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Review

The reciprocal world of MLL fusions: A personal view☆

Rolf Marschalek

Institute Pharm Biology/DCAL, Goethe-University, Max-von-Laue-Str.9, 60438 Frankfurt/Main, Germany

A B S T R A C T

Over the last 15 years the Diagnostic Center of Acute Leukemia (DCAL) at the Frankfurt University has diagnosed and elucidated the *Mixed Lineage Leukemia* (*MLL*) recombinome with > 100 *MLL* fusion partners. When analyzing all these different events, balanced chromosomal translocations were found to comprise the majority of these cases (~70%), while other types of genetic rearrangements (3-way-translocations, spliced fusions, 11q inversions, interstitial deletions or insertion of chromosomal fragments into other chromosomes) account for about 30%. In nearly all those complex cases, functional fusion proteins can be produced by transcription, splicing and translation. With a few exceptions (10 out of 102 fusion genes which were per se out-of-frame), all these genetic rearrangements produced a direct *MLL* fusion gene, and in 94% of cases an additional reciprocal fusion gene. So far, 114 patients (out of 2454 = ~5%) have been diagnosed only with the reciprocal fusion allele, displaying no *MLL-X* allele. The fact that so many *MLL* rearrangements bear at least two fusion alleles, but also our findings that several direct *MLL* fusions were either out-of-frame fusions or missing, raises the question about the function and importance of reciprocal *MLL* fusions. Recent findings also demonstrate the presence of reciprocal *MLL* fusions in sarcoma patients. Here, we want to discuss the role of reciprocal MLL fusion proteins for leukemogenesis and beyond.

1. Introduction and background

Translocations of the *MLL/KMT2A* gene localized at chromosome band 11q23 are of high clinical relevance as they define a group of leukemia patients - despite the many efforts in the last 3 decades - with still a poor outcome.

My laboratory has dedicated their work over three decades on the chromosomal translocation t(4;11)(q21;q23) in order to find clues that may help to find new options for a better treatment. About 40% of all MLL-r leukemia patients are diagnosed with this particular translocation, but we are far away from having a satisfactory treatment option. One of the putative reasons for this situation is the fact that we might have overlooked important contributing factors, such as the role of reciprocal fusion proteins.

For this article I reviewed all our old experimental data that we have gathered over nearly 3 decades, in order to recapture how I made my decisions to investigate not only direct *MLL* fusions, but also reciprocal *MLL* fusion alleles.

When we started in the mid-90's to perform first cell culture experiments with inducible expression vectors we never obtained any phenotypic readout when using *MLL-AF4* expression constructs. Also other labs tried to analyze the *MLL-AF4* fusion in cell culture systems or mouse models, but also failed to see any concrete phenotype indicative for the oncogenic power of an MLL-AF4 fusion protein. By contrast, Caslini and coworkers did show that forced overexpression of an inducible *MLL-AF4* expression construct resulted in a cell cycle arrest and a senescent phenotype [1], indicating that the MLL-AF4 fusion protein functioned in a cell culture model system exactly in the opposite manner than expected from a potent oncoprotein.

At the same time, other investigators were quite successful when testing e.g. the MLL-ENL or MLL-AF9 fusion proteins by introducing their genes retrovirally into murine hematopoietic stem/precursor cells and subsequent transplantation experiments (RTTA: retroviral transduction and transplantation assay). In all these cases, the mice developed leukemias with a myeloid phenotype ([2], reviewed in [3]).

Until today, these quite controversial results are still existing and no rational explanation has been found to explain the non-oncogenic phenotype of the MLL-AF4 fusion. Similar results were obtained in replating assays, where some MLL fusions are able to maintain their colony forming capacity, and in addition, were able to induce acute myeloid leukemia in mouse model systems, while other tested constructs, like e.g. *MLL-LASP1* or others (see below), remained negative [4]. Moreover, most MLL leukemic fusions resulted in disease phenotypes displaying a myeloid commitment, while a lymphoid commitment was not observed - even when fusion constructs were used that were known to cause acute lymphoblastic leukemia in patients.

This skewed the view on the functions deriving from *MLL* fusion alleles in the literature, because most scientific publications were focused on *MLL-ENL* (n = 187) and *MLL-AF9* (n = 437), perhaps because these two fusions were able to easily produce leukemias in most laboratories. This, and the fact that routine diagnosis was sometimes not able to demonstrate the presence of reciprocal fusion transcripts, paved

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E-mail address: rolf.marschalek@em.uni-frankfurt.de.

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the way to put scientific emphasis only on direct *MLL* fusion alleles. Ectopic expression of several tested *MLL* fusion alleles in cell lines or patient cells were then demonstrated to transcriptionally activate *HOXA* genes (*HOXA7* and *HOXA9* in myeloid cells; *HOXA9* and *HOXA10* in lymphoid cells), together with *MEIS1* and PBX factors [5].

By using murine hematopoietic stem/progenitor cells - which can be assumed to still provide an open chromatin conformation – the most prominent MLL fusions were tested. Most of them displayed a clear-cut gene expression signature which was highly similar to that one observed in *MLL*-rearranged (*MLL*-r) leukemia patients [6]. Most, but not all patients, could be classified by their *HOXA* activation signature. The exceptions were again t(4;11) ALL patients which displayed the *HOXA* program only in about 50% of cases [7], while the rest of these t(4;11) patients did show an *IRX1/IRX2* activation [7] associated with a poorer outcome of patients [8,9]. Further studies have revealed that IRX1 overexpression is associated with activation of *HOXB4* and the *EGR1-3* genes [10].

Over decades, many efforts have been made to establish a t(4;11) mouse model system, but no-one has ever been successful in establishing a leukemia model system by using only the human *MLL-AF4* fusion allele or a corresponding *AF4* cDNA knock-in into the endogenous *Mll* locus. When using such transgenic mouse models no leukemia became overt, rather they developed a B cell-type lymphoma after very long latency [11,12]. Also we established a mouse model that displayed a proB ALL, however, we obtained this result with the reciprocal AF4-MLL fusion protein [13], while the direct *MLL-AF4* retroviral construct did not result in leukemia development. Only very recently, when investigators used a human MLL/mouse AF4 chimera (MLL-Af4), they were able to obtain myeloid or B-type leukemias in murine hematopoietic stem/precursors or human cord blood cells after transplantation either into C57Bl6 or NSG mice [14]. However, even this study created more questions than providing answers, and so far no one has been able to reproduce this leukemia model.

All these different results, assay read-outs or mouse models are puzzling many investigators. Have the right hematopoietic stem/precursor cell populations been used in all these experiments? Do we need fetal liver cells for the leukemogenic transformation process? Do we need secondary genetic hits or the activation of the immune system? Do we need even cells upstream of the hematopoietic/endothelial hierarchy? Or do we simply need both reciprocal fusion alleles present in the same cell to initiate a pre-leukemic clone or leukemia [14–20]?

One of the problems when investigating *MLL* fusion alleles are the different constructs used in different laboratories. As one example, *MLL-AF4* fusion alleles were shown to be created by breakpoints localizing mostly within *MLL* intron 9, 10 or 11, while residing in AF4 introns 3 or 4, respectively ($3 \times 2 = 6$ possibilities). In particular, the chromosomal breakpoints in 839 diagnosed t(4;11) leukemia patients were mapped to *MLL* intron 7 ($n = 3$), exon 8 ($n = 1$), intron 8 ($n = 7$), exon 9 (n = 3), intron 9 ($n = 281$), exon 10 ($n = 31$), intron 10 ($n = 166$), exon 11 ($n = 26$), intron 11 ($n = 292$), exon 12 ($n = 10$), intron 12 (n = 16), exon 13 (n = 1) and additional 72 breakpoints downstream of exon 13 (at least additional $10 \times 2 = 20$ possibilities). The complexity of possibilities to create MLL-AF4 fusion proteins is enormous and may hinder comparability between experiments performed in different laboratories.

Another interesting point is statistical differences in breakpoint distribution in different patient cohorts: e.g. infant acute leukemias tend to have their breakpoints in *MLL* intron 11, while adult patients have their breakpoints preferentially localizing in *MLL* intron 9 [21]. This breakpoint bias has also clinical consequences, as breakpoints in *MLL* intron11 are associated with poorer outcome [22]. Whether different breakpoints in *MLL* produce direct or reciprocal fusion proteins with different oncogenic potential still needs to be tested experimentally.

However, this manuscript is not trying to review all the published experiments and arguments from the last 2 decades, rather than is trying to describe "*MLL* research" from a very personal view. The reason for this is clear, because we are the only lab in the world that is systematically working on reciprocal *MLL* fusion alleles. Only very recently, we were asked to share our reciprocal constructs with other investigators, such as Pablo Menendez in Spain, because this group also wanted to investigate the functions deriving from such fusion proteins. In order to introduce potential readers to this additional and quite complex field of *MLL* research, we need to go back to the very beginning of this research field to understand today's situation where only direct *MLL* fusions are still assumed to be the key players. So let's get started with a quite personal journey into the past.

