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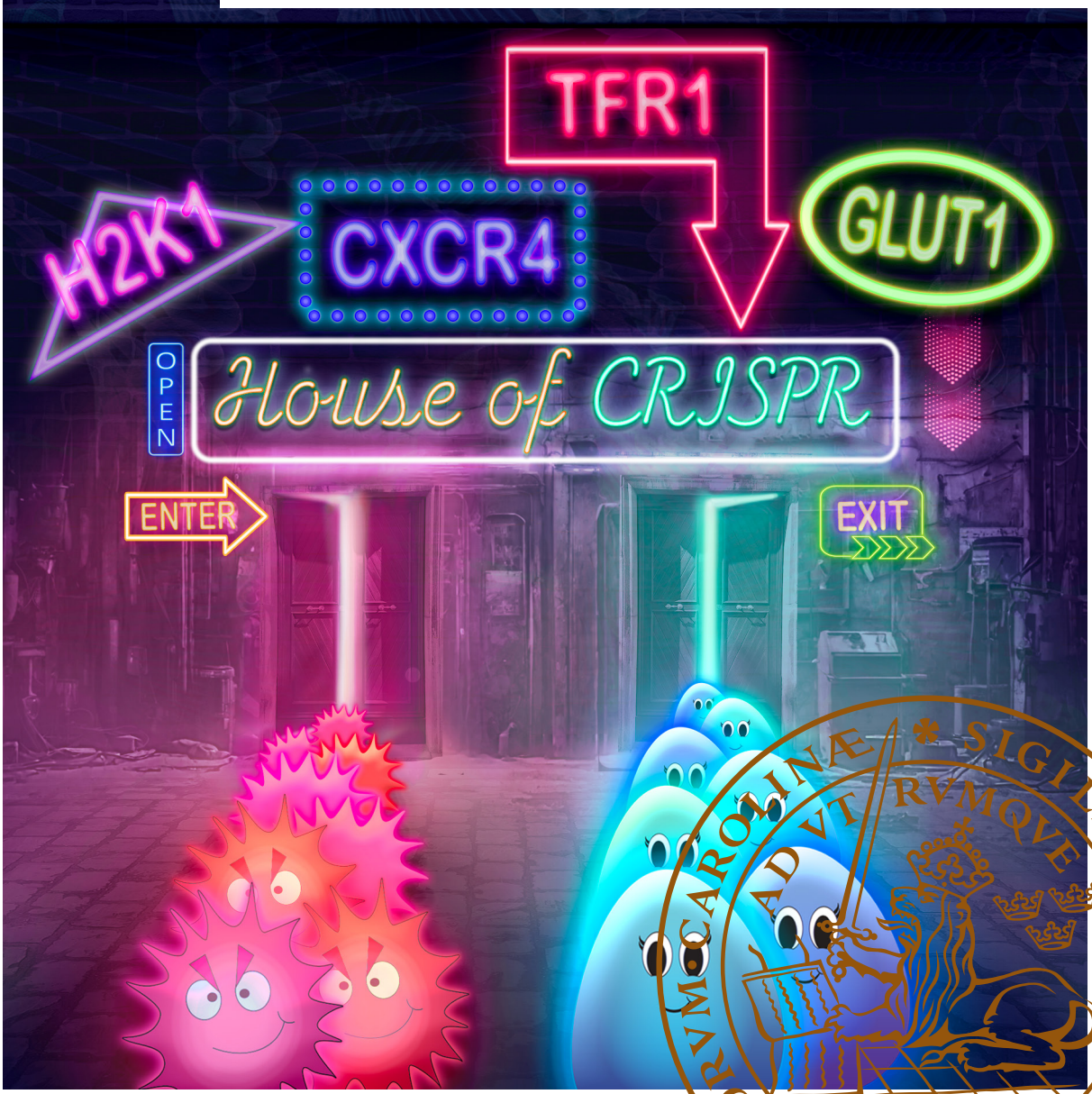

CRISPR Screens Identify Candidate Therapeutic Targets in Leukemia

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LUND
UNIVERSITY

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Abstract

Acute myeloid leukemia (AML) is a complex hematological malignancy marked by proliferation of immature myeloid cells with a dismal 5-year survival. A major challenge is the persistence of leukemia stem cells (LSCs) after standard treatment, leading to relapse. This thesis employs *in vivo* CRISPR/Cas9 screening to investigate critical AML and LSC molecular mechanisms, interrogating the dependancies on cell surface receptors for use as potential therapies.

In our initial study (**Article I**), we identify the chemokine receptor CXCR4 as a crucial dependency of AML cell growth and survival. Using a murine model of AML driven by *MLL::AF9*, we find that CXCR4 loss triggers oxidative stress and differentiation *in vivo*, with CXCL12 ligand signaling being non-essential for leukemia development.

Expanding our study to interrogate nearly one thousand cell surface receptors, we identify three additional AML dependencies. Among these, GLUT1, a primary cellular glucose transporter, emerges as a key regulator of energy metabolism, driving *MLL::AF9* LSC survival (**Article II**). Inhibition of GLUT1 suppresses cellular bioenergetics, prompting autophagy as a metabolic adaptation. Notably, dual inhibition of GLUT1 and oxidative phosphorylation effectively eliminates human AML cells, especially for the *RUNX1*-mutated AML subtype.

Furthermore, our research also reveals iron metabolism as another critical AML dependency (**Article III**). We found that disrupting iron uptake through genetic knockdown of *Tfrc*, encoding the transferrin receptor (TFR1), suppresses leukemia development in a p53-dependent manner, leading to transcriptional repression of antioxidant defense and mitochondrial respiration pathways. Patient-derived AML cells were selectively targeted upon iron chelation treatment.

Additionally, our work uncovers the role of H2K1 in evading NK cell-mediated immune surveillance *in vivo* through disruption of NK cell maturation and activation (**Article IV**). Consistent with this finding, *H2k1* disruption alone suffices to reverse this immune evasion, restoring NK cell-mediated anti-leukemic effects.

In conclusion, this thesis highlights the value of CRISPR/Cas9 screens in identifying physiologically relevant AML dependencies. It offers insights into targetable LSC vulnerabilities, emphasizing the potential of metabolic targeting and combined treatments for improved AML therapies.

Key words: Acute myeloid leukemia, CRISPR screen, leukemia stem cell, cell surface receptor, metabolism, immunotherapy, CXCR4, GLUT1, TFR1, H2-K1.

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2023
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Department of Laboratory Medicine, Lund
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The thesis front cover art encapsulates the essence of my research. It portrays menacing and distressed leukemia cells that clog the bloodstream entering the “House of CRISPR”, a metaphortical garage of genetic repair. Here they undergo transformative repair, and emerging from the exit door are healthy-looking hematopoietic cells, signifying the removal of cancer cells from the bloodstream. The scene is illuminated by the vibrant neon lights of the four proteins central to my study: GLUT1, TFR1, CXCR4, and H2-K1.

On the back cover, the battle between leukemia cells and healthy cells symbolizes the ongoing struggle of the disease within the body, highlighting the importance of cancer research in tipping the scales towards health. The array of diverse weapons that healthy cells employ represent the vast amount of methodologies researchers are using to combat cancer cells.

Front cover art by Lavanya Lokhande

Back cover art by Alexander Svanbergsson

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MADE IN SWEDEN

To my family

"The joy of discovery is the fuel that drives scientific progress, and the thrill of unlocking nature's mysteries is unparalleled."
- Francis Crick

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so we fear less."
-Marie Curie

Table of Contents

Original articles and manuscripts

This thesis is based on the following articles and manuscripts:

Article I

Ramakrishnan, R., Peña-Martínez, P., Agarwal, P., Rodriguez-Zabala, M., Chapellier, M., Högberg, C., Eriksson, M., Yudovich, D., Shah, M., Ehinger, M., Nilsson, B., Larsson, J., Hagström-Andersson, A., Ebert, B. L., Bhatia, R., and Järås, M. **CXCR4 signaling has a CXCL12-independent essential role in murine *MLL-AF9*-driven acute myeloid leukemia.** *Cell Reports*, 2020; 31(8): 107684.

Article II

Rodriguez-Zabala, M., Ramakrishnan, R., Reinbach, K., Ghosh, S., Oburoglu, L., Falqués-Costa, A., Bellamkonda, K., Ehinger, M., Peña-Martínez, P., Puente-Moncada, N., Lilljebjörn, H., Cammenga, J., Pronk, C.J., Lazarevic, V., Fioretos, T., Hagström-Andersson, A.K., Woods, N.B., and Järås, M. **Combined GLUT1 and OXPHOS inhibition eliminates acute myeloid leukemia cells by restraining their metabolic plasticity.** *Blood Advances*, 2023; 7(18):5382-5395.

Article III

Rodriguez-Zabala, M., Reinbach, K., Ghosh, S., Ramakrishnan, R., Hansen, N., Cammenga, J., Pronk, C.J., Lazarevic, V., Fioretos, T., and Järås, M. **TFR1 inhibition has p53-dependent therapeutic efficacy in acute myeloid leukemia.** Manuscript.

Article IV

Ghosh, S., Rodriguez-Zabala, M., Telliam Dushime, G., Reinbach, K., Ramakrishnan, R., Sitnicka, E., and Järås, M. **H2-K1 protects murine *MLL-AF9* leukemia cells from NK cell-mediated immune surveillance.** Manuscript.

Abbreviations

ADC	Antibody-drug conjugate
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANGPT1	Angiopoietin-1
ATP	Adenosine triphosphate
BMSC	Bone marrow mesenchymal stem/stromal cell
CAR	Chimeric antigen receptor
Cas	CRISPR-associated protein
CFU	Colony-forming unit
CHIP	Clonal hematopoiesis of indeterminate potential
CLOUD	Continuum of low-primed undifferentiated
CLP	Common lymphoid progenitor
CXCL12	CXC-chemokine ligand 12
CXCR4	CXC chemokine receptor 4
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
crRNA	CRISPR RNA
CRISPR	Clustered regularly interspaced short palindromic repeats
CRS	Cytokine release syndrome
DART	Dual affinity retargeting
DC	Dendritic cell
DFO	Deferoxamine
DFX	Deferasirox
DGA	Defining genetic abnormalities
DMT1	Divalent metal transporter 1
DNMT	DNA methyltransferase
EFS	Event-free survival
ELN	European Leukemia Net
ES	Embryonic stem
ETC	Electron transport chain
FAB	French American British
FACS	Fluorescence activated cell sorting
FAO	Fatty acid oxidation
FDA	Food and Drug Administration

FPN1	Ferroportin 1
FTH	Ferritin
G6PD	Glucose-6-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GMP	Granulocyte/macrophage progenitor
GvL	Graft-vs-leukemia
GO	Gemtuzumab ozogamicin
GPX4	Glutathione peroxidase 4
	Hematopoietic cell transplantation
HDR	Homology-directed repair
HG	Hydroxyglutarate
HMA	Hypomethylating agent
HSC	Hematopoietic stem cell
HSPC	Hematopoeitic stem and progenitor cell
IC	Immune checkpoint
ICC	International Consensus Classification
IL1RAP	Interleukin 1 receptor accessory protein
IL3	Interleukin 3
iPSC	Induced pluripotent stem cell
IRE	Iron-responsive element
ITD	Internal tandem duplication
LAIP	Leukemia-associated immunophenotyping
LMPP	Lymphoid-primed multipotent progenitor
LIC	Leukemia-initiating cell
LIP	Labile iron pool
LSC	Leukemia stem cell
MHC	Major histocompatibility complex
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cell
MEP	Megakaryocyte/erythroid progenitor
MFC	Multiparameter flow cytometry
MLL-r	Mixed-lineage leukemia rearrangement
MPN	Myeloproliferative neoplasms
MPP	Multipotent progenitor
MRC	Myelodysplasia-related changes
MRD	Minimal residual disease
NHEJ	Non-homologous end joining
NK	Natural killer
NOD	Non-obese diabetic
OS	Overall survival
OXPHOS	Oxidative phosphorylation

PAM	Protospacer adjacent motif
PARP1	Poly-ADP-ribose polymerase 1
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PD-L1	Programmed cell-death ligand-1
PDX	Patient-derived xenograft
PPP	Pentose phosphate pathway
pre-LSC	Pre-leukemic stem cell
PUFA	Polyunsaturated fatty acids
RNAi	RNA interference
RFS	Relapse-free survival
ROS	Reactive oxygen species
R/R	Relapse/refractory
RUNX1	Runt-related transcription factor 1
SEC	Super elongation complex
SCF	Stem cell factor
SCID	Severe combined immunodeficient
scRNA-seq	Single-cell RNA sequencing
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SIRPα	Signal regulatory protein-α
STEAP	Six-transmembrane epithelial antigen of the prostate
TALEN	Transcription activator-like effector nuclease
TAM	Tumor-associated macrophage
TCA	Tricarboxylic acid
TCGA	The Cancer Genome Atlas
TKD	Tyrosine kinase domain
TNF	Tumor necrosis factor
Tf	Transferrin
TFR1	Transferrin receptor 1
TPO	Thrombopoietin
tracrRNA	Trans-activating CRISPR RNA
VAF	Variant allele frequency
WHO	World Health Organization
wt	Wild type
ZFN	Zinc-finger nuclease

Popular scientific summary

Hematopoiesis, the process of blood cell formation, resembles a family tree, with hematopoietic stem cells (HSCs) as the common ancestor at the apex. These HSCs can both self-replicate and give rise to all other blood cell types: red blood cells (oxygen transporters), white blood cells (immune defenders), and platelets (clotting agents). The type of blood cell produced depends on external factors like the bone marrow microenvironment and internal factors acting on HSCs, guiding hematopoiesis down either the myeloid or lymphoid lineage, each yielding distinct blood cell types. Throughout life, blood formation produces over two million blood cells per second, making the blood system highly regenerative but tightly controlled. Occasionally "errors" in the form of DNA mutational changes can lead to uncontrolled cell growth and blood cancer, causing symptoms such as fatigue, fever, infections and bleeding.

Leukemia, a cancer arising from white blood cells and the most common type of blood cancer, is classified into acute and chronic forms based on the speed of disease progression. It can be further categorized according to blood cell type affected into myeloid or lymphoid leukemia, each with distinct prognosis, prevalence and treatment outcomes. Medical advancements have improved five-year survival rates for acute myeloid leukemia (AML), but intensive chemotherapy remains the primary treatment. Disease relapse results from the persistence of chemoresistant leukemia stem cells (LSCs), a specialized cell that perpetuates the disease. Targeting LSCs while sparing normal cells is a promising strategy to enhance long-term survival of AML patients.

Cell surface proteins are pivotal for identifying and targeting LSCs, while enabling communication with their bone marrow niche and other cells. Interactions with molecules such as cytokines, nutrients or growth factors transmit messages through intracellular signals, influencing gene expression. Understanding these signaling networks offers potential for therapeutically disrupting AML cell survival. In this thesis we aimed to identify and characterize cell surface receptors that are vulnerabilities in AML cells by using CRISPR-based screening, a large-scale technique that probes thousands of genes simultaneously. CRISPR, acting like molecular scissors, "turns off" genes, enabling assessment of their role in leukemia progression to be assessed.

In **Article I**, we examined nearly a hundred cell surface proteins overexpressed in leukemia cells compared to normal hematopoietic cells to enhance therapeutic effects and minimize toxicities. By performing a CRISPR screen in an AML mouse model, we

assessed their role in a physiologically relevant context. The chemokine receptor CXCR4 was identified as a critical dependency, as turning off its encoding gene induced AML cells to undergo differentiation and programmed cell death, termed apoptosis. The interaction between CXCL12, the ligand to which the receptor binds to, was dispensable for leukemia development, distinguishing it from normal blood cells.

In **Articles II-IV**, we expanded our study and performed a CRISPR screen to interrogate almost a thousand cell surface proteins present on leukemia cells. This revealed key vulnerabilities of AML within their bone marrow niche, of which we focused on three receptors that regulate distinct biological pathways. In **Article II**, we evaluated the effect of GLUT1, the primary glucose transporter in cells, and found that it was essential for maintaining energy metabolism in LSCs. Genetic disruption of *GLUT1* reduced glycolysis, the process by which energy is obtained from glucose. Under nutrient scarcity, leukemia cells adapted by increasing autophagy, a process by which the cell breaks down and reuses its own constituents for energy. Combining a GLUT1 inhibitor with an alternative metabolic pathway inhibitor led to a strong reduction in patient-derived leukemia cells, particularly in the *RUNX1*-mutated AML subtype, suggesting a potential therapeutic strategy for this sensitive patient group.

In **Article III**, we characterized the dependency of leukemia cells on the transferrin (Tf) receptor TFR1, responsible for facilitating cellular iron uptake. Turning off its coding gene activated apoptosis through p53, a central regulator of multiple cellular signaling pathways referred to as "the guardian of the genome". The disruption of TFR1 also affected additional p53-dependent pathways, all reliant on proteins dependent on iron-sulfur clusters for their activity. Treatment of AML patient-derived leukemia cells with iron-sequestering drugs reduced cell viability, with no effect on healthy hematopoietic cells, highlighting a therapeutic window for iron-targeting treatments.

In **Article IV**, we investigated H2-K1, another AML dependency identified in the screen. We describe a role for H2-K1 as a molecule on AML cells that evades immune-mediated killing, an adaptation frequently seen in cancer cells to sustain their proliferation. The presence of H2-K1 suppressed the cytotoxic ability of natural killer (NK) cells, essential components of the innate immune system that limit tumor expansion and infections. Analyzing the immune cell composition of the bone marrow of leukemic mice, we also found that H2-K1 promoted the maturation of NK cells, offering insights into the strategies AML cells employ to evade immune surveillance.

The work in this thesis represents our contribution to defining new dependencies of AML progression using CRISPR screening. These results advance our understanding of the mechanisms driving LSC activity and form the basis for the development of innovative therapeutic strategies for targeting AML.

Populärvetenskaplig sammanfattning

Hematopoes, processen för blodbildning, kan liknas vid ett släktträd, med hematopoetiska stamceller (HSC) som den gemensamma förfadern i toppen. Dessa anmärkningsvärda HSC har förmågan att både självreplikera och ge upphov till alla andra blodcellstyper: röda blodkroppar (syretransportörer), vita blodkroppar (immunförsvarare) och blodplättar (koaguleringsmedel). Typen av blodcell som produceras beror på mekanismer som påverkar HSC, såsom externa faktorer som benmärgens mikromiljö och interna faktorer. Genom livet skapar blodbildningen mer än två miljoner blodceller varje sekund, vilket gör blodsystemet till ett av våra kroppars mest regenerativa system. Följaktligen behöver denna process kontrolleras noggrant. Ibland uppstår "fel" i form av genetiska förändringar i blodcellernas DNA, vilket kan leda till okontrollerad celltillväxt och utveckling av blodcancer. Leukemi, en cancer som uppstår från vita blodkroppar och den vanligaste typen av blodcancer, klassificeras i akuta och kroniska former beroende på sjukdomens aggressivitet. Den kan ytterligare kategoriseras efter blodcellstyp till myeloid eller lymfoid leukemi, varje med distinkt prognos, förekomst och behandlingar. Cellyteproteiner är avgörande för att identifiera och rikta in sig på leukemistamceller, vilket är en cellpopulation som är resistent mot behandlingar och orsak till sjukdomsprogression. Dessa ytproteiner binder till cytokiner, näringsämnen eller tillväxtfaktorer och sänder meddelanden genom intracellulära signaler, vilket påverkar genuttryck.

Målet med denna avhandling var att identifiera och karakterisera cellytereceptorer som är viktiga för AML-cellerna genom att använda CRISPR-baserad screening. I **Artikel I** undersökte vi nästan hundra cellyteproteiner som överuttrycks i leukemistamceller och identifierade CXCR4 som särskilt viktigt för deras överlevnad. I **Artiklarna II-IV** utvidgade vi vår studie och utförde en CRISPR-screening för att studera nästan tusen cellyteproteiner på leukemiceller. I **Artikel II** identifierade vi GLUT1 som särskilt viktig för leukemistamceller och studerade de underliggande molekylära mekanismerna. I **Artikel III** karaktäriserade vi leukemicellers beroende av transferrinreceptorn TFR1. I **Artikel IV** undersökte vi hur H2-K1 på leukemistamceller påverkar immunförsvaret. Sammantaget beskriver denna avhandling AML-cellernas beroende av specifika ytproteiner som påverkar progression av sjukdomen. Dessa fynd främjar vår förståelse av de mekanismer som driver leukemistamcells-aktivitet och utgör grund för utveckling av innovativa terapeutiska strategier som riktar sig mot AML.

Resumen divulgativo

La hematopoyesis, el proceso de formación de células sanguíneas, puede compararse con un árbol genealógico, con las células madre hematopoyéticas (CMHs) en el vértice como el ancestro común. Estas CMH poseen la capacidad tanto de auto-renovarse como de formar todos los demás tipos de células sanguíneas: glóbulos rojos (transportadores de oxígeno), glóbulos blancos (defensores del sistema inmunológico) y plaquetas (agentes de coagulación). El tipo de célula sanguínea producida depende de mecanismos complejos que actúan sobre las CMHs, los cuales están influenciados por factores externos como el microentorno de la médula ósea y factores internos. Estas señales guían la hematopoyesis hacia la línea mieloide o linfoide, cada una produciendo tipos distintos de células sanguíneas.

A lo largo de la vida, la producción de sangre genera más de dos millones de células sanguíneas por segundo, convirtiendo al sistema sanguíneo en uno de los sistemas más regenerativos de nuestro cuerpo. Por ello, este proceso necesita ser controlado rigurosamente. Sin embargo, ocasionalmente ocurren "errores" en forma de mutaciones, o cambios, en el ADN de las células sanguíneas, lo que puede llevar a un crecimiento celular descontrolado y al desarrollo de cáncer de la sangre. Estas células anormales ocupan espacio en la médula ósea, desplazando a las células sanguíneas funcionales. Esto resulta en síntomas como fatiga, fiebre, infecciones y sangrado.

