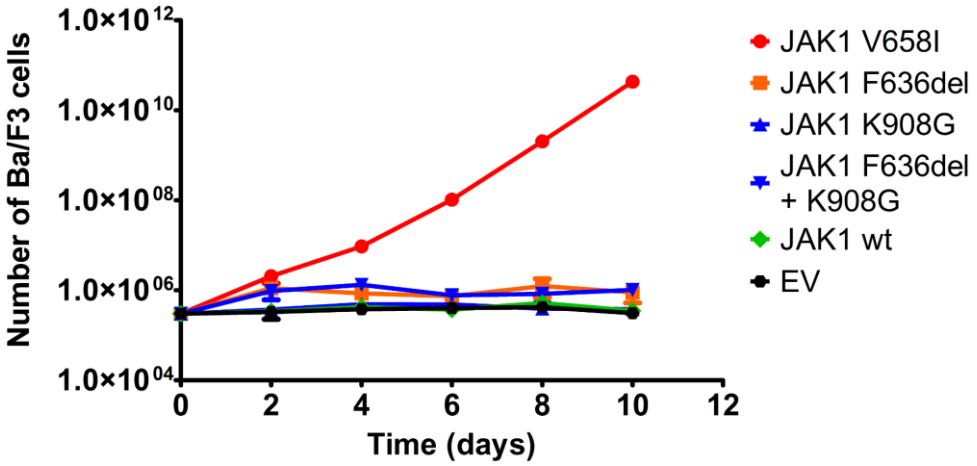


Figure 20: The proliferation of Ba/F3 cells in an IL3 depleted medium shows identical proliferation rates of JAK1 F636del and JAK1 wt. The known activating mutation V658I serves as a positive control. Sorted Ba/F3 were cultured for 10 days in a cytokine deprived medium. Proliferation of the Ba/F3 cells was measured every other day. Dead cells were excluded with the use of trypan blue. EV (empty vector).

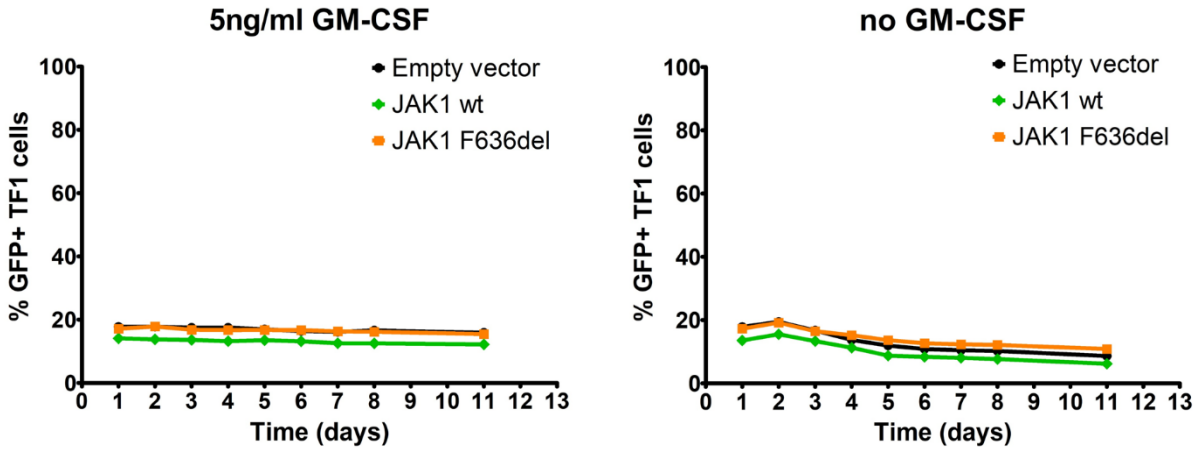


Figure 21: Transduced TF1 cells (with empty vector, JAK1 wt or JAK1 F636del) cultured in the presence (left panel) or absence (right panel) of GM-CSF

Interestingly, we observed a confusing phenomenon of the Ba/F3 assay also recently described by Watanabe-Smith and colleagues (Watanabe-Smith et al., 2017). Strikingly, the JAK1 wild type which served as control in our Ba/F3 assay exhibited cytokine independent growth 15 days after IL3 depletion (Fig. 22A). Sequencing of the transformed JAK1 wild type expressing Ba/F3 cells revealed acquired, previously described, activating mutations L910P and S729C (Kan et al., 2013; Yang et al., 2016). Our data imply that similar unwanted transforming events can appear in wild type genes following Ba/F3 cell selection when cultured in a cytokine depleted medium for a longer time period. Therefore we propose that up to 5 days of culture can give results sufficiently accurate to evaluate the studied mutations transforming potential (Fig. 22B). Moreover transformed cells should always be sequenced, a practice not performed by all laboratories (Watanabe-Smith et al., 2017).

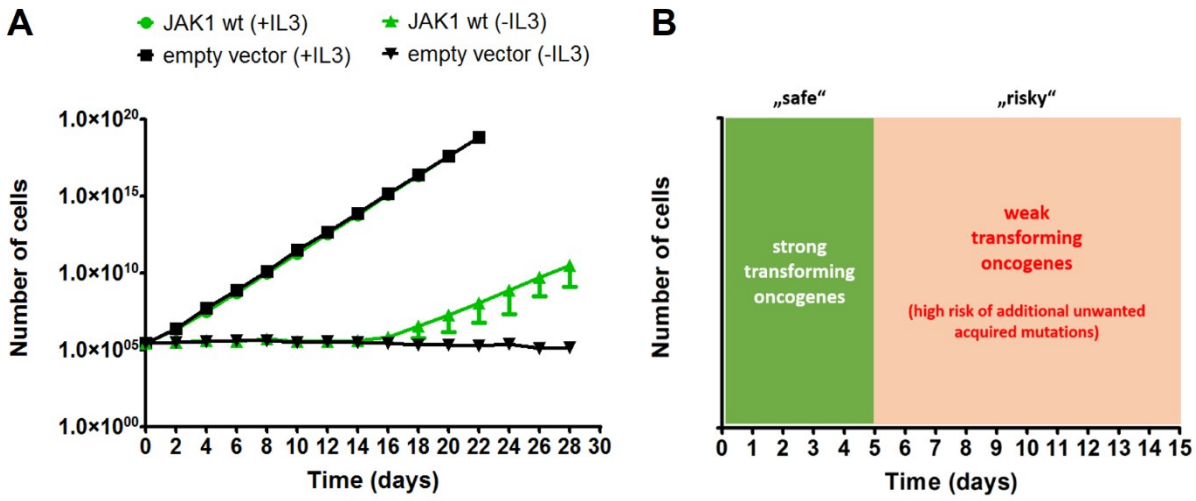


Figure 22: A) Proliferation of Ba/F3 cells transduced with JAK1 wild type or empty vector, cultured either in an IL3 rich medium or in an IL3 depleted environment. Cells harboring the JAK1 wild type construct started proliferating independently of IL3 after 15 days in culture. B) Risk of unwanted acquired mutations occurring in various oncogenes depending on their transforming potential.

7. The colony forming capacity of JAK1 F636del does not differ from wild type in both murine CD34+ bone marrow and fetal liver cells.

To further evaluate the phenotypic impact of JAK1 F636 loss, we introduced empty vector, JAK1 wt or JAK1 F636del into murine bone marrow HSPCs using lentiviruses. Transduced, GFP expressing c-kit positive cells were sorted and used for colony-forming unit (CFU) assays. There was no difference in the colony-forming capacity of JAK1 wt and JAK1 F636del (Fig. 23A). To assess the cell morphology, the percentage of granulocytes, macrophages and immature cells was counted for each construct. There was no difference between the three constructs (Fig. 23B).

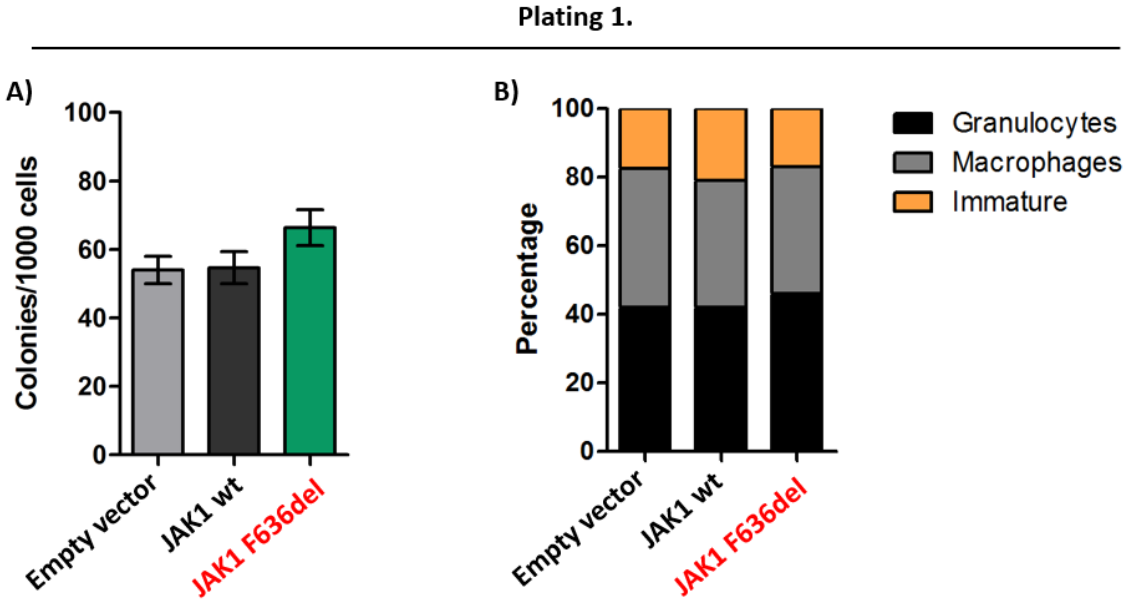


Figure 23: A) Colony-forming unit assays in sorted murine CD34+ bone marrow HSPCs transduced with empty vector, JAK1 wt and JAK1 F636del. B) Percentage of granulocytes, macrophages and immature cells in individual constructs.

Since TMD originates prenatally in the fetal liver we decided to mimic its microenvironment by using fetal liver HSCPs extracted from embryonic day 13.5 mouse fetuses. Transduced, GFP expressing c-kit positive cells were sorted and used for CFU assays. Even in this setting we did not observe any difference in colony-forming capacity between individual constructs (Fig. 24)

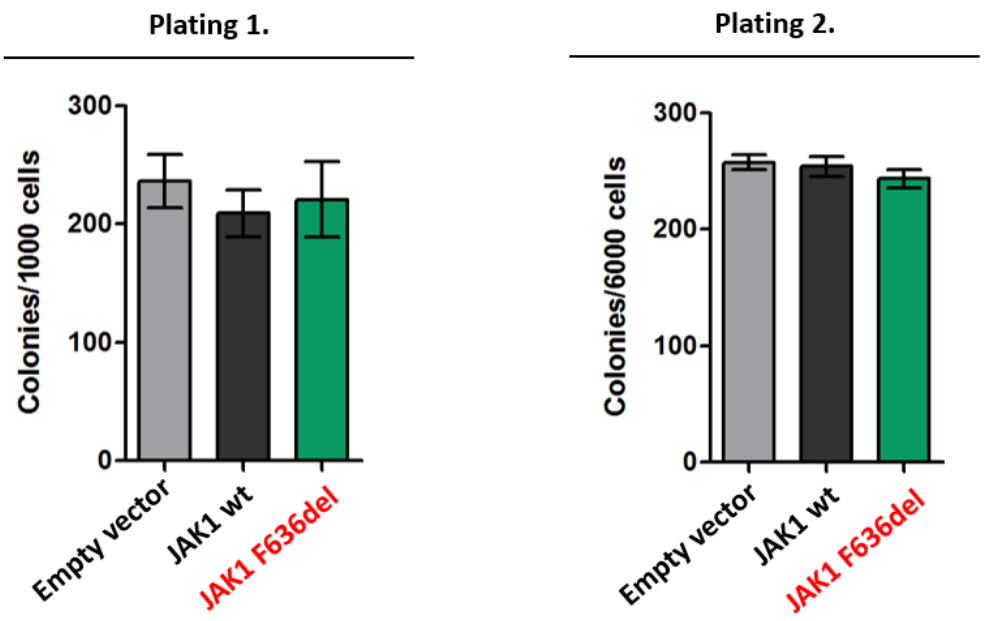


Figure 24: Colony-forming unit assays in sorted murine fetal liver HSPCs transduced with empty vector, JAK1 wt or JAK1 F636del. The number of colonies did not differ between the constructs in both the first and second plating.

8. Mimicking trisomy 21-independent TMD in a murine fetal liver cell model to study the cooperation of JAK1 F636del and GATA1s.

