Aus der Medizinischen Klinik und Poliklinik III
Klinik der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. Dr. Michael von Bergwelt

Akute myeloische Leukämie mit rekurrenten chromosomalen Translokationen, Analyse der Fusionsproteine und kooperierender molekularer Mutationen auf dem Weg zu einer zielgerichteten, personalisierten Therapie.

Dissertation

zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

Sabrina Opatz

aus
Hildesheim

2018

Mit Genehmigung der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

Berichterstatter:	Prof. Dr. med. Stefan Bohlander
Mitberichterstatter:	Prof. Dr. Peter B. Becker
	PD Dr. Christoph Walz
	Prof. Dr. Irmela Jeremias
	Prof. Dr. Stefan Endres
Mitbetreuung durch den promovierten Mitarbeiter:	PD Dr. med. Philipp Greif
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
Tag der mündlichen Prüfung:	03.05.2018

Eidesstattliche Versicherung

Ich, Sabrina Opatz erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

„Akute myeloische Leukämie mit rekurrenten chromosomalen Translokationen, Analyse der Fusionsproteine und kooperierender molekularer Mutationen auf dem Weg zu einer zielgerichteten, personalisierten Therapie“

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 27.07.2018	Sabrina Opatz
Ort, Datum	Unterschrift

Inhaltsverzeichnis

Abkürzungsverzeichnis

AF10	ALL-1 fused gene from chromosome 10, oder MLLT10
AML	Akute myeloische Leukämie
ASXL1	Additional sex combs like transcriptional regulator 1
ASXL2	Additional sex combs like transcriptional regulator 2
CALM	Clathrin assembly lymphoid myeloid leukemia gene
CBFB	Core binding factor beta subunit
CBF	Core binding factor
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase
CFC	Colony forming cell
CR	Complete remission (komplette Remission)
DOT1L	DOT1 like histone H3K79 methyltransferase
ELN	European Leukemia Net
FAB	French-American-British
FACS	Fluorescence-activated cell sorting (Durchflusszytometrie)
FISH	Fluoreszenz-in-situ-Hybridisierung
FLT3	FMS-like tyrosin kinase
H3K79	Histone 3 lysine 79
HOX	Homeobox
HSC	Human stem cell (humane Stammzelle)
HSCT	Hematopoietic stem cell transplantation (hämatopoetische Stammzelltransplantation)
inv	Inversion
ITD	Internal tandem duplikation
KIT	KIT proto-oncogene receptor tyrosine kinase
MRC	Medical Research Council
MRD	Minimal residual disease (minimale Resterkrankung)
MYH11	Myosin heavy chain 11
NGS	Next generation sequencing
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
RAS	Rat sarcoma viral oncogene homolog
RUNX1	Runt-related transcription factor 1
RUNX1T1	Runt-related transcription factor 1 translocated to 1

OS	Overall survival (Gesamtüberleben)
t	Translokation
TET2	Tet methylcytosine dioxygenase 2
TKD	Tyrosinkinase Domäne
TKI	Tyrosinkinase Inhibitor
VAF	Variant allele frequency (Allelfrequenz der pathologischen Variante)
WES	Whole exome sequencing
WHO	World Health Organization
ZBTB7A	Zinc finger and BTB domain containing 7A

Die vorliegende kumulative Dissertation umfasst folgende zwei Manuskripte, die vorab publiziert wurden:

Opatz S, Polzer H, Herold T, Konstandin NP, Ksienzyk B, Zellmeier E, Vosberg S, Graf A, Krebs S, Blum H, Hopfner KP, Kakadia PM, Schneider S, Dufour A, Braess J, Sauerland MC, Berdel WE, Buchner T, Woermann BJ, Hiddemann W, Spiekermann K, Bohlander SK and Greif PA (2013) *Exome Sequencing Identifies Recurring FLT3 N676K Mutations in Core-Binding Factor Leukemia*. **Blood**; 122(10): 1761-9.

Deshpande AJ, Rouhi A, Lin Y, Stadler C, Greif PA, Arseni N, **Opatz S**, Quintanilla-Fend L, Holzmann K, Hiddemann W, Döhner K, Döhner H, Xu G, Armstrong SA, Bohlander SK, Buske C (2011) *The clathrin-binding domain of CALM and the OM-LZ domain of AF10 are sufficient to induce acute myeloid leukemia in mice.* **Leukemia**; 25(11): 1718-27.

1 Einleitung

1.1 Akute myeloische Leukämie (AML)

Die AML ist eine genetisch heterogene, klonale Erkrankung hämatopoetischer Vorläuferzellen im Knochenmark. Sie entsteht durch somatisch erworbene genetische und / oder epigenetische Veränderungen, welche wichtige Prozesse wie Selbsterneuerung, Proliferation und Differenzierung beeinträchtigen.[1] Die betroffene Vorläuferzelle verliert die Fähigkeit auszureifen (Differenzierungsblock) und wird gleichzeitig unsterblich, sie teilt sich unkontrolliert (Proliferationsvorteil) und es kommt zur massiven klonalen Expansion dieser einzelnen defekten Zelle.[2]
Durch den Hinzugewinn weiterer Mutationen wird die klonale Evolution vorangetrieben, der maligne Klon gewinnt weiteren Überlebensvorteil und damit schließlich das Übergewicht in der Hämatopoese.[3, 4] Die gesunden Zellen werden verdrängt. Das klinische Erscheinungsbild der AML wird durch genau diese massive maligne Knochenmarkinfiltration mit der Folge einer zunehmenden hämatopoetischen Insuffizienz bestimmt.
Die individuelle AML zeigte jedoch eine starke klinische Heterogenität, welche Ausdruck der zugrundeliegenden genetischen Veränderung dieser hochmalignen Erkrankung ist. Entsprechend wird die AML seit 2001 anhand der WHO-Klassifikation nach morphologischen, klinischen und genetischen Merkmalen unterteilt.[5] Die Risikostratifizierung erfolgt nach Empfehlungen des ELN und MRC mittels zytogenetischer und ausgewählter molekulargenetischer Veränderungen zum Zeitpunkt der Erstdiagnose (Abb. 1).[6, 7]

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high} Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*, *MECOM* (*EVI1*) -5 or del(5q); -7; -17/abn(17p) Complex karyotype, monosomal karyotype Wild-type *NPM1* and *FLT3*-ITD^{high} Mutated RUNX1 Mutated ASXL1 Mutated TP53

Abb. 1: aus [6] Einteilung von AML Patienten in drei genetische Subgruppen anhand der zytogenetischen und/ oder molekulargenetischen Veränderungen (ELN Klassifikation).

Die einzelnen Subgruppen zeigen signifikante Unterschiede im krankheitsfreien Überleben und Gesamtüberleben (Abb. 2).[6-8]

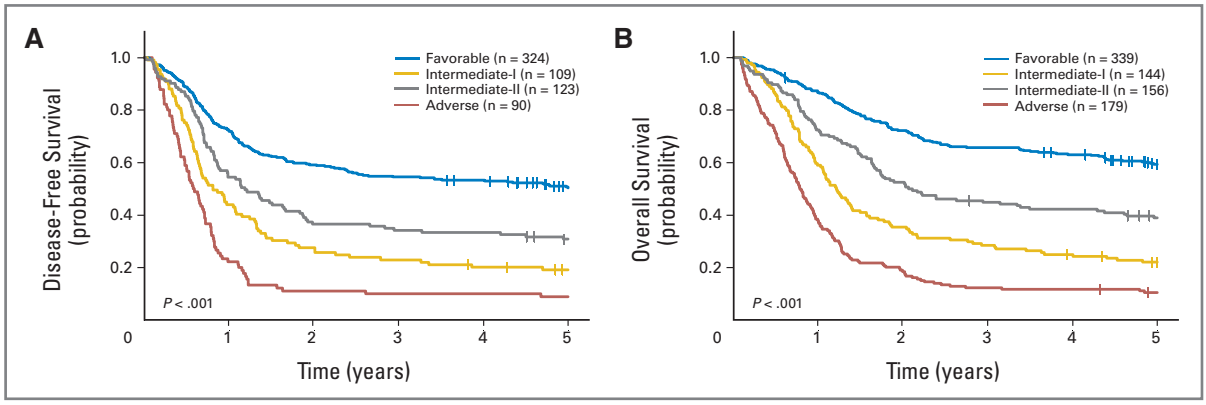

Abb. 2: aus [9] Überleben von AML Patienten entsprechend der vier genetischen Gruppen der European Leukemia Net Klassifikation für Patienten unter 60 Jahren. **A** Krankheitsfreies Überleben **B** Gesamtüberleben. Die blaue Linie repräsentiert prognostisch günstige AML und umfasst unter anderem die CBF Leukämien. CALM-AF10 rearrangierte Leukämien sind eher der roten, also der prognostisch ungünstigen Subgruppe zuzuordnen.

Diese Unterteilung hinsichtlich dem Therapieansprechen ist entscheidend für die Auswahl der Behandlung. Patienten der prognostisch günstigen Subgruppe erreichen meist mit alleiniger Chemotherapie eine dauerhafte Remission (CR: 96%, 3-Jahres-OS: 66%), wohingegen Patienten der ungünstigen Kategorie, deren Rezidiv-Risiko das Risiko der transplantationsassoziierten Mortalität deutlich übersteigt, zeitnah für eine allogene Stammzelltransplantation gebahnt werden sollten (CR: 50%, 3-Jahres-OS: 12%).[9-11]

Die vorliegende Arbeit beschäftigt sich mit ausgewählten Leukämie-assoziierten chromosomalen Translokationen (*CALM-AF10*, *RUNX1-RUNX1T1* und *CBFB-MYH11*), welche jeweils in einer Häufigkeit von 1-10% gefunden werden. Die aus dem genetischen Rearrangement resultierenden Fusionsgene spielen eine wichtige Rolle in der Leukämie-Entstehung, da durch sie die Funktion und Expression einzelner Gene verändert wird. Letztlich definiert diese chromosomale Veränderung, unabhängig von der Blastenanzahl, die AML.

Trotz der großen Bedeutung die den Fusionstranskripten attestiert wird, zeigt sich in den verschiedenen Leukämie-Modellen eine sehr lange Latenz bis zur Leukämie-

Entstehung. Dies lässt vermuten, dass die direkten Effekte dieser onkogenen Rearrangements nicht ausreichen, um eine Leukämie auszulösen. Vielmehr scheint die Akkumulation zusätzlicher genetischer Veränderungen notwendig zu sein.[12-16]

1.2. AML mit *CALM-AF10* Fusion

Das Fusionsgen *CALM-AF10* ist das Resultat einer Translokation zwischen den Chromosomen 10 (AF10) und 11 (CALM) [t(10;11)(p13-14;q14-21)]. Es wird in ca. 1% der AML- und auch ALL-Patienten beschrieben und ist mit einer sehr ungünstigen Prognose assoziiert.[17]

Es konnte gezeigt werden, das dieses Fusionstranskript *in vivo* die maligne Transformation auslösen kann.[12, 18] Die lange Latenz, wie auch die inkomplette Penetranz sprechen jedoch dafür, dass zusätzliche genetische Veränderungen für die endgültige Entstehung einer Leukämie notwendig sind.[12-14]

Der Mechanismus der leukämischen Transformation durch *CALM-AF10* ist zum Teil abhängig von *DOT1L*. DOT1L ist eine Methyltransferase und ausschließlich verantwortlich für die Methylierung von Lysin 79 im Histon H3 (H3K79). AF10 interagiert über seine OM-LZ Domäne mit DOT1L. Durch die Fusion mit *CALM* kommt es zur Fehlsteuerung und einer aberranten H3K79-Methylierung (Abb. 3). DOT1L methyliert unter anderem Promotoren der *HOXA* (homeobox A) Cluster-Gene und führt dadurch zu einer aberranten, konstitutiven Aktivierung dieser Gene.[13, 14, 19] *HOX* Gene spielen eine wichtige Rolle in der Hämatopoese und Leukämogenese. Die für *CALM/AF10*-Leukämien charakteristische Überexpression der *HOXA* Cluster Gene stimuliert die Selbsterneuerung und die leukämische Transformation von HSC.[20]

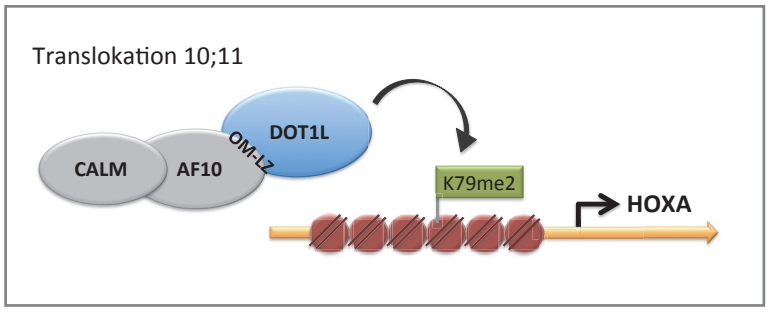

Abb. 3: Fehlsteuerung von DOT1L durch die Fusion von *CALM* und *AF10* mit nachfolgender aberranter H3K79-Methylierung

Am Beispiel der *CALM/AF10* Leukämie konnte zuletzt exemplarisch gezeigt werden wie Veränderungen in epigenetischen Mustern, insbesondere die posttranslationale Chromatinmodifikation, zur Leukämie-Entstehung beitragen. Damit bietet sich nicht zuletzt auch ein gänzlich neuer Therapieansatz abseits der standardisierten Chemotherapieregime und Stammzelltransplantation. Inhibitoren der Methyltransferase DOT1L zeigen eine hohe Wirksamkeit in *CALM/AF10* Leukämie Modellen. Durch selektive Inhibition der S-(5′-adenosyl)-l-methionin (SAM) Bindungsaktivität der Methyltransferase durch die Strukturanaloga EPZ004777 und EPZ-5676 kommt es zu einer dauerhaften Reduktion der H3K79-Methylierung, welche die Expression von Onkogenen (u.a. *HOXA*) eindämmt und auf diesem Weg spezifisch die Proliferation der malignen Zellen unterdrückt.[14]

1.3. AML mit *CBFB/MYH11*- oder *RUNX1/RUNX1T1*-Rearrangement (CBF Leukämie)

In 15-20% aller adulten *de novo* AML ist der Core Binding Faktor (CBF) betroffen, was ihn zu einem der häufigsten Zielestrukturen für Leukämie-assoziierte Veränderungen macht. [21, 22]

Der CBF ist ein heterodimerer Transkriptionsfaktor, zusammengesetzt aus der DNA-bindende Untereinheit RUNX1 (Genlocus auf Chromosom 21) und dem RUNX1-bindenden CBFB (Genlocus auf Chromosom 16). CBFB stabilisiert und interagiert mit RUNX1 und steigert dadurch dessen Affinität zur DNA. Dieser Komplex ist essentiell für eine normale myeloische Ausreifung.

Die CBF Leukämien definieren sich durch den Nachweis von chromosomalen Rearrangements, welche zu Fusionsgenen und Fusionsproteinen führen, welche die physiologische Transkriptionsfaktor-Aktivität des CBF beeinträchtigen; die Expression der Zielgene wird unterdrückt (Transkriptionsrepressor) und es resultiert ein Differenzierungsblock im Sinne einer gestörten myeloischen Ausreifung (Abb4).[21] Nach ELN und MRC-Kriterien zählt dieser Subtyp zu den prognostisch günstigen Leukämien.[6, 7, 9]

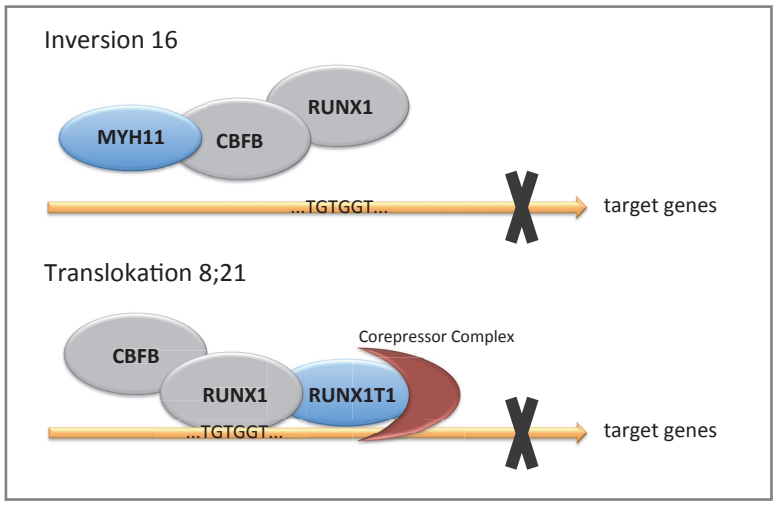

Abb. 4: Die Fusion von CBFB/MYH11, wie auch RUNX1/RUNX1T1 beeinträchtigt die physiologische Transkriptions-faktor-Aktivität des CBF; die Expression der Zielgene wird unterdrückt und es resultiert ein Differenzierungsblock im Sinne einer gestörten myeloischen Ausreifung.

Entsprechend des jeweils zugrunde liegenden Rearrangements lassen sich die CBF Leukämien in zwei Gruppen unterteilen: Durch eine balancierte Translokation zwischen Chromosom 8 und Chromosom 21 bildet sich das Fusionstranskript *RUNX1-RUNX1T1* [t(8;21)(q22;q22)]. Diese zytogenetische Aberration ist mit dem FAB Subtyp M2 assoziiert. Durch eine Inversion des Chromosom 16 [inv(16)(p13;q22)] bzw. eine Translokation zwischen den beiden Chromosomen 16 [t(16;16)(p13;q22)] bildet sich das *CBFB-MYH11* Fusionsgen, welches den FAB Subtyp AML M4 mit Eosinophilie (FAB M4eo) definiert. [21, 22]

Mausmodelle zeigten, dass die Expression dieser Fusionsgene, als AML-definierende Veränderung, nicht ausreichen, um eine Leukämie auszulösen. Zusätzliche Mutationen in bestimmten Signalweg-Proteinen, die sogenannten Proliferations-Driver, wurden beschrieben. Diese kooperieren mit den onkogenen Fusionsgenen, überwinden so den Wachstumsblock und triggern die maligne Transformation.[15, 16, 21] Dies entspricht dem klassischen Modell der Pathogenese der AML, einer Kombination aus einer Typ 1 Mutation (Proliferationssteigerung) und einer Typ 2 Mutation (Differenzierungsblock).[23] In 90% aller CBF Leukämie Patienten konnten Mutationen in *KIT*, *FLT3* und/ oder *RAS* nachgewiesen werden.[24, 25] Nichtsdestotrotz wurde in 10% dieser Patienten bislang keine der bekannten kooperierenden Mutationen gefunden.

1.3.1. Kooperierende Mutationen in CBF Leukämien

Die Pathogenese von CBF Leukämien scheint auf den ersten Blick auf einen überschaubaren Mechanismus aus „nur“ zwei kooperierenden Mutationen zu beruhen.[1, 26] Zum einen konstitutiv aktivierende Mutationen in Tyrosinkinasen (z.B. *FLT3*, *KIT*) oder ihren downstream Effektoren (z.B. *RAS*, *CBL*, *PTPN11*), welche für die ungehemmte Proliferation verantwortlich sind und so den Überlebensvorteil vermitteln. Und zum anderen Veränderungen von Proteinen welche die hämatopoetische Differenzierung und/ oder die Apoptose beeinträchtigen und die Fähigkeit zur Selbsterneuerung vermitteln. Diese werden im Fall der CBF-Leukämien durch die Fusionstranskripte *RUNX1-RUNX1T1* bzw. *CBFB-MYH11* vertreten und stellen die Grundlage für eine maligne Transformation dar.