La leucemia, un cáncer que surge de los glóbulos blancos y el tipo de cáncer sanguíneo más común, se puede dividir en formas agudas o crónicas, según la rapidez de progresión de la enfermedad. También se puede categorizar según el tipo de célula sanguínea afectada en leucemia mieloide o linfoide, cada una con pronósticos y prevalencia distintos. Los avances médicos han mejorado las tasas de supervivencia a 5 años para la leucemia mieloide aguda (LMA), pero la quimioterapia intensiva sigue siendo el tratamiento principal. La recaída de la enfermedad se atribuye a la persistencia de las células madre leucémicas resistentes a la quimioterapia, una célula especializada que perpetúa la enfermedad. Conseguir eliminar las células madre leucémicas mientras se preservan las células normales es una posible estrategia para mejorar la supervivencia a largo plazo de los pacientes con LMA.

Las proteínas en la superficie celular son fundamentales para identificar a las células madre leucémicas, a la vez que permiten la comunicación tanto con su nicho en la

médula ósea como con otras células. Estas proteínas interaccionan con moléculas como las citoquinas, nutrientes o factores de crecimiento, transmitiendo mensajes a través de señales intracelulares e influyendo en la expresión génica. Comprender estas redes de señalización brindan oportunidades para interrumpir esta señalización terapéuticamente, consiguiendo así eliminar las células de LMA.

En esta tesis, nuestro objetivo fue identificar y caracterizar los receptores en la superficie celular que constituyen vulnerabilidades en las células de LMA mediante el uso de técnicas basadas en CRISPR, una técnica a gran escala que analiza miles de genes simultáneamente. La herramienta CRISPR actúa como unas tijeras moleculares, "apagando" genes, lo que permite evaluar su función en la iniciación y progresión de la leucemia.

En el **Artículo I**, examinamos casi cien proteínas en la superficie celular que están sobre expresadas en las células leucémicas en comparación con las células hematopoyéticas normales para mejorar los efectos terapéuticos y minimizar las toxicidades. Realizamos un análisis CRISPR en un modelo de ratón con LMA para evaluar su función en un contexto fisiológicamente relevante. El receptor de quimioquina CXCR4 se identificó como una dependencia crítica, ya que el apagar su gen codificador indujo a las células de LMA a la diferenciación y muerte celular programada, denominada apoptosis. La interacción entre CXCL12, el ligando al que se une el receptor resultó ser prescindible para el desarrollo de la leucemia, lo que la distingue de las células sanguíneas normales.

En los **Artículos II-IV**, ampliamos nuestro estudio y realizamos un análisis CRISPR para investigar casi mil proteínas en la superficie celular presentes en las células de leucemia. Esto reveló vulnerabilidades clave de la LMA dentro de su nicho en la médula ósea, en el que nos enfocamos en tres receptores que regulan vías biológicas distintas. En el **Artículo II**, evaluamos el efecto de GLUT1, el principal transportador de glucosa en las células, e identificamos que es esencial para mantener el metabolismo energético en las células madre leucémicas. La interrupción genética de *GLUT1* redujo la glucólisis, el proceso mediante el cual se obtiene energía a partir de la glucosa. Ante la escasez de nutrientes, las células de leucemia se adaptaron aumentando la autofagia, un proceso mediante el cual la célula descompone y reutiliza sus propios componentes para obtener energía. La combinación de un inhibidor de GLUT1 con un inhibidor de una vía metabólica alternativa condujo a una rápida eliminación de células de leucemia derivadas de pacientes, especialmente en el subtipo de LMA con mutación en el gen *RUNX1*, lo que sugiere una posible estrategia terapéutica para este grupo de pacientes.

En el **Artículo III**, caracterizamos la dependencia de las células de leucemia en el receptor de transferina TFR1, responsable de facilitar la captación de hierro celular. Apagar su gen codificador activó la apoptosis a través de p53, un regulador central de varias vías de señalización celular conocido como "el guardián del genoma". La

interrupción de TFR1 también afectó a otras vías dependientes de p53, todas las cuales dependen de proteínas que requieren compuestos de hierro y azufre para su actividad. El tratamiento de las células de leucemia de pacientes con fármacos secuestrantes de hierro redujo la viabilidad celular, sin afectar a las células hematopoyéticas normales, resaltando un nuevo ángulo terapéutico.

En el **Artículo IV**, investigamos H2-K1, la última dependencia identificada en el análisis. Identificamos una función de H2-K1 como una molécula en las células de LMA que evita la muerte mediada por el sistema inmunológico, una adaptación que se ve con frecuencia en las células cancerosas para mantener su proliferación. La presencia de H2-K1 suprimió la capacidad citotóxica de las células asesinas naturales, componentes esenciales del sistema inmunológico innato que limitan la expansión de tumores y las infecciones. Al analizar la composición de las células inmunológicas en la médula ósea de ratones con leucemia, también descubrimos que H2-K1 promovía la maduración de las células asesinas, lo que ofrece información sobre las estrategias que las células de LMA emplean para evadir la vigilancia inmunológica.

El trabajo en esta tesis representa nuestra contribución a la definición de nuevas dependencias en la progresión de la LMA utilizando la técnica CRISPR. Estos resultados ayudan a comprender los mecanismos que promueven la actividad de las células madre leucémicas, y sientan los cimientos para el desarrollo de estrategias terapéuticas innovadoras para atacar la LMA.

Hematopoiesis

Hematopoiesis, the process responsible for the continuous formation of new blood cells, plays a fundamental role in various physiological functions. These hematopoietic cells are integral components of the immune response, oxygen and nutrient transport, hemostasis, and wound healing. To ensure these functions are met, a high cell turnover rate approximating one trillion cells per day is needed to continuously replenish these specialized mature blood cells. This intricate process is finely tuned, with a complex network of regulating factors that maintain a balance between cell proliferation, differentiation and survival. This plasticity enables rapid cell expansion and modulation during periods of physiological stress such as infections and blood loss. However, if this balance is disturbed, it can lead to various disorders like anemia, hemophilia, or to malignant diseases, such as leukemia.

Hematopoietic stem cells

Hematopoiesis primarily takes place in the bone marrow, where HSCs reside and give rise to all other cellular blood components that become increasingly specialized through differentiation. Under physiologically normal and stress conditions, HSCs or progenitors may be displaced from their native enviroment to the spleen, liver, lymph nodes and para-vertebral regions, refered to as extramedullar hematopoiesis.[1] HSCs are defined by their self-renewal and multipotent potential, which ensures that they can differentiate into all mature cell blood lineages. This balance between self-renewal and differentiation is maintained through symmetrical and asymymetrical cell divisions. During symmetrical divisions, each HSC divides to generate either two daughter HSCs or two differentiated cells. During asymmetrical cell divisions, each HSC generates one daughter HSC and one differentiated daughter.[2,3] Ultimately, all hematopoietic lineages are replenished and maintained by the rapidly proliferating downstream progenitor cells, allowing for HSCs to remain mostly quiescent. By delegating this task to the progenitors, the long-lived HSCs are safeguarded from proliferation-induced mutations and exhaustion, preserving their self-renewal capacity.[4,5] However, the concommitent attenuation of DNA repair pathways in quiescent cells renders HSCs intrinsically vulnerable to accumulation of premalignant mutations.[6,7]

Mice and transplantation assays

Mouse models have been indispensible for the development of the concept of HSCs. It became evident that transplantation of donor bone marrow was capable of reconstituting the irradiation-depleted hematopoeitic system, although the specific repopulating cell identity was unknown. HSCs were later functionally characterised in additional transplantation studies, where donor-derived clonogenic cells were macroscopically detected in the spleen of lethally irradiated recipient mice.[8,9] These colony-forming units (CFU), although not strict HSC populations, allowed for the identification of specialized cells with potential for hematopoietic reconstitution. These cells could form multiple lineages from a single clonal origin (they exhibited a degree of multipotency), and generate daughter cells with the original characteristics of the parental cell (self-renewal), the two ultimately recognized as HSC-defining features.[10] Despite the valuable information yielded from transplantation and colony forming assays, these studies are based on a defective host immune system, thus are limiting in that they are unable to fully recapitulate HSC fate in an unperturbed scenario.[11]

Identification and heterogeneity of the HSC pool

Efforts were next focused on identifying and isolating these murine HSCs from the numerous cellular components in the bone marrow. This proved to be a challenging task, as these cells are exceedingly rare, with estimates of 50,000 to 200,000 HSCs in the bone marrow.[12] Although HSCs were separated with increasing purity based on their functional properties, it was not until they could be defined at the immunophenotypic level that different hematopoietic cell populations could be distinguished with high precision.[13–15] Isolation of cell populations was done based on cell surface marker detection using fluorescence activated cell sorting (FACS), a method that uses antigen-specific antibodies. Since there is no single marker that can define HSC populations, they are purified based on a combination of markers. However, there are fundamental differences between the immunophenotype of human and murine HSCs, including enrichment of $CD34^+$ in humans and the expression of stem cell markers like Sca-1 and c-Kit in mice.[13,14,16] Human adult HSCs have traditionally been defined as $(Lin^-)CD34^+CD38^-CD45RA^-CD90^+CD49f^+$, although this strategy is being challenged by a growing amount of research.[17] The arbitrary cutoff values that are used to facilitate cell compartmentalization in bulk analyses consider the HSC subset to be homogenous. However, single-cell RNA sequencing (scRNA-seq) and single cell transplantation assays are revealing remarkable inter-cellular heterogeneity of the HSC pool, where individual HSCs possess different repopulating and self-renewal abilities.[11,18,19] By combining transcriptional and functional analyses at the single cell

resolution, the otherwise unknown complexities of the HSC compartment are being unraveled, and the classical hematopoietic tree questioned and further refined.

Hematopoietic hierarchy and blood lineages

The hematopoietic system is clasically divided into two branches: the lymphoid- and myeloid lineage. The myeloid lineage comprises of granulocytes (basophils, eosinophils, and neutrophils), monocytes/macrophages, megakaryocytes/platelets, erythrocytes, and dendritic cells (DCs). The lymphoid lineage includes B and T lymphocytes, NK cells, and a subset of DCs.[20] Together, white blood cells constitute the two fundamental and complementary lines of defense of the immune system. Myeloid and NK cells are responsible for the innate immunity, the first line of defence that provides a non-specific response against foreign pathogens. Innate immunity relies on anatomic, physiologic, endocytic, phagocytic and inflammatory defense mechanisms, which facilitates the initiation of adaptive immune responses. B and T cells then take part in the adaptive immunity, which use the capacity for memory to enable the host to mount a rapid and antigen-specific immune response upon subsequent exposure to the pathogen.[21] While traditionally categorized part of the innate system, there is emerging evidence of NK cell memory and memory-like responses following exposure to haptens, viral infections, and cytokine stimulation.[22]

The process of generating hematopoietic cells is built upon a hierarchical structure, where HSCs located at the apex undergo step-wise differentiation to meet the continuous demand for mature blood cells. In the first differentiation step, HSCs lose their self-renewal capacity but retain multilineage differentiation ability producing multipotent progenitors (MPPs).[23,24] A bifurcation occurs downstream of MPPs, separating the common myeloid progenitor (CMP) lineage, which only gives rise to myeloid-restricted progeny, from the lymphoid-primed multipotent progenitor (LMPP), bipotent cells that produce cells from both myeloid and lymphoid lineage.[25,26] CMPs differentiate further into megakaryocyte/erythroid progenitors (MEPs), eventually generating platelets and erythrocytes. Recently, specific transcriptional patterns have revealed lineage-biased MPP subsets, where myeloid-biased MPP3 and lymphoid-biased MPP4 have been identified. As such, MPP3 differentiate into granulocyte/macrophage progenitors (GMPs) belonging to the myeloid lineage, while MPP4 develop into common lymphoid progenitors (CLP), predecessors of NK, B and T cells (**Figure 1**).[23,24,27]

New representations of the classical lineage tree

This classical tree-like hierarchy model of hematopoiesis based on stepwise restriction of lineage potential at binary branch points has been challenged in recent years. This was the case of a study showing that HSCs can commit to megakaryocyte-restricted lineages by bypassing the intermediate maturation stages.[28] These findings highlighted the heterogenous nature of HSCs, as well as their capacity to be primed to differentiate towards a specific lineage. In contrast to the balanced lineage output of HSCs expected from the classical model, HSCs instead display biases towards the generation of specific lineages, findings that have been confirmed as a hallmark of native hematopoiesis through single cells transplantions and *in situ* molecular barcoding methods.[11,29,30]

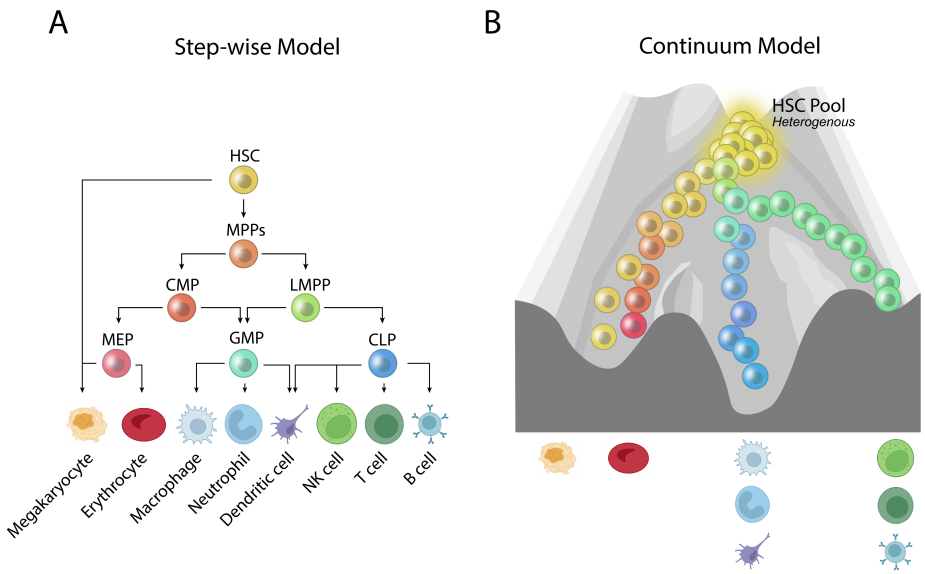

Figure 1 | A) The classical hematopoietic tree. A hierarchical model where HSCs at the apex contribute equally to each lineage. Through stepwise differentiation, cells becoming progressively more lineage-restricted until they produce mature blood cells. **B) Continuum model of hematopoesis**. A dynamic, non-hierarchical model with a heterogeneous HSC pool exhibiting lineage bias, leading to diverse differentiation trajectories in a continuous process. HSCs, hematopoietic stem cells; MPP, multipotent progenitor; CMP, common myeloid progenitor; LMPP, lymphoid/myeloid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; CLP, common lymphoid progenitor. Created with Biorender and adapted from Rodrigues et al.[27]

By integrating flow cytometric, transcriptomic and functional data at single-cell resolution, the roadmap of human HSCs towards lineage commitment is being quantitatively mapped and redefined. An alternative model is being proposed, in which hematopoeitic stem and progenitor cells (HSPCs) exist in a 'continuum of low-primed

undifferentiated' (CLOUD) state, in which the differentiation process does not happen through discrete hierarchically organized cell states, but rather occurs as a continuous process.[11,31,32] Distinct gene expression patterns cooperate to regulate stemness, early lineage priming, and subsequent progression into the different branches of hematopoiesis. Accordingly, transcriptomic profiling of single cell traversing through differentiation landscapes have revealed remarkable cellular heterogeneity within what were otherwise considered distinct clusters.[33–35] scRNA-seq analysis of progenitor cells has also revealed that various mature cell types can originate from more than a single trajectory.[30,35] These findings have led to the refinement of the hematopoietic process, with a proposed representation that appreciates cell heterogeneity, lineage-biased outputs, and diversity in possible routes of differentiation (**Figure 1**).

Regulation of hematopoiesis

Hematopoietic differentiation is fine-tuned by a complex interplay of cell-extrinsic, cell-intrinsic, and stochastic factors.[11] This coordination is critical in steady (normal) state to ensure the maintenance of long-lived repopulating HSCs and the continuous output of proliferative mature cells. Subtle imbalances between various external and internal inputs may lead to the development of severe hematopoietic disorders.

The niche

Amongst the most extensively studied cell-extrinsic factors influencing HSC diversity is the bone marrow stem cell niche, the specific microenvironment that maintains and regulates HSCs.[36] Niche regulatory signals, which can exist in the form of cell-bound or secreted factors, are gradually being identified through technological advancements in bone marrow imaging and genetic manipulation of key regulatory factors.[37] Among the cellular contributors to the perivascular niche are non-hematopoietic cells including endothelial, mesenchymal stromal, adipocytes, osteoblasts, Schwann cells, perivascular cells, as well as other hematopoietic cell types such as macrophages, megakaryocytes and regulatory T cells.[37,38] These cells can secrete cytokines which not only ensure survival of HSCs, but are also capable of instructing cell migration and lineage choices.[38] Some of the key regulatory cytokines are stem cell factor (SCF), thrombopoietin (TPO) and angiopoietin-1 (ANGPT1), which bind to HSC receptors c-KIT, MPL, and TIE2, respectively.[39–41]

Another essential factor is CXC-chemokine ligand 12 (CXCL12), mainly produced by bone marrow mesenchymal stem/stromal cells (BMSCs), which regulates HSCs

through binding to the CXC chemokine receptor 4 (CXCR4). Accordingly, disruption of *Cxcl12* in endothelial cells depleted HSPCs from the bone marrow.[42] A characterization of the role of CXCR4 within the context of myeloid leukemia is discussed in **Article I**. Importantly, neighbouring HSCs within the same microenvironment stimulated by the same regulatory signals may exhibit different responses due to heterogeneity in cytokine receptor expression of individual HSCs.[43] Defining the regulatory role of niche components in hematopoiesis not only offers potential for enhanced regeneration after injury or HSC transplantation, but also aids in understanding how an imbalanced niche function might contribute to disease.[36]

Transcriptional lineage priming

The underlying cell-intrinsic regulatory mechanisms that govern fate decisions in hematopoiesis have remained relatively unknown until recent advances in genomics approaches. Gene expression patterns have defined lineage-biased outputs in multipotent HSCs, thus research has focused on determining the role of master transcriptional factors in this process.[11,44] These specific expression patterns in HSCs have been proposed to result from multilineage priming, a process involving simultaneous, minimal activation or inhibition of expression patterns for alternative lineages. Subsequently, one program "wins out" over the others in a lineage choice, leading to the suppression of alternative programs.[31] This cross-antagonism between pairs of lineage-regulatory transcription factors has been exemplified with PU.1 (SPI1) and GATA1. Increased expression of GATA1 represses PU.1, which can direct cells to either commit to the erythroid lineage through upregulation of KLF1, or favor the megakaryocytic lineage through FLI1 expression.[45,46] Conversely, increased PU.1 levels suppress GATA1, directing cells toward the lymphomyeloid lineage. However, recent studies have challenged the notion that these transcription factors govern HSC lineage commitment, and rather propose that they merely reinforce lineage choice made at an earlier stage.[47]

Epigenetic regulation

Recent studies have however shown that the transcriptome does not consistently correlate with HSC function and fate decisions, suggesting the contribution of other cell-autonomous gene regulatory factors in lineage priming.[27,48] The influence of epigenetic regulation in HSC maintenance, lineage commitment and malignant transformation is becoming increasingly apparent. At the HSC level, multiple epigenetic factors, such as the chromatin remodeler BPTF, instruct cells to either activate or repress transcriptional programs to ensure stemness of the HSC pool is

maintained.[49] Clonal tracking *in vivo* have also suggested that epigenetic programs drive the cell-autonomous functional heterogeneity of individual HSCs.[48] Mapping of DNA methylation, histone modifications and chromatin accessibility landscapes has also shown to successfully capture cell identities down the continuous lineage trajectories. Specifically, these studies reinforce the idea that dynamic alterations in chromatin structure play a pivotal role in coordinating chromatin accessibility during HSC lineage commitment, thereby guiding and strengthening lineage-specific gene expression programs.[11,50,51] This is the case of global DNA hypomethylation skewing commitment towards the myeloid lineage through DNA methyltransferases (DNMTs), or the loss of histone deacetylases such as HDAC1 promoting myeloid lineage specification.[52,53]

Malignant hematopoiesis

Throughout the process of hematopoiesis, mutations are inevitably acquired. Rarely, a mutation arises that confers a selective growth advantage in HSCs or HSPCs, referred to as a "clone". Through Darwinian selection, these clones with augmented fitness progressively expand over time, producing a substantial amount of blood cells arising from the same mutated stem cell. This outgrowth, termed "clonal hematopoiesis", is associated with an increased risk of hematological malignancies, cardiovascular diseases and increased mortality from certain non-hematological cancers.

Clonal Hematopoiesis

With age, HSCs and its derivatives accumulate an increasing number of somatic mutations, making clonal hematopoiesis highly prevalent in the elderly. These age-associated mutations result from spontaneous deamination of DNA bases, errors during nonhomologous end joining, replication errors by DNA polymerase, or structural variation.[54] Large scale studies on mutation-driven clonal hematopoiesis using exome sequencing have confirmed that mutations are largely restricted to a set of genes.[55–57] Some of these include classical oncogenes and tumor suppressor genes responsible for cell growth signaling (like *JAK2*, *GNAS*, *GNB1, CBL*) and the DNA damage response (such as *TP53* and *PPM1D*). However, clonal hematopoiesis is mainly attributed to loss-of-function mutations in two enzymes involved in DNA methylation: *DNMT3A* and *TET2*, accounting for nearly two-third of cases. *ASXL1*, a chromatin regulator, was the third most commonly mutated gene, with other splicing factors (*SF3B1*, *SRSF2*, *PRPF8* and *U2AF1*) also being frequently mutated.[54] Subsequent studies with more sensitive methods have however revealed clonal hematopoiesis to be considerably more widespread than initially anticipated.[58]

Clonal hematopoiesis refers to any clonal outgrowth, regardless of disease state. Thus, the term "clonal hematopoiesis of indeterminate potential" (CHIP) was introduced to describe the presence of non-malignant clonal hematopoiesis linked to a cancer-associated mutation. Having a cancer-associated genetic alteration in blood cells is associated with an elevated risk of eventually developing an overt hematologic malignancy, thus confirming that CHIP is a bona fide premalignant state.[54] For some mutations in CHIP-associated genes, a clear mechanism of clonal expansion has been found. Examples which inherently confer fitness to HSCs are mutations in *Tp53*, which allow entry into the cell cycle bypassing DNA damage repair, or activating mutations in *JAK2*, that allow constitutive signaling leading to enhanced proliferation.[59,60] The functional role of mutations in other genes are less clear, as in *TET2* or *DNMT3A*, suggesting that other factors such as immune-mediated, environmental, heritable genetic variation, and stochastic drift in clones may be affecting the emergence of clonal hematopoiesis.[54]

Leukemogenesis

The mechanisms by which clonal hematopoiesis progresses towards hematological abnormalities or leukemic transformation are not entirely understood. A proposed path is through the sequential acquisition of somatic mutations in the same clone or clones over time, whereby HSCs or HSPCs gain further fitness advantage and undergo clonal expansion. As such, individuals with CHIP are at a three- to fivefold increased risk of developing hematological malignancies as they already have the "first hit" needed for malignant transformation.[54] As leukemia is often preceded by these initial somatic lesions, a so-called "pre-leukemic stem cell (pre-LSC)" with potential for malignant transformation may be present at the time of sample collection with no clinical symptoms arising until many years later.[56,61,62] It is thus critical to assess the clinical significance of these mutations to enable early detection, monitoring and potentially inform intervention prior to progression to overt disease within each individual.