2. *MLL* translocations: a brief history

After the initial discovery and cloning of the *MLL/ALL-1/HRX gene* at 11q23 [23–26], early on we started to work on the gene structures of *MLL* and *AF4*, respectively. After construction of lambda libraries and screening them with cloned cDNAs, we successfully established the complete gene structures of both genes [27,28]. The *MLL* gene exhibits 37 exons with a full coding potential of 4005 amino acids. Be aware that exon 2, coding for 33 amino acids, is not presented in the NCBI database gene structure for *MLL* (nowadays termed *KMT2A*). Moreover, *MLL* exon 2 is present only in about 30% of transcripts [29]. The most commonly produced MLL protein has therefore either 3972 or 3969 amino acids, due to an alternative splice event that occurs additionally between *MLL* exon 15 and 16. Other splice events between these two exons delete 11 or even 14 amino acids of the PHD3 domain. The latter splice events will cause MLL proteins with 3961 and 3958 amino acids. Of interest, changing the amino acid composition of the PHD3 domain toggles CYP33 binding activity to this domain [30], which in turn changes the function of MLL from an activator into a repressor of gene transcription.

The *AF4* gene has three different transcriptional start sites with exon 1a/1 and exon 1a/2, exon 1b and an exon 1c that all splice to exons 2-20. In addition, besides these different full-length transcripts a shorter transcript, named *FelC*, encodes only for *AF4* exons 1-3 has been cloned from an SEM expression library as a polyadenylated cDNA product and was sequenced in our laboratory as well [28].

Concomitantly with others (e.g. C Croce, A Biondi, N Zeleznik-Le), we cloned an *MLL-AF4* but also an *AF4-MLL* expression construct. For this purpose, we used the t(4;11) cell line SEM. This cell line was established in the laboratory of Dr. Johann Greil, working at that time at the Children's Hospital of the Erlangen University, the same University where we started our work on t(4;11) leukemia [31,32].

Our first experiments were conducted in an unbiased fashion with both fusion genes, *MLL-AF4* and *AF4-MLL*, together with the commercially available murine EcR-NIH3T3 cell line that came together with the pIND vector system (Invitrogen). This vector system allowed transgenes to be expressed with the Ecdyson-derivative Ponasterone A, an insect steroid hormone that usually does not act on gene transcription in mammalian cells. NIH3T3 cells were at that time generally accepted as a tool to read out oncogenic activity in so-called focus formation assays [33].

We cloned *MLL-AF4* (MLL exons 1-10::AF4 exons 4-20) and *AF4-MLL* (AF4 exons 1-3::MLL exons 12-37) into the pIND vector system. We also cloned *AF4*, *FelC* [28], an articial *FelC::NLS* construct as well as a mutant *H-RAS** gene (carrying a G12V mutation) as positive control (see Fig. 1A). The results of our first expression experiment revealed some surprising results.

As shown in Fig. 1B, Ponasteron A-induced expression of MLL-AF4 in these EcRNIH3T3 cells grown to confluency in petri dishes did not show any difference to the empty vector construct. However, growth of AF4-MLL expressing cells revealed a phenotype that matched perfectly with a phenotype associated with fibrosarcomas, namely elongated fascicles that are also known as a herringbone pattern (see Fig. 1B). A subsequent focus formation experiment revealed also a loss-of-contact

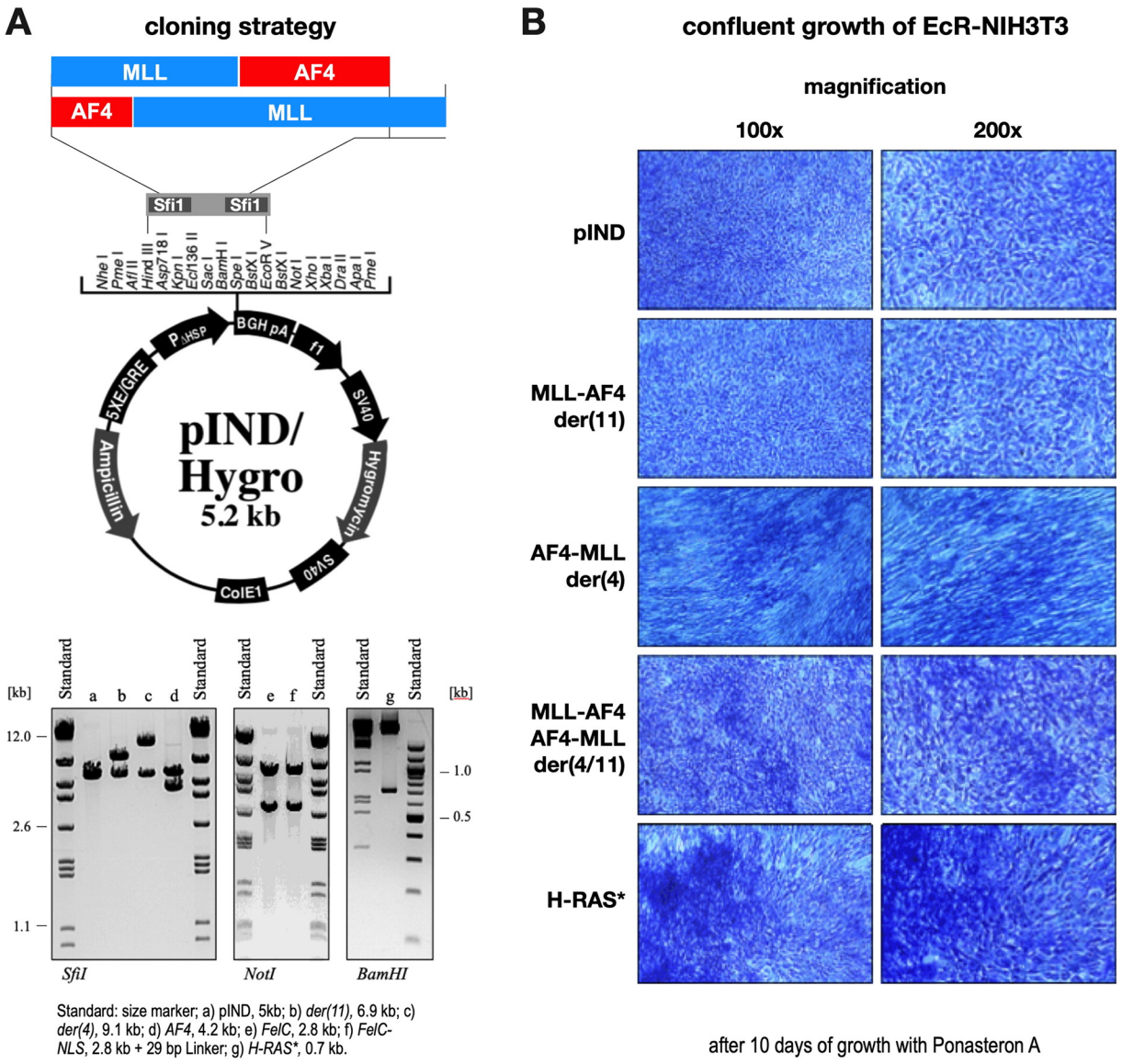

Fig. 1. Phenotype in stably transfected ER-NIH3T3 cells: confluent growth experiments.
A. EcR-NIH3T3 cells were stably transfected with either empty vector (pIND/Hygro) or the same vector containing either *MLL-AF4*, *AF4-MLL*, both fusion genes or the positive control (*H-Ras**). In addition, *AF4*, *FelC* (containing only *AF4* exons 1-3) and the artificial *FelC::NLS* were cloned. Inducibility of all transgenes by appropriate amounts of Ponasterone A has been validated by RT-PCR experiments in all cell lines (data not shown).
B. Ponasterone A was applied for 10 days during confluent growth to induce transgene expression for *MLL-AF4*, *AF4-MLL* and co-transduced cells. Oncogenic *H-Ras* (*H-Ras**) served as a positive control. The resulting phenotypes were loss-of-contact inhibition in case of H-Ras* expression, and a spindle-cell like cell growth phenotype when expressing the AF4-MLL fusion protein alone, which mimicked the phenotype typically seen in fibrosarcomas. Co-expression of both fusion proteins resulted in a denser growth with signs of transformation. Left: construction of vectors is displayed; right: pictures of stained petri dishes with 100 and 200-fold magnification are displayed.

inhibition phenotype for mutant H-RAS*, AF4-MLL but also for AF4 and FelC::NLS expressing cells (see Fig. 2A). Similarly, soft agar experiments revealed clonogenic growth for mutant *H-RAS**, as well as *AF4*, *FelC* and *FelC::NLS* transfected cells (see Fig. 2B). These very early experiments revealed also the importance of the AF4 protein and its derivatives (note: AF4 could be defined by these experiments as proto-oncoprotein), and moreover, the importance of AF4-MLL for subsequent experiments.