Entsprechend dieser Annahme geschieht die klonale Expansion des initiierenden Klons mit *CBFB-MYH11*- oder *RUNX1-RUNX1T1*-Rearrangement also über kooperierende, sekundäre Veränderungen, hauptsächlich in *KIT, FLT3* und *RAS*.[24, 25, 27, 28]

Insbesondere für CBF Leukämien mit *RUNX1-RUNX1T1*-Rearrangement konnte erst kürzlich gezeigt werden, dass das Mutationsprofil dieser Subgruppe komplexer ist, als ursprünglich angenommen und das auch Mutationen auftreten, die nicht als Typ 1 oder 2 klassifiziert werden können. In 50% der Patienten wurden Mutationen in sog. epigenetischen Regulatoren *ASXL1*, *ASXL2* und *TET2* gefunden.[29-31] Weitere 24% tragen Mutationen in Genen des Cohesin Komplex.[32] *ZBTB7A* Mutationen, die den Tumorstoffwechsel beeinflussen, wurden in 23% aller Leukämien dieses Subtyps gefunden.[33]

Aus diesem Wissen ergeben sich nicht nur neue Gesichtspunkte zur Risikostratifikation der klinisch sehr heterogenen Gruppe der CBF Leukämien, sondern auch Möglichkeiten zur personalisierten Therapie. Als Beispiel seien Patienten genannt, die zusätzliche Punktmutationen an Position D816 der Rezeptortyrosinkinase KIT tragen. Für *CBFB-MYH11*-, wie auch *RUNX1-RUNX1T1*-rearrangierte Leukämie zeigt sich hier ein signifikant kürzeres Rezidivfreies-, sowie Gesamtüberleben.[31, 34] In klinischen Phase II und III Studien wird für diese Risiko-

Kohorte aktuell der Einsatz von Dasatinib, einem Tyrosinkinaseinhibitor, als Erhaltungstherapie getestet.[35]

1.4 Darstellung des Forschungsvorhabens und Eigenanteil der Arbeiten

Diese Dissertation beschäftigt sich mit ausgewählten chromosomalen Rearrangements, ihrer Auswirkung auf die hämatopoetische Entwicklung, ihr Zusammenspiel mit zusätzlichen molekulargenetischen Veränderungen und möglichen Behandlungsoptionen im Sinne einer zielgerichteten Therapie der individuellen AML.

Die erste Publikation betrachtet das *CALM/AF10*-Rearrangement, welches eine sehr kleine Subgruppe der akuten Leukämien definiert und mit einer ungünstigen Prognose assoziiert ist. Welche Domänen der beiden Fusionspartner zur Leukämieentstehung beitragen war Fragestellung dieser Veröffentlichung und wurde mittels funktioneller Analysen *in vitro* und *in vivo* im Knochenmark-Transplantationsmodell in der Maus untersucht.

Der Eigenanteil dieser Publikation umfasst vor allem die technische Durchführung der Experimente. Klonierung und Herstellung der diversen *CALM-AF10* Konstrukte, Gewinnung von Knochenmark aus Spendermäusen und Kultivierung *ex vivo*, retrovirale Transduktion von Knochenmarkszellen, Opferung der erkrankten Empfängermäuse und Präparation von Herzblut, Knochenmark und Milz, mit nachfolgender FACS Untersuchung ausgewählter Marker, sowie die Durchführung und Auswertung von CFC-Assays. Die technischen Details sind dem Methodenteil der Veröffentlichung zu entnehmen.

Die zweite Publikation befasst sich mit NGS Methoden, insbesondere der Whole Exome Sequenzierung (WES) zur systematischen Identifikation kooperierender Mutationen in AML Patienten mit *CBFB-MYH11*-Fusion.

Der Eigenanteil umfasst die Mitarbeit bei der Charakterisierung von Patientenproben, anhand zytogenetischer und molekularzytogenetischer (FISH) Untersuchungen und das Zusammenstellen der drei Patientenkohorten. Zudem die

Konzeption des Projekts, Präparation der Proben-Libraries und Auswertung der WES Daten. Die Validierung der Ergebnisse mittels klassischer Sanger-Sequenzierung, das Screening der drei AML-Kohorten auf rekurrente Mutationen im Exon 16 des *FLT3* Gens und die Bestimmung der VAF mittels Hochdurchsatzsequenzierung (targeted resequencing) der mutierten Patienten Proben. Weiterhin die Auswertung und Interpretation der jeweils erhobenen Daten und das Schreiben des Manuskripts, inklusive der grafischen Darstellung der eigenen Ergebnisse.

Die im zweiten Artikel beschriebenen funktionellen Arbeiten wurden von Dr. Polzer durchgeführt. Er demonstrierte das leukämogene Potential der *FLT3* N676K Mutation und zeigten die Möglichkeit einer zielgerichteten Therapie für diese AML Subgruppe auf. Beides steigerte den Wert der eigenen Arbeit, so dass die Erstautorschaft der Manuskripts, entsprechend der gleichwertigen wissenschaftlichen Leistungen, geteilt wurde.

2 Zusammenfassung

Häufig liegen der AML zytogenetische Veränderungen zugrunde. Diese allein sind jedoch nicht ausreichend für die maligne Transformation, bieten aber eine Grundlage für den Hinzugewinn weiterer Mutationen und die endgültige Manifestation der Leukämie. Zudem lassen chromosomale Veränderung Rückschlüsse auf Therapieansprechen und Überleben zu. Auf diese Risikostratifikation fußt die Entscheidung für oder wider eine spezifische Therapie.

Die erste Publikation betrachtet das *CALM/AF10*-Rearrangement, welches eine sehr kleine Subgruppe der akuten Leukämien definiert und mit einer ungünstigen Prognose assoziiert ist. Wir konnten zeigen, das die Fusion der C-terminalen clathrin-binding Domäne von *CALM* an die octapeptid motif - leucin zipper Domäne (OM-LZ) von *AF10* [minimal fusion] notwendig, und gleichzeitig ausreichend ist, eine primäre murine Knochenmarkszelle zu transformieren und eine AML auszulösen. Die Clathrin Binding Region (auf Chromosom 11; *CALM*) und die OM-LZ Domäne

(auf Chromosom 10; *AF10*) sind die ausschlaggebenden Schlüsselstellen dieses hochaggressiven Rearrangements und entscheidend für das transformierende Potential dieses Fusionsonkogens.

Die vorliegende strukturelle Analyse der funktionellen Domänen von CALM/AF10 legte den Grundstein für eine ganze Reihe an Folgeuntersuchungen. Da nun die für die Leukämie wichtigen Domänen feststanden, wurde nach Interaktionspartnern dieser Domänen gesucht, um den Mechanismus der *CALM/AF10* Leukämie zu entschlüsseln. Es fand sich der epigenetische Regulator *DOT1L* als Hauptakteur. Aufbauend auf diese Erkenntnisse konnte zwischenzeitlich mit der selektive Inhibition der S-(5′-adenosyl)-l-methionin (SAM) Bindungsaktivität dieser Methyltransferase durch die Strukturanaloga EPZ004777 und EPZ-5676 eine Therapiemöglichkeit der aberranten Methylierung und Aktivierung von Leukämie assoziierten Genen gefunden werden.

Die zweite Publikation befasst sich mit der systematischen Identifikation kooperierender Mutationen in AML Patienten mit *CBFB-MYH11*-Fusion.

Wie zuvor beschrieben, ist die alleinige Expression der Fusionsgene nicht ausreichend, um eine Leukämie auszulösen. Insbesondere in CBF Leukämien konnten in bis zu 90% der Patienten zusätzliche Mutationen in Genen, welche für Signalproteine codieren (z.B. *KIT*, *FLT3* und/ oder *RAS*) und notwendig sind um den Wachstumsblock zu überwinden, gefunden werden. Nichtsdestotrotz gelang in 10% der Patienten bislang kein Nachweis einer dieser bekannten kooperierenden Mutationen.

Wir untersuchten Knochenmark eines solchen Patienten mit zytogenetisch beschriebener Inversion von Chromosom 16, ohne zusätzliche molekulargenetische und/ oder zytogenetische Veränderungen in der Routinediagnostik. Mittels WES verglichen wir seine DNA (Exome: der Proteine kodierende Anteil der genomischen Sequenz) zum Zeitpunkt der Erstdiagnose mit der DNA zum Zeitpunkt der Kompletten Remission (entsprechend einer Keimbahnkontrolle). Mit diesem Ansatz fanden wir eine neue Mutation in der ersten Tyrosinkinasedomäne von *FLT3*, genauer in Position N676K, welche mit der üblichen Routinediagnostik nicht erfasst

wird. In einer Kohorte von 120 CBF AML Patienten konnten wir diese Veränderung in insgesamt 6 Patienten (5%) nachweisen. Zudem konnten wir in funktionellen Analysen zeigen, dass diese Mutation unabhängiges Wachstum vermittelt und dass sie sensibel gegenüber den bekannten Tyrosinkinaseinhibitoren (TKI) ist. Patienten mit dieser Mutation eröffnet sich somit eine zusätzliche Behandlungsoption mit TKI. Die klinische Signifikanz der neuen *FLT3* N676K Mutation wird unterstrichen durch Untersuchungen von Huang et al. die zeigen konnten, dass das transformierende Potential der *FLT3* N676K Mutation allein auch *in vivo* ausreicht eine akute Leukämie zu induzieren.[36]

In beiden Arbeiten wurde ein wichtiger Beitrag zur den Grundlagen der Leukämie-Entstehung bei Patienten mit spezifischen chromosomalen Translokationen geleistet. Die Identifizierung leukämie-relevanter Proteindomänen und der Nachweis neuer Mutationen in signaling pathway Genen (*FLT3* N676K), die mit den chromosomalen Rearrangements kooperieren, eröffnen nicht zuletzt neue Therapiemöglichkeiten im Sinne einer targeted therapy.

Die Identifikation des individuellen Mutationsprofils einer AML ist nicht nur essentiell für die Diagnose, sondern vielmehr für die biologische Sub-Klassifikation, die Prognose und Risikostratifikation, für die optimale therapeutische Strategie (Therapieentscheidung) und das individuelle klinische Monitoring der Behandlungseffizienz durch Bestimmung der Minimalen Resterkrankung (MRD).

3 Summary

A large number of recurring, balanced cytogenetic abnormalities are found in AML. Even though the gene products of man of these rearrangements are not sufficient to induce leukemia by themselves, they form the molecular basis for accumulation of additional mutations and malignant transformation. Cytogenetic changes are important for prognostic stratification and inform treatment decision and can predict survival.

The present manuscript described specific rearrangements, their impact on

hematopoesis, their cooperation with additional mutations and treatment options regarding targeted therapies.

The *CALM/AF10* fusion gene results from a chromosomal translocation between chromosomes 10 and 11 [t(10;11)(p13-14;q14-21)], a recurrent chromosomal rearrangement observed in patients with poor prognosis acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), especially T-ALL with a T-cell receptor gamma/delta rearrangement. So far, different *CALM/AF10* fusion transcripts have been described in AML patients. To define the contribution of different CALM and AF10 protein domains to *in vivo* leukemogenesis we performed detailed structure-function studies of the CALM/AF10 fusion protein. For this, we generated a fusion protein (termed CALM/AF10 minimal fusion) of the C-terminal 248 amino acids of CALM, which include the clathrin-binding domain, and the octapeptide motif - leucine zipper (OM-LZ) domain of AF10. This minimal fusion protein mediated transformation capabilities in colony forming cell assays *in vitro* and *in vivo*.

Furthermore, leukemias induced by the *CALM/AF10* minimal fusion recapitulated important aspects of full-length *CALM/AF10*-induced leukemia, including enhanced *HOXA* cluster expression. This is the characteristic signature of *CALM/AF10* leukemias due to H3K79 methylation, one of the mechanisms behind leukaemia caused by *CALM/AF10* fusion and aberrant DOT1L function.

In summary, this study indicates that the clathrin-binding domain of CALM and the OM-LZ domains of AF10 are sufficient to induce AML. This finding further suggests that approaches to inhibit *CALM/AF10*-induced transformation should incorporate strategies to block these key domains. Recently, the DOT1L-AF10 interaction with the OM-LZ domain was found to be essential for *CALM/AF10* mediated transformation, establishing a direct link between aberrant H3K79 methylation and leukemogenesis. Thus, disturbing an epigenetic modification can act as a driver of leukemia.

The second publication used next generation sequencing methods to systematically identify cooperating mutations in *CBFB/MYH11*-rearranged leukemia.

Chromosomal rearrangements affecting the core-binding factors *RUNX1* and *CBFB*, are found in 15% to 20%, of adult *de novo* acute myeloid leukemia (AML) cases and are associated with a favorable prognosis.

As mentioned before, expression of a fusion gene alone is most of the time not sufficient to induce malignant transformation. Especially, in CBF leukemias additional molecular mutations were found in up to 90% of patients. These aberrations affect genes of signaling pathways (e.g. *KIT*, *FLT3*, *RAS*), known as proliferation drivers, which are required to overcome the block of differentiation mediated by the fusion genes *RUNX1/RUNX1T1* and *CBFB/MYH11*. However, in up to 10% of CBF AML patients none of the known cooperating mutation could be detected.

To identify mutations, which may collaborate with the *CBFB/MYH11* fusion during leukemogenesis we performed exome sequencing of an AML sample with an inv(16) without additional molecular or cytogenetic aberrations. We discovered a novel N676K mutation in the adenosine triphosphate (ATP)-binding domain (TKD1) of the *FLT3* gene. In a cohort of 84 *de novo* AML patients with a *CBFB/MYH11* rearrangement and in 36 patients with a *RUNX1/RUNX1T1* rearrangement, the *FLT3* N676K mutation was identified in 5 and 1 patients, respectively (5 of 84 [6%]; 1 of 36 [3%]). The *FLT3* N676K mutant alone leads to factor-independent growth in Ba/F3 cells and, together with a concurrent *FLT3* ITD (internal tandem duplication), confers resistance to the FLT3 protein tyrosine kinase inhibitors PKC412 and AC220. Gene expression analysis of AML patients with *CBFB/MYH11*-rearrangement and *FLT3* N676K mutation showed a trend toward a specific expression profile. Ours is the first report of recurring *FLT3* N676K mutations in core-binding factor (CBF) leukemias and suggests a new subgroup of CBF leukemias.

Further experiments investigating molecular mechanisms for leukemogenesis induced by *FLT3* N676K mutation, studies for prognostic significance and clinical evaluation of *FLT3* inhibitors in *FLT3* N676K-positive AML are required.

First evidence for induction of acute leukemia by the novel *FLT3* N676K mutation *in vivo* has recently been provided by Huang et al..[36] In their report, the *FLT3* N676K mutation seems to have remarkable leukemogenic potency and quality comparable to *FLT3* ITD and other *FLT3* TKD mutations.

Identification of additional genetic abnormalities in patients with *CBFB/MYH11*-rearrangement could be helpful in predicting outcome [34, 37], since 60% of CBF leukemia patients relapse after standard chemotherapy.[38, 39] Furthermore, those findings may provide the basis for novel therapeutic targets.

Taken together, defining the mutational landscape of AML is essential to improve biological understanding, prediction of disease outcome, and thus optimization of treatment, and last but not least for the development of novel treatment approaches and individualization of therapy.

4 Originalarbeiten

4.1 The clathrin-binding domain of CALM and the OM-LZ domain of AF10 are sufficient to induce acute myeloid leukemia in mice

Leukemia (2011), 1–10

www.nature.com/leu

ORIGINAL ARTICLE

The clathrin-binding domain of CALM and the OM-LZ domain of AF10 are sufficient to induce acute myeloid leukemia in mice

AJ Deshpande[1,2,3,9], A Rouhi[4,9], Y Lin[5], C Stadler[1,2], P Greif[1,2], N Arseni[1,2], S Opatz[1,2], L Quintanilla-Fend[6], K Holzmann[7], W Hiddemann[1,2], K Döhner[8], H Döhner[8], G Xu[5], SA Armstrong[3], SK Bohlander[1,2] and C Buske[4]

[1]Department of Medicine III, Klinikum Grosshadern, Munich, Germany; [2]Clinical Cooperative Group Leukemia, Helmholtz Center for Environmental Health, Munich, Germany; [3]Division of Hematology/Oncology, Children's Hospital, Harvard Medical School, Boston, MA, USA; [4]Institute of Experimental Cancer Research, Comprehensive Cancer Center, University Hospital of Ulm, Ulm, Germany; [5]State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China; [6]Eberhard-Karls-University of Tübingen, Institute of Pathology, Tübingen, Germany; [7]Chip Facility/ZKF, University Hospital of Ulm, Ulm, Germany and [8]Department of Internal Medicine III, University Hospital of Ulm, Ulm, Germany

The t(10;11)(p13-14;q14-21) translocation, giving rise to the *CALM–AF10* fusion gene, is a recurrent chromosomal rearrangement observed in patients with poor prognosis acute myeloid leukemia (AML). Although splicing of the *CALM–AF10* fusion transcripts has been described in AML patients, the contribution of different CALM and AF10 domains to *in vivo* leukemogenesis remains to be defined. We therefore performed detailed structure-function studies of the CALM–AF10 fusion protein. We demonstrate that fusion of the C-terminal 248 amino acids of CALM, which include the clathrin-binding domain, to the octapeptide motif–leucine-zipper (OM-LZ) domain of AF10 generated a fusion protein (termed CALM–AF10 minimal fusion (MF)), with strikingly enhanced transformation capabilities in colony assays, providing an efficient system for the expeditious assessment of CALM–AF10-mediated transformation. Leukemias induced by the CALM–AF10 (MF) mutant recapitulated multiple aspects of full-length CALM–AF10-induced leukemia, including aberrant *Hoxa* cluster upregulation, a characteristic molecular lesion of CALM–AF10 leukemias. In summary, this study indicates that collaboration of the clathrin-binding and the OM-LZ domains of CALM–AF10 is sufficient to induce AML. These findings further suggest that future approaches to antagonize CALM–AF10-induced transformation should incorporate strategies, which aim at blocking these key domains.

Leukemia advance online publication, 17 June 2011;
doi:10.1038/leu.2011.153
Keywords: CALM; AF10; acute myeloid leukemia

Introduction

The *CALM–AF10* fusion gene results from in-frame fusions of the *CALM* gene on chromosome 11 to the *AF10* gene on chromosome 10. *CALM–AF10* fusions are observed in acute myeloid leukemia (AML), acute lymphoblastic leukemia and malignant lymphoma[1–6] and are especially prevalent in γ–δ lineage T-acute lymphoblastic leukemias.[6] AML patients with this translocation have a significantly poorer prognosis as compared with patients without these translocations.[7] AF10 belongs to a family of proteins that includes AF17 and BR140.[8] These proteins harbor a highly conserved plant homeodomain in the N-terminal portion and an octapeptide motif (EQLLERQW) and leucine-zipper (OM-LZ) domain in the C-terminal region.[8,9] Leukemia-associated translocations consistently fuse the C-terminal OM-LZ domain either to the *MLL* gene (in the case of MLL–AF10 and MLL–AF17 fusions) or to CALM (in the case of CALM–AF10 fusions).[8,10,11] Consistent inclusion of the AF10 and AF17 OM-LZ domains in leukemic fusions highlights the potential importance of these highly conserved motifs in leukemogenesis. Indeed, it has been demonstrated that the interactions of AF10 with critical components of the chromatin modifying machinery are mediated by the OM-LZ domain.[12–14] Importantly, aberrant recruitment of the histone methyltransferase DOT1L by the OM-LZ domain of AF10 is thought to be critical for the leukemogenesis of MLL–AF10 and CALM–AF10 fusions.[13,15,16] Since MLL–AF10 and CALM–AF10-positive leukemias are marked by global hypomethylation of histone H3 lysine 79 (H3K79), as well as *HOXA* cluster-specific local H3K79 hypermethylation, these diseases could serve as excellent models for studying the contribution of aberrant epigenetic marks to neoplastic development.[17] We have demonstrated that the expression of the human *CALM/AF10* fusion gene in murine bone marrow (BM) stem and progenitor cells results in an aggressive AML *in vivo*.[18] Similar results have also been obtained by expressing this fusion gene under the control of the hematopoietically active *Vav* promoter in transgenic mice.[19] In a separate study, continuous expression of this fusion gene was shown to be necessary for leukemia propagation, since suppression of *CALM/AF10* transcripts by shRNA knockdown significantly increased the latency of leukemia induced by the t(10;11)(p13-14;q14-21) positive U937 human leukemia cell line.[16] Data from patients with CALM–AF10-positive leukemia have demonstrated that despite splicing events, key functional domains of the fusion gene are retained.[20] In this study, we define the leukemogenic activity of key domains of CALM–AF10 by appropriate *in vitro* and *in vivo* assays and demonstrate that the leukemogenic activity of CALM–AF10 is due to the function of two regions, the clathrin-binding domain and the OM-LZ domain.