Among the features that correlate with malignant progression are the size of the mutant clone, the number and type of mutations, and the genes affected.[54] These features have been identified through large-scale longitudinal studies in cohorts from cancer patients and healthy volunteers, as well as from patients with hematological malignancies that are either at clinical remission or in relapse. These studies have revealed time patterns of preleukemic mutation acquisition.[56,63] For instance, nonlethal primary mutations such as *DNMT3A*, *TET2, ASXL1* and *IDH1/2* are initially acquired, often leading to successful mutant clonal expansion at the expense of normal HSCs. After a subclinical state is maintained for a long period of time, additional secondary or tertiary mutations in "proliferative" genes such as *FLT3* and *NPM1* occur.[56,63] However this pattern of

mutational acquisition has been deemed incomplete, as subsequent studies have identified mutations that do not fit into either category. The continued accumulation of mutations cooperating together may lead to the production of leukemia stem cells (LSCs) which exhibit malignant characteristics, such as maximizing self-renewal capacity, dormancy, plasticity, and chemoresistance. Terminal myeloid differentiation block can then fuel clonal outgrowth and lead to overt AML (**Figure 2**).[64,65] The co-occurrence of mutations in specific genes has also been observed to synergize to promote malignant transformation, as is the case of *DNMT3A* with co-mutations in *FLT3*, RAS signaling pathway members (*NRAS, KRAS*, and *PTPN11*), or *NPM1*.[66] Similarly, *TET2* mutations cooperate with mutation in *JAK2* and *FLT3* to drive myeloid malignancies.[64]

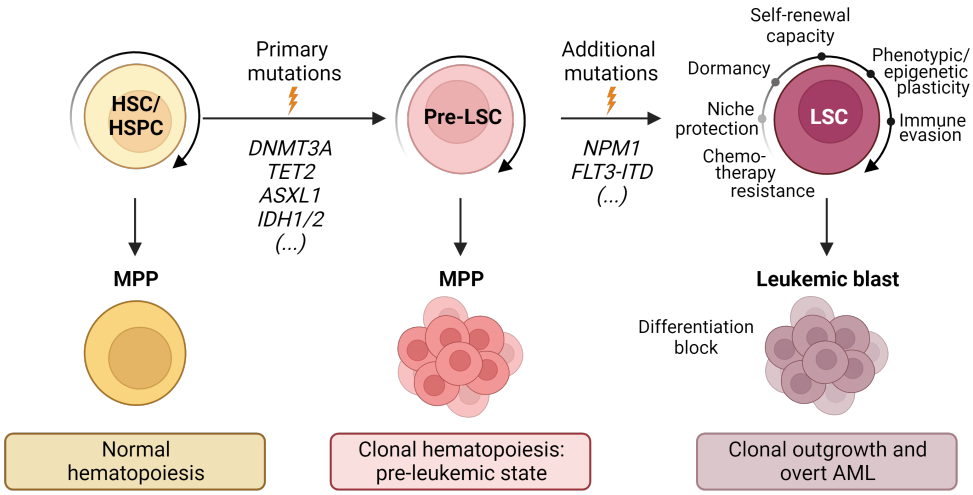

Figure 2 | Model of leukemic transformation. Initial mutations in HSCs/HSPCs lead to the formation of pre-LSCs, which possess self-renewal capacity, undergo clonal expansion, and exhibit multi-lineage differentiation ability. Subsequent mutations transform pre-LSCs into LSCs, driving clonal expansion and the progression to full-blown AML. HSC, hematopoietic stem cell; HSPC: hematopoietic stem and progenitor cell; LSC, leukemia stem cell; MPP, multipotent progenitor. Created with Biorender and adapted from Stelmach et al.[65]

Therapy-induced shaping of clonal landscape

In addition to age-related factors, therapy-induced selective pressures have recently been shown to be critical in shaping the fitness landscape of clonal hematopoiesis. Longitudinal population studies have revealed a correlation between subsequent development of hematological malignancies in individuals who have undergone therapy to either treat non-hematological malignancies, or as a conditional regime prior to stem cell transplantation. Radiation and cytotoxic therapy associated with prevalence

of recurrent mutations in genes encoding DNA damage regulators, such as *TP53*, *PPM1D*, *ATM*, and *CHEK2*.[67–69] These mutations are not thought to be a result of the use of cytotoxic drugs per se. Instead, it is hypothesized that these mutant DNA damage-resistant clones expand following the increased selective pressure from therapy-induced genotoxic stress, but not in its absence.[69] In support of this, mutations in *TP53* were enriched in therapy-induced compared to *de novo* leukemias, suggestive of a clonal advantage of *TP53* mutant clones upon genotoxic insult.[70] It has recently been reported that *TP53* mutations attain clonal dominance in the context of cancer therapy as a result of the acquisition of further genetic alterations, such as chromosomal aneuploidies.[69] As mutant *TP53* is associated with chemoresistant disease, it is clinically relevant to understand the therapy-driven mechanisms that lead to selection of specific mutations.

These findings highlight the caution that must be taken when attempting to eliminate a clone to avoid introducing selective pressures that may promote the emergence of a more aggressive and malignant clone. Nonetheless, these studies provide a rationale for clinical intervention, possibly aimed at developing treatments aimed at eradicating high-risk clones, or adjusting the use of cancer therapy for patients at highest risk of progression to hematological malignancies. This might involve delaying cytotoxic therapy or choosing alternative treatments when clinically appropriate.[69] This represents a step towards precision medicine, potentially enabling tailored screening for clonal hematopoiesis prior to cancer therapy if feasible, allowing molecular-based early detection and prevention of future adverse outcomes.

Leukemia stem cells

The concept of LSCs is based on the idea that a subset of cells is able to entirely replenish the bulk of leukemia cells. These cells are functionally defined by their long-term self-renewal capacity, incomplete differentiation, and their ability to reinitiate leukemia when transplanted into immune-deficient mice.[71,72] LSCs exhibit specific phenotypic, epigenetic, and metabolic features that distinguish them from their HSC counterparts, making them vulnerable to therapeutic targeting. However, some of these features also enable LSCs to adapt in response to therapy-induced stresses, leading to chemotherapy resistance and disease relapse. A better understanding of LSC biology, recently aided by the onset of single-cell multi-omics, is critical to uncovering resistance mechanisms and potential therapeutic vulnerabilities.

Cell of origin in hematological malignancies

Clonal evolution models have attributed the rise of acute leukemia to the so-called "cell of origin". Initially thought to originate from HSCs, recent studies have shown that the cell of origin may instead be an immature HSPC that undergoes acquisition of genetic lesions, as is the case of human AML.[61,73,74] This is however different based on the affected hematopoietic lineage. In the case of acute lymphoblastic leukemia (ALL), the cell of origin varies between HSCs and committed lymphoid progenitors.[75] Identification of the cell of origin is often challenging, yet it holds significance as it can dictate aggressiveness, prognosis and explain the heterogenous nature of acute leukemias.[76] Subsequently, the cell of origin acquires initial mutations giving rise to leukemia-initiating cells (LICs) or pre-LSCs, which exhibit self-renewal potential, drive clonal expansion, and maintain differentiation abilities capable of producing mature blood cells.[65] LICs are functionally identified by their capacity to initiate and sustain leukemia when transplanted into a host.[71,72] Disease progression is then driven by further molecular insults which cooperate to give rise to fully transformed LSCs, which, upon a block in differentiation, result in the accumulation of leukemic blasts. These aggressive subclones eventually undergo clonal outgrowth, leading to an override of the entire hematopoietic system and development of overt leukemia (**Figure 2**).[65]

While the terms LIC and LSC are often used interchangeably due to their similarities, it is important to differentiate them. LICs exhibit mutations not associated with leukemia that are insufficient to induce leukemia alone, and serve as precursors to the later mutations that convert LICs into LSCs.[77] However, the presence of a clonally expanded LIC pool in AML patient samples in remission indicates that LICs surviving chemotherapy may serve as a reservoir for clonal evolution leading to disease.[78]

Characterization of LSCs

Following the pioneering work from John Dick's group, it was thought that the LSC population was restricted to the $CD34^{+}CD38^{-}$ fraction and that it immunophenotypically resembled normal HSCs.[71] The notion that LSCs are solely confined to the $CD34^{+}CD38^{-}$ fraction has since been challenged, as studies in permissive immunodeficient mouse models have revealed engraftment potential of leukemia populations originally thought to be devoid of LSC activity, such as the $CD34^{low/-}$ and $CD38^{+}$ compartments.[79,80] Gene expression patterns have revealed a core transcriptional program underlying "stemness" properties shared between LSCs and HSCs, which has subsequently been shown to correlate with poor clinical outcomes.[81,82] Despite their similarities, more refined methods are identifying differentially expressed cell surface markers distinguishing LSCs and HSCs. Specifically, LSCs exhibit higher levels of CD25, CD32, CD44, CD96, CD123, GPR56, CLL-1, IL1RAP, N-cadherin, and Tie2.[83] By combining sequencing technologies and *in vivo* functional assessments, the expression levels of these LSC markers are being correlated with high-risk genetic subgroups and poor outcome, as is the case with GPR56 expression in AML patients.[80]

Global gene expression analysis in $CD34^{+}$ AML patient samples has also provided additional insights into the LSC population, revealing that their immunophenotype more closely resembles that of normal committed progenitors rather than HSCs.[84] In particular, two molecularly distinct populations with LSC activity were identified to co-exist within the LSC pool. The more immature LSC population was traced to normal LMPPs, whereas the more mature population is functionally similar to normal GMPs. In analogy to normal hematopoiesis, these LSC populations retained a hierarchical structure in that the leukemic LMPPs could give rise to the leukemic GMPs, but not the converse.[84]

Efforts are being directed at identifying a unique immunophenotypic signature that would allow specific isolation and targeting of LSCs. However, due to inter- and intra-patient heterogeneity, there is a lot of variation in the expression pattern, density, and distribution of surface antigens, hindering the use of one identifying marker

combination for all individuals.[85] Similar to HSC regulation, the heterogeneity within the LSC pool is shaped by the interplay between cell-intrinsic mechanisms such as gene mutations and epigenetic modifications, as well as extrinsic signals from the microenvironment.[83]

The leukemic niche

Leukemogenesis was long regarded to be solely driven by cell autonomous processes. This dogma was questioned upon the discovery of donor cell-derived leukemia in patients who had undergone bone marrow transplantation.[86] These findings aligned with the "seed and soil" theory, introduced by Paget in 1889, which postulated that tumor metastasis depended on favorable interactions between tumor cells (the "seed") and their surrounding microenvironment (the "soil").[87] It has now been established that bidirectional signaling between leukemia cells and the bone marrow niche plays a pivotal role in leukemia development.

Role of the microenvironment in leukemogenesis

Alternations in hematopoietic and non-hematopoietic niche constituents have been shown to create favorable conditions for leukemia progression. As a proof of concept, a specific deletion of *Dicer 1* in BMSCs induced myelodysplasia and secondary leukemia.[88] Further supporting the role of the osteolineage compartment in leukemogenesis, an activating mutation in β-catenin in osteoblasts resulted in AML development mediated by aberrant activation of Notch signaling in HSPCs.[89] In both of these settings, the effects are attributed to a deregulation in the inflammatory state of the bone marrow niche.[83]

The hypoxic nature of the niche has also been suggested to promote leukemogenesis. This is due to the tendency of leukemia cells to preferentially home to the most hypoxic areas within the niche, where compromised blood flow reduces exposure to chemotherapy and immune regulators. These regions mainly harbor chemoresistant LSCs in a quiescent state, known to be maintained by a hypoxic environment.[90,91] It is proposed that the activation of hypoxia-inducible factors 1α and 2α (Hif-1α and Hif-2α) may increase the expression of CXCR4 and CXCL12 on both AML cells and endothelial cells, thus facilitating the recruitment and maintenance of LSCs.[90,92,93] As a result, LSC proliferation results in expansion of hypoxic microenvironmental niches.[92]

Niche remodelling by LSCs

In an attempt to compete for the bone marrow niche against its normal counterparts, LSCs can reprogramme the microenvironment at the expense of normal hematopoiesis. By transforming the environment, LSCs favor their hematopoietic reserve by promoting their own survival and proliferation. Consequently, this creates a "malignant niche" that is inhospitable to normal HSCs, being able to promote their displacement.[94] One way in which BMSCs support leukemogenesis is by enhancing the bioenergetic capacity of LSCs through increased tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) activity. This process equips LSCs with essential glutathione-dependent detoxifying enzymes, strengthening their antioxidant defenses to counteract reactive oxygen species (ROS) during leukemogenesis and chemotherapy.[95] LSCs may also stimulate abnormal autocrine and paracrine cytokine secretion to create a pro-inflammatory environment known to favor their survival.[96,97] This was observed in murine myeloid LSCs, where they exhibited constitutive NF-Kβ activity as a result of maintained autocrine tumor necrosis factor-α (TNF-α) secretion.[98] By reprogramming their transcriptional profile, LSCs can aberrantly express cytokine cell surface receptors, as is the case with the interleukin 1 receptor accessory protein (IL1RAP), thereby promoting a pro-inflammatory autocrine loop.[99] Accordingly, inhibition of the IL1-IL1RAP axis has been proposed to reduce inflammation in the bone marrow niche, thereby promoting restoration of normal hematopoiesis over AML proliferation after chemotherapy.[100] LSCs may also create a permissive leukemic niche structurally, both through vascular remodeling and upregulation of adhesion molecules such as CXCR4, retaining them in the protective niche and impairing chemosensitivity.[101–104]

The dependance of this crosstalk for leukemia initiation, progression, and protection from genotoxic insults also highlights vulnerabilities that can be potentially targeted. Thus, characterizing LSC-niche interactions will aid in refining therapeutic targets to overcome acquired drug resistance and prevent relapse post-treatment.[92]

Source of relapse

A major challenge in AML treatment is relapse post-remission, primarily attributed to therapy-resistant leukemia cells known as minimal residual disease (MRD), containing LSCs that drive clonal outgrowth.[105] Detecting these drug-persistent LSCs and their associated resistant mechanisms early may allow patient stratification and potential aversion of relapse.[65]

The origin of leukemia cells in relapse can be traced back by analyzing their mutational profile and by using specific mutations as lineage tracking marks. In this way, cells within diagnostic and relapse samples that share the same mutational profile will most likely have originated from the same founder LSC. Individual variant allele frequencies (VAFs) can then be used to follow LSC clonal evolution from diagnosis to relapse.[65,106,107] Studies with patient-derived xenotransplantations in immune-deficient mice have been instrumental in defining the origins of leukemic relapse, as they can capture even the rare relapse-relevant LSCs. For instance, clonal tracking in non-obese diabetic/severe combined immunodeficient (NOD-SCID) xenotransplant models revealed remarkable heterogeneity in repopulating potential within the LSC pool. Upon serial transplantation, a rare quiescent LIC with high self-renewal potential and an exceptionally low proliferation rate was identified.[108] These studies prompted the notion that only a specific subset of LSCs is responsible for driving relapse.

Historically, the cause of relapse was attributed to chemotherapy-induced insertion of mutations making a subset of cells therapy-resistant.[109] In the last decade however, an increasing number of studies with paired diagnostic and relapse AML samples have instead pointed to the pre-existence of drug-resistant cells. Sequencing efforts have revealed that relapse arises from the re-emergence or clonal evolution of a pre-existing clone already present before treatment, and its selection is influenced by chemotherapy.[110–112] Building on these findings, a recent study on diagnostic/relapse AML samples has identified two distinct types of LSC populations causing relapse. One extremely rare population had features resembling the HSPC phenotype, such as quiescence and self-renewal capacity making them resilient to chemotherapy, whereas the other larger population consisted of committed progenitors displaying a transcriptional stemness signature.[107] The identification of these complex relapse patterns within individual patients is essential to overcome LSC-mediated therapy resistance and may help predict the mutational basis that prevails treatment.

Acute Myeloid Leukemia

AML is a highly aggressive disease that arises from uncontrolled proliferation of immature myeloid precursor cells. It is the most common acute leukemia in adults, accounting for around 80% of all cases, and its incidence continues to increase with age.[113] Out of all leukemia subtypes, AML has the shortest survival, with a median overall survival (OS) rate ranging from 25-30% in adults and 50-80% in children.[113,114] Modest improvements in AML survival have mainly been attributed to the optimization of curative therapies over the years, including intensive chemotherapy and allogeneic hematopoietic stem cell transplantation (HCT).[115] This benefit is however largely confined to younger patients and those without adverse-risk cytogenetics, while older and less fit patients still exhibit poor prognosis.[113] After years of stagnation in therapeutic advances, new molecular insights in AML progression and resistance mechanisms have led to the development of a dozen new Food and Drug Administration (FDA) approved therapies in the last five years.[116] Undoubtedly, these and other targeted therapies in the pipeline will revamp the diagnostic, prognostic, and therapeutic landscape of AML.

Clinical aspects

At diagnosis, a patient with AML may present a large range of symptoms, including bleeding, swollen lymph nodes, fever, joint paint, fatigue, and signs of infection.[117] Following an initial physical examination, several tests are performed to establish a diagnosis, including peripheral blood counts, bone marrow morphology, flow cytometry immunophenotyping, cytogenetics and mutational analyses.[117] In addition to the standard leukemia-associated immunophenotyping (LAIP) performed to distinguish AML from normal hematopoietic cells, an assessment of the amount of residual LSCs is being recommended at the time of diagnosis. Prospective studies have shown that LSC detection improves sensitivity and prognostic impact of MRD. [118,119] Detection of MRD is in turn used to accurately define a complete remission status, generally defined as <5% blasts in the bone marrow and normal peripheral blood counts, as well as assessing post-remission relapse risk.[120] LSCs can be clinically defined as $CD34^{+}/CD38^{low}$ cells, in combination with aberrant markers absent from normal

HSCs.[121] Recently, better characterizations of LSCs based on the expression of 17 genes (LSC17) or clinical transcriptome-based assays are offering great diagnostic returns in terms of predicting prognosis, treatment resistance and adverse-risk subgroups.[82,122] Screening for gene mutations and rearrangements is required to establish diagnosis, recommend a suitable therapy, and identify actionable therapeutic targets.[117]

Classification in AML

The first classification of AML was proposed by the French American British (FAB) system, which stratified the heterogeneity on blast morphology, differentiation status and myeloid cell type affected.[123] However, with the advancements in genomic analysis techniques, the World Health Organization (WHO) system was introduced, further expanding the spectrum of classification by incorporating current genetic and cytogenetic abnormalities.[124] This system better classified AML entities, carried prognostic information, and included other important factors such as a prior hematological malignancies or exposure to therapy. Based on disease etiology, AML was originally proposed to be broadly categorized into *de novo*, therapy-related, and secondary AML, the latter evolving from antecedent myeloid neoplasms such as myelodysplastic syndrome (MDS) or MDS/myeloproliferative neoplasm (MPN).[124] However, according to the European Leukemia Net (ELN) recommendations, these predisposing features (therapy or prior neoplasms) are appended as qualifiers of the diagnosis, rather than being class-defining.[117]

The WHO system

AML classification is rapidly moving from a morphologic to a molecular and genetic basis, as evidenced in the most recent update of the WHO system released in 2022.[125] AML is now mainly divided into AML with defining genetic abnormalities (DGA) or defined by differentiation (**Figure 3**). A major change compared to the previous 2016 edition is the broadening of the *KMT2A* and *MECOM*-rearrangements (-r) groups, as a well as the inclusion of *NUP98*-r. Additionally, mutations in *NPM1* and *CEBPA* are now considered AML-defining groups, whereas the *RUNX1*-mutated group has been removed due to an overlap with molecular features defining existing subgroups.[126,127] There has been a refinement in the definition of AML with myelodysplasia-related changes (AML-MRC), where morphologic criteria of dysplasia are being replaced with mutations in the MDS-related genes *ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1* and/or *ZRSR2* based on relevant studies.[128,129] This group, termed AML myelodysplasia-related (AML-MR) is now within the genetically-defined AML

subgroups.[126] Another significant change is that the previous AML-defining blast threshold of ≥20% myeloid blasts in the peripheral blood or bone marrow has been lowered. The WHO lowered the threshold to 10% in cases where genetic abnormalities define specific AML subtypes. Exceptions to this rule where the 20% is maintained include *CEBPA* mutations and AML-MR, to exclude MDS and MPN, and *BCR::ABL1* to avoid confusion with chronic myeloid leukemia (CML).[117,124,126,130] Cases of AML defined by differentiation still require a 20% blast cutoff to allow distinction from MDS, although it is likely that genetic features rather than arbitrary cutoffs will become more prevalent in the future.[126,131]