Next, we used two Burkitt Lymphoma B cell lines, DG-75 and RAJI, in combination with an episomal vector system. Both cell lines and the episomal vector BC364A were a gift from Georg Bornkamm, Munich. The RAJI cell line is positive for EBV (but negative for the expression of the immediate-early protein BZLF1) and carries a t(8;14) translocation leading to the overexpression of c-MYC. The DG-75 cell line carries only the chromosomal translocation t(8;14). We cloned again the expression cassettes *MLL-AF4* and *AF4-MLL* into this new vector backbone and used for the first time Sfi1 sites for directional cloning. Also here, the EBNA1 based episomal vector system expressing MLL-AF4 revealed no obvious phenotype (as the empty vector, data not shown), while the expression of AF4-MLL led to a very impressive phenotype: big cell agglomerates, similar to 3D organoids, in the presence of expressed AF4-MLL fusion protein. The largest agglomerates were 1 cm in diameter and only the smallest ones could be photographed for documentation. These rather sticky cell clusters occurred only upon expression of the AF4-MLL fusion protein in both cell lines (see Fig. 3). Unfortunately, we had at that time no possibilities to express both fusion cDNAs together in the same cell line, because no second, episomal vector system was available that expressed a different selection marker.

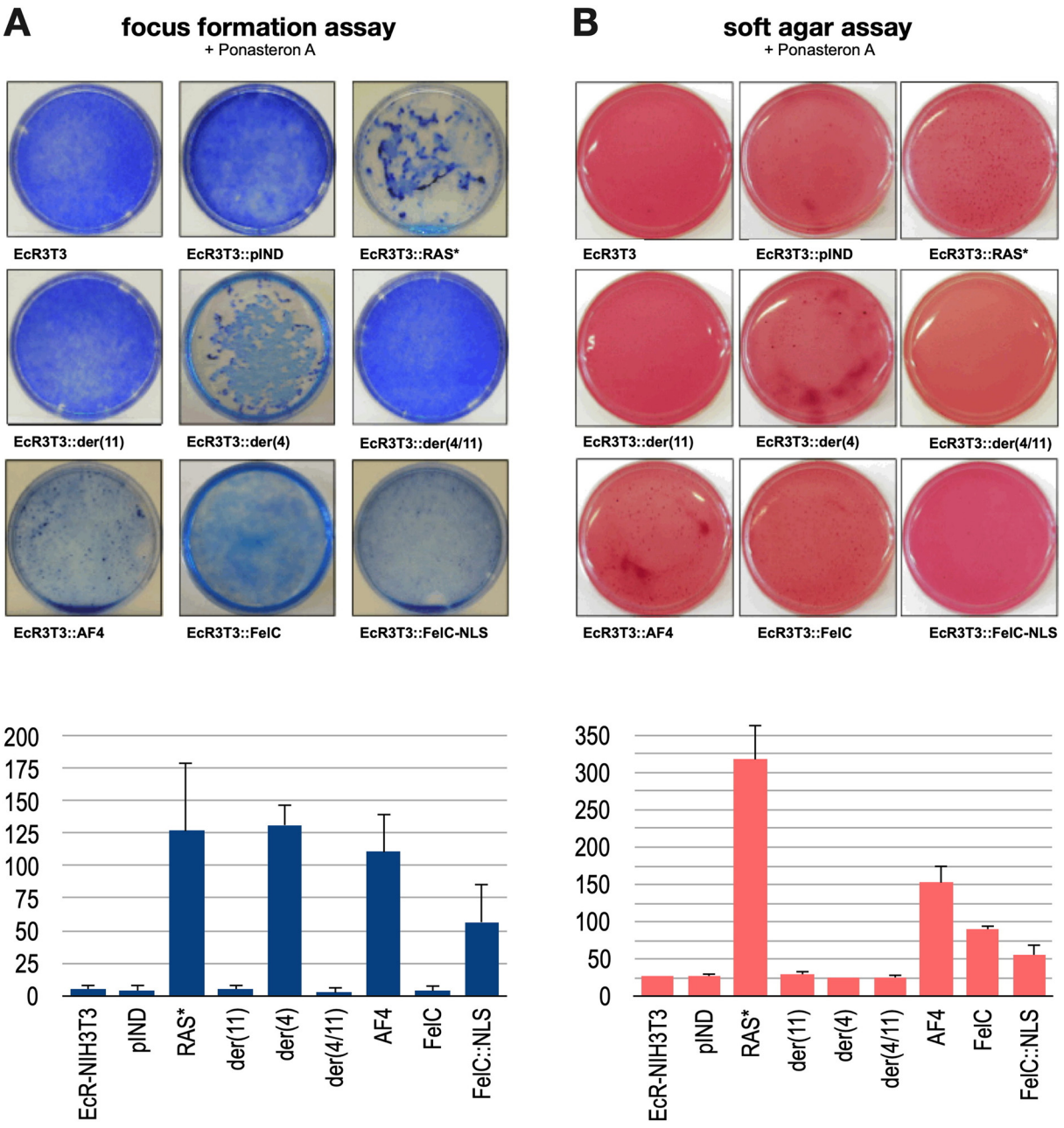

Fig. 2. Phenotype in stably transfected ER-NIH3T3 cells: focus formation and soft agar assay.
Similar experiments were performed to investigate focus formation (**A**) and soft agar experiments (**B**). All petri dishes of each experiment (n = 7) were visually inspected and quantification for the resulting phenotype is displayed below. Focus formation was observed after 21 days in H-RAS*-, AF4-MLL-, AF4- and FelC::NLS-expressing cells (left lower panel). Similarly, growth in soft agar was documented for H-RAS*- AF4-, FelC and FelC::NLS expressing cells (n = 7). All cells grown without Ponasterone A did not show any difference to mock treatment cells.

However, it became clear at this point that we needed to investigate not only the MLL-AF4 fusion protein, but also the putative functions deriving from the AF4-MLL fusion protein. At least in this particular cellular context where the proto-oncoprotein c-MYC is overexpressed, functions deriving from the AF4-MLL fusion protein seem to result in a strong phenotype. This was the basis for our decision that all experiments that we performed in our laboratory over the next two decades were investigating always both reciprocal fusion proteins (alone and in combination), regardless at which MLL translocation we were looking at.

3. Oncogenic concepts

The concept of two cooperating oncoproteins was not new at that time. Former work by Thomas Graf and co-workers at the EMBL in Heidelberg had already shown that that v-erbA and v-erbB, encoded by the chicken avian erythroblastosis virus (AEV), were cooperating proteins that caused either erythroleukemia or sarcomas in the chicken system [34,35]. V-erbA could even cooperate with other viral oncoproteins, like v-ets or a fusion product of v-ets and v-myb to cause a very fast onset of erythroleukemia [36]. Using single oncoproteins, a much longer latency for tumor formation was observed. All these and many other experiments in the 80's paved the way for the generally accepted hypothesis that at least 2 cooperating events (2-hit-rule) are