Our results suggested that JAK1 F636del may exert its impact in TMD pathogenesis only in cooperation with mutated GATA1. Therefore the need for a more precise model was warranted. We established a collaboration with Prof. Jan-Henning Klussman from the Martin-Luther University in Halle, Germany. I conducted the here described experiments during a 3-month stay in Prof. Klusmann's laboratory. We utilized an *in vitro* model recently described by Prof. Klusmann and colleagues (Labuhn et al., 2019). Induction of Gata1s expression in disomic fetal liver HSPCs from embryonic day 13.5 ROSA26:Cas9-EGFPki/wt mice was mediated by the CRISPR/Ca9 gene editing system. In this setting, Gata1s expression leads to the expansion and hyperproliferation of fetal liver HSPCs. A 3-week selection process under low levels of the cytokines thrombopoietin (TPO) and stem cell factor (SCF) facilitate the acquisition of a pure Gata1s positive cell population, whereas the cells negative for Gata1s differentiate and arrest their expansion. Next, we introduced empty vector, JAK1 wt or JAK1

F636del into the Gata1s expressing cells in order to study their joined effect on the fetal liver HSPCs proliferation (Fig. 25-26).

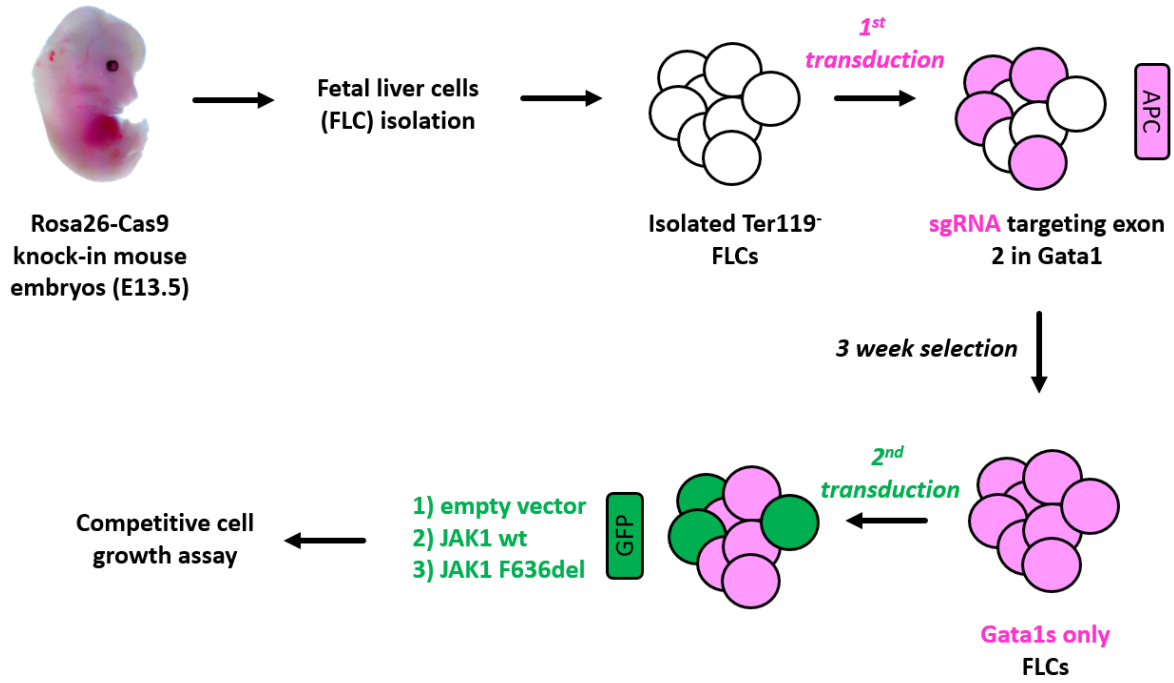


Figure 25: Mimicking Gata1 mutated TMD without trisomy 21 in a murine fetal liver cell model to study the cooperation of Gata1s and JAK1 F636del. sgRNA (single guide RNA), APC (allophycocyanin), GFP (green fluorescent protein).

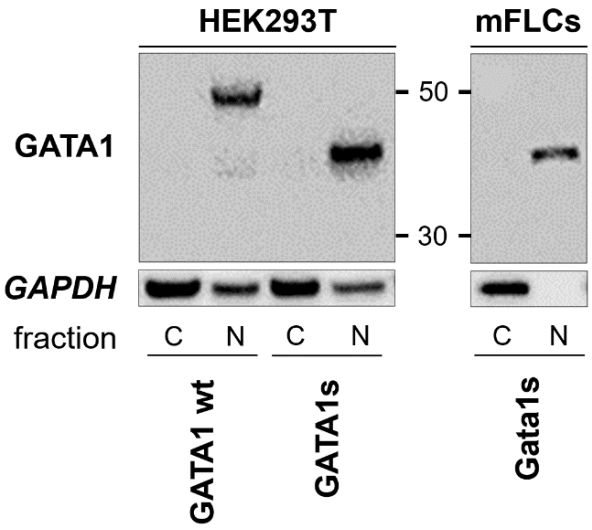


Figure 26: Western blot analysis confirming the sole expression of Gata1s in the CRISPR/Cas9 edited mouse fetal liver cells (mFLCs). GAPDH was used as loading control.

In the competitive growth assay setting were empty vector, JAK wt or JAK1 F636del were lentivirally introduced into the Gata1-edited HSPCs, JAK1 F636del had no additional impact on cell proliferation (Fig. 27) or maturation status (Fig. 28).

Next, we conducted competitive growth assays, focusing on the JAK/STAT pathway. Similarly as in the previous experiment, empty vector, JAK wt or JAK1 F636del were lentivirally introduced into the Gata1-edited HSPCs. The cells were then cultured with various levels of interleukin 6 (IL6) in order to specifically stimulate JAK1 signaling. Overgrowth of the double-positive (Gata1s + JAK1 F636del) cell population was not present in any of the used culturing conditions (Fig. 29).

Additionally, cells were cultured in cytokine-depleted growth-restrictive conditions. Likewise, no difference between JAK1 wt and JAK1 F636del was visible in this experimental setting (Fig. 30).

Moreover, JAK1 wt or JAK1 F636del were introduced into murine fetal HSPCs simultaneously with the GATA1 editing tools in order to monitor their combined effect on cell proliferation instantly after transduction. There was no proliferative advantage of the double positive HSPCs population (Gata1s + JAK1 F636del) (Fig. 31).

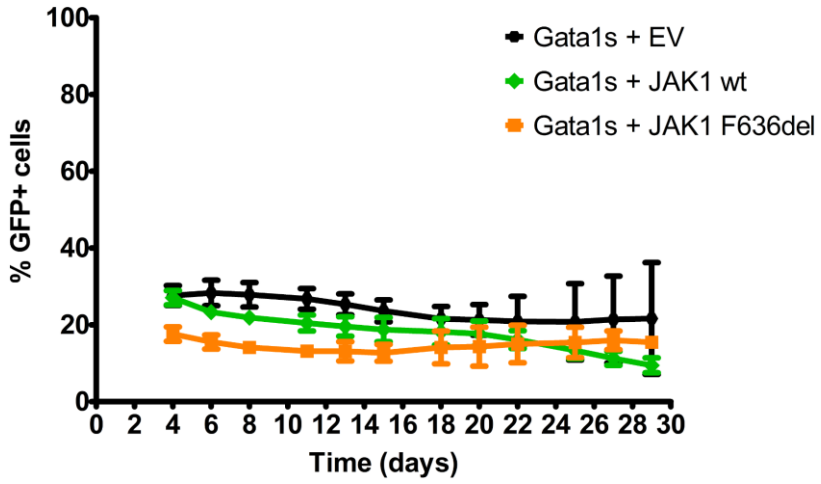


Figure 27: Competitive growth experiment of the Gata1-edited murine fetal liver HSPCs (APC+) transduced at day 0 with empty vector (EV), JAK1 wt or JAK1 F636del (GFP+). The percentage of double positive cells (APC+GFP+) in the whole population of Gata1-edited cells (APC+) is showed on the y-axis.

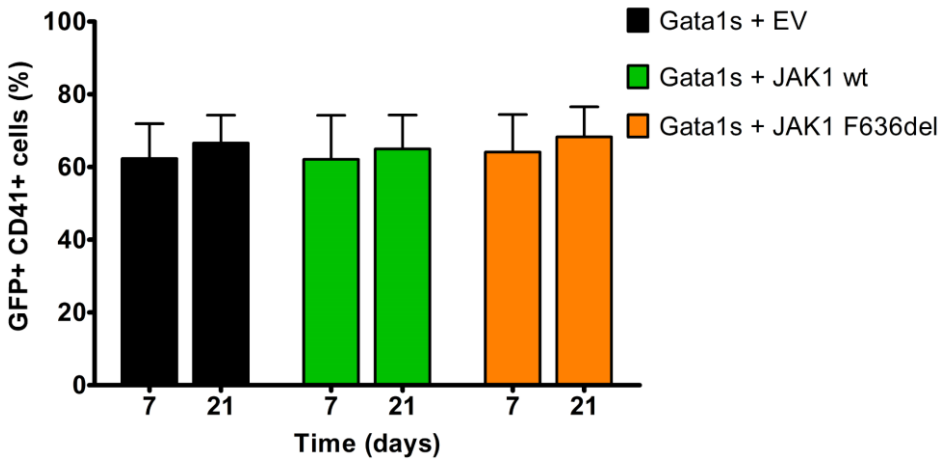


Figure 28: Expression of the megakaryocytic marker CD41 after transduction (day 7 and 21) of the Gata1-edited cells with EV, JAK1 wt or JAK1 F636del. The double positive (APC+GFP+) cell population was used for the analysis.

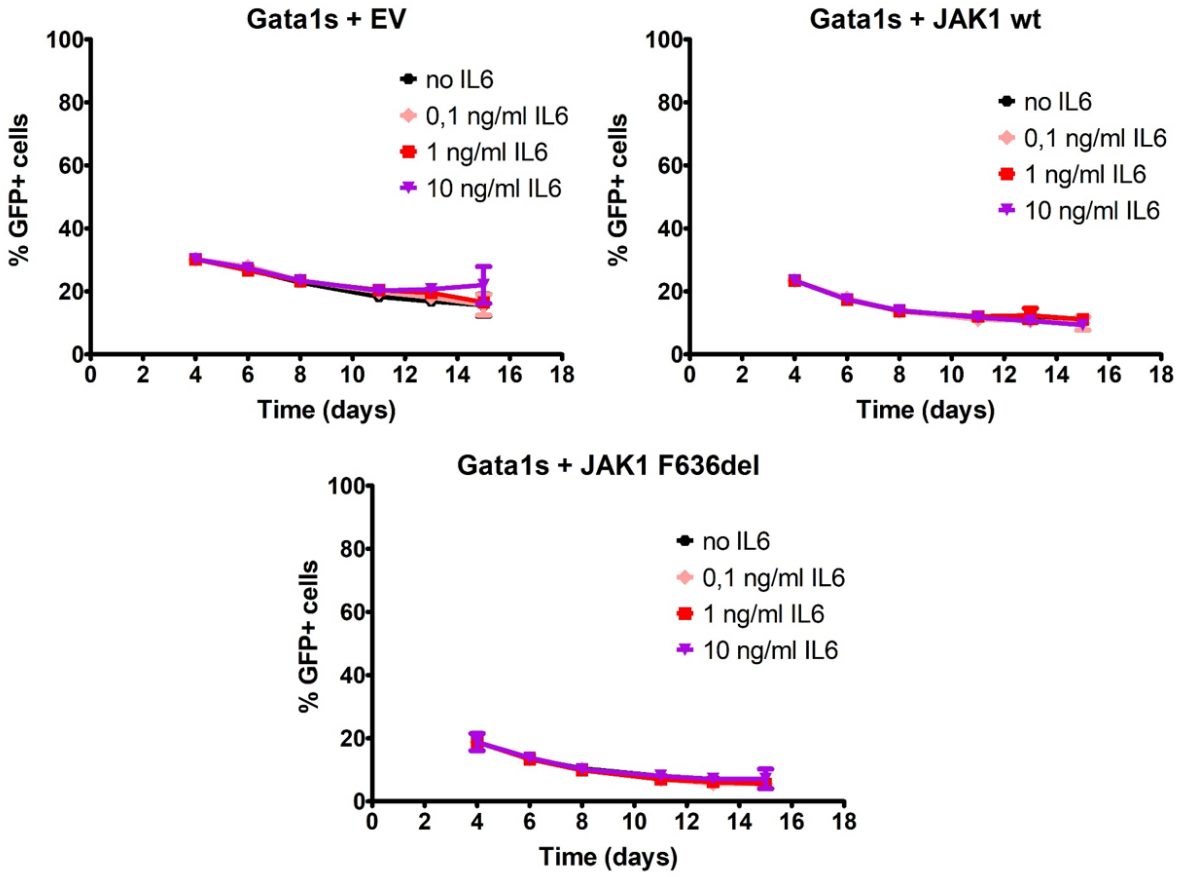


Figure 29: Competitive growth experiment of the Gata1-edited murine fetal liver HSPCs transduced at day 0 with empty vector (EV), JAK1 wt or JAK1 F636del. Cells were treated with either no IL6; 0,1 ng/ml IL6; 1 ng/ml IL6 or 10 ng/ml IL6.