Comparison between different classification systems

In the same year as the updated 2022 WHO classification was published, a separate system known as the International Consensus Classification (ICC) was introduced, adding to alternative classification systems as the one proposed by Papaemmanuil in 2016. [66,126] Despite the many overlapping classification criteria between the WHO and the ICC systems, there are key discrepancies that could influence patient treatment regimes, risk stratification, and clinical trials inclusions (**Figure 3**). Several studies have recently emerged to retrospectively analyze to what extent the new classification systems impact the diagnosis of AML patients.[127,132] These studies revealed that both classifications emphasized the integration of genetic abnormalities to define specific groups, with a large degree of agreement of 86%.[127] The *NPM1* mutation was considered subgroup-defining by both systems due to its rarity in MDS and its association to rapid AML progression.[133] One notable distinction is the separation of the AML-MR group in the WHO classification into three categories in ICC: AML with MR gene mutations (including *RUNX1*), AML with MR cytogenetic abnormalities, and AML with mutated *TP53*.[126,127] There are also differences in the definitions of AML with *CEBPA* mutations, where ICC only includes in-frame bZIP *CEBPA* mutations, while WHO 2022 accepts biallelic *CEBPA* mutations and single mutations of any kind that are located in the bZIP region. Differences in the definitions of AML-*CEBPA* and the exclusion of *TP53* mutated cases in AML-MR are associated with differences in OS rates.[127] ICC also includes *KMT2A*- and *MECOM*-r with partner genes distinct from *MLLT3* or *GATA2*, specifically defining the partner genes as distinct entities. Another notable difference is that in contrast to WHO 2022, ICC sets the blast cutoff for AML-DGA to 10%, assigning cases with 10-19% blasts without DGA to a new disease category, MDS/AML.[126] This inclusion in ICC could provide more flexibility for clinical trials and practice, potentially aligning better with the natural course of high-risk MDS.[134] However, WHO decided against this overlap category, citing concerns about the risk of over-treating some patients.[125] Furthermore,

unlike the WHO classification, ICC does not use a documented history of MDS or MDS/MPN as a diagnostic criterion for AML-MR but rather employs it as a diagnostic qualifier.[117,126,127]

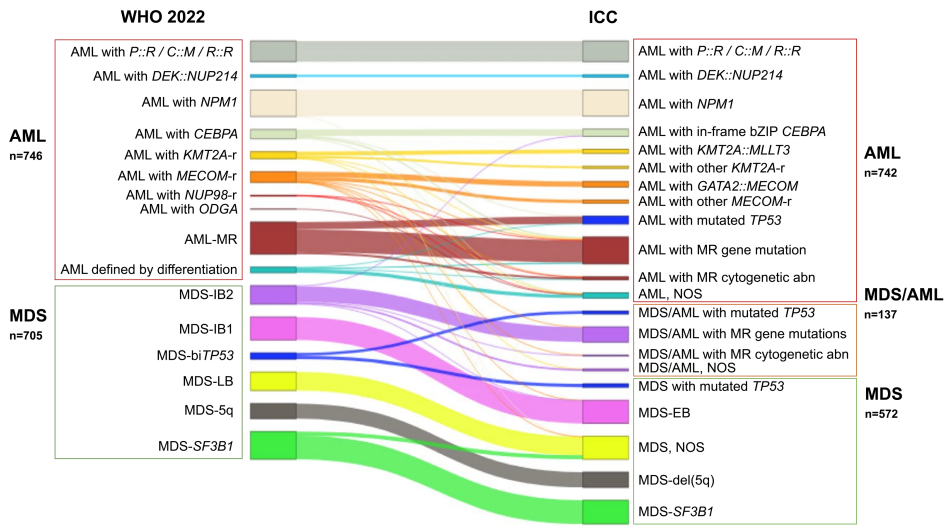

Figure 3 | Changes in AML diagnoses between the WHO 2022 and ICC systems. *P::R* = *PML::RARA*; *C::M* = *CBFB::MYH11*; *R::R* = *RUNX1::RUNX1T1*; -r rearrangement; ODGA Other defined genetic alterations; MR, myelodysplasia-related; IB, increased blasts; bi*TP53*, biallelic *TP53* mutation; LB, low blasts; NOS, not otherwise specified; abn, abnormalities; EB, excess blasts; del(5q), isolated 5q deletion. Adapted from Huber et al.[127]

These changes in AML classification have also been reflected in the recently updated ELN recommendations for AML diagnosis, risk-stratification, and management.[117] AML patients are stratified into favorable, intermediate and adverse risk groups based on the presence of specific genetic abnormalities. Changes in the new classifications have affected all three risk groups, highlighting the need for a common AML classification to ensure comparability of diagnostic data across centers.[117,127132]

Molecular landscape in AML

In comparison to solid cancers, relatively few mutations are needed for AML development, averaging at 13 mutations per patient according to The Cancer Genome Atlas (TCGA) and the Beat AML datasets.[135,136] It is through the serial acquisition of these somatic mutations in HSPCs that the neoplastic clone gains capacity to self-renew and propagate. As discussed in the section "*Leukemiogenesis*", multiple factors can influence the number of mutations including age, the bone marrow niche, epigenetic

regulation and prior exposure to cytotoxic therapy.[54,56,66,135] The predisposing factor of age in genomic instability is evidenced by the marked differences between pediatric and adult AML landscapes, with the former having only 5 somatic mutations per patient.[137] Mutational frequency also varies between AML subtypes, with some initiating events requiring more accompanying mutations, and increases with underlying neoplasms such as MDS or MPN.[138]

The TCGA research network integrated genomic, transcriptomic and epigenomic data from 200 *de novo* AML cases, and categorized recurrent mutations into nine groups based on their biological function.[135] The majority of mutations were identified in signaling genes, including *FLT3, RAS, KIT, CBL, NF1*, and *PTPN11*, followed by mutations in DNA-methylation-related genes like *DNMT3A, TET2, IDH1*, and *IDH2*. Mutations were also detected in genes associated with chromatin modification (*ASXL1* and *EZH2*), myeloid transcription factors (*RUNX1, CEBPA*, and *PU.1*), cohesin-complex genes (*STAG2* and *RAD21*), spliceosome-complex genes (*SRSF2, SF3B1, U2AF1*, and *ZRSR2*), tumor-suppressor genes (*TP53*), and *NPM1*, which stood as its own distinct group.[135] The last category confers transcription-factor fusions, which may arise as a result of chromosomal alterations in the form of insertions/deletions, chromosomal trisomy, inversions and translocations.[135,139] Large chromosomal alterations are common in AML, with 10-14% of patients displaying complex karyotypes with three or more cytogenetic abnormalities.[140] Some of the common fusion genes are associated with a more favorable risk, such as *PML-RARA*, *RUNX1-RUNX1T1*, or *MYH11-CBFB*, whereas mixed-lineage leukemia rearrangements (*MLL*-r)- confer worse prognosis.[141,142] The combinations of mutations that ultimately drive leukemogenesis suggest strong biological cooperativity and mutual exclusivity among several of the genes and categories. In this section, several of these recurrently mutated genes with relevance to the topics covered in this thesis are discussed.

MLL-rearranged leukemia

Translocations involving *MLL* (officially known as histone-lysine N-methyltransferase 2A, *KMT2A)* give rise to one of the most aggressive forms of myeloid, lymphoid, and therapy-induced acute leukemias.[143] Overall, *MLL*-r accounts for about 10% of acute leukemia cases, although clear lineage, subtype, and age tendencies have been observed depending on the *MLL* translocation partner[143–145]. Age was found to play a significant role in *MLL* genomic breakpoints, which in turn correlated with varying etiologies in the different age groups.[143] Additionally, *MLL*-r leukemias rarely co-exist with recurrent mutations, which involve *KRAS, NRAS, PTPN11, BRAF*, and *FLT3.* With an average of 1-2 mutations per patient, often subclonal and lost in relapse, it is thought that *MLL*

fusions may be sufficient to drive leukemia progression in the absence of cooperating mutations.[137,144,146]

MLL encodes a histone methyltransferase, a nuclear protein with a complex domain structure that positively regulates gene expression. Taspase 1-mediated proteolytic cleavage generates C- or N-terminal fragments which cooperate to recruit additional proteins that regulate chromatin accessibility and gene expression.[147] Key transcriptional targets of *MLL* are the *HOX* gene cluster and *MEIS1*, well-established regulators of embryonic development and normal hematopoiesis.[148] MENIN is essential in transcriptional regulation, as demonstrated by the disruption in *HOX9* transcription by both wild type (wt) and *MLL*-r in the absence of it.[149,150] The methyltransferase activity of *MLL*, on the other hand, has been shown to be dispensable in gene regulation.[151]

Translocations arise from monoallelic double-stranded breaks in *MLL*, followed by fusion with one of over 80 partner genes known to date.[143,145] Of these, only 5 fusion partners constitute ~80% of *MLL*-r cases, which include *AF4* (36%), *AF9* (19%), *ENL* (13%), *AF10* (8%) and *ELL* (4%).[152] The molecular mechanisms by which the fusion process drive leukemogenesis have remained elusive. Although the implication of wt *MLL* has been debated, it is known that the truncated *MLL* gene is devoid of transformation ability, suggesting that the fusion partners play a major role.[153–155] The majority of fusion genes causing illegitimate recombinations are part of the super elongation complex (SEC), which include *AF4, AF9, ENL, ELL*, and *P-TEFb*.[156] Upon *MLL* fusion with these genes, SEC is recruited and through interaction with H3K79 methyltransferase DOT1L, transcriptional elongation of *MLL*-r target genes is enabled, a process thought to be one of the major drivers of *MLL*-r leukemogenesis.[157,158]

Several studies have looked into the dependence and biological implication of *MLL*-r leukemias on a deregulated transcriptome to uncover cues about the oncogenic potential of the transforming target cell. For instance, an upregulation of *HOX* and *MEIS1* in *MLL*-r has been shown to contribute to a block in myeloid differentiation and aberrant self-renewal signature, properties conferring a survival advance.[159–161] In this thesis (**Articles I-IV**), our focus was to identify indispensable components driving *MLL::AF9* leukemia, the most common translocation in adult AML, and leverage these dependencies as potential therapeutic targets.[152]

RUNX1 mutation

Runt-related transcription factor 1 *(RUNX1)* is a transcription factor indispensable for the establishment of definitive hematopoiesis.[162] It is a target for chromosomal translocations and somatic mutations, which can lead to a preleukemic state that

predisposes to *de novo*/secondary AML, ALL, MDS, and atypical CML.[163,164] In a study involving 2500 newly-diagnosed AML patients, 10% of cases were defined by *RUNX1* mutations, with the frequency of these mutations increasing with age and in secondary AML evolving from MDS.[165] The latter aligns with the role of mutated *RUNX1* in promoting leukemic transformation progression of myeloproliferative neoplasms (MPNs).[166] Notably, AML patients harboring somatic *RUNX1* mutations tend to have dismal clinical outcomes in terms of rates of event-free survival (EFS), relapse-free survival (RFS) and OS.[117,165,167]

The majority of mutations in *RUNX1* have been mechanistically characterized. Some mutations result in the deletion or truncation of the DNA binding "Runt" domain, consequently inactivating the protein. On the other hand, other mutations confer a weak dominant negative activity. This is the case with mutations resulting in a lack of all or part of the transactivation domain, or those impairing DNA-binding capacity by preventing the interacting subunit partner CBFβ from stabilizing the conformational structure of RUNX1.[163,168,169] Across the broad spectrum of somatic mutations, biallelic *RUNX1* mutations have been associated with a specially poor outcome, suggesting a potential dosage effect.[170] Unlike epigenetic modifiers like *DNMT3A* and *TET2*, which often appear as initiating clonal events in leukemogenesis, *RUNX1* mutations tend to emerge later as intermediate secondary events that drive leukemia development.[63,66,117] In a recent study, it was observed that *RUNX1*-mutations significantly correlate with a distinctive pattern of concurrent genetic lesions involving epigenetic modifiers (A*SXL1, IDH2, KMT2A* and *EZH2*), components of the spliceosome complex (*SRSF2* and *SF3B1*), as well as *STAG2, PHF6, BCOR*.[165] The intricate molecular landscape associated with *RUNX1* mutations has posed challenges in delineating class-defining genetic lesions, a topic that has been subject to recent debate.[127] Consequently, *RUNX1* mutations are no longer class-defining groups according to the most recent WHO classification or the new ICC system, primarily due to the overlap with *ASXL1* and splicing factor mutations that define other AML subgroups.[125,126]

Loss of function mutations in *RUNX1* are associated with refractory disease and adverse outcomes presumably due to chemotherapy resistance.[171] This was evident as *Runx1*-deficiency in murine HSPCs conferred protection against chemotherapeutic agents, providing a selective advantage allowing for expansion in the bone marrow at the expense of normal HSPCs. This resistance to therapy-induced genotoxic stress, as well as endogenous stress, was largely attributed to slower growth, reduced protein translation, and decreased ribosome biogenesis in *Runx1*-deficient HSPCs.[164,169,172] Recent studies have harnessed this susceptibility to ribosomal stress and protein translation inhibition caused by *RUNX1* mutations for selective cytotoxic therapy.[173] AML cells harboring *RUNX1* mutations were sensitized to the lethal activity of the protein translation inhibitor omacetaxine (HHT), and combining HHT with

venetoclax, a BH3 mimetic and B-cell lymphoma 2 protein (BCL-2) inhibitor, or a BET inhibitor, led to synergistic lethality.[173] Contributing to the ongoing efforts to develop alternative targeted therapeutic options for AML, we identify a novel combination strategy that exhibits strong sensitivity in patient-derived mutated *RUNX1* AML cells through disruption of multiple metabolic pathways (**Article II**).

TP53 mutation

TP53 is a tumor suppressor gene that encodes the transcription factor p53, appropriately coined the "guardian of the genome."[174] TP53 is the most frequently mutated gene across all human cancers, and, just like in most cancer types, the presence of *TP53* mutations in AML is associated with adverse prognosis.[174] Although it is arguably one of the most well studied genes to date, mutant p53 is considered "undruggable", thus hindering translation of knowledge into clinical benefits. *TP53* mutations, which are typically characterized by loss of function, present complexities as a therapeutic target. With a dismal median survival of 5 to 10 months regardless of the therapies used, individuals with *TP53*-mutated AML represent one the most pressing patient groups in need of innovative therapeutic solutions.[70,175,176]

Mutations in *TP53* occur in 5-10% of *de novo* AML (and MDS), although frequencies are substantially higher (20 to 40%) in older patients or those with therapy-related myeloid malignancies.[135,176–178] *TP53* mutations further increase to 70-80% in patients with complex karyotypes and abnormalities on chromosome 3, 5, 7, and 17, features particularly linked to poorer prognosis[175,176,178] As previously discussed in the section "*Therapy-induced shaping of clonal landscape*", *TP53* mutations do not necessarily arise from the cytotoxic effects of chemo- or radiotherapy. Instead, therapy exerts selective pressures that lead to the expansion of preexisting *TP53*-mutated small clones resistant to DNA damage which, over time, progress to develop *TP53*-mutated AML.[69,70] A compromised response to DNA damage results in impaired DNA repair, cell cycle arrest and apoptosis, likely contributing factors to the accumulation of genetic aberrations causing resistance to chemotherapy and venetoclax-based therapies reported in *TP53*-mutated AML.[179,180] Detailed characterizations of the *TP53* locus have recently revealed that despite the dominance of missense mutations within the DNA-binding domain, other chromosomal alterations present leading to allelic imbalances may have critical diagnostic and prognostic value.[174,177,178,181] Specifically, the allelic state of *TP53* mutations, whether mono- or bi-allelic, delineates two patient subsets. On one hand, multiple hits consistent with biallelic targeting of *TP53* rarely co-occur with mutations in other myeloid malignancy-related genes, indicating biallelic defects evolve to become dominant clones.[177,178] Conversely, monoallelic mutations often have co-

occurring mutations in other genes, particularly *TET2, SF3B1, ASXL1* and *DNMT3A*, and are considered late subclonal events with varying impacts on outcomes.[177,181]

Increasing recognition of the therapy resistance and unfavorable outcome exhibited in patients with *TP53*-mutated AML has prompted research aimed at identifying p53-directed strategies that can be integrated into current frontline therapies.[181] This research is becoming possible through our increasing understanding of the functional implications of *TP53* mutations, such as their impact on metabolism, ferroptosis, immunoregulation, cytokine milieu, and the microenvironment.[181,182] In **Article III**, we identify a p53-dependent mechanism of therapeutic response to iron deprivation-based strategies, potentially offering insights to optimize treatment strategies for enhancing response durability and survival in *TP53*-mutated AML.

Conventional therapy strategies

The predominant goal of treatment is control and, whenever possible, eradication of the disease. The current treatment paradigm employed to achieve this outcome generally begins with CR-inducing chemotherapy, with anthracyclines and cytarabine (the latter also referred to as Ara-C) remaining the backbone of induction therapy.[117] The mainstay regime is a "7+3" induction therapy, with a 7 day infusion of standard-dose cytarabine plus 3 days of anthracycline. Alternative regime options include fludarabine, granulocyte-colony stimulating factor (G-CSF), idarubicin and mitoxantrone-based cytarabine.[117] Addition of targeted approaches to first-line therapy is based on the genetic profile, exemplified by the inclusion of the kinase inhibitor midostaurin in the treatment regime for patients with *FLT3*-mutant AML.[117,183,184] Other therapeutic options that are currently under randomized evaluation for AML induction therapy are gemtuzumab ozogamicin (GO), an anti-CD33 drug-conjugated humanized antibody, and CPX-351, a dual-drug formulation encapsulating cytarabine/daunorubicin.[117,185–187]

Once CR is achieved, consolidation chemotherapy with intermediate-dose cytarabine and/or allogeneic HCT is given to patients with an estimated risk of relapse of >35% to 40%.[188] Besides risk stratification, consolidation treatment choice is also recently being informed by MRD persistence.[117] To date, allogeneic HCT represents the only potentially curative strategy for patients with intermediate- or adverse-risk AML.[188] Although this approach is efficient at attaining CR and survival benefits in a subset of patients, it is not a viable option for many others based on advanced age, comorbidities and poor performance status.[189] Maintenance therapy given to patients who have achieved CR lacks a universally standardized definition. The FDA describes

it as a course of treatment of limited duration, usually minimally toxic, aimed at minimizing the risk of relapse.[190]

Substantial strides are being made to develop alternative therapeutic strategies for patients that are considered unfit for intensive chemotherapy. The purpose of these therapies is to optimize quality of life and reduce cytopenia-related complications through transfusions or other supportive care measures.[117] Hypomethylating agents (HMAs) such as azacitidine or decitabine are being approved as alternative treatment regiments, with promising combination strategies that are quickly establishing new standards of care.[117] Addition of venetoclax to either azacitidine or decitabine is improving clinical responses in elderly and unfit AML patients.[191,192] Similarly, a randomized study revealed superior clinical benefits for patients with *IDH1* mutation with azacitidine plus the IDH1 inhibitor ivosidenib compared to azacitidine alone.[193] In patients considered too trail for HMA-based therapy, monotherapy with targeted IDH1/IDH2 inhibitors or low-dose cytarabine plus venetoclax represent alternative options.[194,195] Advances in genomic profiling and understanding of AML pathobiology are guiding research aimed at de-intensifying therapy, leading to the approval of novel agents and targeted therapies in the frontline, relapse, and maintenance settings.[116]

Cell surface markers in AML

Immunophenotyping by multiparameter flow cytometry (MFC) has become the basis for accurate AML diagnosis, remission assessment and prognosis prediction.[117] In cases where an aspirate is unobtainable or circulating blasts are absent, myeloid phenotyping is confirmed via immunohistochemistry on a core biopsy.[117] MFC has been implemented in a clinical setting to identifying cell surface and intracellular markers that distinguish leukemia cells from normal cells, along with facilitating subsequent MRD and LSC monitoring.[117] Current research is aimed at identifying cell surface markers as therapeutic targets by leveraging their differential expression between leukemia cells and normal cells to increase therapeutic effects while minimizing toxicities.

Surface immunophenotyping was first introduced to define the cellular hierarchical organization of AML, with disease-initiating LSCs considered to be predominantly confined to the $CD34^{+}CD38^{-}$ compartment.[71,72] The clinical significance of immunophenotyping was originally shown in a study correlating $CD34^{+}CD38^{-}$ LSC at diagnosis with the duration of RFS in AML patients.[196] Later work revealed greater variability in the phenotypes of LSCs than previously suggested, where LSCs were shown to also reside in $CD34^{+}/CD38^{+}$ or $CD34^{-}$ cell fractions.[79,91,197] This is the case

in patients with *NPM1*-mutated AML, where LSCs are equally present in both the $CD34^+$ and $CD34^-$ fractions.[79,198] A lack of CD34 expression and an absence of hierarchy in these fractions have suggested that *NPM1*-mutated AML may have a different cell or origin.[79,199] These studies have shed light on how subtype-defining genetic mutations can influence the immunophenotype of LSCs. Consequently, alternative cell surface markers such as KIT/CD117, GPR56, IL1RAP and CD200 are employed to enrich for LSC activity in CD34 non-expressing AML.[80,198,200]

Table 1 | Cell surface proteins differentially expressed in AML LSCs ($CD34^+CD38^-$) compared to corresponding normal bone marrow cells (NBM) based on expression data from Herrmann et al.[202]

Antigen	CD	Protein name	Expression on NBM $CD34^+$ $CD38^-$ cells
IL1RAP	N/A	Interleukin 1 receptor accessory protein	no
MRP-1	CD9	CD9 molecule	minimal/no
IL-2RA	CD25	Interleukin 2 receptor subunit alpha	no
FCGR2B	CD32	Fc fragment of IgG receptor IIb	no
SIGLEC3	CD33	CD33 molecule	lower than AML LSCs
SCARB3	CD36	CD36 molecule	no
TSPAN26	CD37	CD37 molecule	minimal/no
MER6	CD47	CD47 molecule	lower than AML LSCs
NCAM1	CD56	CD56 molecule	no
GP40	CD7	CD7 molecule	no
CD27L/TNFSF7	CD70	CD70 molecule	no
TSPAN27	CD82	CD82 molecule	lower than AML LSCs
MXR4A/C1QR1	CD93	CD93 molecule	lower than AML LSCs
TACTILE	CD96	CD96 antigen	minimal/no
SLC3A2	CD96	Solute carrier family 3 member	lower than AML LSCs
ADGRE5	CD97	Adhesion G protein-coupled receptor E5	lower than AML LSCs
MIC2	CD99	CD99 molecule	lower than AML LSCs
IL3RA	CD123	Interleukin 3 receptor subunit alpha	lower than AML LSCs
TNFRSF4/OX40	CD134	TNF receptor superfamily member 4	minimal/no
TIM-3/HAVCR2	CD366	Hepatitis A virus cellular receptor 2	no
CCL-1/CLEC12A	CD371	c-type lectin domain family 12 member A	no

There has been an ongoing effort to refine and expand the combinations of cell surface markers for the precise detection of leukemic stem and progenitor cells while minimizing contamination by normal cells.[201] However, LSCs display such phenotypic

heterogeneity that no marker can consistently distinguish LSCs from HSCs across all AML subtypes. This heterogeneity has been attributed to the distinct aberration profiles and signaling machineries induced by different genetic variants in AML subtypes.[202] To address this, MFC has been used to compare AML LSC phenotypes with cell surface marker profiles of HSCs from normal bone marrow and cord blood.[202] While many markers are shared between LSCs and HSCs, a majority of AML patients exhibit LSCs aberrantly expressing at least one of the following markers: CD9, CD25, CD69, CD93, CD96, CD371/CLL-1, and IL1RAP.[202] Therefore, it is recommended to detect $CD34^{+}CD38^{low}$ cells combined with an aberrant marker not present on HSCs, although this has not yet been routinely implemented in clinical settings.[117,121] Co-expression profiles of multiple distinct LSCs markers are also under evaluation.[203,204] These unique phenotypes are facilitating LSC detection and guiding the development of LSC-specific targeted therapies. **Table 1** summarizes cell surface markers differentially expressed in AML LSCs compared to corresponding normal bone marrow cells.