Correspondence: Professor C Buske, Institut für Experimentelle Tumorforschung des CCC Ulm, Universitätsklinikum Ulm und Klinik für Innere Medizin III, Albert-Einstein-Allee 11, 89081 Ulm, Germany.
E-mail: christian.buske@uni-ulm.de
[9]These authors contributed equally to this work.
Received 21 April 2011; accepted 3 May 2011

Materials and methods

Generation of CALM–AF10 mutant constructs

The pMIG-CALM-AF10 construct has been described previously.[18] A PCR amplified fragment corresponding to amino

acids 1–648 of CALM (NCBI accession NP_009097.2) with a stop codon was cloned into the MSCV-IRES-YFP (pMIY) plasmid vector for generating the CALMΔ3′ mutant. For the CALM + OM-LZ mutant, a PCR amplified fragment corresponding to amino acids 1–648 of CALM was fused to a PCR amplified fragment encoding amino acids 677–758 of AF10 (Accession: NP_001182555), comprising the OM + LZ with a stop codon. The CALM–AF10 ΔOM-LZ construct was generated by ligating two separate PCR fragments of CALM–AF10 such that the OM-LZ domain was excluded from between the two fragments, thereby excluding amino acids 677–758 of AF10. Mutants for interrogation of the CALM portion were generated by fusing an *Eco*R1–*Bam*H1 fragment of either amino acids 1–410 or 400–648 of CALM to the AF10 amino acids 677–758 of AF10 in the MSCV-IRES-GFP vector.

Mice and retroviral infection of BM cells

Parental strain mice were bred and maintained at the Helmholtz Centre Munich animal facility. Donors of primary BM cells were 8- to 12-week-old (C57BL/6Ly-Pep3b × C3H/HeJ) F_1 (PepC3) mice, and recipients were 8- to 12-week-old (C57BL/6J × C3H/HeJ) F_1 (B6C3) mice. Donors were treated with 5-fluorouracil and 5 days later, BM from these mice was harvested and plated in BM medium (Dulbecco's modied Eagle's medium, 15% fetal bovine serum, 1% Pen/Strep) + cytokines (100 ng/ml stem cell factor, 10 ng/ml interleukin 6 (IL6), 6 ng/ml interleukin 3 (IL3)). After 48 h of pre-stimulation, the BM cells were transduced with different viruses by overlaying them on virus-producing irradiated (40 Gy) GP^+E86 producers in the presence of cytokines and protamine sulfate (5 μg/ml). The transduction was stopped by removing the BM cells from the GP + E86 cells and plating them in BM medium with cytokines for another 48 h to allow for green fluorescent protein (GFP) expression. Retrovirally transduced cells were sorted based on expression of GFP by using a FACSVantage (Becton Dickinson, Franklin Lakes, NJ, USA). Sorted GFP or yellow fluorescent protein (YFP)-positive cells were used for colony forming cell (CFC) or colony forming unit-spleen (CFU-S) assays or injected directly into recipient mice.

CFU-S assay

The CFU-S assay was performed as previously described.[21] Transduced and sorted 5-fluorouracil-treated BM cells were injected intravenously into lethally irradiated (800 cGy of 137Cs γ-radiation) (C57BL/6J × C3H/HeJ) F_1 (B6C3) mice at cell numbers adjusted to give 5 to 15 macroscopic spleen colonies. Cell doses ranged from 1.5 to 5×10^4 sorted cells. At 12 days after injection, animals were killed and the number of macroscopic colonies on the spleen was evaluated after fixation in Telleyesniczky solution (absolute ethanol, glacial acetic acid and formaldehyde mixed in a 9:1:1 ratio, respectively). For the CALM (400–648) + AF10 (677–758) mutant, mice were injected with fewer cells to ensure scoring resolution (1000 GFP sorted cells per mouse).

BM transplantation and assessment of mice

In all, 8- to 10-week-old recipient mice (C57BL/6J × C3H/HeJ) F_1 (B6C3) were irradiated (800 cGy) from a ^{137}Cs γ-radiation source. Fluorescence activated cell sorting-purified transduced BM cells, or a defined ratio of transduced and untransduced cells was injected into the tail vein of irradiated recipient mice. Hematopoietic engraftment of GFP-positive cells was assessed by flow cytometry of regularly collected peripheral blood samples. Mice were closely monitored for signs of disease manifestation and killed when moribund. BM, peripheral blood and spleen cells of killed leukemic or control mice were analyzed for morphology and flow cytometric assessment of lineage markers as described.[18] Gr-1, *Sca*l, Ter-119, CD4, Mac1, Kit, B220 or CD8 antibodies were used for analysis (all antibodies from BD Biosciences, San Jose, CA, USA). Stained cells were analyzed on a FACSCalibur flow cytometer using the CellQuest-Pro software (BD Pharmingen, San Diego, CA, USA). Histological analysis and immunohistochemistry was performed on fixed organs of representative leukemic mice using standard protocols.

Colony forming cells

For *in vitro* CFC assays, transduced cells were sorted for GFP and directly plated in 1% myeloid-conditioned methylcellulose containing Iscove's modified Dulbecco medium-based Methocult (Methocult M3434; StemCell Technologies, Vancouver, Canada) at a concentration of 1000 cells/ml. Single cell suspensions of colonies were serially replated at the same concentration for upto 6 weeks or until the exhaustion of cell growth.

Immunostaining and confocal laser scanning fluorescence microscopy

For intracellular localization studies, the human osteosarcoma cell line U2-OS was grown on coverslips in six-well plates and transiently transfected with pcDNA3-FLAG-AF10, pcDNA3-FLAG-CALM–AF10 or pcDNA3-FLAG-CALM (400–648) + AF10 (677–758) plasmids using 1 μg plasmid DNA and 1.5 μg polyethyleneimine (Sigma, St Louis, MO, USA). After 24 h, cells were fixed with phosphate-buffered saline 2% paraformaldehyde for 10 min, permeabilized with phosphate-buffered saline 0.1% Triton X for 10 min and blocked with phosphate-buffered saline 10% fetal calf serum for 1 h. Coverslips were incubated overnight with monoclonal mouse FLAG antibodies (Sigma). Following extensive washing with phosphate-buffered saline, Alexa555-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) were added for 1 h. After further washing steps, cells were stained with 4′,6′-diamidine-2′-phenylindole dihydrochloride (Hoechst, Frankfurt, Germany) and mounted using Cytomation medium (DAKO, Glostrup, Denmark). Finally, immunostained species were analyzed in a confocal fluorescence laser scanning system (TCS-SP2 scanning system and DM IRB inverted microscope; Leica, Solms, Germany).

Western blotting

Total protein was extracted from GFP^+ E86 cells to assess the sizes and amounts of CALM–AF10 and CALM (400–648) + AF10 (677–758) proteins via western blotting, as described before.[18] The membranes were incubated overnight with goat polyclonal anti-CALM antibodies A-2 and C-18 (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) used at a concentration of 200 ng/ml. A horseradish peroxidase conjugated donkey anti-goat antibody (100 ng/ml) was used as secondary antibody.

Gene expression profiling and microarray analysis

Mouse BM cells were transduced with CALM–AF10, CALM (400–648) + AF10 (677–758), CALM–AF10 ΔOM-LZ and empty vector (MIG). Three days post-transduction, GFP-expressing cells were sorted by fluorescence activated cell sorting and total RNA (including both long and short RNA fractions) was extracted using Qiagen miRNeasy Mini Kit (Hilden, Germany).

For each construct, three biological replicates were produced. Microarray analysis was performed using Affymetrix GeneChip Mouse Gene 1.0 ST and GeneChip miRNA arrays. For the GeneChip Mouse Gene 1.0 ST Array, analyses were performed using 200 ng total RNA as starting material and 5.5 μg ssDNA per hybridization (GeneChip Fluidics Station 450; Affymetrix, Santa Clara, CA, USA). The total RNAs were amplified and labeled following the whole transcript sense target labeling assay (http://www.affymetrix.com). Labeled ssDNA was hybridized to Mouse Gene 1.0 ST Affymetrix GeneChip arrays (Affymetrix). The chips were scanned with an Affymetrix GeneChip Scanner 3000 and subsequent images analyzed using Affymetrix Expression Console Software (Affymetrix). For the GeneChip miRNA Array, 1 μg of total RNA was labeled using the FlashTag Biotin HSR labeling Kit (Genisphere, Hatfield, PA, USA). In all, 21.5 μl biotin-labeled sample were hybridized to GeneChip miRNA Arrays at 48 °C and 60 r.p.m. for 16 h. After washing Wash at a Fluidics Station 450 using fluidics script FS450_0003 arrays were scanned with an Affymetrix GeneChip Scanner 3000. Raw data were background corrected and quantile normalized using the miRNAQCTool from Affymetrix with default parameters recommended by Affymetrix.

A transcriptome analyses was performed using BRB-ArrayTools developed by Dr Richard Simon and BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html). Raw feature data were normalized and $\log_2$ intensity expression summary values for each probe set were calculated using robust multiarray average[22] using Affymetrix Expression Console Software (Affymetrix). For class comparison, we identified genes/miRNAs that were differentially expressed among the two classes using a two-sample *t*-test. Genes were considered statistically significant if their *P*-value was <0.05 and displayed a fold change between the two groups of at least 1.5-fold.

Our complete mRNA and miRNA array data are accessible at a gene expression omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ltmtnyguieagozi&acc=GSE27514).

Results

CALM–AF10 significantly increases the CFU-S frequency of normal BM progenitors

Sequencing of CALM–AF10 transcripts has revealed splicing events, but has shown that the C-terminal part of CALM with its clathrin-binding domain and the OM-LZ domain are present in patients with AML.[12,20] We confirmed these findings in three patients with CALM–AF10-positive AML, demonstrating that the C-terminal clathrin-binding domain of CALM and the OM-LZ domain of the AF10 part in the *CALM–AF10* fusion gene are not spliced out (data not shown). Based on this finding, we gauged the extent of the contribution of these two domains to the leukemogenic activity of the *CALM–AF10* fusion gene. The full-length CALM–AF10 fusion, which was derived from the t(10;11)(p13-14;q14-21) translocation of the U937 cell line, includes the amino acids 1 to 648 of CALM to amino acids 81–1042 of AF10 (Figure 1a). In order to assess the impact of short-term CALM–AF10 expression on early hematopoietic progenitors *in vivo*, we employed the day-12 CFUs in spleen (d-12 CFU-S) assay[21] (Figure 1b). Upon injection of BM cells expressing the empty MSCV-IRES-GFP (MIG) vector, an average of 3 ± 1.2 d-12 CFU-S colonies could be recovered per 10^5 input cells, whereas sorted CALM–AF10 transduced BM cells generated an average of 81 ± 5 d-12 CFU-S colonies (Figure 1b). CALM–AF10 expression therefore increases the d-12 CFU-S activity by ~27-fold as compared with empty vector ($P<0.0001$). We then used this assay to test several deletion mutants to identify protein domains that contribute to the aforementioned hematopoietic activity of the CALM–AF10 fusion.

The OM-LZ of AF10 is necessary and sufficient for the enhancement of CFU-S activity by CALM–AF10

We initially focused on the AF10 part of the fusion and constructed several deletion mutants of the AF10 region. Expression of the *CALM* gene truncated at the breakpoint of *CALM–AF10* (designated *CALM*Δ3′) or the *CALM–AF10* fusion gene with a deleted OM–LZ domain (designated *CALM–AF10* ΔOM-LZ) gave an average of three and four d-12 CFU-S colonies, respectively, per input 10^5 BM cells. These numbers were similar to d-12 CFU-S colonies obtained from empty vector (MIG) transduced cells, indicating that the AF10 portion of CALM–AF10, especially the OM-LZ domain (amino acids 677–758 of AF10) is necessary for the CFU-S enhancement phenotype. BM cells transduced with a construct harboring the *CALM* gene fused only to the *OM-LZ* region of *AF10* (CALM + OM-LZ) showed an average of 54 colonies per 10^5 input cells (an 18-fold increase compared with MIG; $P=0.0005$). This effect was comparable to the activity of the full-length CALM–AF10 fusion (CALM–AF10 vs CALM + OM-LZ; $P=0.065$) (Figure 2a).

Fusion of the clathrin-binding domain of CALM to the OM-LZ domain of AF10 enhances CALM–AF10-mediated transformation

Having established the OM-LZ domain of AF10 as the important determinant of the enhancement in d-12 CFU-S, we turned our attention to the CALM part of the fusion. The N-terminal amino acids of CALM have a high homology to AP180, the neuronal homolog of the CALM protein. These residues (amino acids 1–413), which include the ENTH or ANTH (Epsin or AP180 N-terminal homology) domain, have been shown to be insufficient for the targeting of CALM to clathrin-coated pits. The binding of clathrin to CALM was shown to primarily involve the C-terminal residues 414–652 of CALM. We therefore interrogated these N- and C-terminal portions of CALM for their contribution to the oncogenicity of the *CALM–AF10* fusion gene. We generated two CALM deletion mutants fused to the AF10 OM-LZ domain. The CALM (aa 1–410) + OM-LZ fusion generated an average of 37 d-12 CFU-S colonies, which was comparable to the CALM + OM-LZ construct. Strikingly, expression of the CALM (aa 400–648) + AF10 (aa 677–758) mutant, henceforth referred to as the CALM–AF10 minimal fusion (MF) or CALM–AF10 (MF) mutant, profoundly augmented the ability of BM cells to form d-12 CFU-S colonies, with an average of 800 colonies per 10^5 input cells (*P*-value of 0.0033 compared with MIG and <0.0001 compared with CALM–AF10) (Figure 2b).

The dramatic increase in colony numbers by the CALM–AF10 (MF) mutant suggested that this mutant might confer a significant proliferative advantage to hematopoietic progenitors. We tested the effect of CALM–AF10 (MF) expression in 5-fluorouracil-treated BM cells using methylcellulose-based CFC assays. Unlike the full-length CALM–AF10 fusion, which fails to transform BM progenitors, under defined conditions *in vitro* (in the presence of medium supplemented with 6 ng/ml IL3, 100 ng/ml stem cell factor, 10 ng/ml IL6), CALM–AF10 (MF) expression led to a rapid immortalization of hematopoietic precursors (Figure 3). Specifically, CALM–AF10 (MF) showed a significant increase in the number of secondary CFCs (32-fold vs MIG; 15.38-fold vs CALM–AF10). While CALM–AF10-expressing BM cells typically lost their replating potential in the second week of culture,

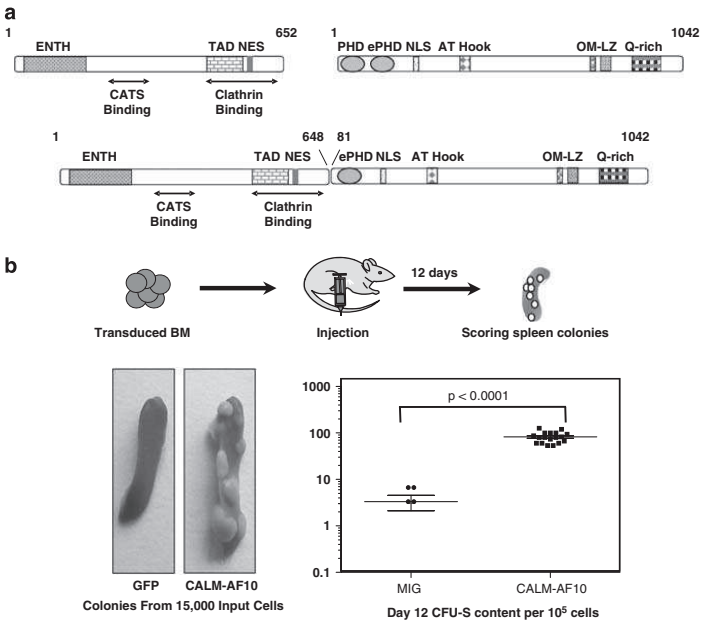

Figure 1 Analysis of the CALM–AF10 fusion protein (**a**) A cartoon depiction of the various domains in CALM, AF10 and the CALM–AF10 fusion. TAD, potential trans-activating domain; NES, nuclear export signal; PHD, plant homeodomain; ePHD, extended PHD domain; NLS, nuclear localization signal. The CATS and clathrin-binding domains in CALM are marked. (**b**) Upper panel shows a brief schematic presentation of the d-12 CFU-S assay. Pictures of representative fixed spleen colonies are shown on the lower left panel, whereas the right panel shows d-12 CFU-S numbers normalized to input 10^5 cells plotted on a $\log^{10}$ scale.

CALM–AF10 (MF)-expressing BM colonies could be serially replated for at least 4 weeks in CFC assays (Figure 3a) and proliferated extensively in culture for at least 8 weeks in medium supplemented only with 6 ng/ml IL3 (data not shown). Colonies obtained from CALM–AF10 (MF)-expressing progenitors were typically compact and hypercellular and composed predominantly of cells with a blast-like morphology (Figure 3b).

Induction of AML by the CALM–AF10 (MF) mutant

In order to ascertain that the transformation potential of the CALM–AF10 (MF) mutant would translate into leukemia generation, we injected CALM–AF10 (MF)-expressing 5-fluorouracil-enriched BM progenitors into lethally irradiated mice. CALM–AF10 (MF) expression induced leukemia in the recipients ($n=9$), with a median survival of 110 days (mean 116 days). The latencies of disease in these leukemias were not significantly different ($P=0.86$) from leukemias initiated by the full-length CALM–AF10 fusion (median survival 138 days, $n=8$). Moreover, similar to the CALM–AF10 mice described previously,[18] CALM–AF10 (MF)-induced leukemias showed diffuse infiltration of large cells with blastic chromatin and prominent nucleolus in the spleen, liver, kidney, intestine, lung and lymph nodes. Myeloperoxidase staining showed massive infiltration of the spleen and other organs with myeloblastic cells (Figure 4a). The disease induced by the CALM–AF10 (MF) mutant could also be transplanted into secondary recipients who died with a median latency of 31 days post-transplantation ($n=8$). Analysis of BM cells from leukemic CALM–AF10 (MF) mice demonstrated the presence of Gr-1/Mac1+ myeloid blasts. Importantly, all the mice analyzed (n = 5) harbored distinct populations of Mac1+/B220–, Mac1+/B220+ and Mac1–/B220+ cells (Figure 4b), similar to the hierarchically arranged cell populations described for the full-length *CALM–AF10* fusion gene.[18] These findings demonstrate the striking similarity in leukemia presentation, disease latency and transplantability between CALM–AF10 and CALM–AF10 (MF)-induced leukemias, despite the lack of larger portions of the fusion gene in the CALM–AF10 (MF) construct.