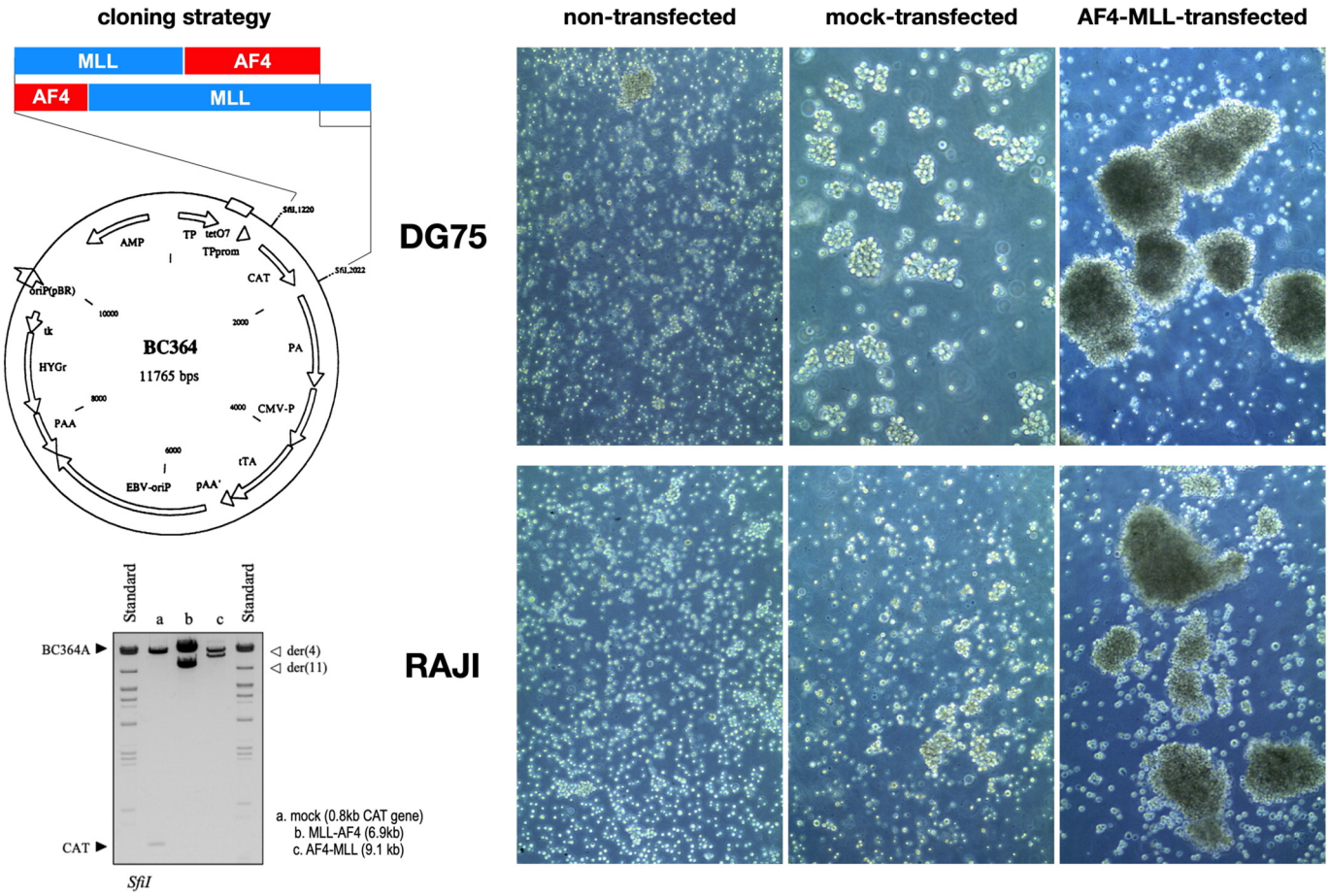

Fig. 3. Phenotype in B-lymphoid cells.
The same expression cassettes of *MLL-AF4* and *AF4-MLL* were cloned in the episomal vector system BC364 that expresses the EBNA1 protein. DG-75 and RAJI cells, both Burkitt lymphoma cell lines, were transfected and grown in suspension cultures, together with non-transfected and mock-transfected cells. No phenotype was observed with MLL-AF4 expressing cells (similar to mock vector), while AF4-MLL expressing cells did show large size 3D-agglomerates (centimeters), of which the smallest ones (millimeters) could be documented by microscopy. Both cell lines, DG-75 and RAJI, displayed identical results although only RAJI cells are EBV positive.

necessary to induce malignant cell growth. This concept is still valid but has been extended tremendously in modern cancer biology [37].

Thus, a chromosomal translocation that occurs between two different genes may allow the simultaneous creation of two cooperative fusion proteins (direct and reciprocal) that are potentially sufficient to cause the onset of cancer. To substantiate this assumption, we have the possibility to look retrospectively into our database of diagnosed MLL-r leukemia patients (n = 2454) [38–42]. As a matter of fact, the majority of patients display two or even 3 fusion alleles (~94%). The only exceptions from the "2-hit-rule" are: (1) genetic fusions between *MLL* and *ENL* occur in about 50% upstream of *ENL* exon 1 (153 out of 302 ENL rearrangements). Thus, an MLL-ENL fusion protein can be produced only by transcription and splicing (termed "spliced fusion") from a C-terminal disrupted *MLL* gene to *ENL* exon 2, but no reciprocal *ENL-MLL* fusion RNA can be generated from such a genetic rearrangement; (2) interstitial deletions involving the *MLL* gene at 11q23.3 and another gene localized telomeric to it may lead to the presence of a single fusion allele, when the deleted region from chromosome 11 is indeed lost and not integrated somewhere else (n = 5 out of 2454). In these precisely defined patient cases (n = 158 out of 2454 which is ~6%) only a single and direct fusion protein could be produced that exhibits the oncogenic power to initiate and maintain a leukemic disease. However, it could well be that these few patients exhibit yet unknown secondary mutations that are complementing for a missing reciprocal allele.

The vast majority of *MLL* recombinations carry 2 or more fusion alleles, and thus, the existence of reciprocal fusion alleles cannot be denied. Moreover, all these reciprocal fusion proteins - including the MLL* protein produced by a gene-internal promoter of the *MLL* gene [43] - bear important catalytic activities, such as e.g. The SET domain complex [44] or the ability to recruit important HATs (CREBBP, MOF/MYST1) [45,46], as well as a PHD1-3/BD domain which is able to bind and read chromatin or toggle the biological activity for gene transcription [47]. This clearly means that reciprocal MLL fusion proteins exert a distinct biological function, and moreover, may compete for binding factors that usually bind to the wildtype protein complexes. To this end, good scientific practice demands also the analysis of reciprocal MLL fusion proteins in an open-minded and unbiased fashion.

4. Experiments performed with reciprocal fusion proteins

In order to analyze direct and reciprocal MLL fusion proteins in a coordinated and comparable fashion, we decided to use the established Sleeping Beauty transposon vector system [48] in combination with cell culture model systems. The Sleeping Beauty transposon system has many advantages over retro- or lentiviral vector systems, as the copy number of vector integration could be kept low (1–10 copies), because the vector coding for the transposing sequence as well as the catalytic active SB transposase vector can be applied at various concentrations. Moreover, the vector backbone has no length restrictions and transposon integration usually does not occur in transcribed regions, because

TA-dinucleotides are targeted. Thus, the risk for integration mutagenesis is lower when compared to retro- or lentiviral vector systems that preferentially integrate into active genes.

We designed all vectors in a way that the corresponding *MLL* fusion cassettes could be Sfi1-cloned and expressed in a doxycycline inducible fashion, while a second constitutive promoter drives a polycistronic transcript encoding the reverse TET repressor (rtTA), with either 3 different fluorescent proteins in combination with 4 different selection markers [49], all separated by 2A cleavage signals. The "universal" direct vector contains the MLL N-terminus (exons 1-9), followed by a short intronic sequence and a cloning site (coexpressing GFP). The "universal" reciprocal contains a cloning site, followed by a short intronic sequence and the MLL C-terminus (exon 14-37, coexpressing RFP). Both vector systems were used to analyze in cell culture models 4 different MLL fusions: *NEBL*, *LASP1*, *MAML2* and *SMAP1* [50]. Corresponding cDNA fragments of all 4 genes were cloned into both universal MLL-N and MLL-C vector backbones and transfected into murine embryonic fibroblasts (MEF cells). Single as well as co-transfections were performed and stable cell lines were selected for all 12 conditions (3 per translocation). Inducible transcription and correct splicing was confirmed by sequencing the cDNA products of the transcribed *MLL* fusion alleles. Subsequently, experiments analyzing changes in cell growth behavior, cell viability, *Hoxa* gene transcription and loss-of-contact inhibition experiments were performed. As positive control, we used again the oncogenic H-RAS* protein, and we also included a vector expressing only the MLL* protein [43].

As published in 2014, the results were quite surprising and unexpected: 3 out of 4 tested reciprocal MLL fusions (*NEBL*, *LASP1* and *MAML2*) displayed oncogenic features, while only 1 out of the 4 tested direct MLL fusions did the same (*SMAP1*). However, all co-transfected cells displayed an oncogenic phenoytpe, underscoring again that both fusions are cooperating with each other [50]. As expected, the mutant H-RAS* protein alone was sufficient to exert oncogenic properties. Unexpectedly, the N-terminal truncated MLL* protein also displayed oncogenic features. To this end, this first pilot study supported our notion that *MLL*-rearranged leukemias should be analyzed individually in an unbiased fashion by using both - direct and reciprocal - MLL fusion protein alone and in combination.

Our data also validated an earlier publication regarding the MLL-LASP1 fusion, derived from a t(11;17) translocation diagnosed in a leukemia patient that exhibited an AML M4 disease phenotype. Retroviral transduction with an *MLL-LASP1* fusion construct alone neither produced colonies in methylcellulose nor leukemia in a retroviral transduction and transplantation setting [4]. Here, we validated these earlier data, but also showed that the reciprocal LASP1-MLL fusion displayed oncogenic features. Thus, the list of direct MLL fusions that failed to show oncogenic features encompassed AF4, LASP1 but also GRAF (now ARHGAP26), FBP17 (now FNBP1) and ABI1.

5. Reciprocal AF4-MLL

My laboratory has also investigated the reciprocal AF4-MLL fusion protein in murine hematopoietic cells. As outlined above, this reciprocal fusion already demonstrated its oncogenic features in very early experiments. Moreover, AF4-MLL is somehow quite special as it exhibits important protein binding modules of AF4 and MLL, which allow one to speculate about potential functions: the MLL domains presented in the fusion protein are capable of reading, activating and writing chromatin while the N-terminal portion deriving from AF4 is able to steer transcriptional elongation. Of interest, the AF4-MLL fusion protein is missing any target specificity due to the missing N-terminus of MLL, where usually MEN1 and LEDGF are complexed and responsible for binding to promotor-bound transcription factors at MLL target genes [51].

We first cloned *MLL-AF4* and *AF4-MLL* into a retroviral vector backbone (PIDE). The PIDE vector is a PINCO derivative that contains an IRES::GFP and known to exert a weaker promoter activity than other retroviral vector systems (a gift from Martin Ruthardt and Elena Puccetti). After in vitro packaging and titration of viral stocks in BAF3 cells, purified $Lin^-/Sca1^+$ hematopoietic stem/precursor cells were transduced and retro-orbitally injected into sublethally irradiated C56BL6 mice (8 Gy) to monitor leukemia development (2×10^5 cells per transplant). Transduction of the hematopoietic stem/precursor cell population was poor, due to the length of both proviruses (11,344 and 13,281 bp, respectively) and the MOI ranged from 10^{-3} to 10^{-4}. Nevertheless, leukemia development was observed for *AF4-MLL*- and co-transduced cells with a penetrance of about 35% and a mean latency of 233 days (~7.7 months) [13]. All those leukemias could be retransplanted into secondary or tertiary mice with a strong progression of leukemia development (mean of 25 days for a full blown leukemia).