Analyzing markers not typically found in normal hematopoiesis is a valuable approach for identifying unique markers for prospectively isolation and therapeutic targeting of LSCs.[201] Unbiased sequencing approaches, such as CITEseq, may enable patient-specific immunophenotyping at the single cell level, offering insights into cellular states and biological functions within AML LSCs.[205,206] However, the clinical implementation of high-throughput sequencing at the single cell resolution is still in progress.

Murine models of AML

Despite advances in *ex vivo* and *in silico* methods to recapitulate the bone marrow microenvironment, predicting the physiological and molecular cues of leukemogenesis is limited by the complexity in whole-organism systems. Animal models offer invaluable tools to investigate disease pathogenesis, uncover molecular contributors, and evaluate novel therapeutic strategies under controlled experimental conditions.[207] The mouse is the most widely used animal model owing to their genetic similarity to humans, their rather short life cycle and cost-efficacy.[207] Several mouse leukemia models have been established, from transgenic animals expressing AML-associated genes, to transfer methods using retrovirally transduced mouse cells transplanted into recipients, and xenotransplantation into immunocompromised mice (**Figure 4**). While complementary to each other, none of these strategies faithfully recapitulate the complex heterogeneity of human AML.[208] Researchers face the task of discerning inherent inter-species disparities and differences between *in vitro* and *in vivo* cellular behavior to ensure clinically translational data is generated.

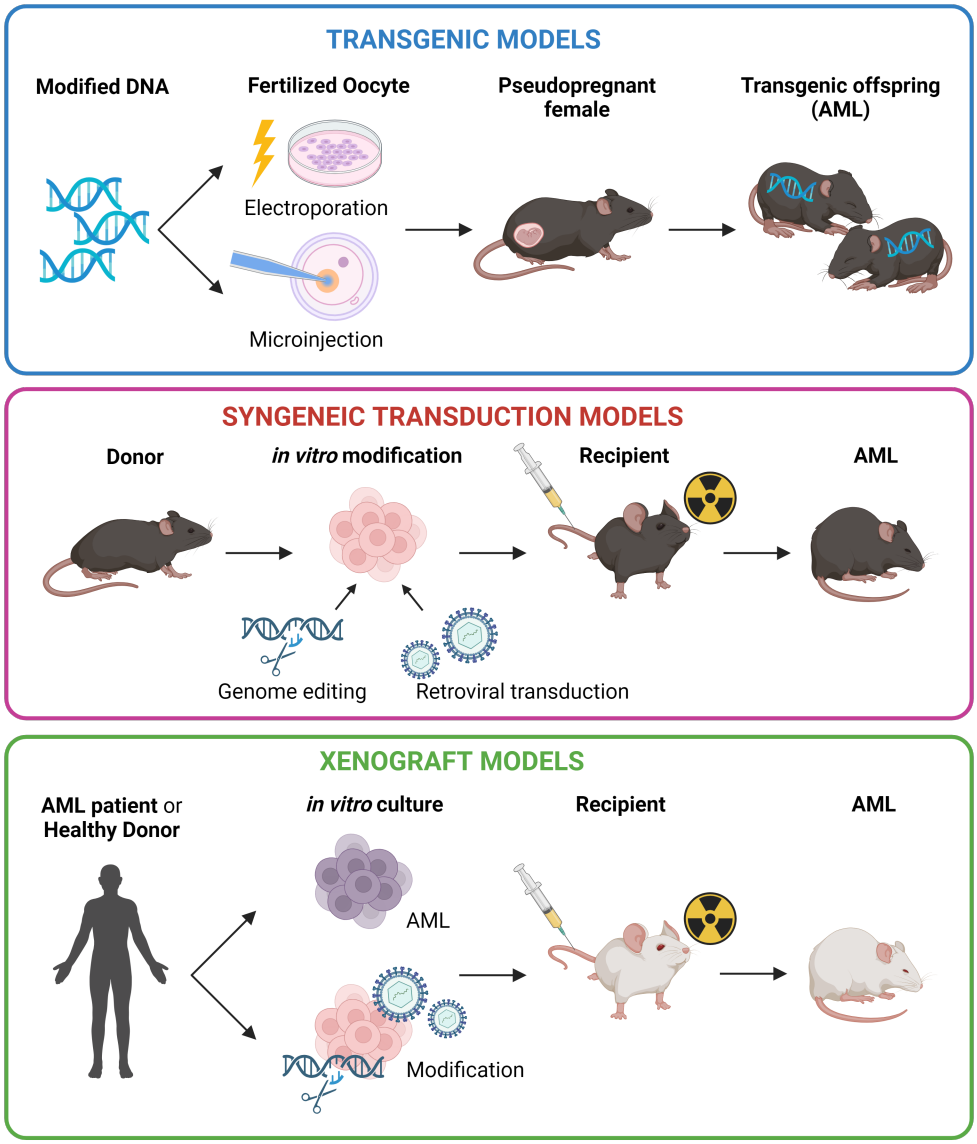

Figure 4| Murine models of AML. **Transgenic approach** involves DNA insertion into the mouse genome, achieved either randomly via pronuclear microinjections into fertilized oocytes or through targeted electroporation and homologous recombination in embryonic stem cells. **Syngeneic approach** involves modification of murine HSPCs *in vitro* using retroviral transduction or genome editing techniques, followed by tail intravenous transplantation into irradiated recipients. **Xenograft approach** involves transplantation of donor-derived leukemic blasts or *in vitro* modified HSPCs into immunocompromised recipients. Created with Biorender and adapted from Almosailleakh et al.[208]

Transgenic models

Genetically engineered, or transgenic mouse models, were amongst the earliest models to be implemented since the revolution in molecular methods in the 70s-80s. These models replicate leukemias driven by genetic aberrations through the introduction of foreign genetic elements into the mouse germline to create transgenic lines.[208] The DNA fragment of interest enclosed in a vector is inserted into the mouse genome either randomly through microinjection into a fertilized egg, or through targeted electroporation into embryonic stem (ES) cells.[208] These models allow the introduction of "knockout" loss-of-function mutations or "knock-ins" of altered genes (**Figure 4**).[209] One early transgenic model, generated by homologous recombination of a short fragment of AF9 cDNA into the mouse *Mll1* locus, established a proof of concept for the *MLL::AF9* fusion gene.[210] This model formed the basis for identifying the stepwise progression of the disease, gene dosage effects, and potential cellular targets driving *MLL::AF9*-associated AML.[211,212]

Refinements of conventional models led to the development of conditional mouse models, whereby expression or ablation of a gene of interest is driven by lineage specific *Cre* expression acting on the inserted floxed gene. Inducible expression of the transgene is regulated by spatially and/or temporally controllable promoters, which can be activated by inducers such as tamoxifen, tetracycline, and doxycycline.[208] Conditional knock-in transgenic models for *NPM1c, FLT3-ITD* and N-/K-*RAS* mutations have been pivotal in demonstrating that they are insufficient to induce AML but act as potential cooperating lesions instead.[213–215] On the other hand, *FLT3*-ITD and *TET2* knockout mutation were found to cooperate to remodel DNA methylation and gene expression, inducing AML.[216] While contributing to our understanding in AML modelling, transgenic approaches are often inefficient, technically challenging, time- and cost-consuming and often fall short in reproducing the leukemic phenotype observed in patients with the genetic lesion.[208]

Syngeneic transduction models

The syngeneic mouse model is based on the adaptive transfer of retrovirally transduced hematopoietic cells expressing AML-associated genetic aberrations into irradiated recipients. Engrafted donor-derived cells may then outcompete normal hematopoiesis in the chimeric host, potentially driving leukemogenesis (**Figure 4**).[208] Zuber et al. used this so-called "mosaic" approach to study the leukemogenic potential of the common AML translocations *AML1::ETO* and *MLL::ENL*, as well as their cooperation with oncogenic *NRAS* mutations. These studies faithfully recapitulated common genetic and pathologic features of human AML.[217] Most notably, the mouse

models accurately replicated the diverse response patterns seen in patients with these mutations when exposed to standard induction chemotherapy. Specifically, *AML1::ETO* murine leukemias, which are associated with a favorable prognosis in patients, displayed a high sensitivity to chemotherapy, while *MLL::ENL* leukemias, known for their poor prognosis in patients, exhibited resistance to chemotherapy.[217]

Several studies have used syngeneic transduction models to evaluate the LSCs responsible for sustaining, expanding, and regenerating *MLL::AF9*-driven AML.[218,219] In this approach, the *MLL::AF9* fusion gene was retrovirally transduced into normal GMPs (Lin^{low}, Sca-1^{-}, c-Kit^{+}, $Fc\gamma RII^{hi}$, $CD34^{hi}$) and transplanted into sublethally irradiated syngeneic recipient mice.[218] Remarkably, LSCs isolated from these committed GMP-initiated leukemias retained the immunophenotypic identity of their parental progenitors, while activating HSC- and self-renewal-associated programmes.[218,219] Further, contrary to the stepwise progression in the pathogenesis of *MLL::AF9* leukemia previously reported in a knock-in mouse model, these studies suggest a progressive temporal increase in the frequency of aberrantly self-renewing stem and progenitor cells.[219] These studies also determined that these cells with LSC activity resided in both the bone marrow and spleen of leukemic mice.[219]

In this thesis (**Articles I-IV**), we have based our work on this *MLL::AF9* AML model, previously generated in a dsRed transgenic background, enabling identification of leukemic cells from normal cells.[220] To enriched for stem cell activity, leukemia cells were serially transplanted through secondary, tertiary, and quaternary recipients, generating leukemias with high penetrance and short latency.[220] Despite the inherent limitations of retroviral models, such as non-physiological gene dosage, the risk of insertional mutagenesis, and cell manipulation, they remain the primary method for investigating the *in vivo* transforming potential of AML-associated genetic lesions, owing to their rapid and efficient methodology.[208]

Xenograft models

There are inherent and adaptive differences between murine and human leukemic phenotypes that require humanized models to better comprehend nuances of human disease (**Figure 4**). While *ex vivo* models of patient-derived AML blasts have been developed to address this need, their expansion and maintenance in long term cultures poses technical challenges.[221] These limitations are being overcome by culturing in engineered humanized 3D niches, which have shown to sustain growth of $CD34^{+}$ cells from AML and MDS patients for up to 3 weeks.[222] Biomimetic bone marrow systems however lack a physiologically relevant context, limiting probing of the interacting signals between primary AML cells and the bone marrow niche.[223] To mitigate these

constraints, patient-derived xenografts (PDXs) are modelled in immunodeficient mice through subcutaneous or intravenous transplantation.

Several immunodeficient mouse strains have been developed with different impairments in immune function, including SCID, NOD-SCID and NOD-SCID-IL2rγ^{null} (NSG) strains.[224] While NSG strains exhibit high engraftment capabilities, they may not be suitable for low and intermediate-risk AML samples that tend to have poorer engraftment.[225,226] Thus, more advanced strains have been developed to transgenically express human cytokines at either supraphysiologic (NSG-S) or physiologic (MISTRG) levels, thereby enhancing the engraftment of human AML cells.[226–228] Another approach to create humanized microenvironments is the implantation of biological scaffolds into immunocompromised mice, which support engraftment of human AML xenografts without altering their functional properties.[229]

More recently, CRISPR-based genome editing and transcription activator-like effector nucleases (TALEN) have been employed to engineer disease-associated mutations into the gene loci of primary cells.[230] For instance, this technique was used to introduce chromosomal translocations of *MLL* and *AF9* in primary human CD34^{+} umbilical cord blood HSPCs. Transplantation of these modified cells into NSG mice induced a leukemic phenotype similar to what is seen in patient cells.[231] A similar model was employed to investigate the therapeutic efficacy to azacitidine treatment in *TET2* and cohesin-mutated hematopoietic cells.[232] The studies demonstrate the potential of generating genetically defined humanized mouse models of AML, and their suitability to accurately model mutational consequences and drug responses. Notably, the immunodeficient nature of recipient mice may limit the assessment of immunotherapies, a field that has recently gained considerable attention in AML.[233]

Novel Therapeutic approaches in AML

The approach to discovering novel treatments for AML differs greatly from that of just a decade ago. Drug development strategies predominantly focused on identifying single agents, primarily for use in the relapse setting, that targeted the genetic aberration driving the disease. This approach has led to the successful approval of multiple targeted therapies such as FLT3 and IDH1/2 inhibitors.[117] However, the advent of single-cell multi-omics is revealing heterogenous co-mutational patterns with important prognostic and treatment implications. With an increased understanding of these pathogenic pathways, the therapeutic landscape is evolving towards the use of combinatorial strategies as duplet or triplet regimes to improve treatment outcomes.[234] Increased understanding of the functional consequences of mutations is also shifting the treatment paradigm from cytotoxic and HMA-based therapies to novel immune and non-immune targeted strategies. Additionally, as genomic data is being integrated into routine standards of care, personalization of treatments will likely become a reality in the near future.[117]

Molecularly targeted therapies

In the last five years, twelve agents have been FDA-approved for the treatment of AML, of which nine target specific molecular or cellular subgroups (**Figure 5**). These therapies are being introduced in induction, consolidation, and/or relapse/refractory (R/R) settings, and are namely suitable for patients of advanced age, unfit, or with mutations in *FLT3* or *IDH1/2.*[235] AML patients frequently exhibit *FLT3* mutations, most commonly as an internal tandem duplication (ITD) and/or in the tyrosine kinase domain (TKD). The type 1 FLT3 inhibitors midostaurin and gilteritinib have been approved, which effectively target both ITD and TKD variants.[235] Midostaurin, a kinase inhibitor now integrated into first-line therapy, has showed increased OS when co-administered with chemotherapy for *FLT3*-mutated AML.[183] Gilteritinib has been approved for *FLT3*-mutated AML patients who do not respond or are unfit for induction treatment, or with relapsed disease.[236] Notably, quizartinib, a type 2 inhibitor targeting *FLT3*-ITD, has recently been incorporated to the first-line treatment armament available for patients with this AML subtype based on promising data from

landmark trials.[237,238] As with *FLT3*-mutated AML, the treatment landscape for *IDH1* or *IDH2*-mutated AML has also been recently transformed. The pathogenesis of *IDH1/IDH2* mutations has been characterized by an epigenetic dysregulation and impaired differentiation mediated by the oncometabolite 2-hydroxyglutarate (2-HG).[239] Ivosidenib and enasidenib, approved inhibitors of mutated *IDH1* and *IDH2*, respectively, suppress 2-HG levels enabling the differentiation block to be overcome. Both drugs have been introduced in the R/R setting, with ivosidenib also being used in patients with mutated *IDH-1* with clinical features precluding the use of standard chemotherapy.[193,195,240] Olutasidenib, an emerging inhibitor for *IDH1*-mutated AML, is currently in a Phase I clinical trial showing meaningful clinical efficacy either as a standalone treatment or in combination with azacitidine.[241]

A large number of investigational agents with therapeutic potential are gaining attention. This is the case for two menin inhibitors, revumenib and ziftomenib, which have exhibited promising anti-leukemic activity in early-phase clinical studies for heavily pretreated R/R patients with *MLL*-r or mutant-*NPM1*.[242–244] Mechanistically, these inhibitors disrupt the interaction of menin and *MLL* fusion proteins, resulting in the downregulation of key leukemia drivers such as *HOXA* and *MEIS1*.[150,245] Similar to *MLL*-r AML, the sensitivity of *NPM1*-mutated AML to menin inhibitors is attributed to dysregulated *HOX* expression.[246] Another promising agent broadening treatment opportunities is eprenetapopt, which has shown early efficacy in *TP53*-mutated AML patients when used in dual or triple combinations with azacidine and venetoclax.[247,248] Several mechanisms of action have been proposed, including restoration of p53 through refolding conformations, or induction of cell-death mechanisms via ROS, ferroptosis, or depletion of deoxyribonucleotides.[181] Collectively, these emerging molecularly-defined therapies are providing unprecedented treatment options for previously untargeted genetic abnormalities in AML.

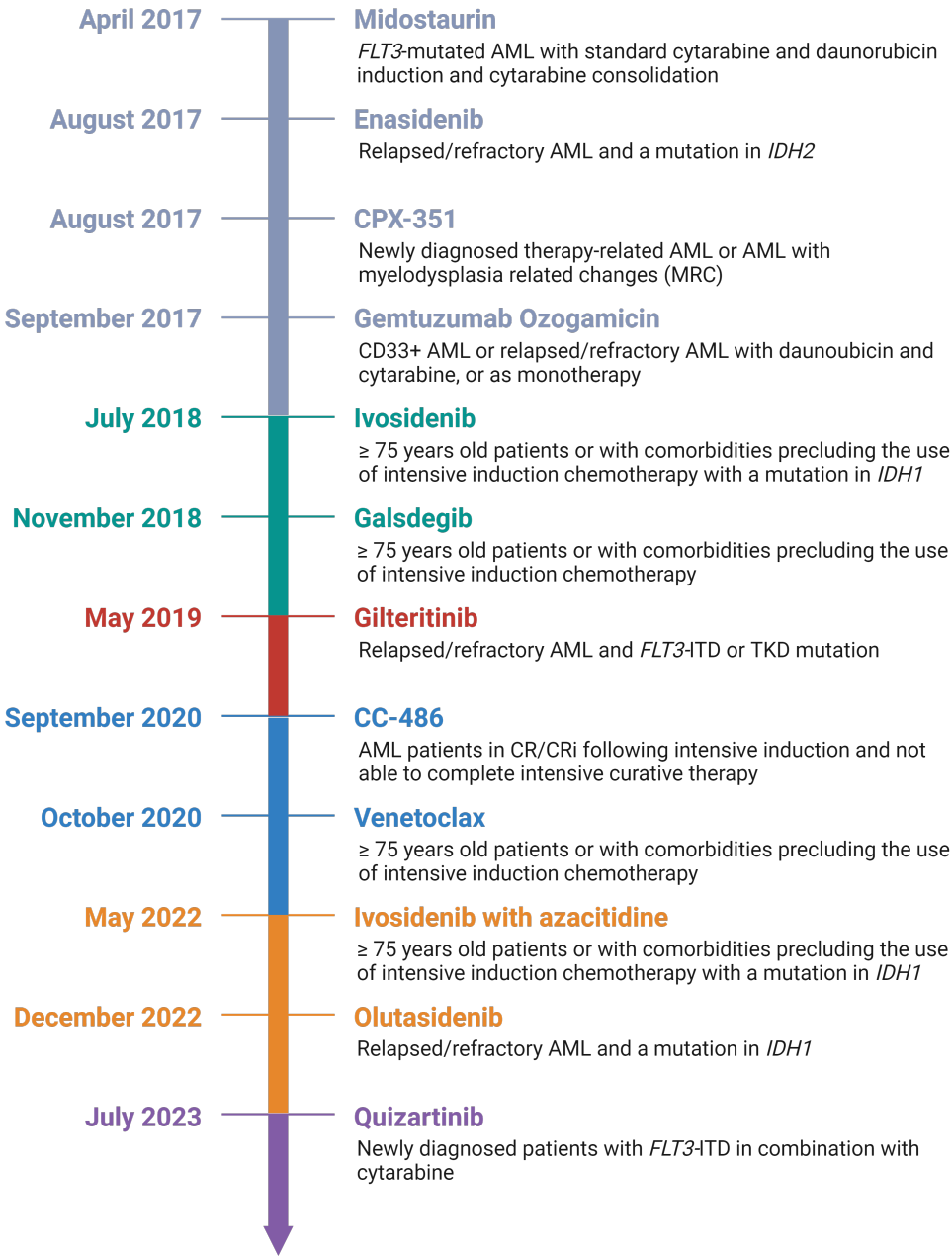

Figure 5 | Timeline of FDA-approved agents for AML for specific molecular or clinical subgroups. Adapted from Lachowiez et al.[235]

Immunotherapy

Immunotherapy traces its roots back to more than 50 years ago when the concept was first introduced in the context of HCT, where immune cells from the donor graft were observed to eradicate leukemia cells. This phenomenon, termed the graft-vs-leukemia (GvL) effect, served as proof-of-principle that even the more resilient LSCs could be vulnerable to immune-mediated eradication.[249] This finding laid the foundation for the development of immunotherapies. Contrary to traditional radiation and chemotherapy treatments, immunotherapies aim to harness and enhance the patient's immune system to mount a targeted response against leukemia cells while sparing healthy tissue. However, their clinical translation has encountered significant challenges.[250] These hindering factors include the relatively low mutation load in AML that result in a low number of neoantigens resulting in low immunogenicity, immune evasion strategies, and antigenic variability among patients.[251,252] Ongoing efforts are aimed at identifying suitable target antigens, augmenting immune surveillance, and overcoming escape mechanisms through the combination of complementary strategies.[252]