Enhanced expression and increased nuclear localization of the CALM–AF10 (MF) mutant

In order to assess the localization and relative expression levels of CALM-AF10 and the CALM-AF10 (MF) mutant, we performed immunofluorescence microscopy using FLAG-tagged, full-length CALM–AF10 or the CALM–AF10 (MF) mutant constructs transfected in U2-OS cells and used wild-type AF10 as a control. As it has been demonstrated previously,[23] AF10 was predominantly nuclear, whereas CALM–AF10 could be detected in a punctuate pattern in the cytoplasm. Moreover, in contrast to the speckled appearance of CALM–AF10, the CALM–AF10 (MF) protein was rather homogeneously distributed throughout the cytoplasm, with some cells showing an increased level of CALM–AF10 (MF) protein expression in the nucleus (Figure 5a). Protein expression of the CALM–AF10 (MF) was also confirmed by western blotting (Figure 5b).

Analysis of global gene expression changes induced by the CALM–AF10 mutants

Our results demonstrated that the CALM–AF10 (MF) mutant can profoundly enhance the proliferative capability of BM

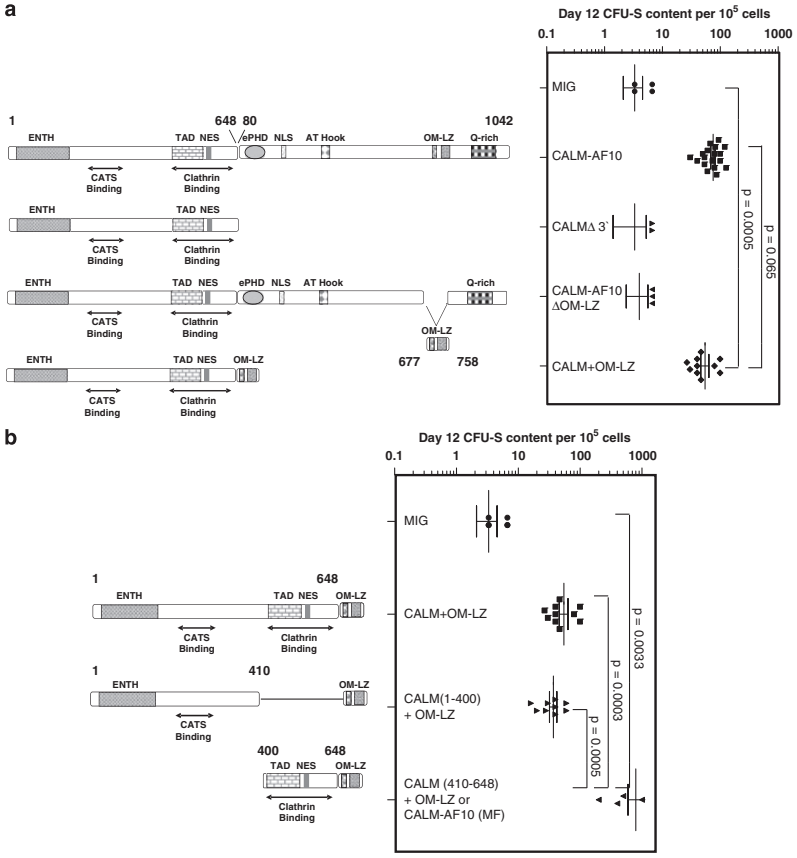

Figure 2 Structure-function analysis of AF10 and CALM. Values for d-12 CFU-S colonies per 10^5 cells are plotted on a log scale for the various mutants of AF10 (**a**) and CALM (**b**). The mean of all values is represented by a vertical line with error bars representing the s.e.m. Schematic representations of the mutants employed in the assays are depicted on the left side of each plotted value. Each experiment includes at least four independent replicates.

progenitors in short-term assays. We therefore sought to analyze the differences in global gene expression between CALM–AF10 and CALM–AF10 (MF) transduced BM cells. We performed microarray analysis of BM cells transduced with empty MIG vector, CALM–AF10, CALM–AF10 (MF) or CALM–AF10(ΔOM-LZ) using Affymetrix GeneChip Mouse Gene 1.0 ST and GeneChip miRNA arrays. Transcripts from 49 mRNAs and 13 miRNA were differentially expressed between the CALM–AF10 and the CALM–AF10 (MF) transduced BM cells (Supplementary Tables S1 and S2, respectively). Most notably, a number of genes known to be highly expressed in CALM–AF10 leukemias, especially genes of the *Hoxa* cluster, were significantly upregulated in the CALM–AF10 (MF)-expressing cells as compared with BM cells expressing the full-length CALM–AF10. Figure 6 depicts the differential expression of the *Hoxa* cluster genes (coding and non-coding) among CALM–AF10, CALM–AF10 (MF) or CALM–AF10 ΔOM-LZ and empty MIG vector. Interestingly, the *Hox* cluster embedded microRNA, miR-196b, which has been shown to be upregulated in the human CALM–AF10 T-cell acute lymphoblastic leukemia,[24] was also highly upregulated in CALM–AF10 (MF)-expressing cells (Figure 6). Supplementary Tables S3 and S4 list the differentially expressed mRNAs and miRNAs between CALM–AF10 and CALM–AF10 ΔOM-LZ, respectively.

Discussion

Breakpoint heterogeneity in chromosomal translocations can be instructive for the identification of domains that are crucial for the transformation potential of oncogenes generated by those fusions. The C-terminal portion of AF10 is retained in all reported CALM–AF10 and MLL–AF10 fusions.[1,20,25] This C-terminal region includes the OM-LZ domain and a glutamine-rich (Q-rich) domain that are both highly conserved. The OM-LZ domain of AF10 has been shown to interact with a number of important proteins such as the Swi/Snf interacting protein Gas41,[14] the lineage determining transcription factor Ikaros[23] and the histone methyltransferase Dot1l.[13] The OM-LZ domain was shown to be critical for the *in vitro* transformation capability of the *MLL–AF10* fusion gene.[15] Okada *et al.*[16] demonstrated that the OM-LZ region of AF10 is important for the interaction of

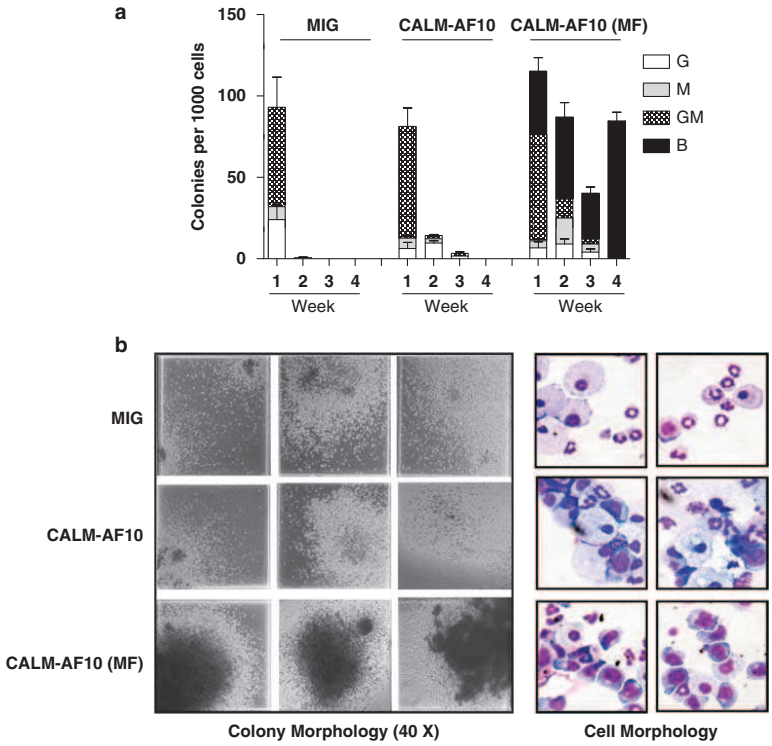

Figure 3 Colony forming potential of the CALM–AF10 (MF) mutant. (**a**) Bar graphs (upper panel) indicate the number of colonies per 1000 sorted cells plotted on a $\log^{10}$ scale. Error bars indicate s.e.m. in each case. B, Blast; G, granulocyte; GM, granulocyte–macrophage; M, macrophage. (**b**) Representative pictures of 1-week colonies or Wright–Giemsa-stained cytospin preparations from pooled colonies are shown in the lower panel.

CALM–AF10 with Dot1l and that the deletion of this region reduces the proliferative capacity of CALM–AF10 transduced BM cells. We now demonstrate that the OM-LZ domain of AF10 is both necessary for the expansion of early hematopoietic progenitors and also sufficient for *in vivo* leukemic transformation by the *CALM–AF10* fusion gene.

Under conditions routinely used to assay the *in vitro* myeloid transformation activity of oncogenes, the *CALM-AF10* fusion gene failed to transform BM cells *in vitro*. This is in contrast to its strong leukemogenic effect *in vivo*.[18,19] However, expression of the CALM–AF10 (MF) mutant rapidly immortalized BM progenitors *in vitro* and dramatically increased the recovery or d-12 CFU-S progenitors. It could be that transformation by the full-length CALM-AF10 protein is dependent on collaboration with *in vivo* niche signaling such as certain growth factors or cellular interactions absent under the aforementioned myeloid *in vitro* culture conditions, whereas transformation driven by CALM-AF10 (MF) is independent of these factors. In this regard, it is pertinent to observe that CALM–AF10 transduced BM cells can proliferate extensively in media supplemented with fetal thymic organ culture and IL3,[16] but not under more defined cytokine supplemented conditions (unpublished observations), supporting the hypothesis that additional, hitherto unknown growth factors are required for transformation by the full-length CALM–AF10 fusion. Alternatively, it is possible that the *in vitro* transformation potential of CALM-AF10 (MF) is caused by a greater abundance in the nucleus as compared with the full-length CALM-AF10 protein. We and others have observed that CALM–AF10 disrupts the physiological activity of the AF10–DOT1L complex, resulting in global H3K79 hypomethylation and *HoxA*-locus-specific hypermethylation although the exact molecular mechanisms of these epigenetic abnormalities remain obscure. Disruption of the H3K79 methyltransferase activity of the AF10–DOT1L complex is believed, *inter alia*, to lead to the aberrant overexpression of the *HOXA* cluster genes typically observed in the CALM–AF10 leukemias. These *Hoxa* cluster genes (including the microRNA miR-196b) were significantly upregulated after short-term expression of the CALM–AF10 (MF) protein, but to a much lesser extent in the full-length CALM–AF10 transduced BM. These results suggest that the CALM–AF10 (MF) protein might more rapidly and efficiently disrupt the AF10–DOT1L complex, leading to the observed *in vitro* transformation phenotype.

Lastly, it is possible that the CALM–AF10 (MF) mutant is more efficient at *in vitro* transformation because the N-terminus (aa 1–410) of the CALM protein, which is not present in the MF, normally impairs the *in vitro* transformation capability of the full-length CALM–AF10 fusion. Interestingly, the expression of the MF mutant did not alter the latency or phenotype of the disease in the BM transplant setting, suggesting that the lack of pronounced transformation *in vitro* by the full-length CALM–AF10 protein is compensated by microenvironmental factors *in vivo*.

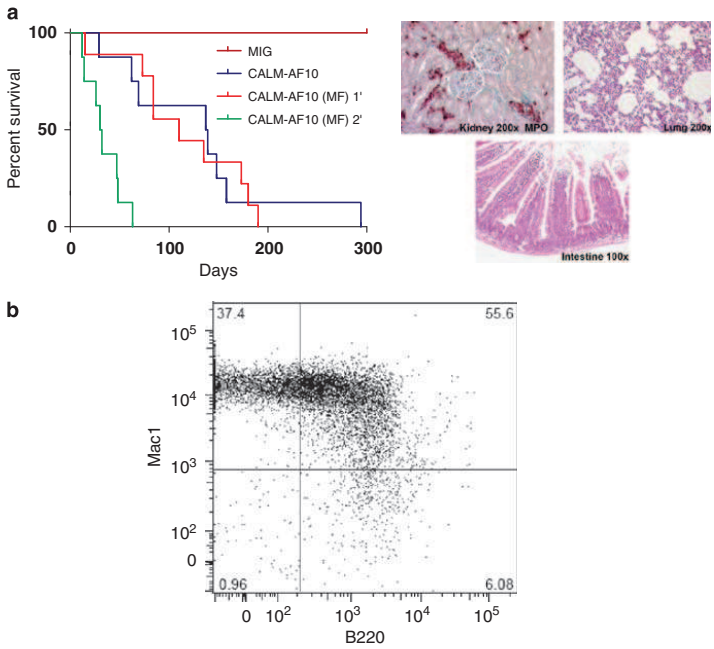

Figure 4 Leukemia initiation by the CALM–AF10 (MF) mutant. (**a**) (Left panel) Survival of mice injected with BM cells transduced with MIG (control vector), CALM–AF10 or CALM–AF10 (MF) plotted on a Kaplan–Meier curve. The survival curve of secondary mice injected with CALM–AF10 (MF) primary leukemias is depicted in green. (Right panel) Wright–Giemsa-stained histological preparations of various organs from leukemic primary CALM–AF10 (MF) mice are shown with respective magnifications inserted at the bottom. (**b**) Flow cytometric analysis of CALM–AF10 (MF) leukemic BM cells co-stained for Mac1 and B220.

Although several prior studies on CALM–AF10 have focused on the role of AF10, the potential contribution of the different CALM domains remains to be elucidated. The CALM protein, which is involved in clathrin-mediated endocytosis, has several defined domains. The N-terminal region of CALM (aa 1–300) is highly homologous to the N-terminal region of Epsin and AP180, important components of the endocytic machinery.[26] This domain, which has been termed the ENTH/ANTH domain, binds inositol phospholipids and contributes to the formation of clathrin coats on cell membranes.[27,28] A large part of this domain is excluded from the MLL–CALM fusion observed in a case of infant AML.[29] Our studies show that the exclusion of the N-terminal amino acids of CALM (which include the E/ANTH domain) from the CALM–AF10 fusion enhances the clonogenic capability of the fusion protein in short-term assays. Furthermore, the presence of the C-terminal amino acids 414–648 of CALM is sufficient to induce leukemia that recapitulates several features of full-length CALM–AF10-induced disease. The C-terminal amino acids (414–648) of CALM have been shown to bind clathrin.[26] A separate study demonstrated that these amino acids possess transcriptional activation potential when fused to a heterologous DNA-binding motif in yeast.[30] Interestingly, since the CALM–AF10 (MF) mutant showed enhanced *in vitro* transformation potential, several plausible contributions of the C-terminal amino acids of CALM could be hypothesized. Either C-terminal CALM-mediated disruption of endocytosis may interfere with cytokine receptor internalization, or alternatively C-terminal CALM domains may transactivate genes at loci targeted by the Af10–Dotll complex. Our results that the OM-LZ domain of AF10 (aa 677–758) is necessary and sufficient for *in vivo* leukemia initiation and phenocopies the full-length CALM–AF10 fusion strongly underline the importance of targeting the oncogenic activity of this domain in CALM–AF10 leukemias. Aberrant recruitment of the H3K79 methyltransferase DOT1L by the oncogenic AF10 fusions has been proposed as a major mechanism of leukemogenesis of AF10-rearranged leukemias. This is based on the observation that the DOT1L protein binds to the AF10 OM-LZ domain and that genes of the *HoxA* gene cluster show H3K79 hypermethylation in CALM–AF10 and MLL–AF10 leukemia cells.[13,16] Exciting new findings have shown that AF10 is crucial for catalyzing Dot1l-mediated H3K79 methylation, since RNAi-mediated suppression of AF10 leads to a significant reduction in this chromatin modification.[17,31] The importance of this process is highlighted by the fact that Dot1l-mediated hypermethylation of H3K79 at the *HoxA* gene cluster could be a critical leukemogenic mechanism in non-AF10 leukemias as well.[32] However, the changes in H3K79 methylation patterns caused by AF10 containing fusion genes are more complicated. We have previously reported a genome-wide hypomethylation of H3K79 in MLL–AF10 and CALM–AF10-expressing leukemias. This genome-wide hypomethylation at H3K79 could be linked to an increase in chromosomal instability.[17]

Eight differentially expressed miRNAs were found between the full-length CALM–AF10 and CALM–AF10(ΔOM-LZ) transduced BM cells. Of note is the downregulation of the pro-apoptotic and anti-proliferative *miR-128* in CALM–AF10 compared with CALM–AF10(ΔOM-LZ).[33,34] There are 28

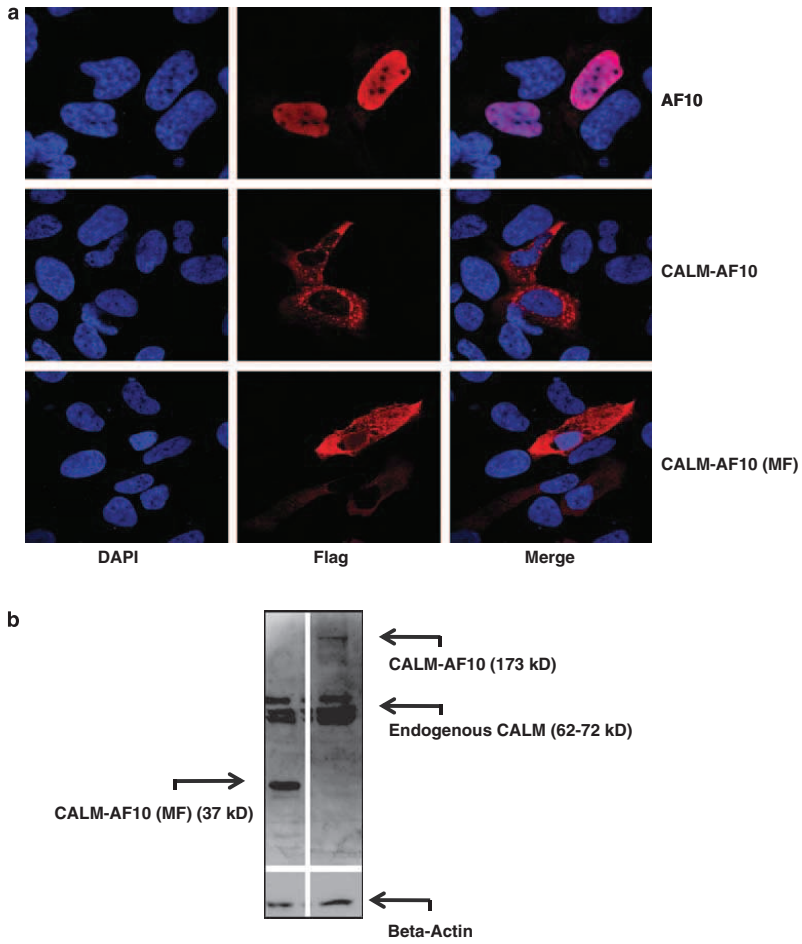

Figure 5 CALM–AF10 (MF) expression and subcellular localization. (**a**) Confocal laser scans of U2-OS cells transfected with FLAG-tagged AF10, CALM–AF10 or CALM (400–648) + AF10 (677–758) probed with anti-Flag monoclonal antibodies, and visualized by Alexa555-conjugated anti-mouse secondary antibodies. Left panels show 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI) stained cell nuclei, middle panels show Alexa555-signals and overlay images are shown in the left panels. (**b**) Western blots showing CALM–AF10 and CALM (400–648) + AF10 (677–758) expression using anti-CALM antibodies A-2 and C-18 (Santa Cruz Biotechnologies Inc.). Bands for endogenous CALM and β-actin were used as loading controls.

mRNAs significantly differentially expressed between CALM–AF10 and CALM–AF10(ΔOM-LZ) samples. Of these 28 differentially expressed mRNAs, none corresponded to the *Hoxa* cluster, indicating that this genomic region is not overtly activated in the 3 days post-transduction of the full-length CALM–AF10 BM, *in vitro*. However, the Hedgehog pathway gene *Smo*, a homolog of the Drosophila smoothened gene,[35] was upregulated upon full-length CALM–AF10 expression compared with CALM–AF10(ΔOM-LZ), suggesting that it may have a role in CALM–AF10-mediated transformation. The Hedgehog pathway has been shown to be activated in chronic myeloid leukemia and the loss of the *Smo* gene was shown to impair chronic myeloid leukemic stem cells. However, it remains to be seen if this pathway is also activated in CALM–AF10 leukemias. Comparison between the full-length CALM–AF10 and CALM–AF10 (MF) transduced BM cells showed that interestingly, in contrast to the full-length CALM–AF10 protein, short-term expression of the CALM–AF10 (MF) mutant significantly upregulated genes of the *Hoxa* cluster (including *miR-196b*). Taken together, these results indicate that the CALM–AF10 (MF) mutant is more efficient in activating some of the CALM–AF10-specific leukemogenic programs but lacks the full capacity to induce a more aggressive leukemia *in vivo* compared with the full-length CALM–AF10.