The resulting immunophenotype was proB ALL (with AF4-MLL alone) or a B/T precursor ALL and mixed-lineage leukemia (MLL) when both constructs were present. All leukemic cells were successfully investigated for the correct expression of their transgenes, indicating that the expression of these fusions was necessary for leukemia maintenance [13]. The low MOI also indicated that these results could not be due to integration mutagenesis. Therefore, this work represented in a certain way a paradigm shift, as it was the first paper that demonstrated leukemia initiation and maintenance by using a reciprocal MLL fusion protein in an in vivo model system. However, it was still puzzling because we had at that time no molecular mechanism that could explain our observations.

However, this made clear that the AF4-MLL fusion protein was causing a developmental arrest at the proB stage, while the combination of both fusions caused the arrest at an earlier upstream stage of lymphoid and myeloid lineage development. The 3 observed immunophenotypes also reflected on the capability of many *MLL*-r leukemias which harbor both potentials (lymphoid and myeloid) and gave the *MLL* gene its original name (*Mixed Lineage Leukemia*). This capacity of *MLL*-r leukemia cells, and in particular in cases with t(4;11) leukemia, can still be seen today, when t(4;11) patients were treated with an anti-CD19 or a Blinatumomab therapy, resulting in a lineage switch and thereby causing therapy escape.

6. Functions deriving from AF4 and AF4-MLL

In order to understand all these data at the functional level, we subsequently purified and analyzed the multiprotein complexes that are formed on the AF4 and AF4-MLL protein backbones. The composition of the MLL complex was already described at that time [52,53] and this knowledge was used to validate the identified proteins binding the C terminal portion of MLL.

Corresponding expression constructs with a C-terminal Strep-tag were expressed in 10^9 HEK293 cells and purified ($n = 30$) in the presence of MG132. Blocking the proteasome was necessary because the AF4 protein is rapidly degraded via the proteasomal pathway by binding to the E3 ligases SIAH1 or SIAH2 [54], while AF4-MLL is rather stable with a half-life of > 90 h. Subsequent analysis by Nano-LC MS/MS technologies revealed a complex pattern of bound proteins that were subsequently all validated by Western blot and Co-IP experiments [55], and confirmed an earlier publication regarding the functional characterization of the murine Af4 complex [56].

Thus, it became clear that human AF4 - as well as murine Af4 - have a central role for transcriptional elongation, a fundamental biological process important to all cells and their tissue-specific expression programs.

AF4 and AF4 family members (AF5, LAF4 and FMR2) in the human system have a distinct function in our cells: they provide a molecular platform for the assembly of multiprotein complexes that execute transcriptional elongation (reviewed in [57–60]). Initiation of gene transcription is carried out by the pre-assembled RNA Pol II, also named PIC (pre-initiation complex) with the help of TFIIH (CDK7 and Cyclin

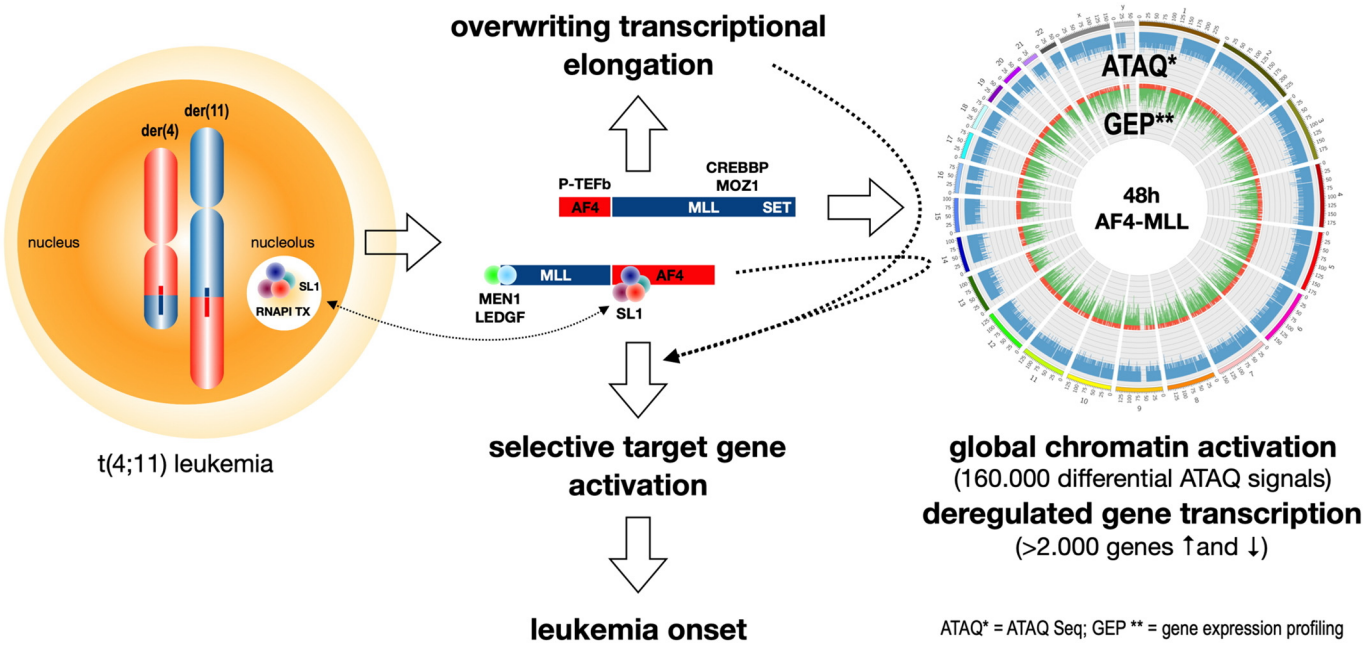

Fig. 4. Potential functions deriving from the cooperating fusion proteins MLL-AF4 and AF4-MLL.
Based on our experimental data, we propose the following hypothesis for the function of the direct and reciprocal fusion proteins deriving from t(4;11) translocations. The AF4-MLL fusion protein is overwriting the cellular transcriptional elongation control in a dominant fashion and causes increased $H3K4_{me3}$ and $H3K79_{me2/3}$ chromatin signatures, as published recently [55]. In addition, uncontrolled gene transcription may lead to a chromatin activation which is reflected by the ATAC Seq experiment (shown for Hela cells). This was accompanied by a strong upregulation of gene transcription, measured by Affymetrix gene expression profiling. Both data sets were combined to demonstrate the effect caused by the AF4-MLL fusion protein which seem to activate all chromosomes apart from centromeric regions. Within such a setting, the MLL-AF4 fusion proteins, bound to MENIN, LEDGF and SL1 [93] - alone or together with endogenous transcription factors - are able to define new genetic programs which might be reflected by the lymphoid and myeloid nature of such cells, as well as by their plasticity to switch lineages upon selective pressure.

H) that phosphorylates serine-5 residues within the many CTD repeats of RNA Pol II ($n = 52$). After transcriptional initiation, a transcriptional pause occurs around nucleotide (nt) +50. Transcriptional pausing is induced by binding of the inhibitory factor DSIF and the NELF complex to RNA Pol II. This promotor-proximal pausing has an important biological function, as it enables the necessary capping process at the 5′-nucleotriphosphate of the initiated transcript.

Subsequently, the paused RNA Pol II needs to be converted from its arrested state (POL A) into the elongating state (POL E), a process which is mainly executed by the AF4 complex (note: in most body tissues, AF4 is the highest transcribed gene of all four AF4 family members). The AF4 complexes contain several histone modifying proteins (DOT1L, NSD1 and CARM1) as well as the P-TEFb kinase (CDK9 and Cyclin T1). P-TEFb kinase is rather important, because it carries out the necessary phosphorylation steps at serine-2 residues within the CTD repeats of RNA Pol II, causing a functional switch from POL A (arrested) to POL E (elongating). In addition, phosphorylation of DSIF and the NELF complex, which lose their inhibitor function or become destroyed thereafter, relieves the transcriptional block. Elongating RNA polymerase II then produces the full-length mRNA, traveling along with AF4 complex as well as additional specific splice and termination factors (reviewed in [61]). P-TEFb also phosphorylates UBE2A, which then forms a complex with RNF20 and RNF40 to ubiquitinylate H2B [62]. This leads to the removal of several nucleosomes from the chromatin to allow transcription to be more efficient.

The histone modifying enzymes DOT1L, NSD1 and CARM1 (PRMT4) are imprinting important signatures on the chromatin: $H3K79_{me1-3}$, $H3K20_{me2}$, $H3K36_{me2}$ and $H3R2_{me1}$, $H3R17_{me1/2}$ and $H3R26_{me1}$, respectively. Therefore, promotor regions (identified by $H3K4_{me3}$) and transcribed gene bodies (identified by $H3K36_{me2}$ and $H3K79_{me2/3}$) are carrying different signatures that mark transcribed genes and allow the generation of a cell-type specific transcriptional memory system under physiological conditions. This way, cells "remember" through the chromatin signatures which genes needs to be transcribed in terminally differentiated cells.