Immune evasion

Immune evasion is a hallmark of cancer, and hematological malignancies are no exception. It has become evident that AML cells employ complex immune dysregulation strategies to enhance their survival, resistance to therapeutic strategies, and relapse.[253,254] AML cells can escape immune recognition by hampering the effector function of T cells, NK cells, macrophages and DCs (**Figure 6**). One strategy involves the aberrant overexpression of inhibitory signals. AML blasts have been found to aberrantly express ligands that inhibit immune checkpoints (IC), essential for T cell-mediated immune surveillance. Well-known IC inhibitory AML ligands include programmed cell-death ligand-1 (PD-L1) and galectin-9, with the latter also contributing to an autocrine loop vital for LSC maintenance.[252] Recently, overexpression of CD200 has been described to be a LSC-specific mechanism to escaping elimination by T cells.[255] AML cells also employ immunoediting strategies, downregulating antigen presenting molecules like HLA class I and II to escape immune surveillance from DCs and macrophages.[254,256] Additionally, AML cells can also promote and expand immunosuppressive cell types, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs).[257–259] Multiple research lines also highlight how AML cells undergo remodeling of the bone marrow niche through alterations of the cytokine milieu, release of inhibitory soluble factors, and metabolic reprogramming to facility immune evasion.[252]

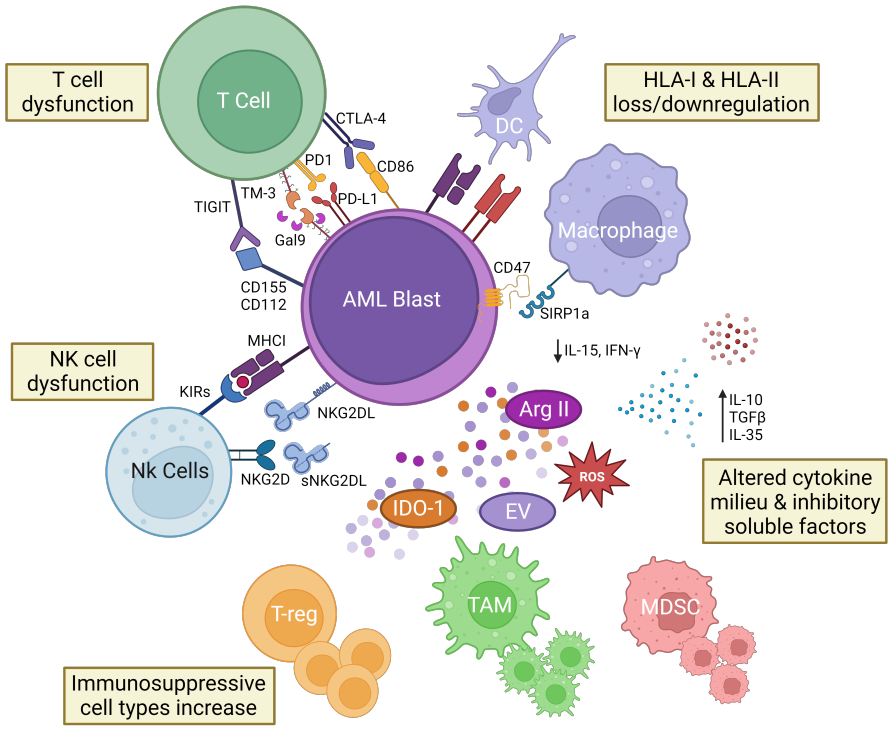

Figure 6 | Immune evasion strategies in AML. AML cells employ multiple strategies to evade immune recognition, impairing effector function and modifying the leukemic microenvironment to enhance their survival and drug resistance. Key molecules involved in this crosstalk are depicted. Created using Biorender and adapted from Tettamanti et al.[252]

In the context of NK cells, AML cells mediate immunosuppressive effects through the disruption of the balance of costimulatory ligands, which is mirrored by concomitant changes on effector cells. During malignant transformation, the expression of ligands for the NK and $CD8^+$ T cell receptor NKG2D are selectively induced on leukemia cells, activating effector cytotoxic functions.[252] To avoid NK-mediated clearance, AML cells mediate transcriptional modification of genes encoding proteins comprising the NKG2D ligandome (NKG2DL), namely *MICA*, *ULBP1*, *ULBP2*, and *ULBP3*.[260] AML cells have also been shown to release a soluble form of NKG2DL, leading to the downregulation of the NKG2D receptor and impairing NK cell cytotoxicity.[261] The role of the NKG2D/NKG2DL axis in immune evasion was exemplified in AML patient-derived xenotransplant models, where NKG2DL-expressing AML cells were cleared by NK cells, while NKG2DL-negative leukemic cells evaded NK-mediated killing.[262] Strikingly, these NKG2DL-negative leukemic cells had an immature morphology, stemness characteristics, and chemoresistance, linking the LSC concept

to immune evasion. Mechanistically, poly-ADP-ribose polymerase 1 (PARP1) was found to repress expression of NKG2DLs, providing a strong rationale for the pharmacological inhibition of PARP1 to render LSCs susceptible to NK cell control that will be evaluated in upcoming trials.[65,262] In **Article IV**, we identify a novel major histocompatibility complex (MHC) class-I molecule-mediated immune evasion strategy employed by LSCs, whereby they disrupt NK cell cytotoxicity and maturation. Our findings support the pseudotime analysis of the NK cell compartment in newly diagnosed AML, which have revealed a disruption of the maturation process with a bifurcation from conventional NK cells toward $CD56^{-}CD16^{+}$ NK cells.[263] Emerging spatially-resolved, high-throughput single-cell transcriptomic, proteomic, and mass cytometry technologies are unravelling the AML immunologic microenvironment offering insights for tailored immunotherapeutic strategies.[252]

Antibody-based treatments

Initially introduced as "magic bullets" to target disease cells, antibody-based treatments have improved the therapeutic landscape of AML due to their high specificity and capacity to active the immune system.[264] Some of the cell surface biomarkers for AML cells listed on **Table 1** have been the focus of antibody-based treatments therapeutic endeavors. Therapeutic antibodies exert anti-leukemic effects through several mechanisms, including direct signaling inhibition of the receptor, delivery of a toxic payload, and recruitment of immune effector cells.[265]

One of the most explored targets is CD33, owing to its presence on leukemic blast in around 90% of AML patients.[266] GO is a humanized anti-CD33 antibody-drug conjugate (ADC) linked to a calicheamicin-based cytotoxic moiety, which is intracellularly released once the GO/CD33 complex is internalized.[267] Following an initial retraction based on questionable benefit, the FDA has reapproved GO for newly diagnosed CD33-positive AML patients, although it comes with connotations[187] CD123 is also an attractive antigen for therapeutic intervention as it is overexpressed in a large portion of AML patients, which tend to correlate with poor outcomes.[268] It is the α chain of the interleukin 3 (IL3) receptor and promotes hematopoietic cell proliferation through activation of the PI3K/MAPK pathway and antiapoptotic protein upregulation.[269] A phase 1b/2 ongoing clinical trial is evaluating novel combinatorial regimes in AML with pivekimab sunirine, an anti-CD123 ADC with a payload that alkylates DNA, yielding promising results for *FLT3*-IDT and *ELN*-mutated AML.[243]

Refinements in antibody engineering led to the development of bispecific antibodies, which simultaneously target two tumor antigens, or target both a tumor antigen and an effector cell receptor, bringing the effector cell into close proximity to its target

cell.[265] The most widely used bispecific antibodies are T cell engagers (BiTEs), with one arm targeting CD3 on T cells and the other binding to the target cell, thereby mounting an immune response against leukemia cells.[270] Several CD33-directed BiTEs are currently in dose-escalation phases, showing modest effects in R/R AML.[270] Since then, alternative constructs have been developed from bispecific antibodies, including dual affinity retargeting (DARTs) antibodies which engage CD3 on T cells, and well as bi- and tri-specific killer cell engagers (BiKEs and TriKEs), which harness NK cells through CD16.[271] Flotetuzumab, a CD123 × CD3ε bispecific DART, has been shown to eradicate CD123-expressing primary AML cells in preclinical studies, and is being evaluated in a clinical trial for patients with primary induction failure or early relapse.[272] Importantly, almost all patients experienced cytokine release syndrome (CRS), a major adverse event frequently observed in immunotherapies.[272] CRS is a non-specific activation of the immune system that triggers pro-inflammatory cytokine release into circulation, further activating lymphoid and myeloid cells. Although it can be mitigated with step-up dosing schedules, temporary dose interruptions and immunosuppressive drugs, it is a potentially life-threatening toxicity that warrants careful consideration in clinical trials.[273]

CD47 has been under thorough investigation since it was first described by the Weissman group to be an adverse prognosis factor and potential therapeutic target on LSCs.[274] CD47 is a transmembrane antiphagocytic protein that binds to the signal regulatory protein-α (SIRPα) on the surface of macrophages and DCs, enabling immune evasion through the inhibition of phagocytosis. This "don't eat me signal" conferred by CD47 is a result of the inhibition of prophagocytic factors such as Fc receptors, complement receptor 3, and SLAMF7.[275] In follow-up studies, CD47 was found to be transiently upregulated in murine HSCs as a marker of self to provide protection against macrophage engulfment. Leukemia cells thus mimic this ability to avoid phagocytosis.[276] Magrolimab is a first-in-class humanized anti-CD47 antibody that stimulates the macrophage-mediated engulfment of cancer cells through blocking the CD47-SIRPα inhibitory checkpoint. Although of limited single agent activity, magrolimab has shown preliminary activity in combination with azacitidine, particularly encouraging among patients with *TP53*-mutated AML.[277] Despite the development of anemia as a result of CD47 expression on erythrocytes, encouraging results have led to several phase III trials with novel triplet regimen of magrolimab, azacitidine plus venetoclax in older/unfit, R/R and high risk AML.[278,279] Numerous other inhibitors of the CD47-SIRPα axis are under various stages of early clinical evaluation, highlighting the therapeutic potential of targeting this macrophage checkpoint (**Figure 7**).[117,280]

Cellular therapies

Other immunotherapies under development involve genetically engineered patient-derived immune cells to enhance their effects as living therapeutic agents. Effector cells undergo genetic modification to express receptor proteins with an extracellular antigen-binding domain and an intracellular signaling domain. Once transplanted back into the patient, these engineered cells can broadly recognize target antigens independent of the HLA background. Despite the success of cellular therapies in lymphoid malignancies, the translation has been challenging in AML, owing to the hostile milieu in the bone marrow niche and the lack of suitable, leukemia-restricted target antigens.[281,282]

Chimeric antigen receptor (CAR) T cells have been incredibly sought after for the treatment of myeloid malignancies after over 90% of patients with ALL achieved CR with anti-CD19 CAR-T cell therapy.[283] Several putative myeloid antigens targeted by CAR-T cells are currently being explored, including IL1RAP, CD33, CD38, CD70, CD123, CD135, CD371, CLL1, FLT3, TIM3, LILRB4, NKG2D and Lewis Y.[181,284] Compound CAR-T (cCAR-T) cells that simultaneously target multiple antigens are enhancing therapeutic effects, as seen for the dual targeting of CD33 together with either CD123 or CLL-1.[281,285] Combinatorial regimes of CAR-T cell therapy with novel targeted agents or chemotherapy are exhibiting synergistic cytotoxicity against AML.[286,287] Importantly, CAR-T cell therapies risk long-lasting suppressive effects on normal myeloid cells. One proposed approach to allow CAR-T cell persistence while limiting myeloablation effects is to genetically disrupt the CAR target antigen from a donor allograft using CRISPR, and then give CAR-T cells directed against this now "leukemia specific" antigen post-allogeneic HCT.[288]

Modified NK cell-based approaches have the potential to provide safer alternatives for AML targeting without the challenges associated with T cell therapies, including CRS and neurotoxicity.[289] Allogeneic NK cells are generally obtained from peripheral blood and umbilical cord, although induced pluripotent stem cells (iPSCs) derived NK cells are also becoming an option. In a phase I trial, iPSC-derived CAR-NK cells expressing noncleavable CD16 have shown early promise in R/R AML, with no dose-limiting or immune-related toxicities reported.[181]

CAR-T and -NK cell therapies are likely to be most effective in AML patients with low-burden disease, such as in the setting of MRD, in frontline, or early salvage combination approaches.[117] The ultimate goal is to engineer universal 'off-the-shelf' allogeneic CARs that could be manufactured in bulk from healthy donors and be readily available on demand.[290,291] **Figure 7** illustrates the main immunotherapies in AML, including antibody-based drugs, CAR therapy, checkpoint inhibitors, and vaccines.

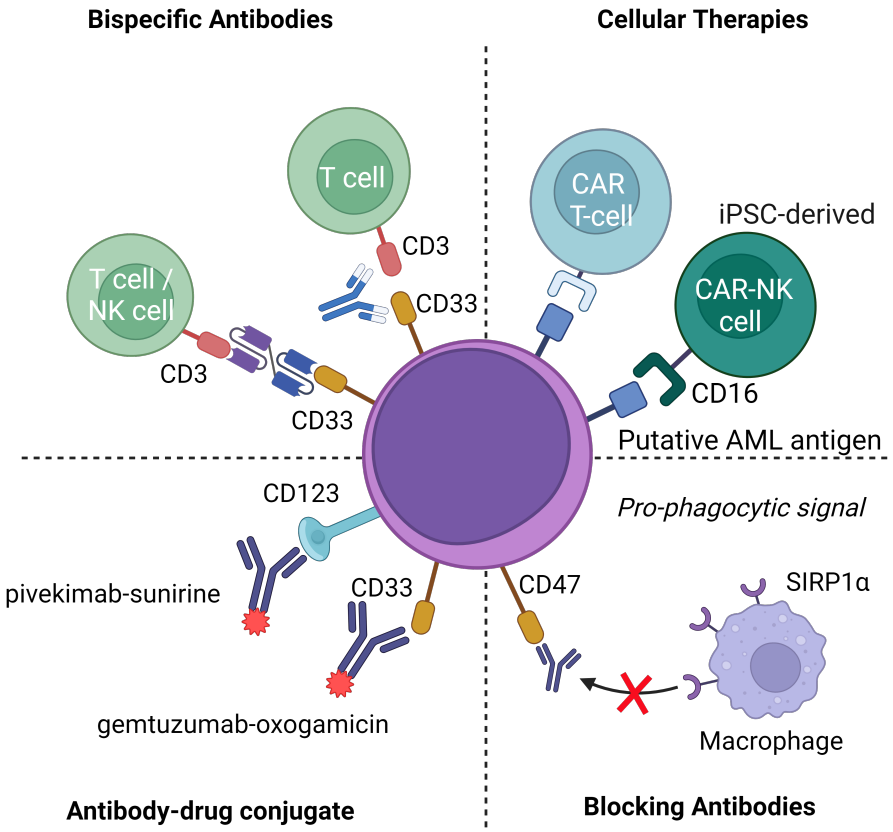

Figure 7 |. **Emerging immunotherapies in AML**. Representative immunotherapeutic strategies are shown, including antibody-drug conjugates (ADCs), bispecific antibodies, blocking antibodies, and cellular therapies. Created using Biorender and adapted from Tian et al.[292]

Metabolism in AML

Cell metabolism comprises intricate multienzyme systems that coordinate to convert nutrients into energy currencies, biomass and building blocks for macromolecules. These metabolic progresses are indispensable for a myriad of cellular biological processes, including proliferation, antioxidant defense, and the overall regulation of the transcriptome and epigenome.[293] It was in the 1950s when Otto Warburg first recognized the propensity of cancer cells to favor glycolysis, a rapid yet less energetically efficient pathway compared to OXPHOS - a phenomenon famously known as the "Warburg effect."[294] Since then, elucidating how cancer cells rewire their metabolism to sustain oncogenic pathways has revealed specific metabolic dependencies that have been the basis for effective therapeutic strategies.[293] A significant milestone in targeting cancer metabolism was reached with the approval of enasidenib and ivosidenib, which specifically target IDH1/2-mutated AML.[193,195,240] More recently, the disrupting energy metabolism drug venetoclax has reshaped the therapeutic landscape for hematological malignancies, setting new standards of care for older or unfit AML patients.[117,191,295] As we gain a better understanding of the metabolic properties in AML, with a particular focus on LSCs, the prospects of translating this knowledge into effective metabolism-targeting therapies with superior clinical outcomes becomes increasingly promising.

Metabolic profiles of HSCs, LSCs and leukemic blasts

Mammalian cells predominantly generate energy currency in the form of adenosine triphosphate (ATP) through two pathways, glycolysis and mitochondrial OXPHOS. OXPHOS is highly metabolically efficient, yielding 36 ATP molecules from glucose as opposed to only two produced by glycolysis.[296] The relative contribution of these pathways to total ATP production varies based on cell type, growth rate and microenvironment.[296] This variability extends to hematopoietic cells, where global metabolic and proteomic profiling of HSCs, LSCs and AML blasts has revealed distinct metabolic profiles (**Figure 8**).[297–299]

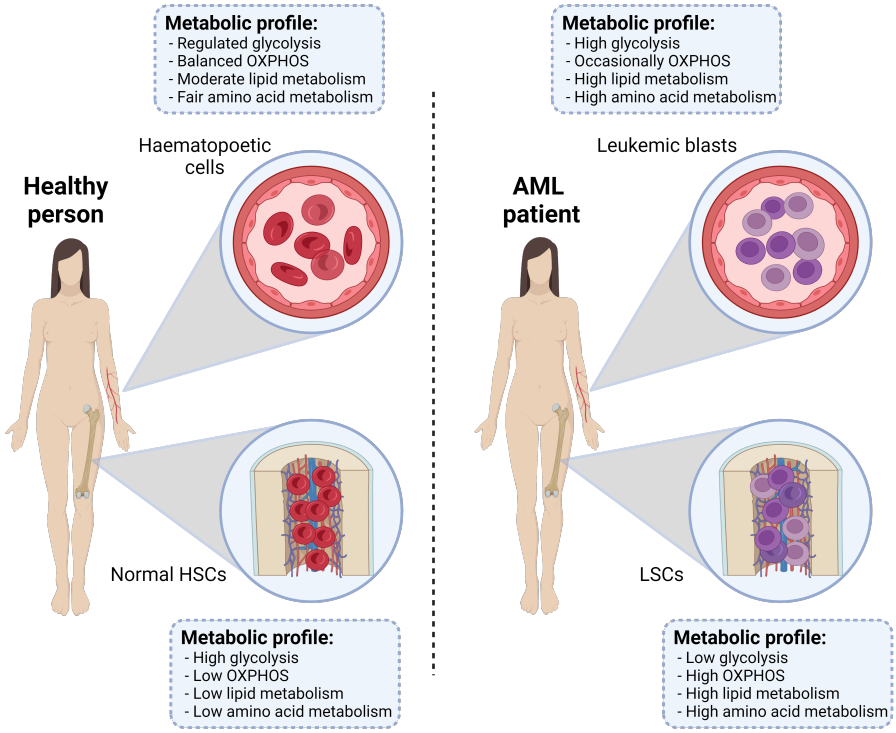

Figure 8 | Key distinctions in metabolic profiles between normal and leukemic cells. Normal hematopoietic cells, HSCs, LSCs and leukemic blasts rely on different energy pathways to produce ATP. While HSCs and mature leukemic blasts are highly glycolytic and are more reliant on glycolysis, LSCs are more dependent on OXPHOS, amino acids metabolism, and fatty acid metabolism. Created using Biorender, adapted from Mesbahi et al.[300]

Generally, bulk AML blasts fit the classical Warburg effect; they are dependent on glycolysis for ATP production and have decreased viability when cultured under limiting glucose conditions.[298] Apart from energy generation, AML blasts upregulate glycolysis to generate building blocks for the macromolecules needed to sustain their proliferative and leukemic state, including nucleotides, amino acids and lipids.[294,301] Interestingly, it has become apparent that stem cells and more mature blasts exhibit distinct metabolic differences, while the nuances of energy metabolism in HSCs and LSCs are less clear.[302] HSCs preferentially rely on glycolysis, however, their metabolic program is dependent on their activation state. During quiescence, HSCs rely on anaerobic glycolysis in their low-oxygen hypoxic niche, and shift to OXPHOS to meet increased energy demands upon differentiation.[303,304] This reliance on glycolysis and the presence of the hypoxic microenvironment niche minimizes production of ROS, which can contribute to stem cell ageing, inducing an exit from quiescence, and compromising their population maintenance.[304] Similarly to HSCs, LSCs also maintain

low ROS levels to maintain their state of dormancy while decreasing their oxygen and energy requirements to promote leukemogenesis.[297] Despite their low spare respiratory capacity for OXPHOS, that is, the amount of ATP that can be produced by the mitochondria upon energy demand, LSCs are paradoxically more reliant on OXPHOS for survival.[297,305] Recent work from the Jordan lab has shown that due to their decreased glycolytic activity, LSCs are dependent on alternative metabolic fuels such as amino acids to provide substrates for OXPHOS. Specifically, they showed that AML patient-derived LSCs are unable to efficiently catabolize both glucose and fatty acids.[298] Additionally, they have demonstrated that while AML blasts and HSCs can upregulate glycolysis to compensate for reduced OXPHOS, LSCs do not have this capacity due to exhibiting little to no glycolytic reserve capacity.[297] These findings argue for an intrinsic metabolic inflexibility of LSCs, a characteristic that provides opportunities for therapeutic intervention.[302]

Nonetheless, the role of glucose and glycolysis in LSC maintenance remains debated. Recent research has shown that a decrease in glycolytic gene expression, as well as diminished pyruvate and lactate production, compromised LSC maintenance without affecting ATP levels, underscoring the dependence of glycolysis in LSC maintenance, but not through energy suppling.[306] Another study revealed that while quiescent LSCs preferentially rely on OXPHOS, LSCs in an active and cycling state, a characteristic found in *MLL*-r leukemia, depend on glucose metabolism.[307,308] Additionally, the notion that LSCs lack metabolic plasticity has also been disputed, where the hypoxic niche has been shown to prompt LSCs to undergo a metabolic switch from OXPHOS to glycolysis in CML.[309] Importantly, increased metabolic flexibility has also been observed in LSCs isolated from relapsed AML patients, which shift their reliance from amino acids to glucose and fatty acids to synthesize TCA cycle intermediates.[298]

In **Article II**, we emphasize the significance of glucose metabolism for murine *MLL::AF9* LSCs, as inhibiting glycolysis had a selective inhibitory effect on LSCs compared to their normal counterparts. Additionally, inhibition of OXPHOS sensitized patient-derived AML blasts to glycolytic inhibition, albeit to different degrees, suggesting potential therapeutic benefits from combining metabolism-targeting drugs.[310] Collectively, these proof-of-principle studies challenge the relevance of the Warburg model for hematological malignancies, and reveal remarkable metabolic heterogeneity within AML blast and LSC populations.