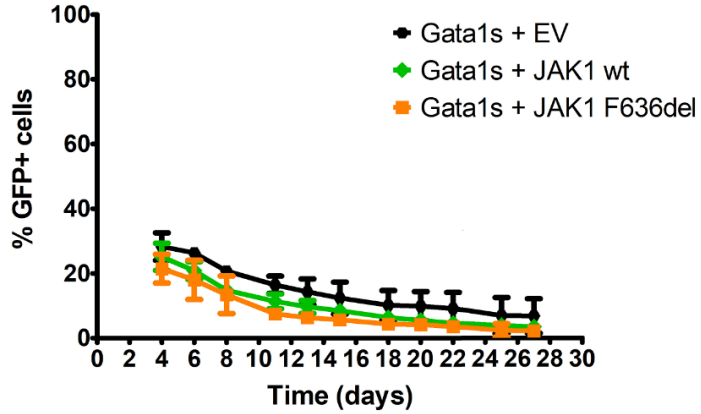


Figure 30: Competitive growth experiment of the Gata1-edited murine fetal liver HSPCs transduced at day 0 with empty vector (EV), JAK1 wt or JAK1 F636del. Cells were cultured in the absence of mouse SFC.

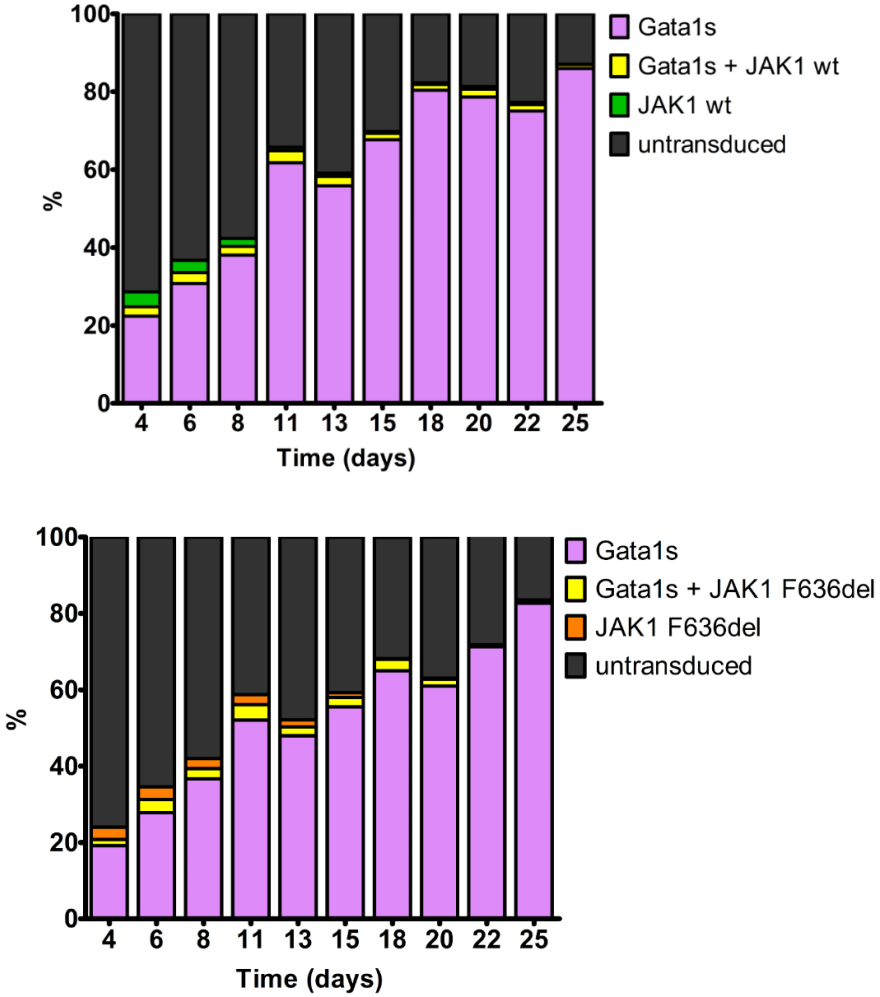


Figure 31: Simultaneous introduction of Gata1s together with JAK1 wt or JAK1 F636del into murine fetal liver HSPCs. The different cell fraction percentage from the total cell population after transduction is shown on the y axis. The x axis shows days after transduction (Day 0).

<u>Project 2.</u> Characterization of two novel fusion genes, AIF1L-ETV6 and ABL1-AIF1L, resulting from a single chromosomal rearrangement in ETV6-ABL1-positive pediatric ALL

1. Novel fusion genes identified in a BCP-ALL harboring an ETV6-ABL1 fusion.

We decided to characterize in detail the molecular background of an interesting BCP-ALL harboring the ETV6-ABL1 fusion, diagnosed in our department in 2016. Both the clinical picture and immunophenotype (Table 4) did not differ from the previously described BCP-ALLs associated with an ETV6-ABL1 fusion gene.

Cytogenetic analysis revealed a diploid male karyotype together with an isodicentric chromosome 7. No rearrangements of ETV6, RUNX1 and KMT2A gene loci were observed. Multiplex RT-PCR was positive for the ETV6-ABL1 fusion transcript and negative for BCR-ABL1, TCF3-PBX1, ETV6-RUNX1 and KMT2A-AFF1 fusion transcripts (Table 4).

To assess the genomic profile of leukemic blasts, SNP array analysis, WES and RNAseq were applied. Four copy number aberrations were detected using SNP array. A 102 kb (kilobase) long monoallelic deletion on 3p affecting the FHIT gene, a 164 kb-long monoallelic deletion on 9p affecting the CDKN2A and CDKN2B genes and a loss of 1 copy of 7p, involving the IKZF1 gene, with gain of 1 copy of 7q corresponding to the presence of the isodicentric chromosome 7 (Lukes et al., 2018).

Five non-synonymous substitutions affecting five genes were identified by WES, namely TPO A477T, SLC25A15 I254L, PCDHB15 R494Q, ELOVL4 A253S and OBP2A R118H. None of the affected genes have been, to the best of our knowledge, associated with ALL so far.

We utilized RNAseq to identify leukemia-specific fusion transcripts. The presence of the type B (exon 5 to exon 2) ETV6-ABL1 fusion was confirmed and additionally, two novel in-frame fusion transcripts were found. The ABL1-AIF1L fusion gene, were exon 1 from ABL1 is fused to exon 5 in AIF1L and the AIF1L-ETV6 fusion gene, were exon 4 in AIF1L is fused with exon 6 in ETV6 (Table 4, Fig. 33-34).

Next, we analyzed the gene expression profile of the leukemic blasts. An in house cohort of 108 B-other BCP-ALL patients, negative for TCF3-PBX1, BCR-ABL1, ETV6-RUNX1 and KMT2A-involving fusions, was used for the gene expression analysis. Hierarchical clustering of the patients from this cohort using a gene set specific for BCR-ABL1-positive or BCR-ABL1-like-positive ALL showed that the leukemic blasts harbored a BCR-ABL1-like gene expression signature (Fig. 32).

2. Three fusion genes resulting from a single chromosomal rearrangement

The AIF1L gene and the ABL1 gene have the same genomic orientation. Moreover, AIF1L is located on 9q downstream of ABL1. We therefore concluded that all three in-frame gene fusions resulted from a single chromosomal rearrangement (Fig. 33) (Lukes et al., 2018). We applied DNA-based long-distance PCR and described the exact intronic junction sequences of AIF1L-ETV6, ABL1-AIF1L and ETV6-ABL1, confirming the predicted chromosomal rearrangement on genomic level (Fig. 33-34). The insertion of a portion of 9p that included parts of the AIF1L and the ABL1 genes into chromosome 12 was balanced. There were no gains or losses at breakpoint sites in intron 5 of the ETV6 and intron 1 of the ABL1 gene. The breakpoint in intron 4 of the AIF1L gene harbored a deletion of 2 base pairs (Fig. 34).

ABL1 gene insertion into the ETV6 gene represents the most frequent mechanism that results in the ETV6-ABL1 fusion in BCP-ALL as has been previously reported by our group (Zaliova et al., 2016; Zuna et al., 2010). In order to clarify, if the breakpoint of the telomeric 9q segment in the AIF1L gene, which results in the ABL1-AIF1L and/or AIF1L-ETV6 fusions, occurs recurrently, we screened a cohort of 10 patients with ETV6-ABL1-positive leukemias from our previous study for the corresponding fusion transcripts. This cohort of patients included 5 cases with an unknown localization of the ETV6-ABL1 fusion and 5 patients with a confirmed insertion of the ABL1 gene into the ETV6 gene by cytogenetic analysis. No fusion transcripts involving the AIF1L gene were detected.

3. The observed chromosomal rearrangement originated prenatally.

The prenatal origin of the ETV6-ABL1 fusion has been previously demonstrated by us in a pediatric ALL case positive for the ETV6-ABL1 fusion gene (Zuna et al., 2010). We optimized a sensitive and specific PCR system in order to detect the patient's ETV6-ABL1 genomic fusion. We used the patient's Guthrie card (neonatal blood spot) for the analysis (Fig. 35). A positive result in the form of a PCR product of an expected length was obtained in 1 out of 7 reactions that contained a portion of the patient's Guthrie card (Fig. 36). The presence of the ETV6-ABL1 fusion gene in the positive PCR product was verified by Sanger sequencing. Our results confirm the prenatal origin of the observed genomic rearrangement, which therefore probably represents the first leukemogenic event, followed by the acquisition of secondary aberrations in later stages of the leukemogenic process. Deletions in the IKZF1 and/or CDKN2A/B most likely represent the secondary hits (Fig. 35-37).

4. Chimeric protein analysis reveals *in vitro* expression of AIF1L-ETV6.

The two fusion genes AIF1L-ETV6 and ABL1-AIF1L have not been previously reported. Importantly, no disruptions of the AIF1L gene have been, to the best of our knowledge, described in leukemias so far. Therefore we decided to study these novel fusion genes in more detail. The allograft inflammatory factor 1 like (AIF1L) gene encodes three protein isoforms (Coordinators, 2017). The isoform 1 (NP_113614) consists of 150 amino acids with a predicted molecular weight of 17 kilodalton (kDa). It contains two centrally located EF-hand calcium-binding domains (EF1, EF2). Its main function is binding and cross-linking actin (Fig. 38).

The predicted molecular weight of the hypothetical chimeric protein encoded by the ABL1-AIF1L fusion gene is 14 kDa. The ABL1 moiety encodes 45 N-terminal amino acids of this chimeric protein that do not form any known functional domains. The AIF1L moiety encodes 83 amino acids of ABL1-AIF1L that include the incomplete EF1 and the complete EF2 domains (Fig. 38).

The predicted molecular weight of the hypothetical chimeric protein encoded by the AIF1L-ETV6 fusion gene is 22 kDa. The AIF1L moiety encodes 67 N-terminal amino acids that include a part of the EF1 domain. The ETV6 moiety encodes 115 C-terminal amino acids that

include the ETS domain which mediates DNA binding of the wild type ETV6 (Fig. 38) (Lukes et al., 2018).

We were not able to analyze the presence of the chimeric proteins ABL1-AIF1L and AIF1L-ETV6 in the patient's primary leukemic cells due to insufficient quality and amount of protein isolated from the patient's diagnostic bone marrow sample. Therefore we decided to study the expression of AIF1L-ETV6 and ABL1-AIF1L hypothetical chimeric proteins in HEK293T cells. The patient's diagnostic bone marrow sample was used for cDNA amplification of the full-length coding regions of both of the fusion transcripts (Lukes et al., 2018). HEK293T cDNA was used for AIF1L wild type amplification. PCR products were analyzed by Sanger sequencing and showed that AIF1L wild type transcript represents the variant 1 of AIF1L, encoding for isoform 1. Moreover it revealed that the AIF1L-ETV6 transcript that is expressed by the leukemic cells lacks exon 3 of AIF1L. The third exon of AIF1L is also spliced out in the AIF1L wild type transcript variant 1 (Lukes et al., 2018).

Coding sequences of the AIF1L-ETV6 and ABL1-AIF1L fusion transcripts were transfected into HEK293T cells. The presence of AIF1L wild type, AIF1L-ETV6 and ABL1-AIF1L proteins was analyzed with two antibodies targeting AIF1L. The AIF1L-N antibody was used for AIF1L-ETV6 chimeric protein detection, hence it detects the near N-terminal region of AIF1L which is involved in AIF1L-ETV6. The AIF1L-C antibody was used for ABL1-AIF1L chimeric protein detection, as it detects the C-terminal part of AIF1L which is involved in ABL1-AIF1L.