AF4 complexes contain also the BET protein BRD4 which recognizes acetylated histone proteins comprising transcriptionally active chromatin. To this end, BRD4 may even enhance transcription. However, BRD4 has additional functions not linked to AF4 complexes, e.g. by binding directly to transcription or histone modifying factors like GATA1, RCF1-5 and JMJD6. Generally, BRD4 has the functions to enhance transcriptional processes in cells.

The purified AF4-MLL protein complex contained all functional important proteins found for AF4 – apart from of BRD4 and SIAH1/2 [54] – and all proteins known to bind to the MLL C-terminus. The fusion protein complex was quite stable – even when MG132 was not used - and able to increase $H3K4_{me3}$ and $H3K79_{me2}$ histone methylation signatures [55].

Similar functions are exerted by the MLL-AF4 fusion protein complex that targets a subset of known MLL target genes and actively recruits the endogenous AF4 complex, similar to what MLL-ENL or MLL-AF9 fusion proteins are doing. To this end, both t(4;11) fusion proteins have similar but also opposite functions: both enhance H3K4 and H3K79 methylation signatures, but MLL-AF4 does so at specific target genes, while AF4-MLL provides this function in a genome-wide and RNA POL II-dependent fashion, because the AF4-MLL complex is assumed to travel along with RNA POLII.

Since MLL fusion proteins are instructive, their function(s) can be investigated in any cell line, however, the experimental read out in terms of "target genes" will be cell-type specific. In order to understand the functional importance of AF4-MLL, we investigated AF4-MLL in stably transfected Hela and HEK293 cells. We induced AF4-MLL expression for exactly 48 h by adding doxycyline and performed an ATAQ sequencing experiment. Along with ATAQ sequencing, a gene expression profiling experiment was performed. Both data sets were aligned to the human genome to understand the function deriving from the

presence of an AF4-MLL fusion protein. It became pretty clear from both data sets that the main function of AF4-MLL is to activate globally chromatin on all chromosomes, and to strongly increase gene transcription (see Fig. 4) [63]. The activated chromatin is reminiscent in a certain way of an iPS experiment by using the Yamanaka factors [64], and may point to the recently promoted concept of super enhancer activation in these leukemias [65]. Increased transcription due to the presence of the AF4-MLL fusion protein may also help to overcome the negative effects exerted by the MLL-AF4 fusion protein already described in the beginning. If such features are true for other reciprocal MLL fusion proteins, then they need to be investigated in future experiments.

However, in terms of clinical implications these findings are important. If these leukemia cells exhibit a status like a stem cell, then these leukemic cells are much harder to treat and have the potential to evade potential tumor therapies which translates into therapy resistance and relapse. This may explain their poor clinical courses and outcome. In addition, it requires both fusion proteins to be addressed in any future targeted therapy approach.

7. New hypothesis for the t(4;11) leukemia pathomechanism

How does this fit into a molecular model for t(4;11) leukemia? If both t(4;11) fusions activate chromatin, either at specific target genes (MLL-AF4) or globally (AF4-MLL), cells will be genetically reset. While AF4-MLL functions as a “chromatin opener”, other factors like MLL-AF4 and/or endogenous transcription factors will then set an “oncogenic program” [63]. This would explain the plasticity of these cells, their biphenotypic character and indicate that different genetic programs are now eligible in such cells. Cells with such an “open chromatin” could be somehow classified as “stem cells”, because this is exactly what happens when stem cells are first produced after fertilization, and may easily explain the known “stem cell-like features” of MLL-r leukemic cells.

The ATAQ Seq results (see Fig. 4) raised an important question about the pathomolecular “mode-of-action” exerted by AF4-MLL: is it a “hit-and-run” mechanism or is AF4-MLL constantly required? A hit-and-run scenario is somehow supported by the extremely short time required to globally activate chromatin, which allows the direct MLL fusions in conjunction with endogenous factors present in hematopoietic cells to quickly set up new genetic programs. In addition, it would explain the low or even absent expression of the reciprocal fusion transcripts in isolated cells of leukemia patients. This important question has to be investigated experimentally in order to understand the biological consequences of reciprocal fusions better. We are currently performing such experiments where the MLL-AF4 fusion protein is constitutively expressed, but the reciprocal AF4-MLL fusion is shut down after 48 h in order to investigate the changes in gene expression (Alex Wilhelm, unpublished data). Hopefully, these experiments will enable us to answer this quite important question.

8. Recent developments: MLL and sarcoma

Two recent publications have provided a link between MLL and sarcomas [66,67]. Both sarcoma research groups have investigated large numbers of round cell sarcomas ($n = 184$) or unclassified sarcoma types ($n = 20$) by RT-PCR or RNA Seq to identify known and unknown gene fusions specific for this class of tumor.

The first study identified in one of the investigated 184 patient samples, case SARC002, an *MLL-YAP1* (*MLL* exon 7::*YAP1* exon 9) and two different *YAP1-MLL* fusions (*YAP1* exon5:: *MLL* exon 5 and *YAP1* exon 6::*MLL* exon 5 (personal communication Franck Tirode, Lyon, France; *MLL* nomenclature according to ref. [27])) with nearly equal read numbers (see Fig. 5A). Based on this information, a recombination event between both genes must have occurred in such a way that *MLL* exons 5 to 7 and *YAP1* exons 7 and 8 were duplicated during the recombination event to explain the identified fusion transcripts. All identified fusions were in-frame fusions (which is visible from Fig. 5A where all introns are color-coded).

The authors of the second paper identified 2 reciprocal *MLL* fusions, namely a *VIM-MLL* and again a *YAP1-MLL* fusion, with breakpoints in *MLL* intron 2 and *VIM* intron 4, resulting in *Vim* exon 4::*MLL* exon 3 fusion in patient 2. The recombination between *YAP1* and MLL occurred in *YAP1* intron 5 and *MLL* intron 4, leading to a *YAP1* exon 5::*MLL* exon 5 fusion in the third patient. RNA Seq data from the second paper counted 112 reads for the reciprocal *YAP1-MLL* fusion transcripts and 647 reads for the *VIM-MLL* fusion transcript, while both direct fusion transcripts, *MLL-YAP1* and *MLL-VIM*, were detected only as a single read in the investigated patients (therefore marked in grey in Fig. 5A). The resulting reciprocal fusion proteins exhibit the coiled-coiled domain of VIM fused to the AT-hook region of MLL, while the other fused the TID and 2 WW-domains of YAP1 to the CXXC domain of MLL. Both tumors were quite aggressive and metastasized into the lung of both patients and both patients died from their disease.

A third paper describes a hypercellular, spindle cell like neoplasm with meningothelial infiltration in the brain of a 22-year-old female patient [68]. Molecular analysis revealed an *MN1* exon 1::*MLL* exon 4 fusion. *MN1* has also been identified in fusions such as *MN1-FLI1* and *MN1-TEL* in myeloproliferative disorders and leukemias. This first description on an *MN1* exon 1::*MLL* exon 4 fusion with a sarcoma phenotype is therefore very interesting. *MN1* is a small gene with 2 exons. Exon 1 consists of a 1213 nt long 5′-NTR and a 3781 nt long coding sequence which is separated from exon 2 by a 45,666 nt long intronic sequence (type I). Exon 2 is composed of 182 nt long coding sequence and a 2638 nt long 3′-NTR.

The protein portion encoded by exon 1 is known to exhibit transcriptional activator potential at the *TBX22* target gene, and disruption of the *MN1* gene - e.g. by the balanced translocations t(4;22) – causes a loss-of-function of the MN1 protein. Disruption of *MN1* is associated with meningioma development [69]. In leukemia, the strong transactivation domain of MN1 is fused to the DNA binding domains of either FLI1 or TEL, and thus, very potent transcription factors are created. The MN1-TEL fusion protein acts as a dominant-negative mutant form of MN1 and blocks RARA-mediated transcription [70], first described in [71]. Similarly, the MN1-FLI1 fusion protein induced acute megakaryocytic leukemia (AMKL) in murine hematopoietic progenitor cells [72]. The resulting fusion proteins formed in all these sarcoma patients are displayed in Fig. 5B.

All these new data point to the fact that sarcomas or a sarcoma-like phenotype might be caused by the presence of reciprocal MLL fusion proteins, similar to the first observation in EcRNIH3T3 cells following transfection with *AF4-MLL* (see Fig. 1B). It is also important because it underscores again the oncogenic potential of reciprocal MLL fusion proteins outside of the leukemia research field. Opening chromatin to allow the establishment of an oncogenic genetic program could explain these type of sarcomas and their potent malignancy as well. More sarcoma cases need to be investigated to validate all these new findings, but it is per se very interesting to see the first solid tumors to be described with *MLL* rearrangements.