Dysregulated metabolism and therapy resistance

Aberrant metabolic signaling not only drives leukemogenesis but also confers resistance to conventional chemotherapy. Indeed, chemotherapy-resistant LSC phenotypes are shaped by various mechanisms that act alone or in cooperation, including genetic aberrations, alternations in gene expression and epigenetics, and metabolic dysregulations.[65]

To identify metabolic contributors of chemoresistance, several studies have characterized the metabolic profiles of persistent AML cells in relapse phase after chemotherapy. In one study, primitive leukemic populations that resisted chemotherapy exhibited low rate of energy metabolism and reduced cellular oxidative status.[297] LSCs metabolically adapt to maintain low ROS levels through cell intrinsic processes such as the activation of the metabolic stress regulator AMPK, or through juxtaposition to hypoxic niches.[311,312] This state of dormancy is a prominent resistance mechanism for LSCs, as conventional chemotherapeutics are more effective against rapidly proliferating AML blasts.[300] Notably, recent research has challenged the view that treatment resistance is limited to LSC persistence, as AML blasts cells with retained OXPHOS activity and high proliferation rates also exhibited resistance to cytarabine.[313] In this study, they correlated the high OXPHOS-related treatment resistance to increased fatty acid oxidation (FAO) mediated by upregulated CD36 expression.[313] In another study from the same group, the extracellular ATPase CD39 was found to play a role in increasing mitochondrial biogenesis and respiration in chemoresistant AML cells.[314]

The microenvironment in which AML cells reside in significantly influences their metabolic properties. In one study, BMSCs were found to support chemoresistance by enhancing the bioenergetic capacity of LSCs through OXPHOS, TCA cycle activity, and glutathione-dependent antioxidant defenses, thereby promoting disease progression.[95] Mitochondrial transfer from BMSCs to AML cells has also been reported to sustain resistance to cytarabine.[315] Another study highlighted transient metabolic adaptations in persisting residual AML cells following chemotherapy, with these cells increasing their glutamine metabolism through assistance of BMSCs. Specifically, BMSCs provided AML cells with aspartate, fueling malignant glutathione and nucleotide synthesis, which contributed to chemoresistance and enabled their survival.[316]

Overall, it is crucial to determine if chemotherapy treatment favors the selection of specific metabolic states, such as OXPHOS-high cells, or if it causes an increase in OXPHOS in all cells. Furthermore, as therapeutic regimes for hematological malignancies evolve towards targeted and non-chemotherapeutic agents, efforts are

focused on understanding whether metabolic alterations are correlated with or responsible for leukemogenesis.[302]

Targeting energy metabolism

With an increased understanding of the intersections between metabolism and leukemogenesis, several strategies to inhibit metabolism have emerged and are in ongoing clinical trials (**Figure 9**).[304] One strategy involves specifically targeting metabolic activities under oncogenic control, which may be categorized into transforming, enabling, or neutral based on their contribution to cancer initiation.[317] Enzymatic mutations that induce metabolic reprogramming to directly drive cancer transforming activities is a prime area of focus, as blocking them might prevent or arrest tumorigenesis in susceptible patients.[317] Recurrent somatic mutations in *IDH1* and *IDH2* are an example of transforming metabolic perturbations that have been successfully targeted in AML by approved inhibitions.[193,195,240] Given that AML cells have distinct metabolic dependencies often irrespective of their genetic background, targeting metabolic states rather than specific genetic lesions is a particularly relevant strategy.[297] This has been exemplified with venetoclax, which has shown therapeutic efficacy across multiple AML subtypes through metabolic perturbations that suppress OXPHOS.[191,192,295] However, as with other cancer therapies, resistance to metabolic inhibitors has been reported and linked to distinct transcriptomic profiles, such as mutations in *RAS* pathway genes and *TP53*.[318–320] These underlying mutations influence energy metabolism and confer a competitive advantage for AML cells in patients, reinforcing the notion that a "one-size-fits-all" approach is not a suitable therapeutic strategy. Thus, recognizing and accounting for this inter-patient metabolic heterogeneity may help identify and overcome the mechanisms driving therapeutic resistance.

Glycolytic metabolism

In cancer cells, glycolysis is the universal pathway for catabolism of glucose into pyruvate, which is preferentially converted into lactate rather than allowing it to enter the TCA cycle under both anaerobic and aerobic conditions.[321] Glucose is sourced from dietary carbohydrates and cellular glycogen, which undergoes a series of 10 cytosolic reactions during glycolysis. The first 5 reactions constitute the "investment" phase, as they consume ATP molecules, while the remaining "pay-off" reactions yield ATP.[321] Besides generating energy, glycolysis also supplies glucose to other coupled pathways to provide molecular building blocks, such as the pentose phosphate pathway (PPP) which

is needed for nucleotide synthesis and generation of NADPH as the universal electron carrier.[322] Given that glycolytic dysregulation has been implicated in leukemogenesis, targeting components of the glycolytic pathway has been explored therapeutically (**Figure 9**).[323]

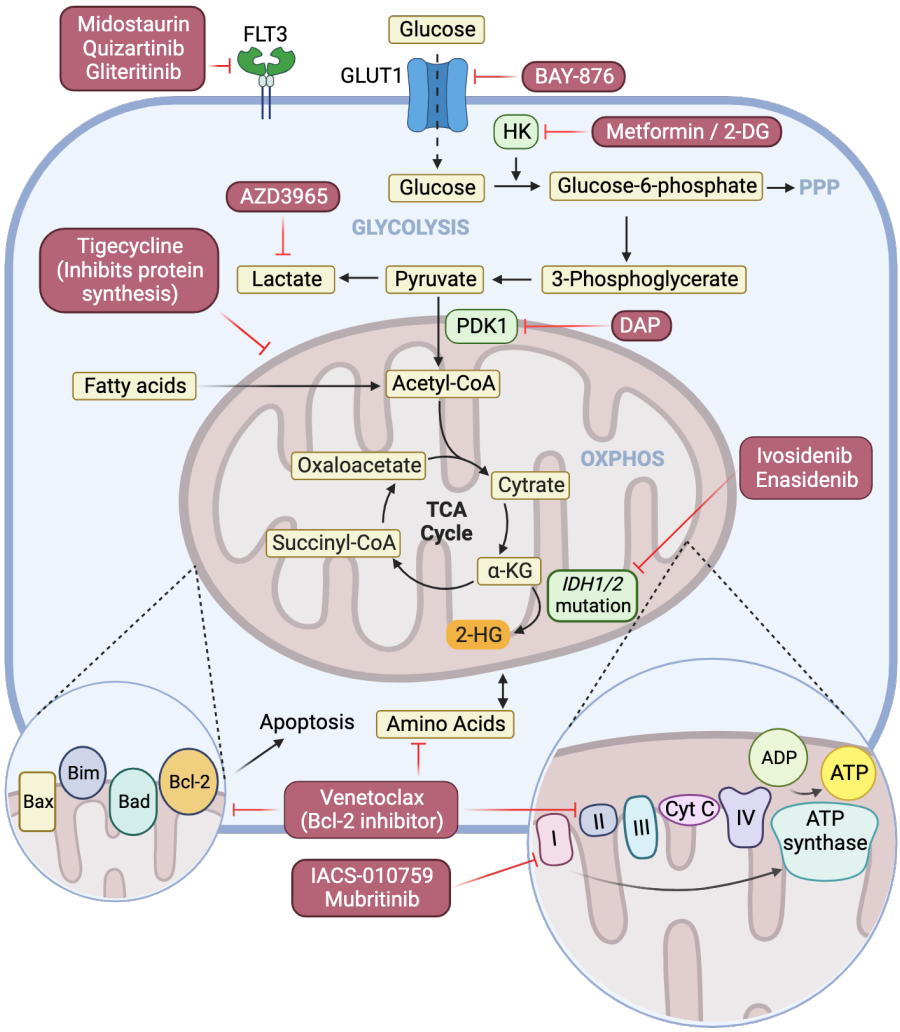

Figure 9 | Targeting energy metabolism in AML. Potential therapeutic strategies targeting components of glycolysis and mitochondrial metabolism are shown. Metabolites are represented by yellow boxes, metabolic enzymes by green boxes, and therapeutic agents by red boxes. HK, hexokinase; PDK1, pyruvate dehydrogenase kinase 1; α-KG; α-ketoglutarate; 2-HG, 2-hydroxyglutarate. Created using Biorender and adapted from Stelmach et al.[65]

Several independent studies have reported that upregulated glycolysis confers chemoresistance in patients with AML, however the prognostic value of increased glycolytic metabolism has been disputed.[323–325] On one hand, high aerobic glycolysis in AML blasts at diagnosis were predictive of an improved outcome to induction therapy; on the other, a high prognosis risk score based on expression of 6 glycolytic metabolites predicted a poor outcome independent of other prognostic factors.[324,325] Nonetheless, the use of glycolytic inhibitors as anti-leukemic agents or as modulators of chemoresistance in AML have been reported.[323] In a recent study, the cytotoxic effects of seven metabolic inhibitors were tested on patient-derived AML cells, three of which suppressed glucose metabolism. The molecular targets for these inhibitors ranged from MCT1 that regulates lactate transport (AZD3965), to the first rate-limiting glycolytic enzyme hexokinase (HK; metformin and 2-DG). Despite the heterogenous response between individual patients, inhibition of glycolysis was reported to have the strongest cytotoxic effect in the majority of patients irrespective of genotype, karyotype or other known risk factors.[326] However, as these inhibitors are affected by both intracellular and systemic levels of glucose, they are predicted to exhibit strongest sensitivity in glycolysis-driven leukemia cells.[326] Indeed, the relevance of system glucose availability in supporting leukemia cell growth has been recently reported. AML cells are thought to adopt a parasitic state whereby they simultaneously hijack glucose from normal cells depriving them from glycolytic fuel, while increasing glucose availability to drive their own growth.[327] Disrupting this adaptive homeostasis suppressed leukemia progression, highlighting the need to approach glucose metabolism from a holistic perspective, considering the organism as a whole rather than viewing solely as a cell intrinsic process.[327]

Several other studies have examined how modulating glucose metabolism through inhibition of other glycolytic enzymes downstream of HK affect leukemic cell populations. Specifically, inhibition of PFKFB3 (phosphofructokinase) and PKM2 suppressed AML in several *in vitro*, *in vivo*, and xenograft models.[328–330] Additionally, in a late phase clinical trial, the serine/threonine kinase mTOR complex (mTORC1) was found to be critical in promoting glycolysis and glucose addiction in AML cells.[331] This dependency on glucose was essential for maintaining a continuous energy supply through the PPP, serving as a pro-survival mechanism. These findings rationalize the potential therapeutic targeting of glucose-6-phosphate dehydrogenase (G6PD), the key-limiting enzyme within the PPP.[331]

Another effective strategy for suppressing glycolysis is by blocking substrate uptake through inhibition of the main glucose transporters.[332] As glucose is hydrophilic, it needs to be transported across the lipid bilayer through specialized membrane carrier proteins. The genes encoding these glucose transporters are classified into three families: SLC2A genes that code sodium-independent glucose transporters (facilitated

transport, GLUT proteins), SLC5A genes, encoding sodium-dependent glucose symporters (secondary active transport, SGLT proteins), and SLC50A genes, which encode the recently discovered SWEET protein.[333] The GLUT family has been the most well studied, comprising 14 members (GLUT1-14) differing in substrate selectivity, tissue expression, and subcellular localization. These carriers can be further divided based on substrate specificity and sequence homology into class I, which have high selectivity for glucose (GLUT1-4 and 14), class II, with selectivity for glucose and fructose (GLUT5, 7, 9, and 11), and the rather uncharacterized class III (GLUT6, 8, 10, 12, and 13). Within the Class I group, GLUT1 and GLUT3 exhibit the highest affinity for glucose and have been recognized to be overexpressed in many types of cancer to maintain an unhindered supply of glucose for fueling glycolysis. As such, GLUT1 dependency in particular represents an important hallmark in cancer. [333,334]

The proven therapeutic potential of inhibiting GLUT1 in cancer has led to the exponential development of anti-GLUT1 antibodies, small molecules, antisense cDNA, short hairpin RNAs (shRNAs), and microRNAs (miRNAs), among others.[333,335] In AML, several natural and synthetic GLUT1 inhibitors have exhibited strong anti-leukemic effects when tested in cell lines *in vitro*.[336–339] Furthermore, targeting GLUT1 also sensitized human AML cell lines to cytarabine, findings of clinical significance as overexpression of GLUT1 is associated with poor chemotherapy response in patients with AML (**Figure 9**).[339]

Overall, inhibiting glycolysis has mainly been investigated in AML patients at diagnosis or in AML blasts, needing further investigation in the context of AML relapse and LSCs.[300] In **Article II**, we explore the therapeutic value of inhibiting GLUT1 either by single guide RNA (sgRNA)-mediated genetic disruption or by pharmacological inhibition using BAY-876 in *MLL::AF9*-driven AML.[310] We observed significant growth inhibitory effects in murine LSCs *in vivo* and in patient-derived AML cells *ex vivo*, supporting the feasibility of GLUT1 inhibition as a therapy for AML patients. Furthermore, we characterized the otherwise unexplored mechanisms regulating GLUT1 dependency in AML cells, as well as examine potential factors contributing to increased sensitivity to GLUT1 inhibition.[310]

Mitochondrial metabolism

Mitochondria, often referred to as cellular powerhouses, serve as a central metabolic hub where diverse metabolic pathways fueled by glucose, amino acids, and fatty acids converge to feed into the TCA cycle. For the TCA cycle to function, a constant supply of acetyl-CoA is needed, which is derived from various catabolic processes involving these substrates.[340] The TCA cycle generates reducing equivalents (NADH and FADH2) that transfer high-energy electrons to the electron transport chain (ETC).

This process creates a proton gradient across the mitochondrial membrane, which in turn, drives ATP synthesis through OXPHOS.[340] The heightened reliance of LSCs on substrates to fuel OXPHOS has drawn attention to the pharmacological inhibition of mitochondria-related pathways or direct perturbation of mitochondrial components as an attractive therapeutic strategy in AML (**Figure 9**).[295,297,298]

Pyruvate is irreversibly decarboxylated to acetyl-CoA by pyruvate dehydrogenase complex (PDC) and as such, lies at the intersection between glycolysis and OXPHOS defining a critical bioenergetic step. Pyruvate dehydrogenase kinases (PDKs) inhibit PDC activity through phosphorylation, inactivating the conversation of pyruvate into acetyl-CoA and inducing a concomitant glycolytic shift.[341] Regulation of pyruvate flux through inhibition of the PDC/PDK axis has thus become an attractive anticancer strategy.[341] Inhibition of PDK1 with 2,2-dichloroacetophenone (DAP) was shown to block AML proliferation and induce apoptosis *in vitro* and in injection-based xenografts.[342]

In another study, loss of PDK1 drives glycolytic cells into an OXPHOS state, resulting in mitochondrial stress-mediated toxicity in AML cells but not in normal counterpart cells.[343] Interestingly, high levels of PDK1 correlated with low OXPHOS and stemness transcriptional signatures, suggestive of a potential therapeutic avenue to eradicate LSCs, particularly in patients exhibiting low mitochondrial respiration activity.[343]

Direct targeting of ETC complexes has also exhibited significant anti-leukemic efficacy in pre-clinical AML models, findings that have recently translated into testing of several inhibitors in clinical trials.[302] For instance, inhibition of the ETC complex I using mubritinib, a canonical inhibitor of the tyrosine kinase ERBB2, elicited strong anti-AML effects *in vitro* and *in vivo*.[344] Similarly, IACS-010759, a highly selective small-molecule inhibitor of complex I, mediated its cytotoxic effects through a combination of energy depletion, reduction of aspartate production, and ultimately an impairment in nucleotide biosynthesis.[345] Importantly, both inhibitors suppressed OXPHOS, elicited the strongest effects in primary leukemias exhibiting OXPHOS hyperactivity, and specifically targeted LSCs while leaving normal stem and progenitor cells unaffected.[344,345] However, in two phase I trials in patients with R/R AML and solid tumors, IACS-010759 was found to have a narrow therapeutic window with emergent dose-limiting toxicities.[346] These toxicities involved a compensatory increase in glycolysis as evidenced by elevated lactate levels.[346] This metabolic adaptation implies a potential therapeutic benefit of using combination strategies involving glycolytic-suppressing agents to mitigate the neurotoxic effects reported. In **Article II**, we investigated a synergistic combination treatment using IACS-010759 and identified a specific AML subtype that could potentially benefit from it.[310] Our research aligns with previous studies that have reported synergistic effects when combining IACS-010759

with either venetoclax or the FLT3 inhibitor AC220 (quizartinib) in AML cell lines and patient-derived samples.[347,348] In another study, inhibition of the ETC complex II was found to be particularly effective in primary *FLT3*-ITD-mutated AML patients cells, attributed to elevated succinyl-CoA ligase levels and an OXPHOS-driven metabolic state. Notably, AML cells metabolically rewired to import extracellular lactate to fuel OXPHOS, a vulnerability that was effectively exploited by co-inhibiting ETC complex II and lactate transport.[349]

Indirect targeting of OXPHOS has included strategies such as inhibiting the translation of mitochondrial-encoded ETC proteins. One notable example is tigecycline, an antibiotic that suppresses the synthesis of mitochondrial ETC components including mtCOXI and mtCOXII, effectively inhibiting OXPHOS and selectively targeting primary AML LSCs.[350] These promising findings led to a phase I trial evaluating intravenous infusion of tigecycline in refractory AML patients, although no clinical responses were observed and limitations related to drug pharmacokinetics were reported.[351] Additional strategies for indirectly disrupting OXPHOS involve the inhibition of mitochondrial proteases, such as ClpP, and modifications in post-translational processes, such as glutathionylation. Both of these approaches impair ETC activity and LSC survival (**Figure 9**).[352,353]

In light of the distinct use of amino acids, fatty acids, and glucose to fuel and provide TCA cycle intermediates between normal cells and LSCs, the potential anti-leukemic effect of limiting substrate availability is under active investigation. Among the 20 amino acids, 9 are essential for normal cellular function, and cannot be synthesized *de novo* or are inadequately synthesized relative to metabolic demands. The remaining 11 are considered nonessential and participate in oxidation-reduction reactions, macromolecule biosynthesis, and signaling.[354] Until recently, several essential (branched-chain amino acids) and nonessential (glutamine and cysteine) amino acids had been shown to be important in several hematological malignancies.[355–357] However, it was not until the metabolome of LSCs from *de novo* AML patients was interrogated that the role of amino acid metabolism in powering OXPHOS represented a point for therapeutic intervention.[298] In this study, pharmacological inhibition of amino acids uptake was shown to reduce TCA cycle flux and induced selective LSC death.[298] Importantly, relapsed LSCs exhibited decreased reliance on amino acids, compensating for this by upregulating fatty acid metabolism to generate TCA cycle intermediates.[298] Fatty acids are metabolized through β-oxidation, producing acetyl-CoA, NADH and FADH2, thereby fueling OXPHOS to generate ATP.[340] However, both amino acid and fatty acid metabolism have been implicated in driving OXPHOS in relapsed AML. Indeed, inhibiting both pathways significantly reduced OXPHOS and eliminated relapsed LSCs more efficiently than blocking only one pathway.[319] In line with these findings, increased energy metabolism pathways, including amino acid

catabolism, FAO, and glycolysis, have been reported in relapsed LSCs, all of which were supported by an increased synthesis of NAD^+. [358] The targeting of FAO and its role in fueling OXPHOS represents an exciting new direction in overcoming LSC-mediated therapeutic resistance in AML.[298,304,319] Nevertheless, understanding the specific patterns of AML relapse and the dependence of leukemia cells on OXPHOS as a metabolic reprogramming pathway is crucial for tailoring therapies to effectively disrupt persistent LSCs.