Both the AIF1L-N and the AIF1L-C antibody detected the AIF1L wild type protein (Fig. 39). We confirmed the expression of the chimeric protein AIF1L-ETV6 in HEK293T cells transfected with this particular construct using the AIF1L N-terminal antibody. The AIF1L-ETV6 chimeric protein was located in both the nucleus and the cytoplasm, similarly to AIF1L wild type. We did not detect the expected chimeric protein ABL1-AIF1L in transfected HEK293T cells using the AIF1L C-terminal antibody (Fig. 39).

Patient characteristics	
Age (at diagnosis)	2 years 10 months
While blood cell count (at diagnosis)	49,4/µl
Blasts	50% (peripheral blood) 91% (bone marrow)
Clinical trial	AIEOP-BFM ALL 2009
CNS involvement	No
TKI used in treatment	No
Response to prednisone prephase	Good
Immunophenotype	Pre-B
Positive	CD10, CD19, CD20, CD22, cytoplasmic IgM, cytoplasmic CD79a
Partial weak positivity	CD15, CD66C
Negative	CD3, cytoplasmic CD3, Ig kappa, Ig delta
Genetics	
Multiplex PCR (fusion gene screening)	ETV6-ABL1
RNAseq	ETV6-ABL1, AIF1L-ETV6, ABL1-AIF1L
WES	TPO A477T, SLC25A15 I254L, PCDHB15 R494Q, ELOVL4 A253S, OBP2A R118H
SNP array	IKZF1 del, CDKN2A/B del, FHIT del
Minimal residual disease	3 targets used
Day 33 (end of induction)	3 targets negative
Week 12	1 target positive ($<10^{-4}$)

Table 4: Characteristics of the BCP-ALL harboring the ETV6-ABL1 fusion.

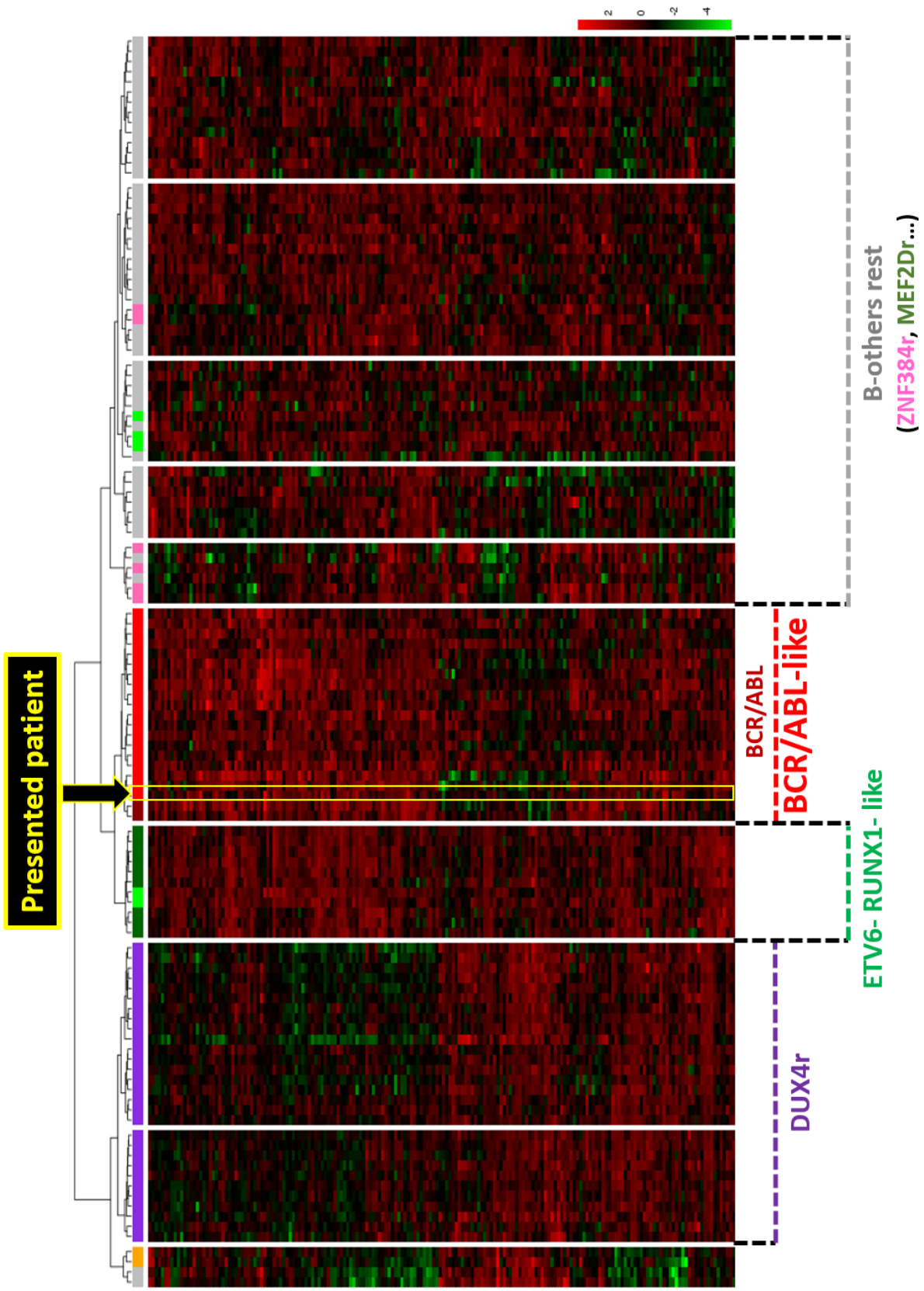


Figure 32: Gene expression analysis. Hierarchical clustering of 108 pediatric B-others BCP-ALL patients using a gene set specific for BCR-ABL1-positive or BCR-ABL1-like-positive ALLs. The patient leukemic blasts harbored a BCR-ABL1-like gene expression signature.

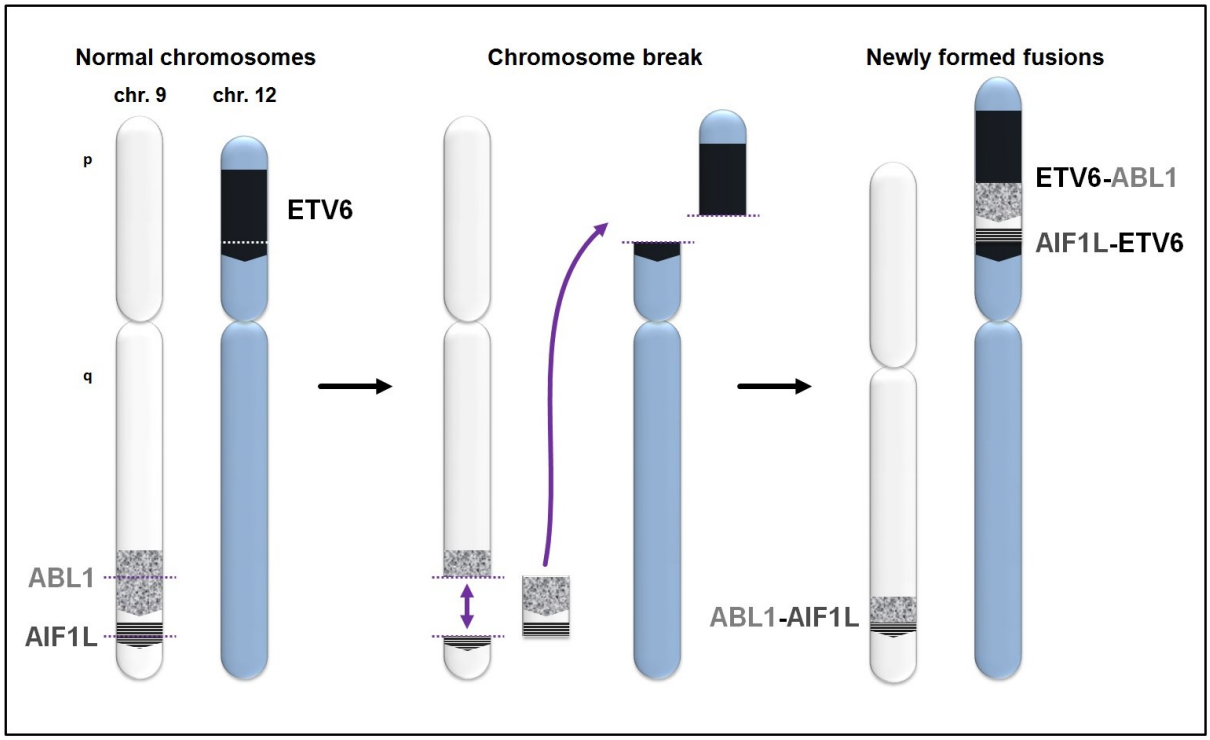
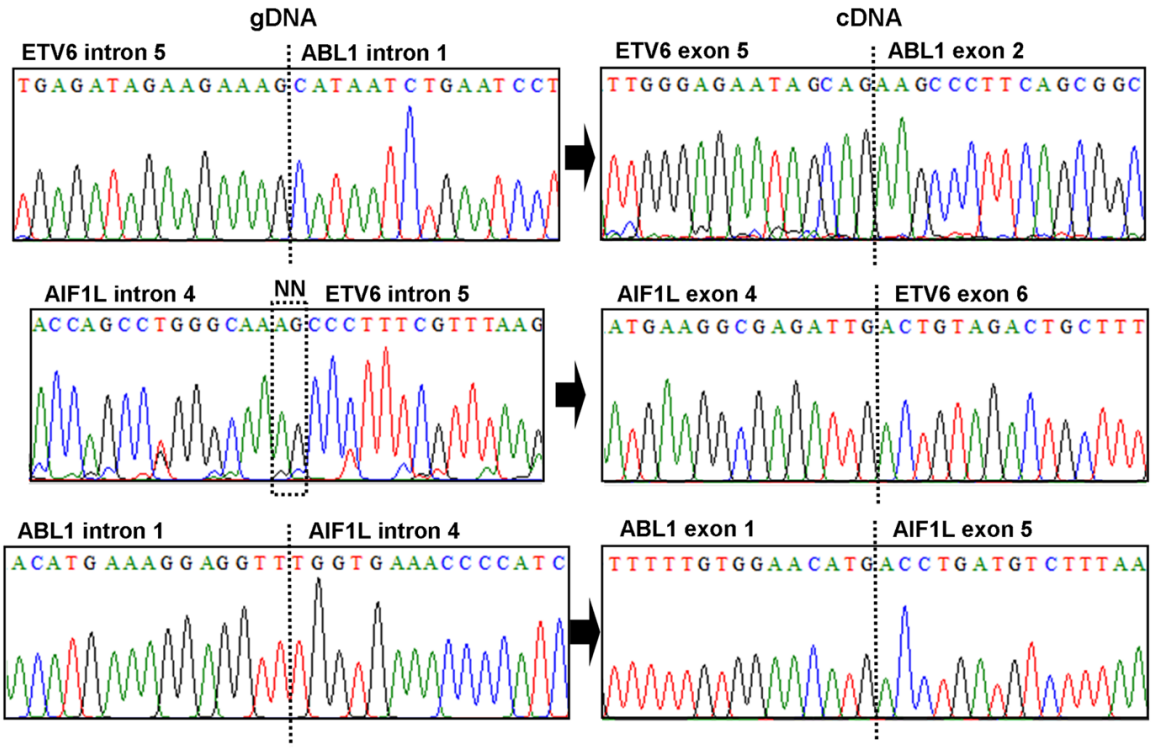


Figure 33: A single chromosomal rearrangement resulted in three fusion genes, two of them novel. A portion of 9p that included parts of the AIF1L and the ABL1 genes was inserted into chromosome 12 into the ETV6 gene. This insertion and subsequent fusion on chromosome 12 resulted in the ETV6-ABL1 and the AIF1L-ETV6 fusion genes. The remaining parts of chromosome 9 fused together and generated the ABL1-AIF1L fusion gene.

A



B

ETV6 intron 5 **chr12:12032615-12032664**

CCATGTGCCTTGAGATAGAAGAAAGCCCTTTCGTTTAAGAAAAATGCACT

AIF1L intron 4 **chr1:113361171-133361220**

GGAGTTCGAGACCAGCCTGGGCAACATGGTGAAACCCCATCTTTACTAAA

ABL1 intron 1 **chr9:133646284-133646333**

TCACTTACTAACATGAAAGGAGGTTCATAATCTGAATCCTCCTGAAGGAA

Figure 34: (A) Fusion gene junctional sequences at genomic DNA level (gDNA) and at RNA level (cDNA). The junctional regions of the fusion genes were amplified by PCR in the case of gDNA and by RT-PCR in the case of cDNA. The PCR products were sequenced by Sanger sequencing. Non-templated inserted nucleotides (NN). (B) Reference sequences of the fusion gene breakpoint regions. The genomic coordinates correspond to GRCh37/hg19 reference genome (Lukes et al., 2018). Vertical red lines indicate specific breakpoints.