Since most leukemic fusion products are poor targets for pharmacologic inhibition, biochemical changes brought about by these fusions offer promising targets for rational drug design. Even as murine models of CALM–AF10 leukemia recapitulate aspects of their corresponding human malignancies, mechanistic or therapeutic studies on these leukemias could be

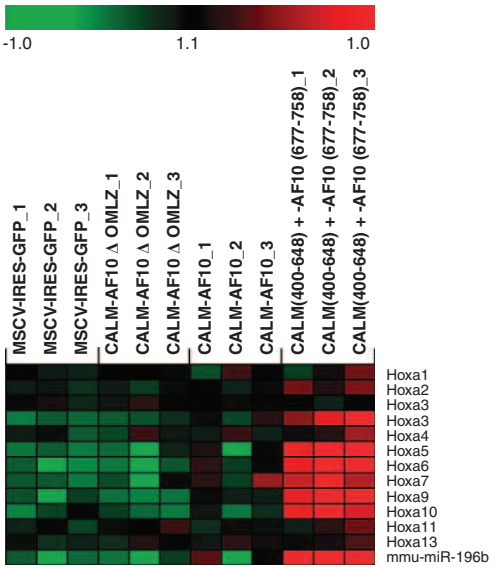

Figure 6 Analysis of differential regulation of the *Hoxa* cluster genes. Heatmap of the differentially expressed mRNA and microRNA transcripts associated with CALM–AF10 leukemias was generated using the Affymetrix platform. Relative expression levels of *Hox* genes and the *Hoxa* cluster embedded microRNA *miR-196b* are shown for full-length CALM–AF10, CALM–AF10(ΔOM-LZ) and CALM-AF10 (MF) in comparison to the empty MIG vector (three independent biological replicates each).

hampered by the prolonged latency of leukemia initiation in the CALM–AF10 retroviral BM transplantation model[18] and incomplete disease penetrance in the CALM–AF10 transgenic model.[19] In this study, we have demonstrated that the d-12 CFU-S assay can be used as a relatively rapid *in vivo* assay for CALM–AF10 activity. Using this method, we screened for domains essential for CALM–AF10 function and identified a potent leukemogenic mutant of CALM–AF10, which can immortalize BM progenitors *in vitro*, and phenocopy leukemia generated by the full-length CALM–AF10 fusion *in vivo*. The CALM–AF10 (MF) mutant, therefore, lends itself very well to expeditious assessment of transformation in the CALM–AF10 leukemias from mechanistic as well as therapeutic perspectives.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Bianka Ksienzyk and Lisa Kaiser for their excellent technical assistance and the members of the animal facility at the Helmholtz Centre for their tremendous support with mouse experiments. AJD and the work were supported by a grant of the Deutsche Krebshilfe (10-1838-Bu 2 to CB). This work was also supported by a grant from the Deutsche Forschungsgemeinschaft DFG (SFB 684, A6) and a grant from the German Ministry of Education and Research (BMBF) National Genome Research Network (NGFNplus) (PKL-01GS0876-6) to SKB. AR was supported by a fellowship from the Canadian Institutes of Health Research (CIHR) and a grant from the Baustein Startförderung at University Hospital Ulm. PAG was supported by a stipend from the Deutsche Jose-Carreras-Leukämie-Stiftung (F05/06). YL was supported by grants from the Ministry of Science and Technology of China (2005CB522400, 2006CB943900 and KSCX2-YWN-019), the Max-Planck-Gesellschaft of Germany and the National Science Foundation of China to G-LX.

References

1 Bohlander SK, Muschinsky V, Schrader K, Siebert R, Schlegelberger B, Harder L *et al.* Molecular analysis of the CALM/AF10 fusion: identical rearrangements in acute myeloid leukemia, acute lymphoblastic leukemia and malignant lymphoma patients. *Leukemia* 2000; **14**: 93–99.
2 Morerio C, Rapella A, Tassano E, Rosanda C, Panarello C. MLL-MLLT10 fusion gene in pediatric acute megakaryoblastic leukemia. *Leuk Res* 2005; **29**: 1223–1226.
3 Morerio C, Rapella A, Rosanda C, Lanino E, Lo Nigro L, Di Cataldo A *et al.* MLL-MLLT10 fusion in acute monoblastic leukemia: variant complex rearrangements and 11q proximal breakpoint heterogeneity. *Cancer Genet Cytogenet* 2004; **152**: 108–112.
4 Kumon K, Kobayashi H, Maseki N, Sakashita A, Sakurai M, Tanizawa A *et al.* Mixed-lineage leukemia with t(10;11)(p13;q21): an analysis of AF10-CALM and CALM-AF10 fusion mRNAs and clinical features. *Genes Chromosomes Cancer* 1999; **25**: 33–39.
5 Kobayashi H, Hosoda F, Maseki N, Sakurai M, Imashuku S, Ohki M *et al.* Hematologic malignancies with the t(10;11)(p13;q21) have the same molecular event and a variety of morphologic or immunologic phenotypes. *Genes Chromosomes Cancer* 1997; **20**: 253–259.
6 Asnafi V, Radford-Weiss I, Dastugue N, Bayle C, Leboeuf D, Charrin C *et al.* CALM-AF10 is a common fusion transcript in T-ALL and is specific to the TCRgammadelta lineage. *Blood* 2003; **102**: 1000–1006.
7 Dreyling MH, Schrader K, Fonatsch C, Schlegelberger B, Haase D, Schoch C *et al.* MLL and CALM are fused to AF10 in morphologically distinct subsets of acute leukemia with translocation t(10;11): both rearrangements are associated with a poor prognosis. *Blood* 1998; **91**: 4662–4667.
8 Chaplin T, Ayton P, Bernard OA, Saha V, Della Valle V, Hillion J *et al.* A novel class of zinc finger/leucine zipper genes identified from the molecular cloning of the t(10;11) translocation in acute leukemia. *Blood* 1995; **85**: 1435–1441.
9 Saha V, Chaplin T, Gregorini A, Ayton P, Young BD. The leukemia-associated-protein (LAP) domain, a cysteine-rich motif, is present in a wide range of proteins, including MLL, AF10, and MLLT6 proteins. *Proc Natl Acad Sci USA* 1995; **92**: 9737–9741.
10 Prasad R, Leshkowitz D, Gu Y, Alder H, Nakamura T, Saito H *et al.* Leucine-zipper dimerization motif encoded by the AF17 gene fused to ALL-1 (MLL) in acute leukemia. *Proc Natl Acad Sci USA* 1994; **91**: 8107–8111.
11 Dreyling MH, Martinez-Climent JA, Zheng M, Mao J, Rowley JD, Bohlander SK. The t(10;11)(p13;q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. *Proc Natl Acad Sci USA* 1996; **93**: 4804–4809.
12 Silliman CC, McGavran L, Wei Q, Miller LA, Li S, Hunger SP. Alternative splicing in wild-type AF10 and CALM cDNAs and in AF10-CALM and CALM-AF10 fusion cDNAs produced by the t(10;11)(p13-14;q14-q21) suggests a potential role for truncated AF10 polypeptides. *Leukemia* 1998; **12**: 1404–1410.
13 Okada Y, Feng Q, Lin Y, Jiang Q, Li Y, Coffield VM *et al.* hDOT1L links histone methylation to leukemogenesis. *Cell* 2005; **121**: 167–178.
14 Debernardi S, Bassini A, Jones LK, Chaplin T, Linder B, de Bruijn DR *et al.* The MLL fusion partner AF10 binds GAS41, a protein that interacts with the human SWI/SNF complex. *Blood* 2002; **99**: 275–281.

15 DiMartino JF, Ayton PM, Chen EH, Naftzger CC, Young BD, Cleary ML. The AF10 leucine zipper is required for leukemic transformation of myeloid progenitors by MLL-AF10. *Blood* 2002; **99**: 3780–3785.
16 Okada Y, Jiang Q, Lemieux M, Jeannotte L, Su L, Zhang Y. Leukaemic transformation by CALM-AF10 involves upregulation of Hoxa5 by hDOT1L. *Nat Cell Biol* 2006; **8**: 1017–1024.
17 Lin YH, Kakadia PM, Chen Y, Li YQ, Deshpande AJ, Buske C *et al*. Global reduction of the epigenetic H3K79 methylation mark and increased chromosomal instability in CALM-AF10-positive leukemias. *Blood* 2009; **114**: 651–658.
18 Deshpande AJ, Cusan M, Rawat VP, Reuter H, Krause A, Pott C *et al*. Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of CALM/AF10-positive leukemia. *Cancer Cell* 2006; **10**: 363–374.
19 Caudell D, Zhang Z, Chung YJ, Aplan PD. Expression of a CALM-AF10 fusion gene leads to Hoxa cluster overexpression and acute leukemia in transgenic mice. *Cancer Res* 2007; **67**: 8022–8031.
20 Narita M, Shimizu K, Hayashi Y, Taki T, Taniwaki M, Hosoda F *et al*. Consistent detection of CALM-AF10 chimaeric transcripts in haematological malignancies with t(10;11)(p13;q14) and identification of novel transcripts. *Br J Haematol* 1999; **105**: 928–937.
21 Helgason CD, Antonchuk J, Bodner C, Humphries RK. Homeostasis and regeneration of the hematopoietic stem cell pool are altered in SHIP-deficient mice. *Blood* 2003; **102**: 3541–3547.
22 Irizarry RA, Ooi SL, Wu Z, Boeke JD. Use of mixture models in a microarray-based screening procedure for detecting differentially represented yeast mutants. *Stat Appl Genet Mol Biol* 2003; **2**: Article1.
23 Greif PA, Tizazu B, Krause A, Kremmer E, Bohlander SK. The leukemogenic CALM/AF10 fusion protein alters the subcellular localization of the lymphoid regulator Ikaros. *Oncogene* 2008; **27**: 2886–2896.
24 Schotte D, Lange-Turenhout EA, Stumpel DJ, Stam RW, Buijs-Gladdines JG, Meijerink JP *et al*. Expression of miR-196b is not exclusively MLL-driven but is especially linked to activation of HOXA genes in pediatric acute lymphoblastic leukemia. *Haematologica* 2010; **95**: 1675–1682.
25 Carlson KM, Vignon C, Bohlander S, Martinez-Climent JA, Le Beau MM, Rowley JD. Identification and molecular characterization of CALM/AF10fusion products in T cell acute lymphoblastic leukemia and acute myeloid leukemia. *Leukemia* 2000; **14**: 100–104.
26 Tebar F, Bohlander SK, Sorkin A. Clathrin assembly lymphoid myeloid leukemia (CALM) protein: localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. *Mol Biol Cell* 1999; **10**: 2687–2702.
27 Itoh T, Koshiba S, Kigawa T, Kikuchi A, Yokoyama S, Takenawa T. Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science* 2001; **291**: 1047–1051.
28 Legendre-Guillemin V, Wasiak S, Hussain NK, Angers A, McPherson PS. ENTH/ANTH proteins and clathrin-mediated membrane budding. *J Cell Sci* 2004; **117**(Part 1): 9–18.
29 Wechsler DS, Engstrom LD, Alexander BM, Motto DG, Roulston D. A novel chromosomal inversion at 11q23 in infant acute myeloid leukemia fuses MLL to CALM, a gene that encodes a clathrin assembly protein. *Genes Chromosomes Cancer* 2003; **36**: 26–36.
30 Archangelo LF, Glasner J, Krause A, Bohlander SK. The novel CALM interactor CATS influences the subcellular localization of the leukemogenic fusion protein CALM/AF10. *Oncogene* 2006; **25**: 4099–4109.
31 Mohan M, Herz HM, Takahashi YH, Lin C, Lai KC, Zhang Y *et al*. Linking H3K79 trimethylation to Wnt signaling through a novel Dot1-containing complex (DotCom). *Genes Dev Mar* 15; **24**: 574–589.
32 Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU *et al*. H3K79 methylation profiles define murine and human MLL-AF4 leukemias. *Cancer Cell* 2008; **14**: 355–368.
33 Godlewski J, Nowicki MO, Bronisz A, Williams S, Otsuki A, Nuovo G *et al*. Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res* 2008; **68**: 9125–9130.
34 Adlakha YK, Saini N. MicroRNA-128 downregulates Bax and induces apoptosis in human embryonic kidney cells. *Cell Mol Life Sci* 2010; **68**: 1415–1428.
35 Zhao C, Chen A, Jamieson CH, Fereshteh M, Abrahamsson A, Blum J *et al*. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature* 2009; **458**: 776–779.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

4.2 Exome sequencing identifies recurring FLT3 N676K mutations in Core-Binding Factor leukemia

Regular Article

MYELOID NEOPLASIA

Exome sequencing identifies recurring *FLT3* N676K mutations in core-binding factor leukemia

Sabrina Opatz,[1-3] Harald Polzer,[1,4] Tobias Herold,[1,4] Nikola P. Konstandin,[1] Bianka Ksienzyk,[1] Evelyn Zellmeier,[1] Sebastian Vosberg,[1,4] Alexander Graf,[5] Stefan Krebs,[5] Helmut Blum,[5] Karl-Peter Hopfner,[6] Purvi M. Kakadia,[1,7] Stephanie Schneider,[1] Annika Dufour,[1] Jan Braess,[8] Maria Cristina Sauerland,[9] Wolfgang E. Berdel,[10] Thomas Büchner,[10] Bernhard J. Woermann,[11] Wolfgang Hiddemann,[1-4] Karsten Spiekermann,[1-4] Stefan K. Bohlander,[4,7] and Philipp A. Greif[1-4]

[1]Department of Internal Medicine 3, University Hospital Grosshadern, Ludwig-Maximilians-Universität, Munich (LMU), Germany; [2]German Cancer Consortium (DKTK), Heidelberg, Germany; [3]German Cancer Research Centre (DKFZ), Heidelberg, Germany; [4]Clinical Cooperative Group Leukemia, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany; [5]Laboratory for Functional Genome Analysis, and [6]Department of Biochemistry at the Gene Center, Munich, Germany; [7]Center for Human Genetics, Philipps University Marburg, Marburg, Germany; [8]Oncology and Hematology, St. John of God Hospital, Regensburg, Germany; [9]Institute of Biostatistics and Clinical Research, and [10]Department of Medicine A, Hematology, Oncology and Pneumology, Universität Münster, Münster, Germany; and [11]German Society of Hematology and Oncology, Berlin, Germany

Key Points

- *FLT3* N676K mutations without concurrent internal tandem duplication (ITD) are associated with core-binding factor leukemia.
- N676K activates FLT3 and downstream signaling pathways.

The t(8;21) and inv(16)/t(16;16) rearrangements affecting the core-binding factors RUNX1 and CBFB, respectively, are found in 15% to 20% of adult de novo acute myeloid leukemia (AML) cases and are associated with a favorable prognosis. Since the expression of the fusion genes *CBFB/MYH11* or *RUNX1/RUNX1T1* alone is not sufficient to cause leukemia, we performed exome sequencing of an AML sample with an inv(16) to identify mutations, which may collaborate with the CBFB/MYH11 fusion during leukemogenesis. We discovered an N676K mutation in the adenosine triphosphate (ATP)-binding domain (tyrosine kinase domain 1 [TKD1]) of the fms-related tyrosine kinase 3 (*FLT3*) gene. In a cohort of 84 de novo AML patients with a *CBFB/MYH11* rearrangement and in 36 patients with a *RUNX1/RUNX1T1* rearrangement, the *FLT3* N676K mutation was identified in 5 and 1 patients, respectively (5 [6%] of 84; 1 [3%] of 36). The FLT3-N676K mutant alone leads to factor-independent growth in Ba/F3 cells and, together with a concurrent FLT3-ITD (internal tandem duplication), confers resistance to the FLT3 protein tyrosine kinase inhibitors (PTKIs) PKC412 and AC220. Gene expression analysis of AML patients with *CBFB/MYH11* rearrangement and *FLT3* N676K mutation showed a trend toward a specific expression profile. Ours is the first report of recurring *FLT3* N676 mutations in core-binding factor (CBF) leukemias and suggests a defined subgroup of CBF leukemias. This trial was registered at www.clinicaltrials.gov as #NCT00266136. (*Blood*. 2013;122(10):1761-1769)

Introduction

The inversion inv(16)(p13;q22), the translocation t(16;16)(p13;q22), and the translocation t(8;21)(q22;q22) are recurring rearrangements in acute myeloid leukemia (AML), which result in the fusion genes *CBFB/MYH11* or *RUNX1/RUNX1T1*, respectively. These rearrangements are found in 15% to 20% of adult de novo AML cases and represent recognized World Health Organization entities that are associated with a favorable prognosis.[1,2]

CBFB and RUNX1 form the core-binding factor (CBF), a heterodimeric transcription factor essential for normal hematopoiesis. The CBFB/MYH11 and RUNX1/RUNX1T1 fusion proteins disrupt the physiologic activity of CBF, leading to the repression of CBF target genes and resulting in a block of differentiation and impaired hematopoiesis. Since knock-in mouse models have demonstrated that the expression of CBFB/MYH11 and RUNX1/RUNX1T1 by themselves is not sufficient to cause leukemia, it is highly likely that additional mutations are required for malignant transformation.[1,3,4] Leukemogenesis is a multistep process. Mutations associated with myeloid malignancies have been found in genes involved in several functional classes: signaling pathways (eg, *FLT3*, *KIT*, *RAS*), transcription factors (eg, *RUNX1/RUNX1T1*, *CBFB/MYH11*), epigenetic regulators (eg, *DNMT3A*, *IDH1*, *IDH2*, *TET2*), tumor suppressors (eg, *TP53*, *WT1*), and splicing machinery (eg, *SF3B1*, *SRSF2*).[5] In CBF leukemia, mutations in genes coding for signaling proteins (so-called proliferation drivers) are commonly found to collaborate with the CBF fusion genes.[6] Mutations in *KIT*, *FLT3*, or *NRAS/KRAS* have frequently been detected in CBF leukemia.[7-9] Up to 90% of AML patients with a CBFB/MYH11 fusion have either a mutation in a receptor tyrosine kinase (RTK) or in RAS.[10,11]

Submitted January 18, 2013; accepted July 8, 2013. Prepublished online as *Blood* First Edition paper, July 22, 2013; DOI 10.1182/blood-2013-01-476473.

S.O. and H.P. contributed equally to this work

Presented as an oral presentation at the 54th annual meeting of the American Society of Hematology, Atlanta, GA, December 8-11, 2012.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

In general, these signaling pathway mutations are mutually exclusive.[5] However, about 10% of AML patients with a CBFB/MYH11 fusion do not carry any of the currently known mutations.