9. Future directions

I will not end this manuscript without mentioning some ongoing projects on reciprocal *MLL* fusions in our lab. One project deals with t(6,11) leukemia where breakpoints are localized either in the major ($n = 90$) or in the newly identified minor ($n = 4$) breakpoint cluster region [42]. Of interest, all patients with a t(6;11) translocation and the breakpoint in the minor BCR displayed a T-ALL phenotype, while t(6,11) patients with breakpoints in *MLL* intron 9 (major breakpoint region) were diagnosed with an AML disease phenotype ($n = 68$) or a T-ALL ($n = 22$). We thought that this could be a very nice system to analyze the instructive functions of these 4 fusion proteins MLL-AF6,

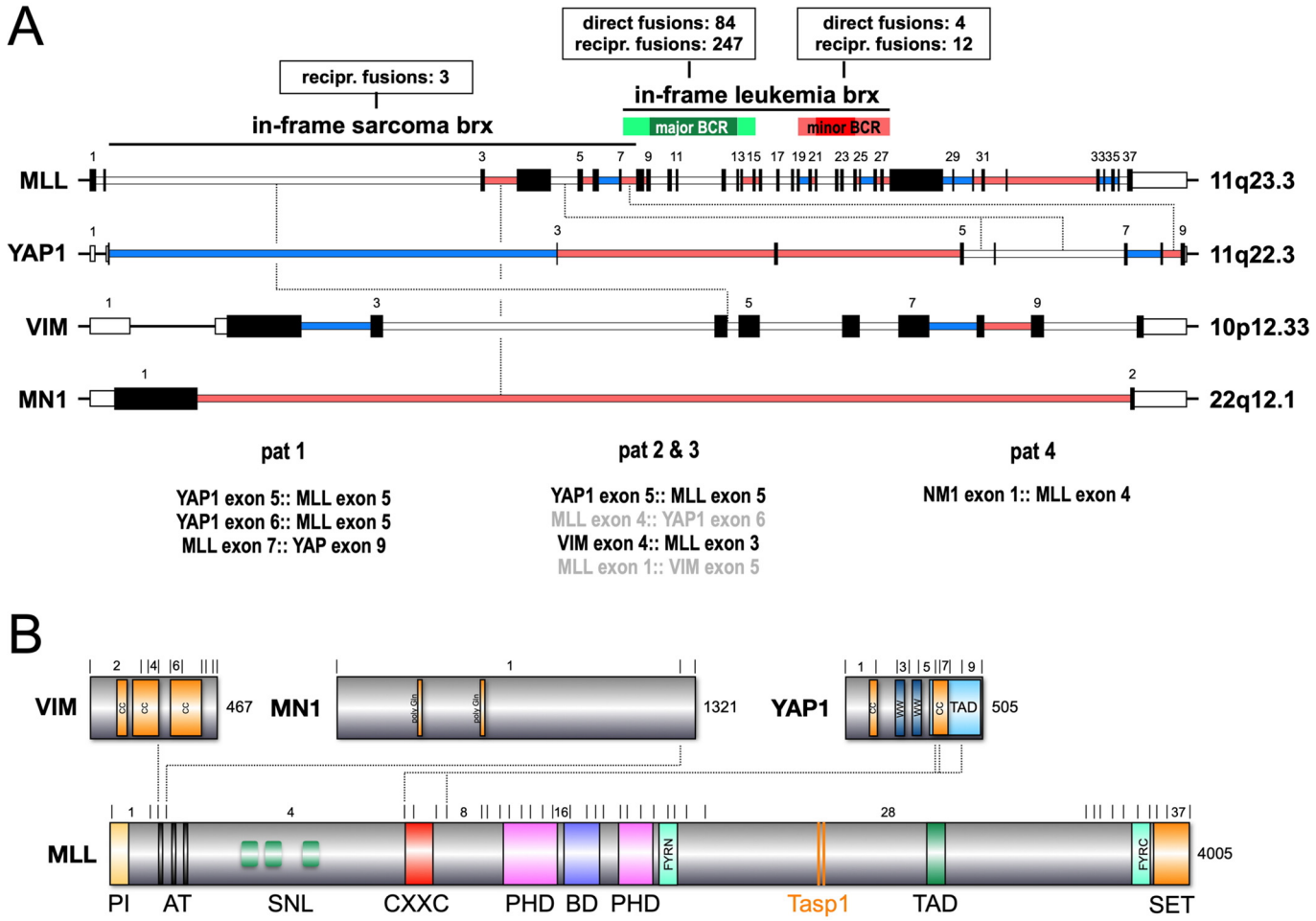

Fig. 5. Intron types of MLL and sarcoma fusion partner genes.
A. The gene structures of *MLL*, *YAP1*, *VIM* and *MN1* are presented. Depending on how introns interrupt the open reading frame, introns can be classified as type 0 (between two codons, white), type 1 (after a codon +1 nt, red) or type 2 (after a codon +2 nt, blue) introns. Recombination events leading to in-frame-fusions occur always in introns of the same color, otherwise would out-of-frame. All described sarcoma like transcripts (see below) from 4 patients represent in-frame fusions. Recombination events were occurring in *MLL* intron 2 to intron 7, in *YAP1* intron 5, 6 and 8, in *VIM* intron 4 as well as in *MN1* intron 1. Thus, the MLL N-terminal portion from intron 2 to intron 7 is recombined in sarcoma or sarcoma-like disease by producing mainly reciprocal fusion proteins, while leukemias display direct and reciprocal fusion proteins and breakpoints localizing in the major and minor breakpoint cluster region. Functionally, all reciprocal fusions - in sarcoma and leukemia patients - are losing their MLL target-specificity, because *MLL* exon 1 encodes the domain which enables MLL to bind to MEN1 and LEDGF. Therefore, they are functionally equivalent, although reciprocal sarcoma fusions retain the CXXC domain as a common feature. **B**. The protein domain structures of MLL, YAP1, VIM and MN1. The VIM protein exhibits 3 coiled-coiled domains (CC), while the MN1 protein is less classified apart from the poly-GLN stretches which are important for its function. The YAP1 protein exhibits 2 CC domains, two WW domains and a transactivation domain (TAD). The MLL protein exhibits an important protein interaction domain at its very N-terminus (binds MEN1 and LEDGF), 3 AT-hooks, subnuclear localization sequences (SNL), the CXXC domain, the PHD1-3/BD domain, the PHD4 domain, The FYRN domain, two Taspase1 cleavage sites, a TAD. The FYRC domain and the C-terminal SET domain. The fusion sites in all 4 proteins are indicated.

reciprocal AF6-MLL, exMLL-AF6 and AF6shMLL, by analyzing their effects on gene transcription. Studies with these 2 direct and 2 reciprocal fusion proteins and their combinations are ongoing, however, are too preliminary to be presented here in detail (Arpita Kundu, unpublished data).

We also want to find out the functional difference between the chimera *MLL-Af4* and the human *MLL-AF4* counterpart in conjunction with the reciprocal *AF4-MLL*. The chimeric MLL-Af4 fusion protein was published to cause leukemia in murine und human cells [14]. However, the chimeric fusion was unable to activate known target genes (e.g. *HOXA* genes) although the human counterpart does (hematopoietic cells usually displays a *HOXA* gene activation). We had the suspicion that the murine Af4 C-terminal portion, which differs from the human sequence at certain domains, may have different capabilities, e.g. in recruiting the SL1 complex to the pSer domain or the mouse sequence interferes with the TBP loading capacity [73,74]. In order to investigate this experimentally, we substituted the 138 amino acid-long human pSer domain by the homologous sequence of murine Af4 protein. As displayed in Fig. 6A, this region displays 1 amino acid that is present only in the human sequence, 3 amino acids only present only in the murine sequence, as well as 16 synonymous and 17 non-synonymous amino acids exchanges. We tested both constructs, human *MLL-AF4* and a murinized *MLL-AF4* derivative, alone and in combination with *AF4-MLL* (Fig. 6A). Results for some of the investigated target genes are summarized in Fig. 6C. The murinized MLL-AF4(Af4) fusion protein (orange bars) did activate the *EGR1-3* and *p21* gene much more strongly than the human counterpart (green bars), however, the cooperative effect with AF4-MLL is completely absent (purple vs. red). The presence of AF4-MLL alone activated all tested genes (except *EGR1* and *TERT*), but strongly enhances transcription of *EGR2*, *EGR3* and *p21* when co-expressed with its cognate counterpart. This was not the case when combined with the murinized construct. The *p21* gene activation was p53-independent, most likely because *p21* is activated via EGR1-3 as described recently [10]. Further work is currently ongoing to