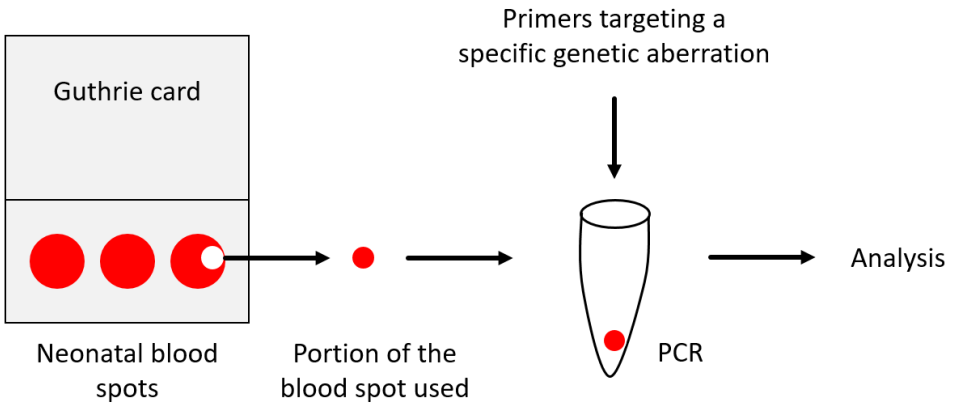


Figure 35: Graphic representation of backtracking studies from Guthrie cards. First, a PCR system able to detect the studied aberration is optimized. Portion of the neonatal blood spot is used for the reaction. Results are validated by gel electrophoresis and Sanger sequencing. Emphasis should be given on limiting the possibility of sample contamination.

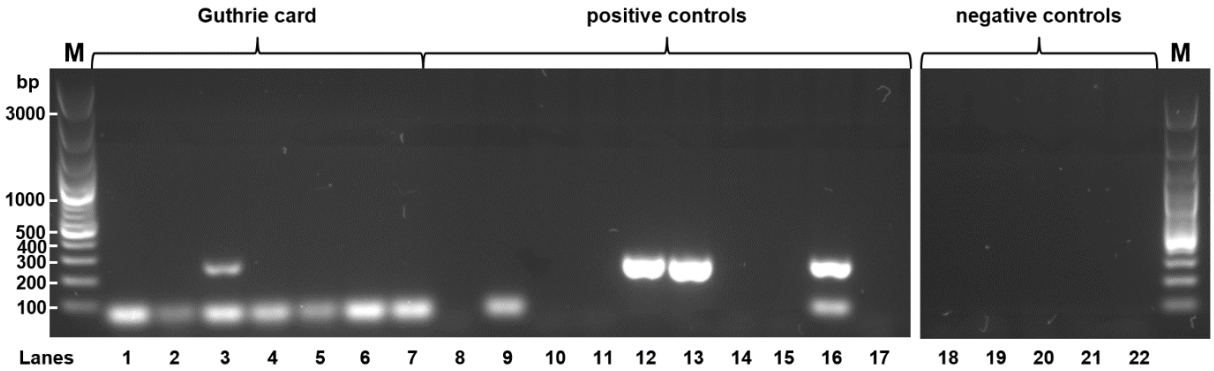


Figure 36: Backtracking analysis of the ETV6-ABL1 fusion gene on the patient's Guthrie card. Lanes 1-7 show PCR products where pieces of the patient's Guthrie card containing samples of his newborn blood were included into the reaction. Lanes 8-17 represent positive control reactions and lanes 18-22 negative control reactions. For positive control reactions, DNA from patient's diagnostic bone marrow sample was diluted into control ("healthy") DNA to a final concentration of 0.005% or 0.001% and used as template (lanes 8–12 and 13–17, respectively) (Lukes et al., 2018). In negative control reactions DNA of a healthy donor was applied. Both the positive and the negative control reactions contained a portion of the Guthrie card from a healthy donor without any blood, therefore achieving same PCR conditions in all reactions. The ETV6-ABL1 fusion was confirmed in the patient's newborn blood by Sanger sequencing of PCR product from lane 3. M (molecular weight markers).

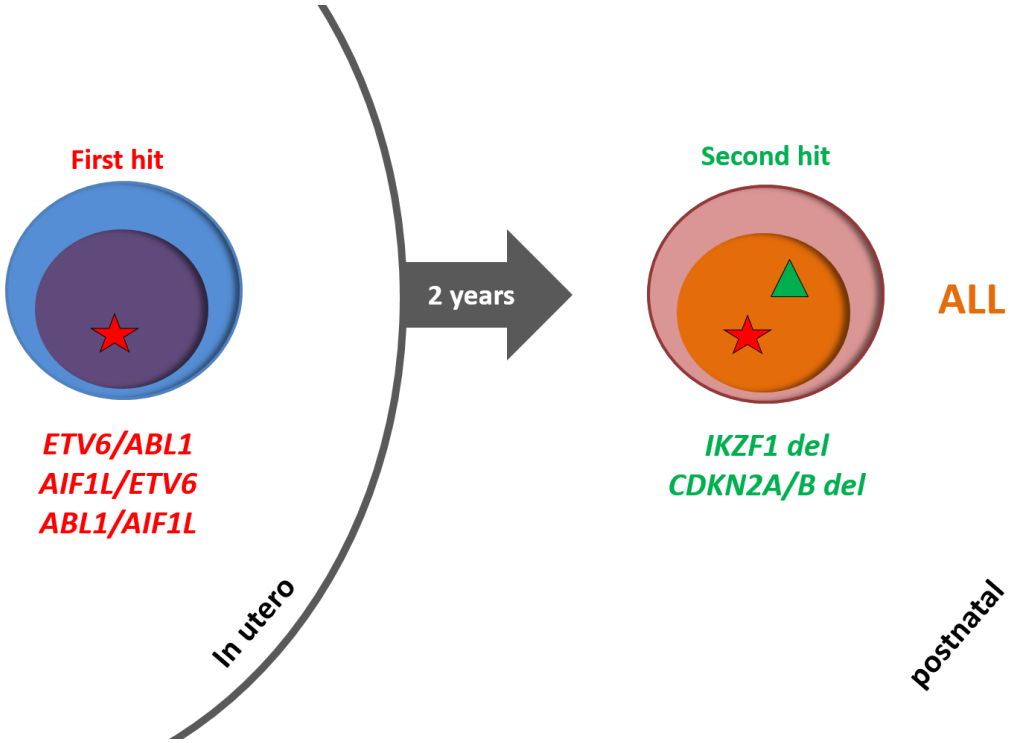


Figure 37: The single chromosomal rearrangement resulting in 3 fusion genes, ETV6-ABL1, AIF1L-ETV6 and ABL1-AIF1L, probably represents the first leukemogenic hit which occured already in utero. The second hit is most likely represented by deletions in the IKZF1 and/or CDKN2A/B genes which probably occured postnatally.

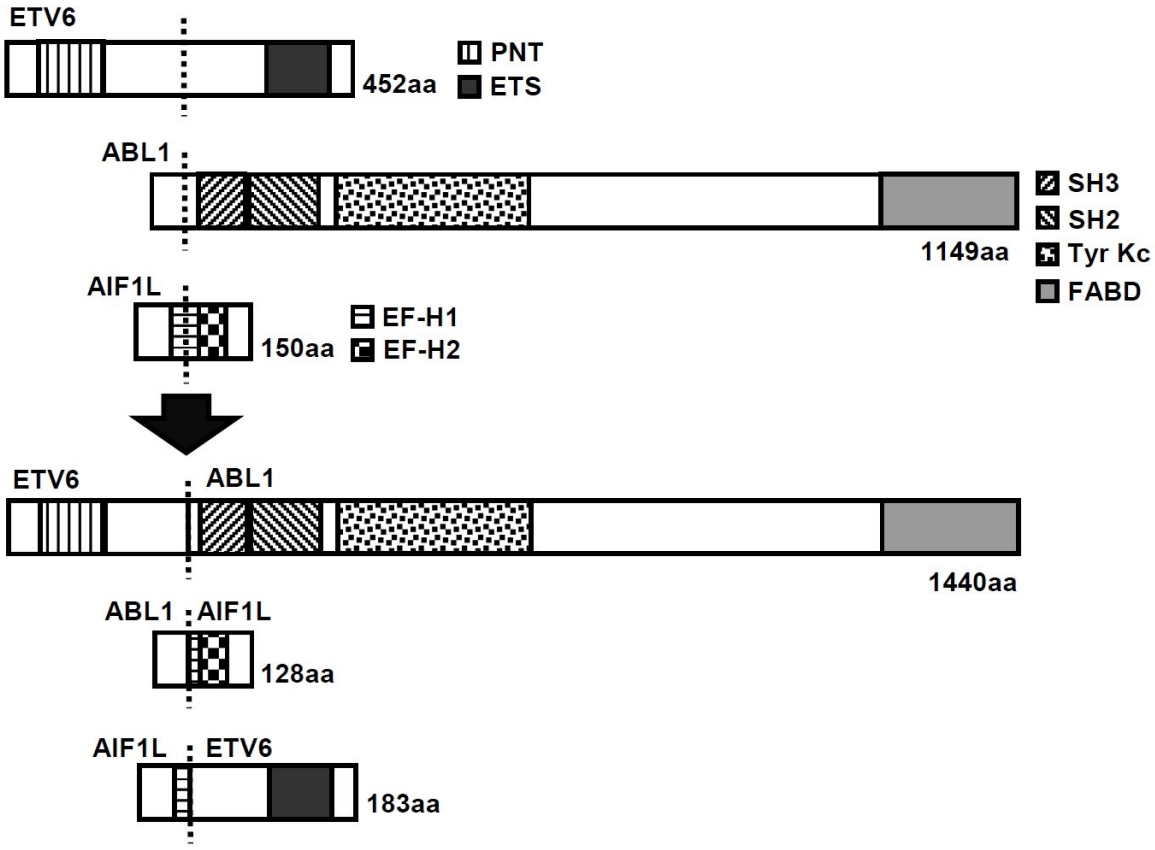


Figure 38: Representation of wild type (top scheme) and chimeric proteins (bottom scheme). Known functional protein domains are shown. The protein length is depicted in number of amino acids (aa). Positions corresponding to genomic breakpoints or junctions are displayed by dashed lines. Reference: ETV6-NM_001987 (NP_001978), ABL1-NM_007313 (NP_009297), AIF1L-NM_031426 (NP_113614). PNT (pointed domain), ETS (ETS domain), SH3 (SH3 domain), SH2 (SH2 domain), Tyr Kc (Tyrosine protein kinase, catalytic domain), FABD (F-actin binding domain), EF-H1 (EF-hand1), EF-H2 (EF-hand2).

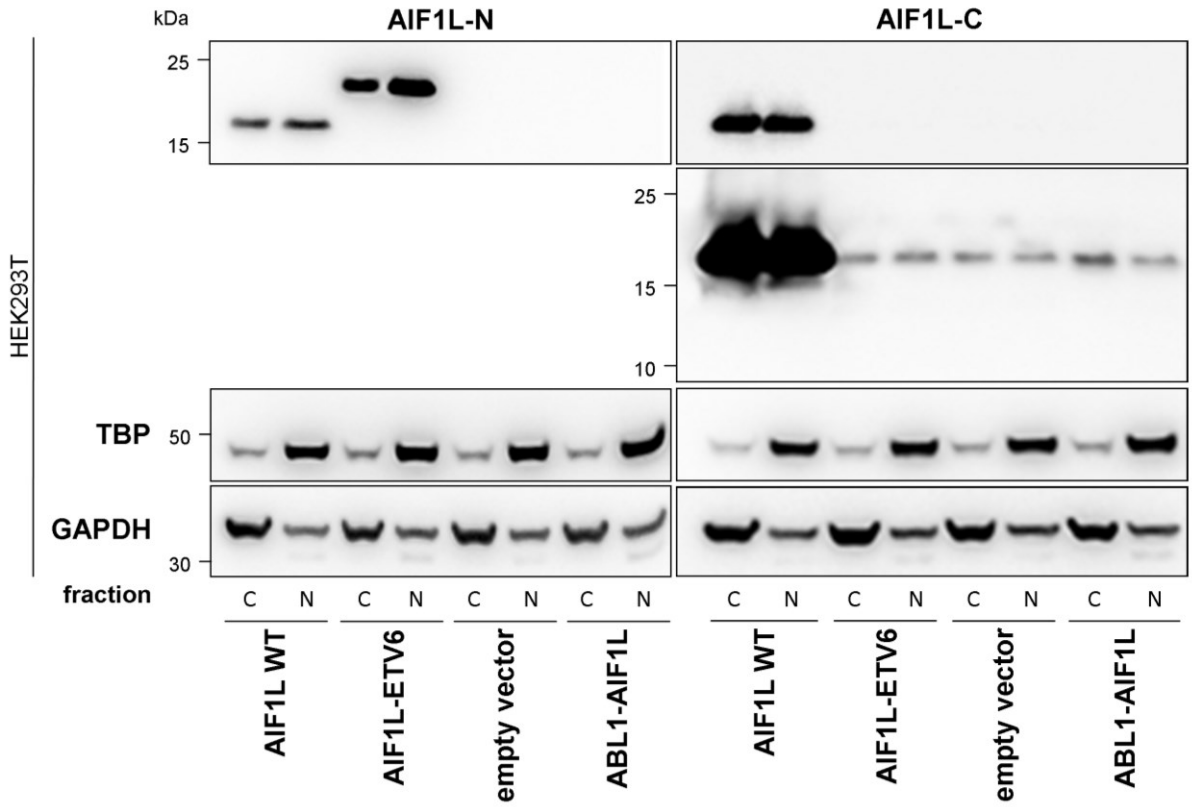


Figure 39: The expression of expected chimeric proteins in HEK293T cells. Cytoplasmic (C) and nuclear (N) protein fractions were analyzed. Plasmid vector with coding sequences of empty vector, AIF1L wild type (wt), AIF1L-ETV6 and ABL1-AIF1L were transiently transfected into HEK293T cells. Antibodies recognizing N-terminal (AIF1L-N) and C-terminal (AIF1L-C) epitopes of AIF1L were used. AIF1L wild type expression together with the AIF1L-ETV6 chimer protein expression was confirmed with the AIF1L-N antibody. The AIF1L-C antibody was successfull in detecting AIF1L wild type, however no band corresponding to the expected ABL1-AIF1L chimeric protein was observed. Not even after the application of long signal acquisition (see lower plot). GAPDH and TBP were used as loading controls. kDa (kilodalton)