To systematically identify additional collaborating mutations in *CBFB/MYH11*-positive AML patients, we performed exome sequencing of an AML with an inv(16) without any additional known genetic alterations. Using this approach, we identified an *FLT3* N676K mutation. Screening a cohort of 120 CBF AML patients, we discovered the *FLT3* N676K mutation to be present in 6 of these patients. Mutations affecting the ATP-binding pocket, in particular position N676, resulting in variable amino acid changes (N676D or N676S), were initially discovered in a screen for resistance to tyrosine kinase inhibitors (TKIs) in *FLT3* internal tandem duplication (ITD)-expressing Ba/F3 cells.[12,13] To the best of our knowledge, an *FLT3* N676K point mutation has been reported just once before in a cytogenetically normal (CN) AML patient with an *FLT3*-ITD mutation who was screened to determine the cause of the acquired TKI resistance after PKC412 therapy.[14] In this study, we report recurring *FLT3* N676K mutations at first diagnosis of CBF AML without concurrent *FLT3*-ITD. Importantly, Ba/F3 cells expressing the FLT3 N676K mutation show factor-independent growth and sensitivity toward commonly used TKIs, suggesting that the presence of the *FLT3* N676K mutation in CBF leukemia patients might open up new treatment options including TKI therapy.

Materials and methods

Patient samples

A diagnostic bone marrow sample was collected from an 18-year-old patient diagnosed with AML M4eo according to standard French-American-British and World Health Organization criteria in December 2003. The inv(16)(p13;q22) was detected by standard cytogenetics analysis (karyotype: 46, XY,inv(16)(p13;q22)[10]). The *CBFB/MYH11* fusion transcript was confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR). No additional genetic alterations were detected at this time. The patient was enrolled in the AMLCG-1999 trial of the German AML Cooperative Group (NCT00266136), and written informed consent was obtained in accordance with the Declaration of Helsinki. The samples were obtained under AMLCG study protocols approved by the ethics committees of the participating centers. After induction chemotherapy and autologous peripheral blood stem cell transplantation, complete remission was achieved (<5% bone marrow blasts; *CBFB/MYH11* transcripts no longer detectable by RT-PCR). A bone marrow sample at complete remission was used as normal control for exome sequencing.

In total, bone marrow or peripheral blood samples from 84 adult patients with newly diagnosed and untreated AML M4eo (*CBFB/MYH11* fusion–positive, including the case analyzed by exome sequencing), from 36 patients with t(8;21) and from 90 patients with CN AML were used for targeted mutation screening.

Sample preparation and high-throughput sequencing

Genomic DNA was extracted from patients' bone marrow or peripheral blood samples using QIAcube technology (Qiagen, Hilden, Germany). For exome sequencing of the index patient, 3 μg of genomic DNA was fragmented to an average size of 150 bp by using the Bioruptor sonicator (Diagenode, Liège, Belgium). Paired-end sequencing libraries were prepared using DNA sample prep reagent set 1 (NEBNext). Library preparation included end repair, adapter ligation, and PCR enrichment and was carried out as recommended by Illumina protocols. Exon-coding sequences were then captured by using SureSelect human all exon 50Mb kit version 3 (Agilent, Santa Clara, CA) according to the manufacturer's instructions. Exome libraries were sequenced by performing 76-bp paired end reads on a Genome Analyzer IIx platform (Illumina, San Diego, CA). Sequence alignment and variant detection was performed as described previously.[15]

Sanger sequencing

The nonsynonymous somatic variant in the *FLT3* gene (detected in the AML but not in the remission sample) was verified by sequencing both DNA strands using ABI 3100-Avant technology (Applied Biosystems) after PCR amplification of *FLT3* exon 16. PCR and sequence analysis of genomic DNA was performed with forward primer 5′-TGCAGATTGACTCTG AGCTG-3′ and reverse primer 5′-CACTGTGACTGAGAAAAGACAA AG-3′, located in the 5′ and 3′ flanking introns, spanning the complete exon 16 and yielding a 327-bp PCR product corresponding to AA 649 to 685 of the human FLT3 protein (National Center for Biotechnology Information reference sequence NM_004119). The same assay was used on a total of 209 de novo AML patients (*84 CBFB/MYH11*- rearranged, 36 *RUNX1/RUNX1T1*-rearranged, and 90 CN-AML patients). Routine diagnostic tests included mutation analysis at defined positions of *FLT3, KIT, KRAS, NRAS, NPM1, MLL* and *WT1* of all 84 AML M4eo samples (supplemental Table 3).

DNA constructs and vectors

The human FLT3-wild-type (WT) and the FLT3-ITD-NPOS constructs containing a 28 AA duplicated sequence (CSSDNEYFYVDFREYEYDLK WEFPRENL) inserted between AA 611/612 of human FLT3-WT were kindly provided by Gary Gilliland (Harvard Medical School, Boston, MA). The FLT3 constructs were subcloned into the MSCV-IRES-EYFP retroviral expression vector (kindly provided by R. K. Humphries, Terry Fox Laboratory, University of British Columbia, Vancouver, BC, Canada).

In vitro mutagenesis

The N676K mutation was introduced into the FLT3-WT and the FLT3-ITD-NPOS vectors by using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutant FLT3 D835Y construct was generated by using the QuikChange Site-Directed Mutagenesis Kit.[16] The correct sequence of all constructs was confirmed by sequencing.

Cell lines, reagents, and antibodies

Phoenix Eco cells were purchased from Orbigen (San Diego, CA) and cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin. Low-passage murine Ba/F3 cells and WEHI-3B cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and maintained in RPMI-1640 medium containing 10% fetal bovine serum, 0.5% penicillin/streptomycin, and 10% WEHI-3B conditioned medium as a source of interleukin-3 (IL-3). Recombinant human FLT3 ligand and recombinant murine IL-3 were obtained from Immunotools (Friesoythe, Germany). FLT3 inhibitor PKC412 was obtained from Novartis (Basel, Switzerland) and AC220 was obtained from SYNthesis Med Chem (Cambridge, United Kingdom).

The following antibodies were used: anti-AKT (9272), anti-pAKT (4060), anti-MAPK (9107), anti-pMAPK (9101), and anti-pSTAT5 (9351) (Cell Signaling Technology, Danvers, MA); anti-FLT3 (sc-480), anti-pTyr (sc-7020), anti-STAT5 (sc-835), and anti-GAPDH (sc-32233) from Santa Cruz Biotechnology (Santa Cruz, CA); and CD135-PE (IM2234U) and IgG1-PE isotype control (A07796) from Immunotech (Marseille, France). Stable transduction of Ba/F3 cells, western blot analysis, and detection of surface markers were performed as described previously.[17,18]

Proliferation and apoptotic cell death of Ba/F3 cells

Proliferation and apoptosis assays were carried out as described previously.[17] For long-term proliferation assays, cells were seeded at a density of 2×10^5/mL in growth medium containing 0.1% WEHI-conditioned medium as source of murine IL-3 and as control in the presence of 10 ng/mL IL-3. After 72 hours, Ba/F3 cells were cleared from IL-3 by two centrifugation steps

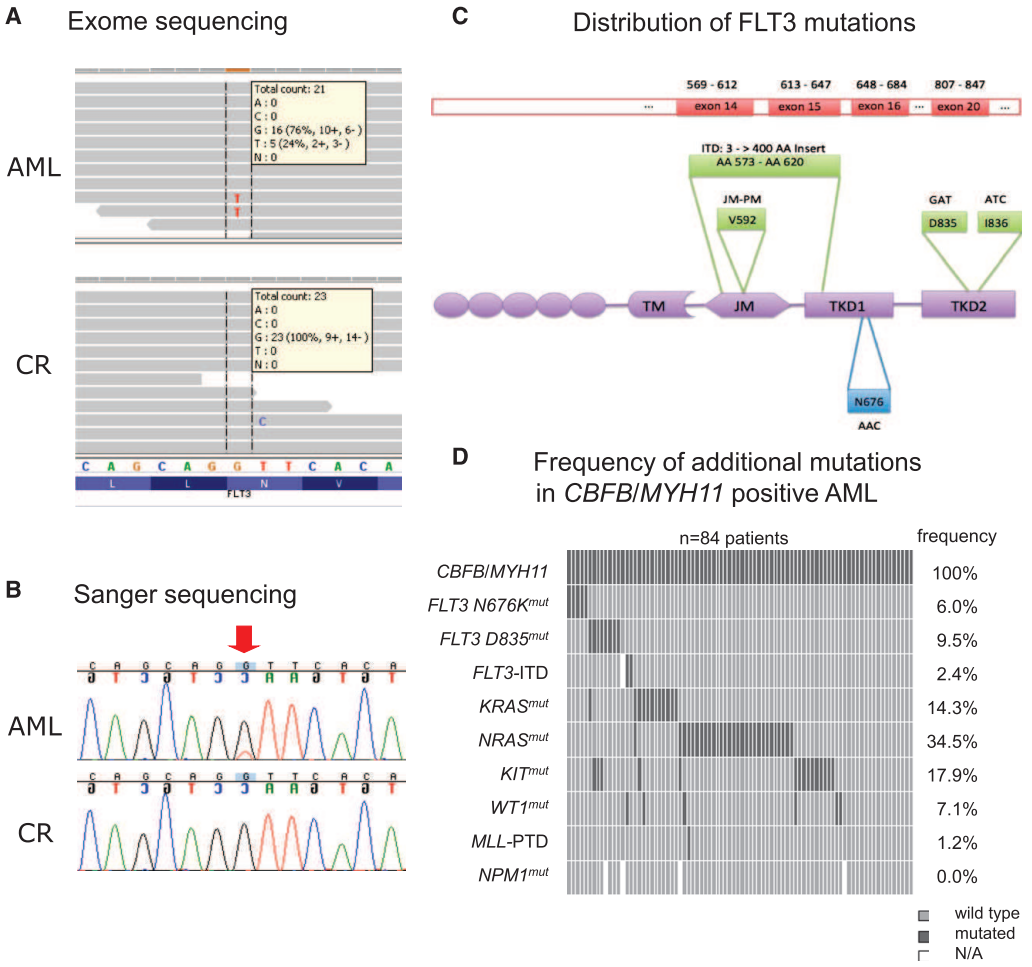

Figure 1. *FLT3* N676K mutations identified in *CBFB/MYH11*-rearranged AML. (A) Exome data sets of a *CBFB/MYH11*-positive AML sample (upper panels) and the corresponding follow-up sample from the same patient (lower panels) are displayed using the integrative genomics viewer.[24] Horizontal gray bars symbolize the 76-bp reads aligned to the reference sequence. The frequency of 24% of the mutant nucleotide T in the diagnostic leukemia sample indicates a heterozygous point mutation causing an amino acid substitution (NM_004119.2:c.2028C>A; p.N676K), whereas in the follow-up sample, only the wild-type nucleotide G is detected at this position. Read depth and base count are indicated for the affected positions, respectively. (B) Sanger sequencing confirmed the *FLT3* N676K mutation found initially by exome sequencing. Chromatograms are shown for both the diagnostic AML sample and the corresponding follow-up sample at complete remission (CR) from the same patient. (C) The structure of the human FLT3 protein includes the transmembrane domain (TM), the juxtamembrane domain (JM), and TKD1 and TKD2. Amino acid positions targeted by known recurrent mutations in AML are indicated in green above the corresponding domains. N676 is indicated in blue below the TKD1 domain. (D) Frequency distribution of additional genetic aberrations in 84 *CBFB/MYH11*-rearranged patients. Each column indicates one patient. Dark gray boxes indicate patients who are positive for the respective mutation; light gray boxes indicate wild-type status. Missing information is shown as a white space (N/A, not available). Gene names and types of mutations are indicated on the left. Mutation frequencies are indicated on the right. *MLL*-PTD, partial tandem duplications in the *MLL* gene.

with phosphate-buffered saline and resuspended in medium without IL-3. Control cells were cultivated in the presence of 10 ng/mL IL-3. Viable cells were counted every day, and a cell density of 2.5×10^6 was not exceeded.

Gene expression profiling and microarray analyses

Pretreatment bone marrow samples from 33 patients (data deposited in GSE37642) were analyzed by using Affymetrix HG-U133 A/B oligonucleotide microarrays (Affymetrix, Santa Clara, CA) as described previously.[19,20] For probes to probe set annotation, we used custom chip definition files based on GeneAnnot version 2.0, synchronized with GeneCards Version 3.04 (http://www.xlab.unimo.it/GA_CDF/).[21] Normalization was carried out by the robust multichip average method as described by Irizarry et al.[22] The Linear Models for Microarray Data (Limma) package was used to compute differentially regulated probe sets by comparing patients with *CBFB/MYH11* rearrangement and mutations affecting *FLT3* D835, *NRAS*, *KRAS*, *KIT*, or *FLT3*-ITD to patients with *CBFB/MYH11* rearrangement and *FLT3* N676K. Gene set enrichment analysis (GSEA) was performed with GSEA software (Broad Institute of Massachusetts Institute of Technology and Harvard) to assess significant changes in gene expression levels.[23] The GSEA was run with 1000 permutations and compared with the "c2_kegg" collection from the Molecular Signatures Database (MsigDB 3.0) consisting of 186 gene sets. All statistical analyses were performed by using R 3.0.1 software and routines from the biostatistics software repository Bioconductor.

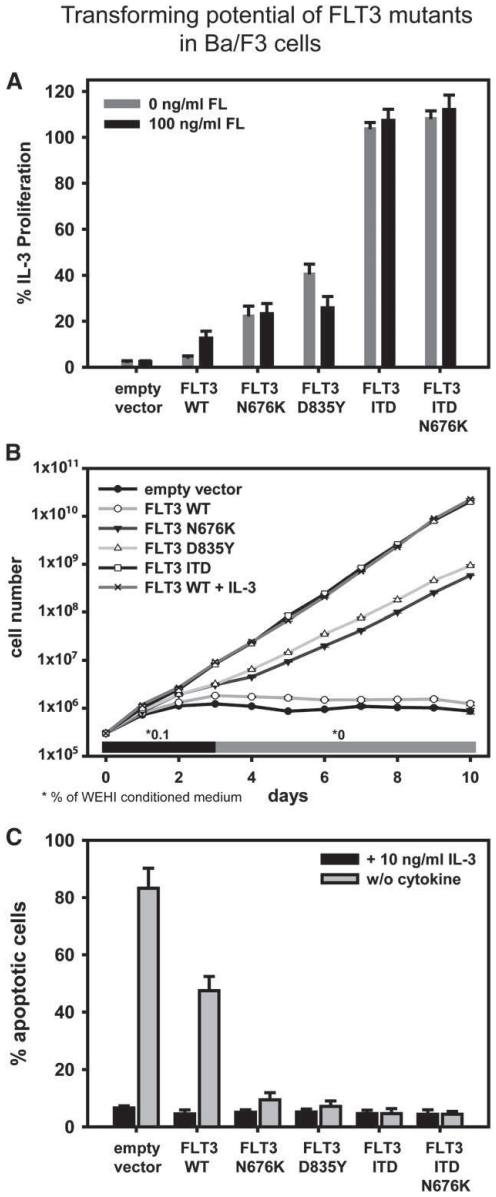

Figure 2. Transforming potential of FLT3 mutants in Ba/F3 cells. All experiments were performed in triplicates. Error bars represent standard deviation of the mean. (A) Ba/F3 cells expressing indicated FLT3 constructs were seeded at a density of 4×10^4 cells per mL in the presence or absence of 10 ng/mL IL-3 and 100 ng/mL FL. Viable cells were counted by trypan blue exclusion after 72 hours. (B) Ba/F3 cells transduced with the indicated FLT3 constructs were seeded at a density of 2×10^5 cells per mL in 0.1% WEHI-conditioned medium and cultured for 10 days. After 72 hours, cells were cleared from previous medium and resuspended in 0% WEHI-conditioned medium. Control cells were cultured in 10 ng/mL IL-3–supplemented medium. (C) Cells were cultured in the presence or absence of 10 ng/mL IL-3 for 72 hours and stained with Annexin V and 7-aminoactinomycin D. The percentage of apoptotic cells was determined by fluorescence-activated cell sorter analysis.

Results

Exome sequencing of an AML M4eo patient

To systematically identify mutations that may collaborate with CBFB/MYH11 during leukemogenesis, we performed exome sequencing of an AML sample with inv(16). The sample was selected on the basis of sample availability and the absence of additional genetic alterations (*FLT3*-ITD, *MLL*-PTD [partial tandem duplication], *FLT3*-TKD, *NPM1*, *NRAS*, *KRAS*, *KIT*, and *WT1* mutation negative). We sequenced the exome (protein coding regions) of the diagnostic sample and a remission sample from the same patient, generating at least 4 Gbp of sequence from each exome. This allowed us to cover more than 80% of RefSeq coding exon positions with a minimum read depth of 10 (supplemental Table 1). By comparing both exome sequences and excluding known polymorphisms, we were able to identify somatically acquired, leukemia-specific sequence variants. Nonsynonymous coding mutations were confirmed by using Sanger sequencing. We found a total of 2 somatic mutations, namely an N676K missense mutation in the ATP-binding domain (TKD1) of *FLT*3 (NM_004119.2: c.2028C>A; Figure 1A-C)[24] and an A251V missense mutation in the *CAT* gene, which encodes the cytoplasmic enzyme catalase (supplemental Table 2).

Recurring *FLT3* N676K mutations in CBF AML

We sequenced *FLT3* exon 16 (containing the codon of N676) in a cohort of 84 AML patients with *CBFB/MYH11* rearrangement (71 patients with inv(16) and 13 patients with t(16;16)). Strikingly, we detected heterozygous missense mutations (N/K) at position 676 of *FLT3* in 5 patients (6%) with inv(16) or t(16;16) (4 [6%] of 71 and 1 [8%] of 13, respectively). Thus, in AML with a CBFB/MYH11 fusion, *FLT3* N676K mutations have a frequency similar to *FLT3* D835 mutations (Figure 1D).

In 36 AML samples with a t(8;21)(q22;q22) and an RUNX1/RUNX1T1 fusion, 1 patient with an *FLT3* N676K mutation could be identified (1 [3%] of 36). None of the CBF AML patients with an *FLT3* N676K mutation had an additional *FLT3*-ITD or a D835 mutation. In contrast, in 90 AML patients with normal karyotype, we detected only a single patient with an *FLT3* N676K mutation, and this patient had a concurrent *FLT3*-ITD similar to that of the patient described by Heidel et al.[14] The incidence of *FLT3* N676K without concurrent ITD in CBF AML (6/120) was compared with the incidence in CN-AML (0/90) by using a two-tailed Fisher's exact test ($P = .039$). These results suggest a specific association between *FLT3* N676K mutations and CBF leukemias.

To determine whether the *FLT3* N676K mutations are somatically acquired, we sequenced remission samples where available. Paired diagnostic and remission material was available only from the N676K-positive patient with t(8;21) and from 1 patient with inv (16). In both patients, the N676K mutation could be detected at diagnosis but not in the remission sample (supplemental Figure 1). Deep amplicon sequencing of N676K-positive cases confirmed variable allele frequencies ranging from 11% to 44% indicating clonal heterogeneity (supplemental Table 5).