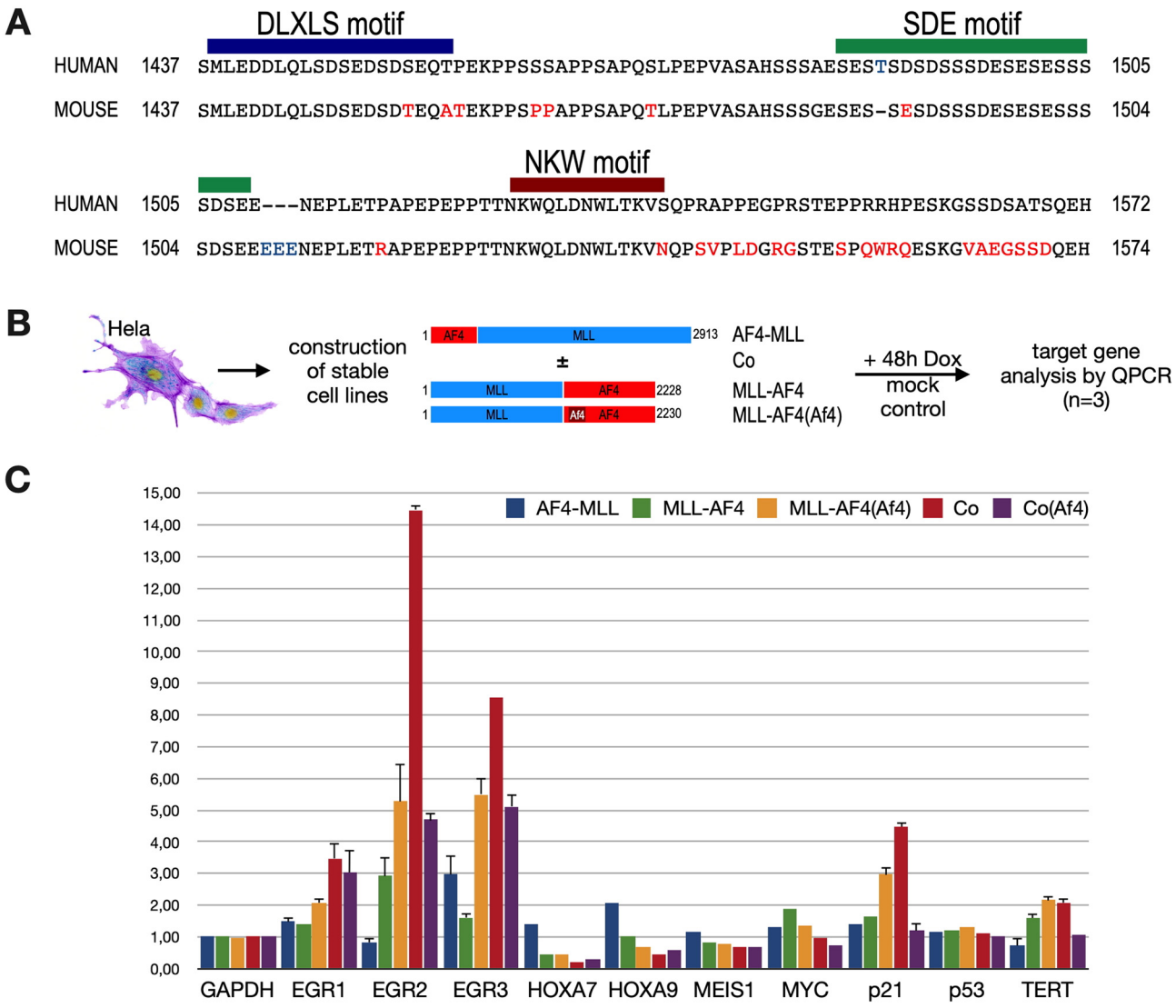

Fig. 6. Comparison of MLL-AF4 with a murinized version of MLL-AF4.
A. The pSer domain of AF4 and Af4. Missing amino acids are displayed by a dash, additional amino acids are shown in blue, while synonymous and non-synonymous amino acids are displayed in red. The DLXLS, SDE and NKW motifs are indicated. According to refs. [73,74], these domains are recruiting the Mediator complex, SL1 complex and are required for TBP loading, respectively.
B. Several stable cell lines (Hela) were constructed (AF4-MLL, MLL-AF4, MLL-AF4(af4), AF4-MLL & MLL-AF4 = Co and AF4-MLL & MLL-AF4(Af4) = Co(Af4)) and analyzed after a 48 h induction by Doxycycline at selected target genes by QPCR experiments which were normalized against GAPDH and mock-transfected cells.
C. Results of target gene transcription under all 5 condition. Please note that *HOXA* genes in Hela cells are already highly expressed, and thus, seem to be slightly downregulated by the tested fusion proteins.

understand the precise molecular mechanism on how a murine pSER domain impairs functions exerted by the MLL-AF4 fusion protein (Anna Siemund, unpublished data). Specifically, we will investigate the recruitment process of SL1 and TBP to the human and murine pSER domain of the AF4 protein.

10. Final note

I am personally quite convinced that *MLL* translocations are an extremely good tool to investigate tumor onset and maintenance mechanisms. Historically, mutant oncoproteins have been divided into different groups according to their cellular functions (receptors and signaling factors vs. nuclear transcription factors) that were shown to cooperate with each other in order to accelerate tumor cell development. Products of chromosomal translocations resemble a third class of oncoproteins, because these “shuffled proteins” already contain cooperating and/or novel functions. They represent either complex signaling machineries, altered transcription factors or chromatin-modifying complexes. Understanding their functions in detail has facilitated the design of targeted therapies and allowed us to learn more about new concepts in cancer biology (e.g. how altering the epigenetic layer may cause cancer). BRC-ABL is a constitutive oncogenic signaling machinery that could be targeted successfully by available kinase inhibitors. PML-RARA could be treated by ATRA and Arsenic. MLL fusions are changing the epigenetic layer in cells and allow “oncogenic reprogramming”, but can be targeted specifically as well (see below).

Of interest, most fusions proteins display different kinetics with regard to their half-lives that depends critically on their complex formation capability. Acquiring more binding partners due to the fusion with other protein sequences prolongs their half-life or impairs their natural degradation process. Most of the known fusion proteins form dimers, tetramers or oligomers [75–77], which when disrupted, directly lead to proteasomal degradation [78–80]. To this end, many leukemic fusions such as *BCR-ABL*, *PML-RARA*, *PLZF-RARA*, *AML1-ETO* or *AF4-*

MLL all carry intrinsic degradation mechanisms which can be targeted by expressed peptides or specific drugs.

In the past, the scientific community has put emphasis mainly on a few direct MLL fusions, however, too many missing pieces of information are hindering clear-cut progress in our understanding. The ability to create mouse models with certain direct fusions (*MLL-AF9*, *MLL-ENL*, *MLL-ELL*, *MLL-AF10*, *MLL-AF17*), but also the inability to create similar models for *AF4*, *LASP1*, *GRAF*, *ABI1 or FBP17* is dividing the community into two factions that have different opinions about the role of direct and reciprocal fusion proteins. Experimental data pointing to the importance of the involvement *HOXA* genes in conjunction with *MEIS1* are true for myeloid leukemias, but are presumably not correct for *MLL*-r leukemias displaying a lymphoid lineage (see above). It is also important to validate all these transplantation mouse models by transgenic approaches, since retroviral gene transfer always harbors a high risk of insertional mutagenesis, and thus, of false-positive results. According to my knowledge, this has been done so far only for MLL-AF9 [81,82], arguing again in favor of MLL-ENL and its functional homologue MLL-AF9 being able to act on their own and exhibit a similar mode-of-action. However, the MLL-ENL mouse from the same lab has both fusions already present [83]. All these mouse models have a mean latency of about 4–9 months before they developed leukemia.

Similarly, a CRISPR/Cas9 mediated genomic MLL-ENL translocation, mimicking a t(11;19) translocation, in human $CD34^+$ hematopoietic stem/precursor cells caused a monocytic leukemia when transplanted into NSG mice [84], indicating again that these models are presumably better than retroviral technologies. Not surprising to me was the fact that secondary transplantation experiments using this CRISPR/Cas9-mediated t(11;19) cells resulted also in B-ALL development. It could well be that the presence of the reciprocal *ENL-MLL* allele in these cells may support a higher plasticity of the leukemic cells, as mentioned already for AF4-MLL fusion protein.

The increased H3K79 methylation signature observed for MLL-AF4 (and AF4-MLL) has led to the development of a DOT1L inhibitor [85], which turned out in clinical trials to be less effective than anticipated [86]. New combinations of several inhibitors targeting different domains of MLL or its derivatives (BETi [87,88], SETi [89], MENi [90], HDACi [91], TASPi [92]) may be presumably more effective, but need to be tested again in pre-clinical models and clinical trials.

I hope that this article will put reciprocal MLL fusions back into the focus of new research programs in order to understand the complexity of *MLL*-r leukemias. These translocations are so powerful in their pathomolecular actions that a simple balanced translocation is sufficient to cause the onset of a hematological tumor, while solid tumors often harbor > 60–80 somatic mutations (of which 15–20 are the important driver mutations). We need to understand in detail the functions derived from direct as well as reciprocal MLL fusion proteins, in order to proceed with new strategies. Such new strategies are absolutely necessary in order to successfully treat these type of acute leukemias in the future.

Nomenclature

We are well aware about all the changes in the HUGO gene nomenclature over the past years. However, for the readability of the text, we use the following gene nomenclature throughout the text: *MLL* (*KMT2A*), *AF4* (*AFF1*), *FMR2* (*AFF2*), *LAF4* (*AFF3*), *AF5* (*AFF4*), *ENL* (*MLLT1*), *AF9* (*MLLT3*), *AF6* (*MLLT4*), *AF17* (*MLLT6*), *AF10* (*MLLT10*), *GRAF* (*ARHGAP26*), *FBP17* (*FNBP1*) *TEL* (*ETV6*), *AML1* (*RUNX1*).

CRediT authorship contribution statement

Rolf Marschalek: Conceptualization, Supervision, Methodology, Validation, Data curation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

There is no conflict of interest.

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