Discussion

Childhood acute leukemias are genetically diverse entities. The one technological breakthrough that precipitated major improvements in molecular genetics in general, and in diagnostics and dissection of childhood leukemias in particular within the last decade were high-throughput sequencing technologies. They helped identify novel and recurrent molecular aberrations in both acute myeloid and lymphoblastic leukemias, as well as allow fast and highly accurate investigation of individual cases (Iacobucci and Mullighan, 2017; Moorman, 2016; Papaemmanuil et al., 2016; Yohe, 2015). A significant number of these aberrations seem to play a major role in disease classification, management and risk stratification. Moreover, subsequent functional studies characterizing these molecular events help us gain insight into their role in the transformation of a normal hematopoietic cell into a malignant one. Understanding the pathogenesis of these processes is crucial for developing novel targeted therapies.

Focus of my thesis rests mainly in a thorough identification and characterization of genetic events in TMD and BCP-ALL. It includes both a detailed dissection of novel genetic mutations and fusion genes, as well as presentation of numerous experimental *in vitro* approaches that focus on the functional description of these particular aberrations. The main aim was to decipher an alternative pathogenesis of TMD development in the absence of trisomy 21 and to characterize a chromosomal rearrangement in BCP-ALL resulting in the production of multiple fusion genes (Lukes et al., 2020; Lukes et al., 2018).

Moreover, I have also participated on a project aiming to study the feasibility of the identification of PML-RARA, CBFB-MYH11 and RUNX1-RUNX1T1 genomic fusion sequences using targeted sequencing. The identified fusion gene breakpoint sequences subsequently served for the design of patient-specific qPCR systems for MRD monitoring. We showed that fusion gene-based MRD monitoring represents a superior tool for therapy response evaluation than the widely used fusion-transcript based approach. By applying the fusion gene-based approach a higher sensitivity was reached. Importantly, we show that fusion gene breakpoint identification is feasible and enables unambiguously interpretable monitoring of MRD in AML patients harboring the PML-RARA, CBFB-MYH11 and RUNX1-RUNX1T1 fusions (Lukes et al., manuscript under consideration).

Last but not least, I participated in studying the frequency of H1038/Q1072 ZEB2 mutations in pediatric B-other ALL and the impact of these aberrations on patient outcome (Zaliova et al., manuscript in revision).

TMD is a leukemia-like disease that originates from fetal hematopoietic cells. In the past two decades substantial effort has been invested into our understanding of the pathogenesis of TMD. Trisomy 21 seems to be a necessary requirement for the development of this preleukemic condition (Banno et al., 2016; Carpenter et al., 2005; Kruger, 2007). However, the exact mechanism of its contribution still remains elusive. A minimal amplified region together with particular genes, namely RUNX1, ETS2, ERG and miR-125b-2, on chromosome 21 have been proposed to be responsible for the expansion of early hematopoietic progenitors, represented mainly by megakaryocytic progenitors (Banno et al., 2016; Chou et al., 2008; Klusmann et al., 2010). Most patients suffering from TMD have constitutional trisomy 21, in the form of Down syndrome or mosaic Down syndrome (Malinge et al., 2009; Roberts and Izraeli, 2014). Rarely TMD also occurs in non-DS patients. However, all described cases of this nature harbored somatic trisomy 21 (Carpenter et al., 2005; Magalhaes et al., 2005; Yumura-Yagi et al., 1992).

We identified, to the best of our knowledge, the first case of TMD with no involvement of chromosome 21. To rule out mosaicism for trisomy 21 or a partial trisomy 21, two independent cytogenetic analyses were performed, both of which revealed a normal karyotype without any numerical aberrations of the chromosome in question. Furthermore, the SNP array analysis of the blast population reliably excluded the presence of trisomy 21. The sequential acquisition of uniform genetic events has established TMD in the past as a perfect model of myeloid leukemogenesis (Garnett et al., 2020; Hitzler and Zipursky, 2005). Our observations, discussed below, point to additional mechanisms that may participate in TMD pathogenesis. More importantly, the absence of trisomy 21 in the blast population questions the (absolute) necessity of the additional chromosome 21 in TMD origin.

To further characterize the genetic background of this exceptional TMD, we performed next generation sequencing and identified novel somatic mutations in the GATA1, JAK1, SPIRE2 and FN1 genes. Normal definitive hematopoiesis is not only maintained by two copies of the 21 chromosome, but also by full-length GATA1 (Crispino, 2005). Mutations in the GATA1

gene are the second essential factor that was postulated as necessary for TMD development (Banno et al., 2016; Carpenter et al., 2005; Gialesaki et al., 2018; Xu et al., 2003). These aberrations are usually located in the second exon and lead to the exclusive production of an N-terminally truncated variant called GATA1s (Shimizu et al., 2008). The dysregulation of GATA1, perturbs a complex transcriptional network regulating HSCs development, that eventually causes an accumulation of immature progenitors (Banno et al., 2016; Shimizu et al., 2008; Shimizu et al., 2009) in a stage specific manner (Gialesaki et al., 2018). When GATA1s expression was induced in human fetal, neonatal and adult HSPCs, terminal differentiation and progenitor cell accumulation was present only in the fetal ontogeny (Gialesaki et al., 2018). The distinct effects of GATA1s in individual developmental stages of hematopoiesis could explain the temporary nature of TMD, which is a self-limiting disease usually resolving spontaneously within the first few months after birth (Klusmann et al., 2008). The spontaneous remission coincides with the gradual transition of hematopoiesis from fetal liver into the bone marrow (Dzierzak and Speck, 2008; Hitzler and Zipursky, 2005). When hepatic hematopoiesis ceases, it may result in loss of necessary microenvironment crucial for TMD blast growth. Possibly, unknown factors in the bone marrow microenvironment may stop the blast proliferation (Miyauchi and Kawaguchi, 2014). The novel GATA1 mutation, GATA1 D65_C228del, described by us, differs significantly from the common mutations resulting in the production of GATA1s, usually represented by small duplications, insertions and deletions (Rainis et al., 2003). GATA1 D65_C228del results in a large deletion spanning between multiple exons. We proved the production of the GATA1 chimeric protein in a cell model and tracked its localization into the nucleus, similarly to GATA1 wt and GATA1s. Interestingly the GATA1 D65_C228del protein lacks the whole NZF and a part of TAD. Conversely, GATA1s lacks only TAD. Point mutations in the NFZ are commonly present in benign congenital anemias (Freson et al., 2001; Freson et al., 2002; Mehaffey et al., 2001; Nichols et al., 2000). However, we have not found any published information about cases of TMD or other hematological malignancies harboring aberrations in the GATA1 gene leading to complete NZF loss. The two major functions of NZF are to enable and subsequently stabilize GATA binding to DNA and to mediate the interaction between GATA1 and its essential cofactor FOG1 (Lowry and Mackay, 2006; Trainor et al., 2000; Trainor et al., 1996; Tsang et al., 1997). Interestingly, abrogation of the interaction between GATA1 and FOG1 results in loss of differentiation, however proliferation of immature megakaryocytes is conserved (Kuhl et al., 2005). The association of GATA1 with

FOG1 is crucial during embryonic hematopoiesis (Shimizu et al., 2004). Mutations perturbing this interaction lead to essential thrombocytopenia (Chang et al., 2002; Nichols et al., 2000). Moreover, the indispensability of NZF was demonstrated previously in two pivotal rescue studies. In transgenic mice, NZF was required for definite erythropoiesis (Shimizu et al., 2001), and in GATA1-null erythroid cells, NZF rescued erythroid differentiation (Weiss et al., 1997). It has been postulated that NZF loss may affect the function of GATA1 more severely than TAD loss (Shimizu et al., 2001). When expressed at high levels, GATA1s was able to rescue definite erythropoiesis, however the NZF lacking GATA1 variant had no rescue effect, regardless of expression levels (Shimizu et al., 2001). We therefore hypothesize, that the novel GATA1 mutation identified in our patient, resulting in complete loss of NZF together with partial loss of TAD, negatively influences GATA1 function in a more severe way when compared with GATA1 aberrations found in trisomy 21-associated TMDs. The aberrant GATA1 D65_C228del isoform is predicted to lose transactivation potential, together with partially losing the ability to recognize DNA binding sites. Conversely, the GATA1s isoform loses only transactivation potential, therefore possibly compromising fetal hematopoiesis to a lesser extent than GATA1 D65_C228del. Nevertheless, the effect of GATA1 D65_C228del remains limited to fetal hematopoiesis, parallel to GATA1s. Interestingly, GATA1s alone, similarly to trisomy 21 alone, is insufficient to cause TMD. This has been exemplified by the presence of a germline GATA1 mutation, resulting in GATA1s production, in a family without TMD occurrence (Hollanda et al., 2006). In comparison, the GATA1 D65_C228del isoform may possibly be able to induce the non-DS-TMD alone.

Tumorigenesis however only rarely results from a single genetic hit and usually requires multiple cooperating aberrations (Inaba et al., 2013). The novel JAK1 deletion, JAK1 F636del, identified by us represented the most probable candidate involved in TMD induction, together with the aberrant GATA1. Mutated JAK genes are recurrently found in various hematological malignancies, importantly also in AML and AMKL (Chen et al., 2012; Jeong et al., 2008; Nikolaev et al., 2013; Xiang et al., 2008; Zhang et al., 2012). JAK1 F636del results in the loss of one highly conserved phenylalanine on position 636 located in the pseudokinase domain a region frequently harboring activating mutations (Flex et al., 2008; Haan et al., 2010). F636 belongs to a triad of aminoacids which, together with V658 and F575, likely controls the catalytic activity of JAK1 (Toms et al., 2013). Hence, we expected that its loss will

significantly impact the structure and possibly function of JAK1. However, homology modeling suggested the compatibility of F636 loss with both the active and inactive conformations of JAK1, together with a third possible "alternative" conformation, resembling the inactive state. We showed that catalytic activity of JAK1 F636del is preserved, however phosphorylation levels of JAK1 and STATs, its downstream signaling molecules, were lower when compared to their wild type counterparts, implicating that F636del results in decreased kinase activity. Typical JAK pseudokinase activating mutations, including JAK2 V617F, which is present in the majority of polycythemia vera patients, cause activation of the kinase domain (James et al., 2005). Similarly, the JAK1 V658F variant which is homologous to JAK2 V617F, activates the JAK1/STAT3 pathway (Hornakova et al., 2009; Jeong et al., 2008; Mullighan et al., 2009c). Furthermore, the activating potential of various aberrations affecting JAK1 via the Ba/F3 cell assay has been demonstrated (Arulogun et al., 2017; Li et al., 2017). In the case of JAK1 F636del, IL3 independent growth was not achieved, therefore questioning the oncogenic potential of this particular deletion. We hypothesized that JAK1 F636del may only exert its effect in the context of mutated GATA1, due to the delicate interplay between these two aberrations in a site and cell specific manner. Therefore, we assessed the cooperation of JAK1 F636del with GATA1s by utilizing a mouse fetal liver HSPC model, successfully mimicking the trisomy 21-independent TMD. However, even in this setting no impact on cell maturation and proliferation was registered. The involvement, if any, of JAK1 F636del in the pathogenesis of the trisomy 21-independent TMD remains elusive. Our findings regarding JAK1 complement the recently published data from the largest sequencing study of TMD and myeloid leukemia of Down syndrome (ML-DS) patients conducted so far. Labuhn and colleagues showed that tyrosine kinase mutations, most prevalently JAK mutations, are very common in both patients with TMD and in patients who progress to ML-DS (Labuhn et al., 2019). Interestingly, the vast majority of JAK aberrations found in ML-DS patients were either already documented as gain-of-function mutations (Baxter et al., 2005; Bercovich et al., 2008; Kiyoi et al., 2007; Malinge et al., 2008), or were proved activating in various cell assays by Labuhn and colleagues (Labuhn et al., 2019). On the other hand, none of the JAK mutations found in this large cohort of TMD patients was shown to be activating (Labuhn et al., 2019). Moreover, another study identified JAK3 loss-of-function mutations in DS-TMD and AMKL (De Vita et al., 2007). Some of these mutations were also previously found in patients with severe combined immunodeficiency (De Vita et al., 2007; O'Shea et al., 2004). Due to their abundance in TMD, it seems that JAK

gene mutations, are not merely passenger mutations and may therefore play a role in the pathogenesis of this preleukemia, however the mechanism of their contribution may significantly differ from that of previously described gain-of-function mutations.