Additional mutations in *CBFB/MYH11*-rearranged AML

We analyzed mutational hotspots (see Materials and methods) of several commonly mutated genes (*FLT3*, *KIT*, *KRAS*, *NRAS*, *NPM1*, *MLL*, and *WT1*) in our 84 *CBFB/MYH11*-positive cohort. The

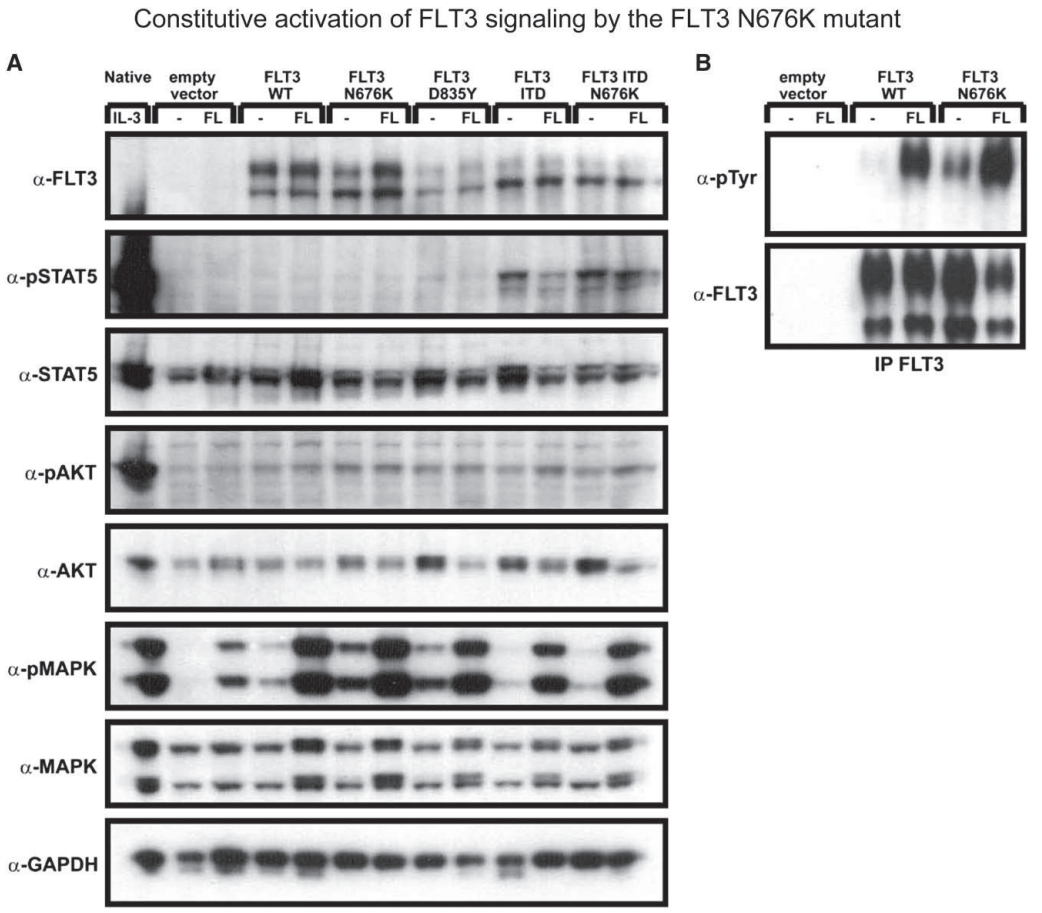

Figure 3. Constitutive activation of FLT3 signaling by the FLT3 N676K mutant. Ba/F3 cells expressing indicated constructs were starved for 24 hours in media containing 0.3% fetal calf serum. Cells were left untreated or were stimulated with 100 ng/mL FL for 10 minutes. Crude cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by western blot for phosphorylation of signaling molecules. (A) STAT5, AKT, and MAPK activation was analyzed by using phospho-specific antibodies, and then stripped and reprobed with antibodies against total STAT5, AKT, and MAPK. Ba/F3 native cells were stimulated with 100 ng/mL IL-3 for 5 minutes; control and an antibody against GAPDH were used as loading control. (B) FLT3 receptor was immunoprecipitated with polyclonal FLT3 antibody, analyzed for tyrosine phosphorylation status by immunoblotting with a phospho-tyrosin antibody, stripped, and reprobed with FLT3 antibody.

mutation frequency of these genes in our cohort (Figure 1D) was similar to that in previous reports.[7,8,25-27] We found *KIT* mutations in 18% of *CBFB*/*MYH11*-positive AMLs (14% exon 8 frameshift mutations, known to be frequent in inv(16) AML,[7,9] and 4% D816 missense mutations). RAS missense mutations were present in 51% of the patients (14% *KRAS*, 37% *NRAS*). As expected, the *FLT3*-ITD mutation was rare in our cohort (2%), whereas the *FLT3*-TKD (D835) mutation had a frequency of 10%. Together with the 6% *FLT3* N676K-mutated patients, a total of 18% (15/84) of the CBFB/MYH11-positive patients had an *FLT3* mutation.

In addition to the common signaling pathway mutations, we found 7% of samples with WT1 mutations causing a frameshift in exon 7. *MLL* PTDs (1%) and *NPM1* mutations (0%) were rare in our *CBFB*/*MYH11*-positive patients. In 18% of the patients, no mutation was detected in the mutational hotspots analyzed. In 12 patients (14%), more than one mutation was present. Seventy-nine percent of the patients (66/84) carried a mutation in *FLT3*, *KRAS*, *NRAS*, or *KIT*.

FLT3 N676K is strongly expressed on the cell surface of Ba/F3 cells

To analyze the transforming potential of the FLT3 N676K mutant, Ba/F3 cell lines stably expressing various FLT3 constructs were established. The expression of WT and mutant (mut) FLT3 receptors was confirmed by immunoblotting or flow cytometry (supplemental Figure 2). Like FLT3-WT, the FLT3 N676K receptor was highly expressed on the cell surface (mature receptor, 160 kDa) compared with FLT3-ITD and FLT3 D835Y. The FLT3-N676K-ITD double mutant showed the weakest cell surface expression. A weak cell surface expression was correlated with an enhanced expression of the immature receptor with a molecular weight of 130 kDa.[28]

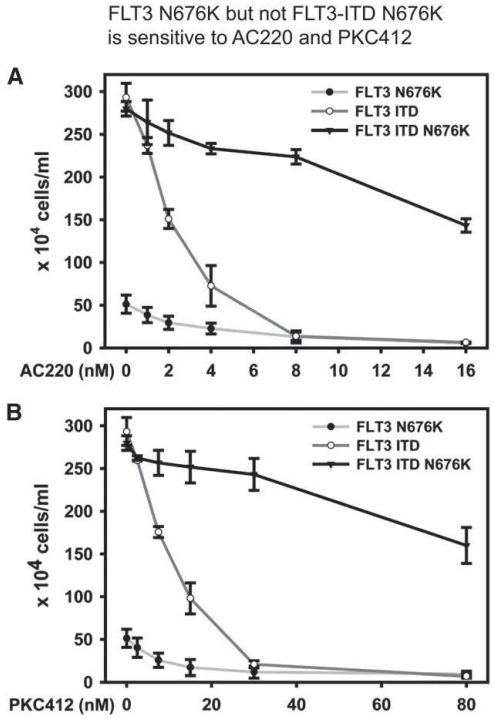

Figure 4. FLT3 N676K but not FLT3-ITD N676K is sensitive to AC220 and PKC412. Ba/F3 cells expressing indicated FLT3 variants were seeded at a density of 4×10^4 cells per mL and counted by trypan blue exclusion after 72 hours. All experiments were performed in triplicate. Error bars represent standard deviation of the mean. (A) Cells were treated with increasing nontoxic concentrations of selective TKI AC220. (B) Cells were treated with increasing nontoxic concentrations of TKI PKC412.

The FLT3 N676K mutant receptor leads to cytokine-independent growth and resistance to apoptosis

Proliferation assays of Ba/F3 cells expressing FLT3 mutant receptors revealed a cytokine-independent growth. As described before, FLT3-ITD was able to fully transform Ba/F3 cells reaching 100% of IL-3–mediated growth. FLT3 D835Y-expressing cells reached 41%. The mutant FLT3 N676K receptor led to IL-3 and FLT3-ligand (FL) independent cell growth, and the Ba/F3 cells reached about 25% of the IL-3 reference proliferation rate at 72 hours of culture time (Figure 2A). This pro-proliferative phenotype increased over time and, eventually, FLT3 N676K-expressing cells reached a proliferation rate similar to that of FLT3 D835Y-expressing cells (Figure 2B). In addition to an enhanced proliferation, Ba/F3 cells expressing the various FLT3 mutants showed a strong resistance to apoptosis after cytokine deprivation (Figure 2C). This antiapoptotic phenotype was strongest in FLT3-ITD–expressing cells (only 4.5% apoptotic cells) followed by FLT3 D835Y (7%) and FLT3 N676K (10%)-expressing cells.

Constitutive activation of FLT3 signaling in FLT3 N676K-expressing cells

To determine critical pathways for the transforming potential of the FLT3 mutants, we analyzed the activation of 3 key signaling molecules downstream of FLT3: the mitogen-activated protein kinase (MAPK), protein kinase B (AKT), and signal transducer and activator of transcription 5 (STAT5). Protein lysates of unstimulated and FL-stimulated Ba/F3 cells expressing FLT3 and the mutants were immunoblotted (Figure 3A). MAPK was strongly activated in all FL-stimulated FLT3 or FLT3 mutant-expressing cells. In contrast to FLT3-ITD–expressing cells, STAT5 was not phosphorylated in FLT3-WT or in the FLT3 N676K or the FLT3 D835Y mutant-expressing cells. MAPK was constitutively phosphorylated in unstimulated cells of the two TKD mutants N676K and D835Y compared with WT and ITD cells. To determine the activation of the FLT3 N676K mutant receptor, the protein was immunoprecipitated and analyzed for tyrosine phosphorylation by immunoblotting. The FLT3 N676K receptor showed a fivefold stronger constitutive phosphorylation as well as a twofold stronger phosphorylation after ligand stimulation compared with FLT3-WT, taking into account the total protein loaded on the gel (Figure 3B). In conclusion, FLT3 N676K mutant-expressing cells showed an enhanced signaling through the MAPK pathway but no aberrant activation of STAT5. Thus, the increased MAPK activation is most likely responsible for the mutant phenotypes.

FLT3 N676K-induced proliferation can be abrogated by selective PTK inhibition

The *FLT3* N676K mutation was previously described only in combination with an *FLT3*-ITD to mediate resistance to PTKIs.[14] In previous studies it was not tested whether *FLT3* N676K alone might be sufficient to confer protein tyrosine kinase inhibitor resistance. To address this question, we used PKC412 and AC220 as selective FLT3 inhibitors in increasing nontoxic concentrations (Figure 4). Nontoxicity of the inhibitors was confirmed in FLT3-WT–expressing Ba/F3 cells (supplemental Figure 5). Both compounds potently inhibited FLT3-ITD–expressing cells with a half maximal inhibitory concentration (IC_{50}) of 13 nM for PKC412 and 2.5 nM for AC220, respectively. FLT3 N676K-expressing cells were also sensitive to FLT3 inhibitors with an IC_{50} of 7.5 nM for PKC412 and 3 nM for AC220. FLT3-ITD-N676K double mutants showed a strong resistance to both inhibitors (IC_{50} greater than 80 nM for PKC412 and greater than 16 nM for AC220). Taken together, the FLT3 N676K mutation with an ITD is very resistant to FLT3 inhibitors. However, cell proliferation driven by FLT3 N676K alone can be inhibited rather effectively.

Differential gene expression in *FLT3* N676K-mutated *CBFB/MYH11*-rearranged AML

To assess the impact of *FLT3* N676K mutations on gene expression, we analyzed the gene expression profiles of 33 patients with *CBFB/MYH11* rearranged AML. Four patients with *FLT3* N676K mutations were compared with 29 patients with *FLT3* D835 (n = 4), *NRAS* (n = 15), *KRAS* (n = 3), *KIT* (n = 3), *WT1* (n = 1), and *FLT3*-ITD (n = 2) mutations or no mutations in any of these genes (n = 5). Some patients had no (n = 5) or more than 1 (n = 3) mutation. Finally, all unique probe sets with $P < .005$ and log fold-change >1.5 were selected for unsupervised clustering (n = 18). Interestingly, all cases with *FLT3* N676K clustered together (supplemental Figure 3). Of these 18 genes, six were highly correlated with sex, since all cases with N676 mutation in our analysis were discovered in male patients. Interestingly, genes with high association and elevated levels in the N676K cluster were CCNA1 (cell cycle), PRG3 (immune response), and HLA-DQA1 (immune response). Genes with negative correlation to the N676K cluster were MEST

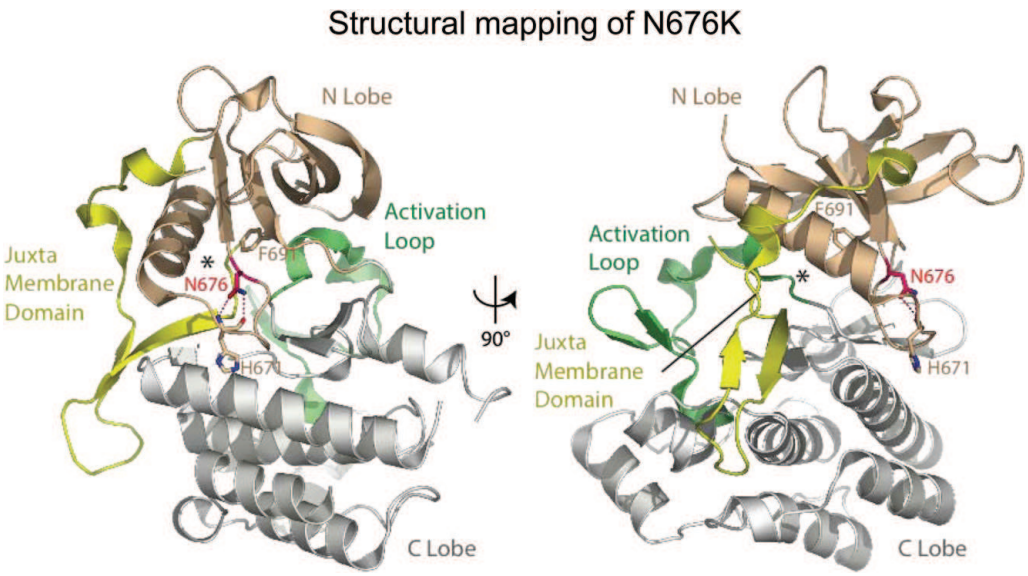

Figure 5. Structural mapping of N676K. Structure of the autoinhibited FLT3 kinase (Protein Data Bank accession number 1RJB) is shown as a ribbon model with highlighted secondary structure and color-coded domains. N676 forms hydrogen bonds to the backbone of H671, stabilizing a loop at the back of the substrate and inhibitor-binding pocket (asterisk). N676K will remove these hydrogen bonds, likely destabilizing the loop and the nearby substrate-binding pocket. This structural effect can explain resistance against TKIs, which target the nearby pocket. However, the mutation could also lift the autoinhibition of FLT3, providing a possible explanation for the observation that this mutation alone shows transforming potential.

(imprinting) and ARG1 (metabolism). To evaluate which pathways were associated with *FLT3* N676K mutations, we compared the 4 patients with this mutation to 29 patients without this mutation. Nine gene sets were significantly enriched at a false discovery rate of <25% and $P < .05$ including metabolic, inflammation and degradation pathways (supplemental Table 4).

Structural mapping of the FLT3 N676K receptor mutation

Since we could demonstrate that a single point mutation in the ATP-binding domain is sufficient to constitutively activate the receptor and increase downstream signaling, we performed structural modeling of the FLT3 N676K mutant to gain further insights into the consequences of the mutation (Figure 5).

Mapping of the FLT3 N676K onto the crystal structure of FLT3 showed that this mutation destabilizes the fold of the kinase domain between the juxtamembrane domain (JMD) and a hydrophobic pocket that is the target of FLT3 inhibitors. The crystal structure of the inactive conformation of FLT3 showed that the JMD serves as a key autoinhibitory element regulating the kinase activity.[29,30] N676K mutations might therefore interfere with the FLT3 autoinhibition by reducing the stability of the JMD, thus, suggesting a structural basis for the transforming activity observed in our experiments with Ba/F3 cells.

Clinical characteristics associated with *FLT3* N676K mutations

Fifty-six of the 84 *CBFB/MYH11*-rearranged AML patients screened for mutations in this study were enrolled in the multicenter AMLCG-1999 trial of the German AML Cooperative Group (NCT00266136). Among these patients, five carried *FLT3* N676K mutations. In this cohort, which was homogeneous with regard to both treatment and cytogenetics, the mutation was significantly associated with higher leukocyte counts ($P = .02$), elevated lactate dehydrogenase ($P = .02$), and male sex ($P = .02$) (Table 1). There was no significant difference in survival of patients with *FLT3* N676K (n = 4) compared with patients with *FLT3* N676 wt (n = 47) (supplemental Figure 4A). However, there was a trend toward reduced complete remission rates associated with *FLT3* N676K mutations (Table 1).

We also compared *CBFB/MYH11*-rearranged AML patients with *FLT3* point mutations affecting residues N676 or D835 (n = 9) to all other *CBFB/MYH11*-rearranged patients (n = 42) and did not observe a significant difference in survival (supplemental Figure 4B).

Discussion

Ours is the first report of recurring *FLT3* N676K mutations in CBF leukemia. Despite the overall rather favorable prognosis associated with CBF rearrangements, almost one third of patients relapses within the first year after intensive chemotherapy and only 60% of CBF AML patients are still alive after 5 years. This heterogeneous clinical outcome of CBF AML patients may reflect the heterogeneity of additional genetic lesions in this subgroup and underscores the need for further investigation. Understanding the pathogenesis of AML is challenging because of the multitude of genetic events. By sequencing known mutational targets, we and others have demonstrated that between 80% and 90% of *CBFB/MYH11*-rearranged patients have mutations that activate either RAS signaling or RTK signaling (*FLT3* and *KIT*), while other

Table 1. Characteristics of *CBFB/MYH11*-rearranged patients

	FLT3 N676 wt		*FLT3* N676K mut		
Variable	**No.**	**%**	**No.**	**%**	***P***
No. of patients	51		5		
Age, years					N/S
Median	42		54		
Range	20-75		18-61		
Male sex	22	43.1	5	100	.02
White blood cells × 10^9/L					.02
Median	38.5		134		
Range	1.3-316		54.1-259		
Hemoglobin, g/dL					N/S
Median	8.6		9.1		
Range	4.4-14.6		8-14.1		
Platelets × 10^9/L					N/S
Median	32		44		
Range	0.01-370		32-47		
LDH (U/L)					.02
Median	666		1326		
Range	143-1870		717-2508		
Bone marrow blasts (%)					N/S
Median	80		60		
Range	25-95		10-90		
ECOG performance status ≥ 2 (%)	14	29.2	1	20	N/S
de novo AML	46	90.2	3	75	N/S
NPM1 mut	0	0	0	0	N/S
FLT3-ITD	2	3.9	0	0	N/S
FLT3-D835	5	9.8	0	0	N/S
MLL-PTD	1	2	0	0	N/S
KRAS mut	7	13.7	0	0	N/S
NRAS mut	21	41.2	0	0	.15
KIT mut	10	19.6	0	0	N/S
WT1 mut	4	7.8	0	0	N/S
Complete remission	39	76	2	40	.11
Deceased	19	37.2	2	40	N/S

All patients were enrolled in the AMLCG-99 trial and received intensive induction treatment. Categorical clinical variables of the *FLT3* N676K-mutated (mut) and *FLT3* N676 wild-type (wt) cohorts were compared by Fisher's exact test. The continuous variables were compared by Mann-Whitney *U* test. $P < .05$ was considered significant. ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase.

common AML-related gene mutations (eg, in *NPM1*, *WT1*, and *MLL*) are rarely found.[7-11] The discovery of *FLT3* N676K mutations adds another piece to the puzzle of CBF-related leukemogenesis, suggesting that the proportion of CBF leukemia with activating RTK mutations has been underestimated. Our observation of concurrent activating mutations in different genes (eg, *KRAS* and *NRAS* or *NRAS* and *KIT*; Figure 1D) suggests either clonal heterogeneity or multiple additive hits in synergistic pathways within *CBFB/MYH11*-rearranged AML.

The specific occurrence of recurring *FLT3* N676K mutations in the *CBFB/MYH11*-rearranged AML subgroup, which accounts for only 6% of AML, might explain why *FLT3* N676K mutations have remained undetected in previous full-length *FLT3* mutation screens of unselected AML patients.[31] Other large studies limited *FLT3* mutational screening to *FLT3*-ITD mutations (exon 14/15) and TKD2 mutations (exon 20; eg, D835) and thus would have missed *FLT3* N676K mutations (exon 16).[7,26]

Even though FLT3 N676K in combination with FLT3-ITD had previously been shown to lead to PTKI resistance,[12,14] we show in this report that the FLT3 N676K mutant on its own exhibits gain-of-function properties. Notably, FLT3 N676K alone has direct transforming potential in Ba/F3 cells through increased downstream signaling similar to that of the FLT3 D835 mutant but weaker than FLT3-ITD (Figures 3 and 4). The power of the gene expression analysis of the *FLT3* N676K-mutated patients is limited by the small sample size. However, *FLT3* N676K-mutated cases clustered together after unsupervised clustering analysis of gene expression in *CBFB/MYH11*-rearranged AML with different mutations affecting *FLT3*, *NRAS*, *KRAS*, *KIT*, and *WT1* (supplemental Figure 3). These findings suggest a distinct biologic subgroup within *CBFB/MYH11*-rearranged AML characterized by *FLT3* N676K mutations.

On the basis of the crystal structure of FLT3, Cools et al[12] proposed that mutation of N676 destabilizes the conformation of the hinge segment, which makes H-bonds with the lactam ring of the PTKI PKC412. We suggest that a mutation at position N676 may also activate FLT3 by disturbing its autoinhibition capacity (Figure 5). Taken together, mutations at position N676 most probably have two consequences: activating FLT3 and, together with a concurrent FLT3-ITD, conferring PTKI resistance. The additional N676K mutation on the ITD background might change the conformation of the FLT3-ITD protein in a way that the binding site of the PTKIs is masked, since the ITD results in an extension of the JMD, which leads to a conformational change of the kinase domain. The fact that the FLT3 N676K alone (without concurrent ITD) does not confer PTKI resistance, might also be related to its localization on the cell surface, in contrast to the mostly intracellular localization of the ITD-N676K double mutant. Hence, N676K-mutated FLT3 might be exposed to higher inhibitor concentrations at the cell surface, possibly allowing efficient inhibition of the TKDs directly beneath the cell membrane. It was shown by others that there is higher intracellular accumulation of PTKIs in the more sensitive AML cells lines than in the less sensitive ones.[32]

In contrast to the initial report of an *FLT3* N676K mutation as a late arising, disease-modifying event, detected at the time of clinical relapse while on PKC412 monotherapy,[14] all of our N676K mutations were detectable at initial diagnosis. Since cell proliferation driven by FLT3 N676K alone could be greatly reduced by FLT3 inhibitors, N676K-positive patients without concurrent ITD may actually benefit from treatment with FLT3 inhibitors.

Our clinical data did not show a significant impact of *FLT3* N676K mutations on survival within *CBFB/MYH11*-rearranged AML patients, but there was a trend toward reduced complete remission rates (Table 1). The significant association of *FLT3* N676K mutations with higher leukocyte counts, elevated lactate dehydrogenase levels, and male sex (Table 1), suggests a distinct biology of these leukemias. Given the small number of *FLT3* N676K–positive patients (n = 4) in our patient cohort, the prognostic significance of the *FLT3* N676K mutation needs to be investigated in larger patient cohorts.

The varying allele frequencies of the FLT3 N676K mutation ranging from 14% to 44% in those cases in which the presence of the *CBFB/MYH11* rearrangement was detected by fluorescence in situ hybridization in the majority of the bone marrow cells (supplemental Table 5) indicate that the FLT3 N676K mutation did not always represent the dominant leukemic clone at diagnosis. It would be interesting to study the clonal evolution in those cases by assessing the *FLT3* N676 status at relapse; unfortunately, no relapse samples from our patients were available.

Although *FLT3* has been known for more than a decade to be mutated in about one third of AML patients, it appears that the spectrum of *FLT3* mutations is still not fully understood. In

particular, defined genetic subgroups of AML might harbor specific *FLT3* mutations. Unbiased mutation screening using exome sequencing allows the detection of novel sequence variations even in extensively studied genes such as *FLT3*.

Acknowledgments

We thank the participating centers of the German Acute Myeloid Leukemia Cooperative Group 1999 (AMLCG-1999) trial.

This work was supported by grant 109031 from the German Cancer Aid (P.A.G and S.K.B.); by National Genome Research Network Plus grant PKL-01-GS0876-6 from the Federal Ministry of Education and Research (BMBF) (S.K.B.); and by the Collaborative Research Center 684 Molecular Mechanisms of Normal and Malignant Hematopoiesis, projects A3, A6, A12 and knock-on funding 2011 from the German Research Foundation (DFG) (K.-P.H., S.K.B., K.S., and P.A.G.).

Authorship

Contribution: S.O., H.P., K.S., S.K.B., and. P.A.G. conceived and designed the experiments; S.O., H.P., N.P.K., B.K., E.Z., and S.K. performed experiments; S.O., H.P., and T.H. analyzed data; K.-P.H. performed structural modeling; S.V. and A.G. provided bioinformatics support; H.B. managed the Genome Analyzer IIx platform; S.O., B.K., A.D., E.Z., P.M.K., S.S., J.B., S.K.B., and K.S. characterized patient samples; M.C.S., J.B., W.E.B., T.B., B.J.W., and W.H. coordinated the German Acute Myeloid Leukemia Cooperative Group clinical trial; P.A.G., K.S., and S.K.B. supervised the project; and S.O., H.P., T.H., S.K.B., and P.A.G. wrote the manuscript.

Conflict-of-interest disclosure: P.A.G. and S.K. received honoraria from Illumina. The remaining authors declare no competing financial interests.

Correspondence: Philipp A. Greif, Marchioninistrasse 25, 81377 München, Germany; e-mail: pgreif@med.uni-muenchen.de.

References

1. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. *Nat Rev Cancer.* 2002;2(7):502-513.
2. Mrózek K, Bloomfield CD. Clinical significance of the most common chromosome translocations in adult acute myeloid leukemia. *J Natl Cancer Inst Monogr.* 2008;(39):52-57.
3. Castilla LH, Perrat P, Martinez NJ, et al. Identification of genes that synergize with Cbfb-MYH11 in the pathogenesis of acute myeloid leukemia. *Proc Natl Acad Sci USA.* 2004;101(14): 4924-4929.
4. Schessl C, Rawat VP, Cusan M, et al. The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. *J Clin Invest.* 2005;115(8):2159-2168.
5. Murati A, Brecqueville M, Devillier R, Mozziconacci MJ, Gelsi-Boyer V, Birnbaum D. Myeloid malignancies: mutations, models and management. *BMC Cancer.* 2012;12(1):304.
6. Gilliland DG. Molecular genetics of human leukemias: new insights into therapy. *Semin Hematol.* 2002;39(4 Suppl 3):6-11.
7. Boissel N, Leroy H, Brethon B, et al; Acute Leukemia French Association (ALFA); Leucémies Aiguës Myéloblastiques de l'Enfant (LAME) Cooperative Groups. Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). *Leukemia.* 2006;20(6):965-970.
8. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. *Blood.* 2006; 107(10):3847-3853.
9. Paschka P, Marcucci G, Ruppert AS, et al; Cancer and Leukemia Group B. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. *J Clin Oncol.* 2006;24(24):3904-3911.
10. Valk PJ, Bowen DT, Frew ME, Goodeve AC, Löwenberg B, Reilly JT. Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16). *Haematologica.* 2004; 89(1):106.
11. Haferlach C, Dicker F, Kohlmann A, et al. AML with CBFB-MYH11 rearrangement demonstrate RAS pathway alterations in 92% of all cases including a high frequency of NF1 deletions. *Leukemia.* 2010;24(5):1065-1069.
12. Cools J, Mentens N, Furet P, et al. Prediction of resistance to small molecule FLT3 inhibitors: implications for molecularly targeted therapy of acute leukemia. *Cancer Res.* 2004;64(18): 6385-6389.
13. Pauwels D, Sweron B, Cools J. The N676D and G697R mutations in the kinase domain of FLT3 confer resistance to the inhibitor AC220. *Haematologica.* 2012;97(11):1773-1774.
14. Heidel F, Solem FK, Breitenbuecher F, et al. Clinical resistance to the kinase inhibitor PKC412 in acute myeloid leukemia by mutation of Asn-676 in the FLT3 tyrosine kinase domain. *Blood.* 2006; 107(1):293-300.
15. Greif PA, Dufour A, Konstandin NP, et al. GATA2 zinc finger 1 mutations associated with biallelic CEBPA mutations define a unique genetic entity of acute myeloid leukemia. *Blood.* 2012;120(2): 395-403.
16. Reindl C, Bagrintseva K, Vempati S, et al. Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML. *Blood.* 2006;107(9):3700-3707.
17. Polzer H, Janke H, Schmid D, Hiddemann W, Spiekermann K. Casitas B-lineage lymphoma mutants activate AKT to induce transformation in cooperation with class III receptor tyrosine kinases. *Exp Hematol.* 2012;41(3):271-280.
18. Reindl C, Quentmeier H, Petropoulos K, et al. CBL exon 8/9 mutants activate the FLT3 pathway and cluster in core binding factor/11q deletion acute myeloid leukemia/myelodysplastic syndrome subtypes. *Clin Cancer Res.* 2009;15(7): 2238-2247.
19. Herold T, Jurinovic V, Metzeler KH, et al. An eight-gene expression signature for the prediction of survival and time to treatment in chronic lymphocytic leukemia. *Leukemia.* 2011;25(10): 1639-1645.
20. Li Z, Herold T, He C, et al. Identification of a 24-gene prognostic signature that improves the European LeukemiaNet risk classification of acute myeloid leukemia: an international collaborative study. *J Clin Oncol.* 2013;31(9):1172-1181.
21. Ferrari F, Bortoluzzi S, Coppe A, et al. Novel definition files for human GeneChips based on GeneAnnot. *BMC Bioinformatics.* 2007;8:446.
22. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics.* 2003;4(2):249-264.
23. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA.* 2005;102(43):15545-15550.
24. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. Integrative genomics viewer. *Nat Biotechnol.* 2011;29(1):24-26.
25. Bowen DT, Frew ME, Hills R, et al. RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients younger than 60 years. *Blood.* 2005;106(6):2113-2119.
26. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood.* 2002;99(12):4326-4335.
27. Schnittger S, Schoch C, Dugas M, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood.* 2002;100(1):59-66.
28. Schmidt-Arras DE, Böhmer A, Markova B, Choudhary C, Serve H, Böhmer FD. Tyrosine phosphorylation regulates maturation of receptor tyrosine kinases. *Mol Cell Biol.* 2005;25(9): 3690-3703.
29. Griffith J, Black J, Faerman C, et al. The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. *Mol Cell.* 2004;13(2): 169-178.
30. Walter M, Lucet IS, Patel O, et al. The 2.7 A crystal structure of the autoinhibited human c-Fms kinase domain. *J Mol Biol.* 2007;367(3): 839-847.
31. Fröhling S, Scholl C, Levine RL, et al. Identification of driver and passenger mutations of FLT3 by high-throughput DNA sequence analysis and functional assessment of candidate alleles. *Cancer Cell.* 2007;12(6): 501-513.
32. Hu S, Niu H, Minkin P, et al. Comparison of antitumor effects of multitargeted tyrosine kinase inhibitors in acute myelogenous leukemia. *Mol Cancer Ther.* 2008;7(5):1110-1120.

5 Literaturverzeichnis

1. Frohling, S., et al., *Genetics of myeloid malignancies: pathogenetic and clinical implications.* J Clin Oncol, 2005. **23**(26): p. 6285-95.
2. Estey, E. and H. Döhner, *Acute myeloid leukaemia.* The Lancet, 2006. **368**(9550): p. 1894-1907.
3. Jan, M., et al., *Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia.* Sci Transl Med, 2012. **4**(149): p. 149ra118.
4. Shlush, L.I., et al., *Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia.* Nature, 2014. **506**(7488): p. 328-33.
5. Arber, D.A., et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia.* Blood, 2016. **127**(20): p. 2391.
6. Döhner, H., et al., *Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel.* Blood, 2017. **129**(4): p. 424.
7. Grimwade, D., et al., *Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials.* Blood, 2010. **116**(3): p. 354-65.
8. Grimwade, D., A. Ivey, and B.J.P. Huntly, *Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance.* Blood, 2016. **127**(1): p. 29-41.
9. Mrozek, K., et al., *Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia.* J Clin Oncol, 2012. **30**(36): p. 4515-23.
10. Dohner, H. and V.I. Gaidzik, *Impact of genetic features on treatment decisions in AML.* (1520-4383 (Electronic)).
11. Cornelissen, J.J., et al., *The European LeukemiaNet AML Working Party consensus statement on allogeneic HSCT for patients with AML in remission: an integrated-risk adapted approach.* (1759-4782 (Electronic)).
12. Caudell, D., et al., *Expression of a CALM-AF10 fusion gene leads to Hoxa cluster overexpression and acute leukemia in transgenic mice.* Cancer Res, 2007. **67**(17): p. 8022-31.
13. Lin, Y.H., et al., *Global reduction of the epigenetic H3K79 methylation mark and increased chromosomal instability in CALM-AF10-positive leukemias.* Blood, 2009. **114**(3): p. 651-8.
14. Chen, L., et al., *Abrogation of MLL-AF10 and CALM-AF10-mediated transformation through genetic inactivation or pharmacological inhibition of the H3K79 methyltransferase Dot1l.* Leukemia, 2013. **27**(4): p. 813-22.
15. Castilla, L.H., et al., *Identification of genes that synergize with Cbfb-MYH11 in the pathogenesis of acute myeloid leukemia.* Proc Natl Acad Sci U S A, 2004. **101**(14): p. 4924-9.
16. Schessl, C., et al., *The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice.* J Clin Invest, 2005. **115**(8): p. 2159-68.
17. Dreyling, M.H., et al., *MLL and CALM are fused to AF10 in morphologically distinct subsets of acute leukemia with translocation t(10;11): both rearrangements are associated with a poor prognosis.* Blood, 1998. **91**(12): p. 4662-7.

18. Deshpande, A.J., et al., *Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of CALM/AF10-positive leukemia.* Cancer Cell, 2006. **10**(5): p. 363-74.
19. Deshpande, A.J., et al., *AF10 regulates progressive H3K79 methylation and HOX gene expression in diverse AML subtypes.* Cancer Cell, 2014. **26**(6): p. 896-908.
20. Argiropoulos, B. and R.K. Humphries, *Hox genes in hematopoiesis and leukemogenesis.* Oncogene, 2007. **26**(47): p. 6766-76.
21. Speck, N.A. and D.G. Gilliland, *Core-binding factors in haematopoiesis and leukaemia.* Nat Rev Cancer, 2002. **2**(7): p. 502-13.
22. Mrozek, K. and C.D. Bloomfield, *Clinical significance of the most common chromosome translocations in adult acute myeloid leukemia.* J Natl Cancer Inst Monogr, 2008(39): p. 52-7.
23. Gilliland, D.G., *Molecular genetics of human leukemias: new insights into therapy.* Semin Hematol, 2002. **39**(4 Suppl 3): p. 6-11.
24. Haferlach, C., et al., *AML with CBFB-MYH11 rearrangement demonstrate RAS pathway alterations in 92% of all cases including a high frequency of NF1 deletions.* Leukemia, 2010. **24**(5): p. 1065-9.
25. Valk, P.J., et al., *Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16).* Haematologica, 2004. **89**(1): p. 106.
26. Murati, A., et al., *Myeloid malignancies: mutations, models and management.* BMC Cancer, 2012. **12**(1): p. 304.
27. Dohner, K. and H. Dohner, *Molecular characterization of acute myeloid leukemia.* Haematologica, 2008. **93**(7): p. 976-82.
28. Boissel, N., et al., *Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML).* Leukemia, 2006. **20**(6): p. 965-70.
29. Krauth, M.T., et al., *High number of additional genetic lesions in acute myeloid leukemia with t(8;21)/RUNX1-RUNX1T1: frequency and impact on clinical outcome.* Leukemia, 2014. **28**(7): p. 1449-1458.
30. Micol, J.-B., et al., *Frequent ASXL2 mutations in acute myeloid leukemia patients with t(8;21)/RUNX1-RUNX1T1 chromosomal translocations.* Blood, 2014. **124**(9): p. 1445-1449.
31. Cher, C.Y., et al., *Next-generation sequencing with a myeloid gene panel in core-binding factor AML showed KIT activation loop and TET2 mutations predictive of outcome.* Blood Cancer J, 2016. **6**(7): p. e442.
32. Gedman, A.L., et al., *The landscape of somatic mutations in the core-binding factor acute myeloid leukemias.* Cancer Res, 2013. **74(20 Suppl)**: p. Abstract nr A15.
33. Hartmann, L., et al., *ZBTB7A mutations in acute myeloid leukaemia with t(8;21) translocation.* Nat Commun, 2016. **7**: p. 11733.
34. Paschka, P., et al., *Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study.* J Clin Oncol, 2006. **24**(24): p. 3904-11.
35. Boissel, N., et al., *Dasatinib in high-risk core binding factor acute myeloid leukemia in first complete remission: a French Acute Myeloid Leukemia Intergroup trial.* Haematologica, 2015. **100**(6): p. 780-5.
36. Huang, K., et al., *Leukemogenic potency of the novel FLT3-N676K mutant.* Ann Hematol, 2016. **95**(5): p. 783-91.

37. Paschka, P. and K. Döhner, *Core-binding factor acute myeloid leukemia: can we improve on HiDAC consolidation?* ASH Education Program Book, 2013. **2013**(1): p. 209-219.
38. Marcucci, G., et al., *Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): a Cancer and Leukemia Group B study.* J Clin Oncol, 2005. **23**(24): p. 5705-17.
39. Schlenk, R.F., et al., *Individual patient data-based meta-analysis of patients aged 16 to 60 years with core binding factor acute myeloid leukemia: a survey of the German Acute Myeloid Leukemia Intergroup.* J Clin Oncol, 2004. **22**(18): p. 3741-50.

6 Danksagungen

An dieser Stelle möchte ich mich ganz herzlich bei allen bedanken, die zum Gelingen dieser Arbeit beigetragen und mich auf dem langen Weg begleitet haben.

Mein besonderer Dank gilt meinem Doktorvater, Professor Stefan Bohlander, der mir diese Dissertation ermöglich hat und mir jederzeit, in außergewöhnlichem Maße, mit hilfreichen Anregungen und wertvollem Rat zur Seite stand.

Ich danke Dr. med. Philipp Greif für die Betreuung dieser Arbeit. Seine Geduld, seine Aufgeschlossenheit und sein Vertrauen ermöglichten es mir die Fragestellungen dieser Arbeit in diesem Umfang selbständig zu entwerfen und zu bearbeiten.

Mein Dank gilt zudem allen Koautoren für die gute Zusammenarbeit zur Publikation der gemeinsamen Daten, insbesondere Dr. Aniruddha Deshpande, Dr. med. Klaus Metzeler und Dr. med. Tobias Herold.

Ich danke Herrn Professor Wolfgang Hiddemann für die Möglichkeit diese Arbeit in der Medizinischen Klinik und Poliklinik III der Ludwig-Maximilians-Universität München zu verfassen.

Mein Dank gilt nicht zuletzt den Mitgliedern der ehemaligen KKG Leukämie und den Kollegen aus dem Labor für Leukämiediagnostik. Die freundschaftliche Arbeitsatmosphäre und die stete Unterstützung ob technisch oder moralisch, haben diese Arbeit in ihrem Umfang und Erfolg erst möglich gemacht. Mein ganz besonderer Dank gilt hier Frau Bianka Ksienzyk – die oft schon vor mir wusste wann ich Hilfe am dringendsten brauchte.

Und ich danke meiner Familie und meinen Freunden, für den Rückhalt und die Motivation.

Danke!