Alternatively, the novel mutations in SPIRE2 (SPIRE2 R471W) and FN1 (FN1 R2420C) genes, identified by WES, may also contribute to the development of this unique TMD. SPIRE2, which encodes Spire type actin nucleation factor 2, plays a role in asymmetric oocyte division. It mediates asymmetric spindle positioning by assembling the actin network and drives polar body extrusion by promoting assembly of the cleavage furrow (Pfender et al., 2011). The FN1 gene encodes fibronectin 1, a glycoprotein involved in cell migration and adhesion processes like wound healing, metastasis and blood coagulation (Barbazan et al., 2017; Grinnell, 1984; Li et al., 2019; Wang and Ni, 2016). However, current knowledge about the involvement of SPIRE2 and FN1 in hematological malignancies is very limited and we can therefore only speculate about their involvement in trisomy 21-independent TMD pathogenesis.

Chromosomal rearrangements resulting in fusion gene production represent a hallmark of pediatric ALL (Iacobucci and Mullighan, 2017; Mitelman et al., 2007). The ETV6-ABL1 fusion gene is a rare, but recurrent, genetic event in both children and adults diagnosed with ALL (Zaliova et al., 2016). Our laboratory focused its research on the characterization of patients harboring the ETV6-ABL1 fusion and published a number of pivotal articles regarding this topic in the past (Zaliova et al., 2016; Zuna et al., 2010). In the presented study we characterize a unique childhood BCP-ALL positive for the ETV6-ABL1 fusion gene. ETV6-ABL1 originates either from the insertion of a part of the ABL1 gene into the ETV6 gene, which is located on chromosome 12, or from the insertion of a part of ETV6 into ABL1 located on chromosome 9 (Zaliova et al., 2016). Rarely, additional chromosomes are involved in the rearrangement (La Starza et al., 2002; Tirado et al., 2005). The fusion identified by us was localized on chromosome 12, supporting the fact that ABL1 insertion into ETV6 represents a more common mechanism over the opposite event (Zaliova et al., 2016). The insertion was cryptic, similarly to the majority of previously described cases. Therefore, no abnormalities were detected during routine FISH analysis with the ETV6 probe.

In leukemias, commonly one or two in-frame fusion genes originate from a single chromosomal rearrangement. We identified three in-frame fusion genes, namely ETV6-ABL1,

ABL1-AIF1L and AIF1L-ETV6. By detecting the exact intronic junction sequences of all three in-frame fusions we confirmed that they originated from a single rearrangement, which represents a rare event. Reciprocal in-frame fusion transcripts can be detected in recurrent fusions like BCR-ABL1, KM2TA-AFF1, ETV6-RUNX1, PML-RARA, CBFB-MYH11 and RUNX1-RUNX1T1 represented by ABL1-BCR, AFF1-KMT2A, RUNX1-ETV6, RARA-PML, MYH11-CBFB and RUNX1T1-RUNX1, respectively (Kowarz et al., 2007; Loncarevic et al., 2002; Romana et al., 1995a, Lukes et al., manuscript under consideration). Despite the fact that these additional fusion genes are undetectable in a proportion of patients, certain studies suggest that they might contribute to leukemia phenotype or perhaps have even oncogenic potential, and therefore are not mere passenger aberrations (Bursen et al., 2010; Gaussmann et al., 2007; Rafiei et al., 2015; Zheng et al., 2009). This has been nicely demonstrated on a number of reciprocal KMT2A fusion proteins (Marschalek, 2020). For example, in mice the AFF1-KMT2A fusion protein, reciprocal to KMT2A-AFF1, was capable of inducing ALL, even without the direct KMT2A-AFF1 fusion protein (Bursen et al., 2010). Moreover, the oncogenic potential of the NEBL-KMT2A fusion protein, reciprocal to KMT2A-NEBL, has been shown in transfected cells (Emerenciano et al., 2013). The BCR-ABL1 fusion gene is a molecular hallmark of CML (Zhou et al., 2018). Its reciprocal counterpart, the ABL1-BCR fusion gene, has been proposed to exhibit leukemogenic potential (Zheng et al., 2009). The ABL1-BCR chimeric protein increased short term stem cell capacity of murine hematopoietic stem cells and the proliferation of early progenitors. Interestingly, BCR-ABL1 exclusively assigned the cells a myeloid phenotype, whereas ABL1-BCR forced the B-cell commitment. By influencing the lineage commitment, ABL1-BCR could possibly contribute to leukemia phenotype determination (Zheng et al., 2009). However, the exact role of ABL1-BCR in CML still remains to be defined.

Moreover, we demonstrated that the fusion genes likely represent the first leukemogenic event in this BCP-ALL case by revealing the prenatal origin of the fusions by backtracking ETV6-ABL1 into archived neonatal blood withdrawn from the patient right after birth. These data support our previous findings suggesting that prenatal origin of ETV6-ABL1 is not uncommon in childhood ALL (Zuna et al., 2010). Leukemia manifestation occurred almost 3 years after birth in the studied patient. From this we can assume that the combined effect of the ETV6-ABL1, ABL1-AIF1L and AIF1L-ETV6 is insufficient to launch overt leukemia,

similarly to ETV6-ABL1 alone. Additional aberrations that cooperate with ETV6-ABL1 are necessary for leukemia development. In 80% of ALL cases positive for the ETV6-ABL1 fusion these lesions are represented by deletions in the CDKN2A/B and IKZF1 genes, as has been demonstrated previously by us (Zaliova et al., 2016; Zuna et al., 2010). Importantly, CDKN2A/B and IKZF1 deletions are also recurrently found in BCR-ABL1-positive ALLs. Aside from similarities in their genomic profiles, these two entities share an analogous gene expression profile (Mullighan et al., 2007; Roberts et al., 2014b; Zaliova et al., 2016). In the presented BCP-ALL both deletions in CDKN2A/B and IKZF1 were identified, likely representing the second hit aberrations contributing to the process of leukemogenesis. Moreover, these findings support the notion, that CDKN2A/B and IKZF1 silencing is a common feature of ETV6-ABL1-positive leukemia.

The ETV6 protein plays an important role in hematopoiesis, especially in the bone marrow, and during embryonic development (De Braekeleer et al., 2012; Wang et al., 1997; Wang et al., 1998). The main functions of the ABL1 gene concern cell adhesion and motility, autophagy, receptor endocytosis and actin binding (Colicelli, 2010; De Braekeleer et al., 2011). The ETV6-ABL1 fusion gene can be found not only in ALL, as described here, but also in AML and myeloproliferative neoplasms (Zaliova et al., 2016). It effects cell survival, proliferation and transforming capacity similarly as BCR-ABL1 (Hannemann et al., 1998; Okuda et al., 1996). Their effect varies in mice, were BCR-ABL1 induces leukemia and ETV6-ABL1 a chronic myeloproliferation (Million et al., 2002). In comparison with the ETV6 and ABL1 genes, information about the biological role and function of AIF1L, except its involvement in actin bundling, remains unclear (Lu et al., 2017; Schulze et al., 2008). Physiologically, AIF1L is expressed in a variety of tissues including the hematopoietic system. Importantly, it is also expressed in the majority of ALLs, according to our RNA sequencing data. However, its expression levels vary significantly. The here described AIF1L-ETV6 and ABL1-AIF1L fusion genes represent, to the best of our knowledge, the first leukemia associated disruptions of the AIF1L gene. Insufficient amount of available material prevented us from the direct analysis of chimeric AIF1L protein expression in the leukemic blast population. Therefore we utilized an *in vitro* approach and successfully localized the AIF1L-ETV6 fusion protein in the nucleus of transfected HEK293T cells. The ETV6 DNA-binding domain is preserved in this chimeric protein, therefore possibly enabling the recognition of ETV6 binding motifs and subsequent

DNA binding. It may play a similar role as in the MN1-ETV6 fusion protein, which can be found in patients with myelodysplastic syndrome and AML (Buijs et al., 1995), where the ETV6 DNA-binding domain is also preserved, and is together with the MN1 moiety capable of transforming murine fibroblasts (Buijs et al., 2000). In this case MN1 probably functions as a transcriptional co-activator, instead of serving as a transcription factor capable of binding to a specific DNA sequence (van Wely et al., 2003). Moreover, in translocations involving the BTL and PAX5 genes, the ETV6 DNA-binding domain is also part of the fusion protein, suggesting possible similarities in the mechanisms involved (Bohlander, 2005; Cazzaniga et al., 2001; Cools et al., 1999; Fazio et al., 2008). However, additional functional studies would be required to examine this hypothesis and reveal the exact function of AIF1L-ETV6. We did not detect the ABL1-AIF1L chimeric protein, despite the fact that the antibody showed a robust signal for AIF1L wild type protein. These data imply that ABL1-AIF1L is unstable or is not expressed at all.

In conclusion, I have participated in the description and detailed characterization of the molecular background behind two unique hematological entities: a TMD that evolved in a trisomy 21-independent setting and a ETV6-ABL1-positive BCP-ALL resulting from a single chromosomal translocation of prenatal origin. Deciphering the role of these molecular events helps us better understand the process of leukemogenesis in both childhood AML and ALL.

During my medical and doctoral studies I had the honor of personally meeting both Prof. Koutecký and Prof. Hrodek, the two pioneers, who introduced pediatric hematology and oncology in late 1960s into general medical practice in the Czech Republic, formerly Czechoslovakia. The initial protocols that they implemented gave a mere 30% disease-free survival of ALL (Kavan et al., 1997; Koutecky, 1990). Advances in diagnosis, disease monitoring and treatment have risen this bar as high as to 90% in the 21st century (Stary et al., 2014). I am glad that during my Ph.D. studies I was able to be part of a team of leading scientist and clinicians facilitating these improvement and could also slightly contribute to this positive trend.

Conclusions

The identification and characterization of genetic aberrations in childhood leukemias plays a pivotal role in understanding the process of leukemogenesis which subsequently helps us in developing novel therapeutic strategies and tailoring patient-specific treatments. In this study we identified novel mutations in protein coding genes together with a chromosomal aberration resulting in the production of previously undescribed fusion genes, providing significant information on the genetic background of two childhood acute leukemia entities, the transient myeloproliferative disorder and the ETV6-ABL1-positive B-cell precursor acute lymphoblastic leukemia.

We described the first case of trisomy 21-independent GATA1 mutation-positive TMD. Our findings contradict the generally accepted claim that this preleukemic condition, which presents an ideal model to study the individual steps of leukemogenesis, requires the extra chromosome 21 during its development. We identified novel molecular aberrations in the JAK1 and GATA1 genes which we functionally characterized. JAK/STAT signaling studies together with various cell based models question the contribution of JAK1 F636del in the pathogenesis of TMD. We hypothesize, that the large in-frame GATA1 deletion which results in the production of an aberrant protein lacking the N-terminal zinc finger, impacts fetal hematopoiesis more severely when compared to GATA1s and may therefore trigger the trisomy 21-independent TMD condition alone.

Moreover, we described two novel fusion genes, AIF1L-ETV6 and ABL1-AIF1L, which result from a single chromosomal rearrangement in an ETV6-ABL1-positive BCP-ALL. We demonstrated the prenatal origin of this unique rearrangement and hence its inability to cause overt leukemia.

Last but not least, we showed that fusion gene-based MRD monitoring is superior to fusion transcript-based MRD monitoring in pediatric AML patients positive for the PML-RARA, CBFB-MYH11 and RUNX1-RUNX1T1 fusion genes. Importantly we demonstrate that fusion gene breakpoint sequence identification by targeted sequencing is efficient and feasible.

List of publications and presentations

Publications:

Lukes J Jr, Danek P, Alejo-Valle O, Potuckova E, Gahura O, Heckl D, Starkova J, Stary J, Mejstrikova E, Alberich-Jorda M, Zuna J, Trka J, Klusmann J-H, Zaliova M. Chromosome 21 gain is dispensable for transient myeloproliferative disorder driven by a novel GATA1 mutation, Leukemia, 2020 February 24, doi: 10.1038/s41375-020-0769-1, **IF=9,94**

Lukes J Jr, Potuckova E, Sramkova L, Stary J, Starkova J, Trka J, Votava F, Zuna J, Zaliova M. Two novel fusion genes, AIF1L-ETV6 and ABL1-AIF1L, result together with ETV6-ABL1 from a single chromosomal rearrangement in acute lymphoblastic leukemia with prenatal origin. Genes Chromosomes Cancer, 2018 May 4, doi: 10.1002/gcc.6., **IF=3,36**

Manuscripts in revision/under consideration:

Lukes J Jr, Winkowska L, Zwyrtkova M, Starkova J, Sramkova L, Stary J, Trka J, Zuna J, Zaliova M. Identification of fusion gene breakpoints is feasible and facilitates accurate sensitive MRD monitoring on genomic level in patients with PML-RARA, CBFB-MYH11 and RUNX1-RUNX1T1 (*manuscript under consideration*)

Zaliova M, Potuckova E, **Lukes J Jr**, Winkowska L, Starkova J, Janotova I, Sramkova L, Stary J, Zuna J, Stanulla M, Zimmermann M, Bornhauser B, Jenni S, Tsai Y-Ch, Bourquin J-P, Eckert C, Cario G, Trka J. Frequency and prognostic impact of ZEB2 H1038 and Q1072 mutations in childhood B-other acute lymphoblastic leukemia (*manuscript in revision*)

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Lukes J Jr., Danek P., Alejo-Valle O., Potuckova E., Gahura O., Heckl D., Starkova J., Stary J., Mejstrikova E., Alberich-Jorda M., Zuna J., Trka J., Klusmann J-H., Zaliova M., Chromosome 21 gain is dispensable for transient myeloproliferative disorder driven by a novel GATA1 mutation, XXVI. Parizek days, Ostrava, CZ, ***Oral presentation, Best presentation award*** (2020)

Lukes J Jr., Danek P., Alejo-Valle O., Potuckova E., Gahura O., Heckl D., Starkova J., Stary J., Zuna J., Trka J., Klusmann J-H., Zaliova M., Chromosome 21 gain is dispensable for transient myeloproliferative disorder driven by a novel GATA1 mutation, 20. Prague hematology days, Hematology 2020 Post-ASH, Prague, CZ, ***Oral presentation*** (2020)

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Lukes J Jr., Danek P., Potuckova E., Starkova J., Stary J., Zuna J., Trka J., Klusmann J-H., Zaliova M., Chromosome 21 Gain Is Dispensable for Transient Myeloproliferative Disorder (TMD) Development, LEGEND iBFM HGV committee joint meeting, Prague, CZ, ***Oral presentation*** (2019)

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List of abbreviations

ABL1	Abelson Murine Leukemia Viral Oncogene Homolog 1
ACK	ammonium chloride potassium
AF750	alexa fluor 750
AFF1 (AF4)	ALL1-Fused Gene From Chromosome 4 Protein
AIEOP	Associazione Italiana di Ematologia e Oncologia Pediatrica
AIF1L	Allograft Inflammatory Factor 1 Like
AIF1L-N	N-terminus of Allograft Inflammatory Factor 1 Like
AIF1L-C	C-terminus of Allograft Inflammatory Factor 1 Like
AML	acute myeloid leukemia
AMKL	acute megakaryoblastic leukemia
ALL	acute lymphoblastic leukemia
APC	allophycocyanin
APL	acute promyelocytic leukemia
ARID1B	AT-Rich Interaction Domain 1B
ARID5B	AT-Rich Interaction Domain 5B
ATRA	All-trans retinoic acid
Ba/F3	murine interleukin 3 dependent pro-B cell line
B-ALL	B cell lineage acute lymphoblastic leukemia
BCL9	B-Cell Lymphoma 9 Protein
BCP-ALL	B cell precursor acute lymphoblastic leukemia
BCR	Breakpoint Cluster Region
BFM	Berlin-Frankfurt-Munster
bp	base pair
BTL	Brx-like Translocated in Leukemia
BTG1	B-cell Translocation Gene 1
C	cytoplasmic
Cas9	CRISPR associated protein 9
CBF	Core-Binding Factor
CBFB	Core-Binding Factor Subunit Beta
CBL	CBL Proto-Oncogene
CCR	complete clinical remission
CD	cluster of differentiation
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B
cDNA	complementary DNA
CEBPA	CCAAT Enhancer Binding Protein Alpha
CEBPE	CCAAT Enhancer Binding Protein Epsilon
c-kit	CD117; stem cell factor receptor
CFU	colony forming unit
chr	chromosome
CML	chronic myeloid leukemia
CNA	copy number aberration
CNV	copy number variation
CNS	central nervous system
CREBBP	CREB Binding Protein

CRISPR	clustered regulatory interspaced short palindromic repeats
CRLF2	Cytokine Receptor Like Factor 2
CTCF	CCCTC-Binding Factor
CZF	C-terminal zinc finger
DAPI	4’,6-diamidino-2-phenylindole
DEK	DEK proto-oncogene
del	deletion
DNMT3A	DNA Methyltransferase 3 Alpha
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxid
DUX4	Double homeobox 4 gene
DS	Down syndrome
DS-AMKL	Down syndrome acute megakaryoblastic leukemia
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E10.5	embryonic day 10.5 of development
E11.5	embryonic day 11.5 of development
E13.5	embryonic day 13.5 of development
EF1	EF-hand1 calcium-binding domain
EF2	EF-hand2 calcium-binding domain
EFS	event free survival
EGFP	enhanced green fluorescent protein
EML1	Echinoderm Microtubule-Associated Protein-Like 1
ELOVL4	Elongation Of Very Long Chain Fatty Acids Protein 4
ETP	early T-precursor
ETV6	ETS Variant Transcription Factor 6
EP300	E1A Binding Protein P300
EPOR	Erythropoietin Receptor
ERG	ETS Transcription Factor ERG
ERK	ERK kinases; extracellular signal-regulated kinases
ETS2	ETS Proto-Oncogene 2
ETS	ETS domain
ETV6 (TEL)	ETS Variant Transcription Factor 6
EV	empty vector
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
F	female
FAB	French-American-British
FABD	F actin binding domain
FACS	fluorescence assisted cell sorter
FBS	fetal bovine serum
FERM	FERM domain; 4.1 protein, ezrin, radixin, moesin
FHIT	Fragile Histidine Triad Diadenosine Triphosphatase
FITC	fluorescein isothiocyanate
FISH	fluorescent in situ hybridization
FLC	fetal liver cell
FLT3	Fms Related Receptor Tyrosine Kinase 3
FN1	Fibronectin 1
FOG1	Friend Of GATA1

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA1	GATA-Binding Factor 1
GATA1s	GATA1 short; lacks 83 N-terminal amino acids
GATA2	GATA-Binding Factor 2
GATA3	GATA-Binding Factor 3
GATA4	GATA-Binding Factor 4
GATA5	GATA-Binding Factor 5
GATA6	GATA-Binding Factor 6
G-CSF	granulocyte-colony stimulation factor
gDNA	genomic DNA
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulation factor
GRCh37	Genome Reference Consortium Human Build 37
HDAC9	Histone Deacetylase 9
HEK293T	human embryonic kidney 293 cell line
hg19	human genome 19
HOXA	Homeobox A
HSA21	copy number variations to chromosome 21
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplanation
HSPC	hematopoietic stem and progenitor cell
chr	chromosome
Ig	immunoglobulin
IGH	Immunoglobulin Heavy Chain
IKZF1	Ikaros Family Zinc Finger 1
IL3	interleukin 3
IL6	interleukin 6
IMDM	Iscove's Modified Dulbecco's Medium
iPS	induced pluripotent stem
JAK1	Janus Kinase 1
JAK2	Janus Kinase 2
JAK3	Janus Kinase 3
K562	human chronic myeloid leukemia in blast crisis cell line
KANSL1	KAT8 Regulatory NSL Complex Subunit 1
kb	kilobase
kDa	kilodalton
KIT	KIT Proto-Oncogene
KMT2A	Lysine Methyltransferase 2A
KRAS	KRAS Proto-Oncogene
LMO	LIM Domain Only
LMO1	LIM Domain Only 1
LMO2	LIM Domain Only 2
LSI	locus specific identifier
LYL1	Lymphoblastic Leukemia Associated Hematopoiesis Regulator 1
m	mouse
M	male
MACS	magnetic activated cell sorting

MDS	myelodysplastic syndrome
MEF2D	Myocyte Enhancer Factor 2D
MFC	multiparametric flow cytometry
miR-125b-2	microRNA 125b-2
ML-DS	myeloid leukemia of Down syndrome
MLL	mixed lineage leukemia
MLLT1 (ENL)	MLLT1 Super Elongation Complex Subunit
MLLT3 (AF9)	MLLT3 Super Elongation Complex Subunit
MNX1	Motor Neuron And Pancreas Homeobox 1
MRD	minimal residual disease
MYB	MYB Proto-Oncogene
MYC	MYC Proto-Oncogene
MYH11	Myosin Heavy Chain 11
N	nuclear
NN	non-templated nucleotides
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
NIH3T3	murine embryonic fibroblast cell line
NOTCH1	Notch Receptor 1
NPM1	Nucleophosmin 1
NRAS	NRAS Proto-Oncogene
NUP214	Nucleoporin 214
NZF	N-terminal zinc finger
OBP2A	Odorant Binding Protein 2A
p	short arm of the chromosome
P2RY8	P2Y Receptor Family Member 8
PacBlue	pacific blue
PAX5	Paired Box 5
PBS	phosphate buffered saline
PBX1	Pre-B-Cell Leukemia Homeobox 1
PCDHB15	Protocadherin Beta 15
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy7	phycoerythrin-cyanine 7
p-gag-pol	plasmid-group specific antigen-reverse transcriptase
Ph	Philadelphia
pIRES2-EGFP	plasmid
PMA	phorbol-12-myristate-13-acetate
PML	Promyelocytic Leukemia
PNT	pointed domain
PTPN11	Protein Tyrosine Phosphatase Non-Receptor Type 11
PU.1	transcription factor PU.1; encoded by SPI1 gene
p-VSV-G	plasmid-vesicular stomatitis virus G
pWCC19	plasmid
q	long arm of the chromsome
RAD21	RAD21 Cohesin Complex Component
RAF	RAF kinases; rapidly accelerated fibrosarkoma

RAG	Recombination Activating Gene
RAG1	Recombination Activating Gene 1
RAG2	Recombination Activating Gene 2
RARA	Retinoic Acid Receptor Alpha
RAS	RAS family of proteins
RNA-seq	whole transcriptome sequencing
ROSA26	Gt(ROSA)26Sor locus
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	real-time quantitative polymerase chain reaction
RUNX1	RUNX Family Transcription Factor 1
RUNX1T1	RUNX1 Partner Transcriptional Co-Repressor 1
SCF	stem cell factor
sgRNA	single guide RNA
SH2	Src Homology 2 domain
SH2-PK	Src Homology 2 domain-protein kinase
SH3	Src Homology 3 domain
SLC25A15	Solute Carrier Family 25 Member 15
SMC1A	Structural Maintenance Of Chromosomes 1A
SNPa	single nucleotide polymorphism array
SNV	single nucleotide variant
Sp1	transcription factor Sp1; encoded by SP1 gene
SPIRE2	Spire Type Actin Nucleation Factor 2
STAG2	Stromal Antigen 2
STAT	Signal Transducer And Activator Of Transcription
STAT3	Signal Transducer And Activator Of Transcription 3
SUZ12	SUZ12 Polycomb Repressive Complex 2 Subunit
TAD	transactivation domain
TAM	Transient Abnormal Myelopoiesis
T-ALL	T cell lineage acute lymphoblastic leukemia
TAF15	TATA-Box Binding Protein Associated Factor 15
TAL	T-Cell Acute Lymphocytic Leukemia
TAL1	T-Cell Acute Lymphocytic Leukemia 1
TAL2	T-Cell Acute Lymphocytic Leukemia 2
TBP	TATA-Box Binding Protein
TCF3	Transcription Factor 3
Ter119	Ter119 antigen
TF1	human erythroleukemia cell line
TKI	tyrosine kinase inhibitor
TLX1	T Cell Leukemia Homeobox 1
TLX3	T Cell Leukemia Homeobox 3
TMD	Transient Myeloproliferative Disorder
TP53	Tumor Protein P53
TPO	thrombopoietin
TRA	T-cell receptor alfa
TRB	T-cell receptor beta
TRD	T-cell receptor delta

Tri21	trisomy 21
Tyk2	Tyrosine Kinase 2
Tyr Kc	tyrosine protein kinase; catalytic domain
UPD	uniparental disomy
V(D)J recombination	variable (diversity) joining gene segment rearrangement
WES	whole exome sequencing
WHO	World Health Organization
wt	wild type
ZNF384	Zinc Finger Protein 384

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Attachments