Venetoclax - a success story

Venetoclax is a highly selective BH3 mimetic BCL-2 inhibitor that can restore activation of apoptosis in AML.[359] The drug neutralizes BCL-2 and releases a break on the pro-apoptotic effector proteins BAX and/or BAK. These effectors oligomerize, creating pores in the mitochondrial outer membrane, causing a release of cytochrome c and caspase activation, ultimately inducing the apoptosis pathway.[359] Given that BCL-2 has been implicated in hematological malignancies as a way of evading apoptosis by sequestering pro-apoptotic proteins, BCL-2 represented a putative therapeutic target for AML.[360] Preclinical testing of venetoclax in AML exhibited has yielded promising results in combination with hypomethylating and chemotherapeutic agents.[359] The proven benefit of venetoclax-based therapies led to the recent FDA approval of the combination of venetoclax and HMA azacytidine for treatment-naïve elderly patients or those unfit for chemotherapy.[191] With over 60 trials evaluating the therapeutic effect in AML as a single agent or in combination, venetoclax is rapidly changing the treatment paradigm for this disorder.[359,361]

In a pivotal study, Pollyea et al. hypothesized that the deep and durable remissions induced by venetoclax + azacytidine in AML patients were attributed to targeting LSCs. They showed that venetoclax toxicity was related to metabolic perturbations that suppressed OXPHOS in LSCs, in particular, by disrupting the TCA cycle as evidenced by suppressed α-ketoglutarate and increased succinate levels.[295] Building on these studies, ongoing efforts have focused on further characterizing the mechanisms by which venetoclax selectively eradicates LSCs through disruption of the metabolic machinery driving energy metabolism. Importantly, despite an impressive rate of response to venetoclax + azacytidine of 70% in AML, there is upfront resistance as well as relapse following initial response in a large amount of patients.[304] This has been linked to LSCs exhibiting molecular and metabolic plasticity in both intra- and inter-patient settings, becoming resistant to BCL-2 inhibition, ultimately becoming the drivers of relapse.[65] Several drivers of venetoclax resistance have been described, ranging from metabolic states, to genetic diversity, mitochondrial structural changes and supporting microenvironment components. Within LSCs, increased FAO mediated by

upregulation of fatty acid transporter CD36 and mutations in TP53 and/or RAS pathway genes have been shown to contribute to venetoclax resistance.[319,320] Accordingly, a combination of venetoclax + azacitidine together with a CD36 inhibitor or through inhibition of fatty acid metabolism in the mitochondria restored the sensitivity of relapsed LSCs to treatment.[298,319] These $CD36^{+}$ LSCs can also metabolically adapt to source fuels for FAO from the adipose tissue niche within the microenvironment, sparing the cost of energy synthesis.[362] Synergistic anti-leukemic effects have also been reported with venetoclax in combination with statins, which inhibit the mevalonate pathway, through upregulation of pro-apoptotic modulators, emphasizing the role of lipid metabolism in treatment response.[363] Targeting glutaminolysis, the process by which the nonessential amino acid glutamine is converted into TCA cycle metabolites, has also been reported to synergize with BLC-2 inhibition via suppression of the glutaminase isoform GLS1.[364] Interestingly, using a metabolically focused loss of function CRISPR screen, heme biosynthesis was recognized as a key apoptosis-modifying pathway that cooperated with BCL-2 to sustain survival. Indeed, inhibition of heme biosynthesis suppressed ETC activity, potentiating venetoclax-induced permeabilization of the mitochondrial outer membrane and apoptosis.[365] Additionally, genes involved in mitochondrial structure remodeling and integrated stress response also contributed to resistance, and their inhibition sensitized AML cells to venetoclax treatment.[366,367]

Developmental stage has also been reported to influence response, where monocytic AML but not primitive AML is resistant. These resistant monocytic AML exhibited decreased levels of BCL-2 and relied on MCL1 to maintain elevated OXPHOS for survival. This differential sensitivity drove a selection of monocytic subpopulations at relapse.[318] In a recent follow up study, this monocytic disease progression was tracked back to a distinct type of LSC different from the well-described primitive LSC, which displayed a unique immunophenotype and transcriptional state, and a reliance on purine metabolism.[368]

Taken together, these studies demonstrate that combinatorial approaches are needed to target the inherent and adaptive metabolic reprogramming in LSCs to effectively overcome resistance to standard and venetoclax-based therapies. Given the heightened adaptability of relapsed LSCs in using multiple fuel sources and their increased heterogeneity, including LSC-targeting strategies in first line regimens is expected to be more effective. Moreover, there is a need to address the challenge of identifying biomarkers that can help categorize patients who would benefit from venetoclax-based therapy.[65]

Iron metabolism

Cellular metabolism also critically relies on iron due to its catalytic properties, making it an indispensable nutrient. It enables the activities of various enzymes involved in fundamental cellular processes, including ATP production, DNA synthesis, antioxidant defense, as well as oxygen transport and sensing, among others.[369] The ability of iron to undergo electron exchange between its oxidized ferric ($Fe3^+$) and ferrous ($Fe2^+$) forms enables its involvement in free radical-generating reactions. Among these processes is the Fenton reaction, where ferrous iron donates an electron to hydrogen peroxide to yield hydroxyl radicals, thereby inducing highly invasive ROS.[370] While ROS plays an essential role in multiple cellular pathways regulating cell survival, proliferation and differentiation, the aberrant accumulation of iron and subsequent excess ROS production can be detrimental. This scenario can induce oxidative stress, damage to DNA, proteins or lipid, or ultimately cause cell death.[371] Consequently, maintaining iron homeostasis to mitigate iron toxicity requires coordinated regulation of iron acquisition, utilization, storage and efflux, as a dysregulation of this balance can contribute to cancer development.[372] Notably, leukemia cells exhibit an increased demand for iron compared to normal cells in order to sustain their rapid growth and proliferation.[373] Iron has also been found to influence the course of clonal myeloid disorders and cause alterations in the leukemic microenvironment, thereby increasing disease morbidity.[374] This marked dependency on iron in the pathogenesis of leukemia renders leukemia cells susceptible to perturbations in iron metabolism, a vulnerability that has been exploited as a therapeutic target in AML (**Figure 10**).[369,374,375]

Physiological iron absorption and regulation

Circulating iron in the body is regulated by several cell types. Dietary iron, primarily in the $Fe3^+$ form, is converted to $Fe2^+$ via ferric reductase and absorbed in the duodenum by enterocytes through the divalent metal transporter 1 (DMT1).[376] Erythrocyte precursors in the bone marrow incorporate iron into hemoglobin, which constitutes the majority of the total body iron (60-70%).[377] Macrophages in the liver, spleen, and bone marrow recycle iron by phagocytosing senescent erythrocytes and releasing the iron back into the circulation. Iron absorbed by enterocytes is either released into circulation through the iron efflux pump ferroportin 1 (FPN1) or stored in ferritin (FTH). On the basolateral membrane, $Fe2^+$ is oxidized by ferroxidase hephaestin, to then be loaded onto transferrin (Tf) in the plasma.[376] Although Tf-bound iron represents less than 0.1% of total body iron, it is the most critical iron pool with the highest turnover rate.[377] The holo-Tf complex Tf-$Fe3^+$ circulates in the plasma

in a redox-inert state, delivering iron to its sites of utilization, mainly the bone marrow for erythropoiesis.[376]

Cellular uptake of Tf-bound iron primarily occurs through the transferrin receptor 1 (TFR1), also known as CD71, which serves as the main source of cellular iron import. The transmembrane TFR1 binds diferric Tf, forming a Tf-$Fe3^{+}$/TFR1 on the cell surface, which is then taken in by receptor-mediated endocytosis. Within the endosome, acidification driven by the proton-pump vacuolar-type ATPase (v-ATPase) promotes a conformational change in Tf, leading to iron release. Iron is subsequently reduced to $Fe2^{+}$ by six-transmembrane epithelial antigen of the prostate (STEAP) proteins and transported into the cytoplasm via DMT1. Simultaneously, the apo-Tf/TfR1 complex is recycled to the cell surface, where apo-Tf is released into the plasma.[376] The released $Fe2^{+}$ constitutes the cytosolic labile iron pool (LIP), a pool of chelatable and redox-active iron that can serve various metabolic needs within the cell or be stored in FTH. Excess cellular iron can be exported through FPN1 after re-oxidation.[376] Iron in the LIP, whether in simple ionic form or incorporated into heme or iron-sulfur (Fe-S) clusters, functions as a cofactor for numerous enzymes such as cytochromes, catalase and other iron-containing proteins.[378] At the cellular level, iron homeostasis is post-transcriptionally regulated through the binding of regulatory proteins IRP1 and IRP2 to iron-responsive elements (IREs) within mRNAs. The role of IRPs is to sense intracellular iron levels and adapt by controlling the expression of proteins that regulate iron import (TFR1, DMT1), storage (FTH), and export (FPN1).[379]

TFR1-targeted therapies

Various approaches have been explored to target iron metabolism, including the modulation of iron regulatory proteins, iron reduction through chelation, and iron overload (**Figure 10**).[374] Given that TFR1 is overexpressed in several hematopoietic malignancies and constitutes the rate-limiting step to cellular iron uptake, targeting TFR1 represents a promising therapeutic target in AML.[375,380] The extracellular accessibility of TFR1 has been harnessed in cancer therapeutics, employing blocking agents to antagonize receptor function and delivering immune effectors or therapeutic molecules to malignant cells.[380,381] The anti-tumor effects of TFR1 inhibition were first explored more than 40 years ago.[382] Since then, several strategies have been developed, such as using Tf, targeting peptides, anti-TFR1 antibodies, and antibody fragments.[380] Anti-TFR1 antibodies, for instance, have demonstrated potent anti-leukemic effects *in vitro* and in murine models.[383–386] These antibodies employed different modes of action, including preventing receptor internalization through surface cross-linking or inducing receptor degradation, impairing recycling and consequently decreasing cell surface

TFR1.[381] These approaches ultimately block iron uptake, leading to impaired TFR1 function and lethal iron deprivation. [381,383–386] Other novel therapeutic approaches have been investigated in AML, including Tf-conjugated nanoparticles for delivering microRNAs targeting mutated genes *(DNMTs*, *CDK6*, *SP1*, *KIT* and *FLT3*) or for enhancing the effectiveness of specific inhibitors.[387,388]

Notably, the prognostic value of TFR1 expression in AML remains debated. In one study, TFR1 levels were observed to increase as cells progress from myeloid dysplasia to leukemia, correlating with the degree of differentiation. TFR1, together with CD34 expression, were thus proposed as cues for understanding clonal development in leukemia.[389] On the other hand, another study found that elevated TFR1 levels, even when linked to complex karyotypes or stemness markers like CD117, did not impact survival outcomes.[390] Concerns have also arisen regarding TFR1-targeted therapies due to the crucial role of iron in hematopoiesis, particularly in erythropoiesis, which is a process that consumes the majority of circulating iron.[391] Indeed, *Tfr1* deficiency and subsequent cellular iron deficiency impaired the proliferation and differentiation of hematopoietic precursor cells and reduced the regeneration potential of HSCs.[392] Thus, further studies are required to establish an optimal therapeutic window that avoids iron-deprivation toxicities. Encouragingly, a phase I clinical trial demonstrated a favorable safety profile for a new human monoclonal anti-TFR1 antibody (PPMX-T003) in healthy individuals. PPMX-T003 is currently being tested in a Phase I/II dose-escalation study in patients with aggressive NK-cell leukemia. Subsequent trials will need to establish both the safety and therapeutic efficacy of TFR1 inhibition in clinical settings (clinical-trials.gov identifier NCT05863234).

From iron chelation to iron overload

In leukemia, iron chelators are primarily employed to manage chronic iron overload arising from frequent blood transfusions, which can result in cardiac, hepatic, or endocrine damage if left untreated.[393] Deferoxamine (DFO) and deferasirox (DFX) have been FDA approved for this purpose.[374] Pre-transplantation use of iron chelators in patients with MDS or acute leukemia undergoing myeloablative HCT has also been speculated to improve OS.[394] Recently, their role has expanded beyond iron overload management, with a growing focus on their potential as anti-leukemic agents.[369,374,375]

These iron-chelating compounds, whether naturally occurring or synthetic, exhibit unique modes of action and are characterized by distinct biochemical and pharmacological properties.[374] For instance, DFO and DFX have demonstrated anti-leukemic activity by reducing the LIP and suppressing ROS production.[395,396] However, the effect of iron chelators on ROS homeostasis in AML cells has been conflicting. In another study, DFX selectively induced cell death in primitive $CD34^{+}CD38^{-}$ patient-

derived AML cells through inhibition of HIF1α expression and induction of ROS.[397] Similarly, both DFO and DFX had ROS-inducing effects in AML blast cells, which led to monocytic differentiation.[398] In this same study, a patient with refractory AML/MDS who received oral DFX exhibited decreased AML cell counts and increased differentiation, suggesting the potential of using iron chelators to induce ROS-dependent differentiation.[398,399] The effect of iron chelator treatment on ROS appears to depend on factors such as the binding properties of the chelator, treatment duration and concentration.[398] These iron chelators have also been shown to sensitize leukemic cells to conventional chemotherapy drugs (doxorubicin and cytarabine), demethylating agents (decitabine), differentiating agents (vitamin D3) and DNA repair inhibitors.[395,398,400–402] Additionally, newer iron chelators like the synthetic di-2-pyridylketone thiosemicarbazone Dp44mT, have exhibited potent and selective antitumor activity, through sequestration of iron and redox cycling of its iron complex to generate ROS.[403] Despite its high affinity to iron, Dp44mT can also bind to copper, forming redox-active complexes that contribute to its potent cytotoxicity.[404] Dp44mT has shown broad activity against several subtypes of acute leukemia, inhibiting the proliferation of cell lines *in vitro*, inducting cell cycle arrest, and promoting caspase-mediated apoptosis.[405] There have been two documented clinical trials investigating the therapeutic potential of iron chelators in AML. One trial involved the use of DFX alone in chemotherapy-naïve individuals (NCT02413021), while the other assessed DFX in combination with azacytidine and vitamin D3 in newly diagnosed elderly patients (NCT02341495). However, these trials were either halted due to insufficient patient enrollment or have yet to disclose results.

Another way to leverage the increased iron dependency of leukemia cells is to consider the vulnerability that this creates. Ferroptosis, a novel iron-mediated form of regulated cell death, has emerged as a paradoxical yet promising strategy to target leukemia cells. It is a progress dependent on intracellular iron and differs from apoptosis, necrosis, and autophagy in terms of morphology, biochemistry, and genetics.[406] Mechanistically, ferroptosis induces iron-mediated ROS accumulation, leading to membrane lipid peroxidation, compromised cellular integrity, and ultimately oxidative cell death.[406] Ferroptosis can be inhibited by iron chelators, lipophilic antioxidants, lipid peroxidation inhibitors, and polyunsaturated fatty acids (PUFA) depletion agents. Conversely, it can be induced by iron accumulation, PUFA-phospholipid buildup, and a deficiency in endogenous inhibitors like GSH, NADPH, GPX4, or vitamin E.[407] Recent studies have highlighted the anti-leukemic potential of ferroptosis inducers like erastin and RAS-selective lethal molecule 3 (RSL-3), which synergize with chemotherapeutic agents.[408,409] The activation of ferroptosis by erastin or RSL-3 hinges on the inactivation of glutathione peroxidase 4 (GPX4), an antioxidant enzyme

that converts toxic lipid hydroperoxides into non-toxic lipid alcohols, thus causing lipid peroxidation and cell death (**Figure 10**).[410]

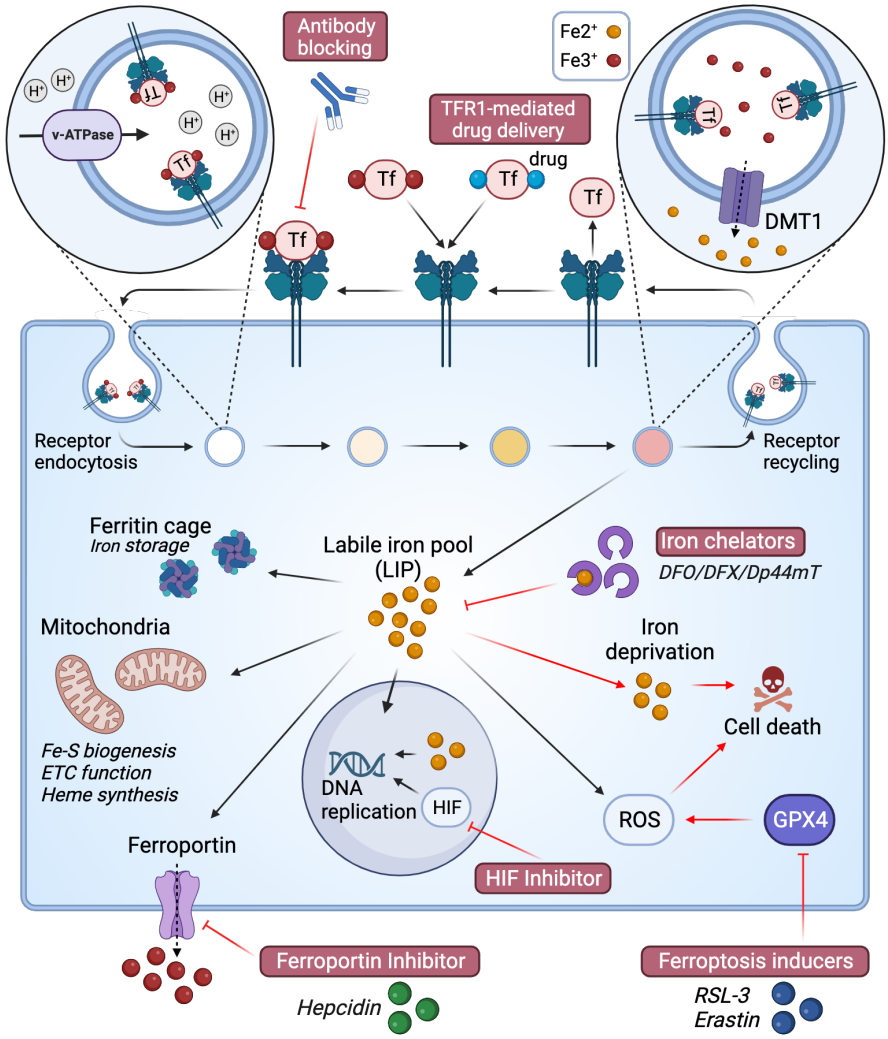

Figure 10 | **Targeting iron metabolism in AML**. Iron deprivation can be mediated through TFR1-targeting therapies, iron chelators and inhibition of HIFs and their target genes. Inhibition of hepcidin-ferroportin axis can increase cellular iron export also depriving the cell of iron. Iron overload and ferroptosis is induced by inhibition of cellular antioxidant defenses such as GPX4, rendering the cell prone to ROS accumulation from iron metabolism and leading to lipid peroxidation. Created using Biorender and adapted from Morales et al.[412]

In **Article III**, we uncover the previously unknown role of TFR1 in regulating iron metabolism in *MLL::AF9*-driven AML cells, with a focus in studying the anti-leukemic effects of TFR1 disruption within LSCs. Our findings align with the growing recognition of the value of targeting iron metabolism in cancer stem cells.[411] Moreover, we demonstrate that iron depletion mediated by DFO and Dp44mT induces remarkable sensitivity in patient-derived AML cells, while having minimal effects on normal bone marrow cells. Our research provides deeper mechanistic insights into the role of iron metabolism in AML and underscores the potential of iron-based treatments. These treatments are likely to benefit from combination strategies with other anti-leukemic agents, effectively lowering the sensitivity threshold of cancer cells to therapy.

CRISPR/Cas9 screens

The pursuit of understanding cellular phenotypes at the molecular level has traditionally involved extensive experimentation, often without the certainty of revealing the crucial molecular determinants. This longstanding challenge has driven the search for innovative tools and technologies to simplify the complex process of unraveling cellular intricacies. Among these innovations, the CRISPR/Cas9 system emerges as a transformative genome editing tool that has revolutionized the field of molecular biology. CRISPR screens exploit the efficiency and flexibility of CRISPR/Cas genome editing and have enabled the unbiased interrogation of gene function in a broad range of applications and species.[413] They have accelerated the pace of molecular descriptions of cellular systems by offering an efficient and highly targeted approach to manipulate the genetic makeup of living organisms. Researchers can design RNA sequences to guide the non-specific endonuclease Cas9 enzyme to specific DNA sequences, facilitating target gene activation, knockout, or manipulation with unprecedented precision.

CRISPR screening has already uncovered numerous molecular mechanisms in diverse files, including basic biology, medical genetics, cancer research, immunology, infectious diseases, and microbiology.[413–415] However, a particularly promising application lies in the realm of drug discovery, where high-content CRISPR screens have streamlined the process of drug target identification and validation, simplifying the initial phases of drug development and opening doors to the era of personalized medicine (**Figure 11**).[415]

CRISPR as a genome editing tool

Our capacity to manipulate DNA through genome engineering plays a pivotal role in elucidating the functions of both coding and non-coding regions of the genome. The first genome editing techniques relied on homologous recombination, which proved to be laborious, time consuming and had inefficient editing ability.[416] These methods were preceded by two programmable nucleases, zinc-finger nucleases (ZFNs) and TALENs. While they enabled precise genome editing, these techniques still posed challenges such

as off-target effects, technical complexities, and resource intensiveness.[417] The most recent addition to this arsenal is the reprogrammable CRISPR/Cas9 endonuclease system, renowned for its high editing efficiency, ease of use, and cos-effectiveness.[417]

This system, comprising of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas), was originally discovered as an inheritable adaptive immune system in prokaryotes to prevent phage infection more than 30 years ago.[418,419] In 2012, Nobel laureates professor Emmanuelle Charpentier and professor Jennifer Doudna recognized a programmable target-site recognition and DNA cleavage guided by dual-RNAs, highlighting the potential of the CRISPR/Cas9 system in RNA-programmable genome editing.[420] The CRISPR/Cas9 naturally relies on two key RNAs for Cas9 specificity: CRISPR RNA (crRNA), containing a sequence complementary to the target site, and a trans-activating crRNA (tracrRNA), which facilitates Cas9 binding. The crRNA contains a recognition sequence, approximately 21 nucleotides long, that determines target specificity. This specificity is further defined by the presence of a protospacer adjacent motif (PAM), in the case of *Streptococcus pyogenes* Cas9, is defined as an NNG motif. To simplify the system for laboratory use, these targeting RNAs have been engineered into a single molecule known as a sgRNA, capable of performing the functions of both crRNA and tracrRNA.[420]

Targeted gene knockout mediated by the CRISPR/Cas9 system involves double strand DNA breaks in a site-specific manner. Subsequently, the cellular "error-prone" DNA repair machinery introduces mutations such as frame-shift mutations or premature stop codons. DNA repair mechanism are divided into non-homologous end joining (NHEJ) and homology-directed repair (HDR).[421] While HDR can repair DNA with high fidelity, it requires sequence homology to either side of the DNA break to mediate the repair. HDR-mediated repaired allows for precise genome edits such as point mutations or insertions of DNA pieces through recombination of the target locus with exogenous donor DNA template. NHEJ is the most common mode of DNA repair and has no requirement for sequence homology.[421]

Drop out functional screens

In a typical CRISPR screen, a library of sgRNAs is designed to target either a predefined set of genes (in pooled CRISPR screens) or the entire genome (in genome-wide CRISPR screens).[422] A CRISPR sgRNA library is introduced into target cells such that each cell receives a single sgRNA to ensure each cell is only perturbed according to the sgRNA received. These sgRNAs are normally delivered into the target cells through lentiviral transduction, where they become integrated into the DNA of the host cell.

This integration enables efficient identification of the perturbations induced based on the sgRNA sequence, serving as molecular barcode.[413] Cas9 is either stably expressed in the cells or ectopically delivered as a plasmid, virus, mRNA or protein, leading to gene knockout in the presence of both sgRNA and Cas9. The edited cells exhibiting single gene ablations are challenged to selective pressures such as drug treatment, viral infection, or cell proliferation, causing them to compete with each other based on the effect on fitness conferred by the genetic disruption. By comparing the sgRNA representation between the pool of cells retained between different challenges or time points, the genes conferring a sensitization (depleted sgRNAs) or a selective advantage (enriched sgRNAs) to the challenge can be identified. The quantification of sgRNA abundance is typically conducted using high-throughput sequencing techniques (**Figure 11**).[413]

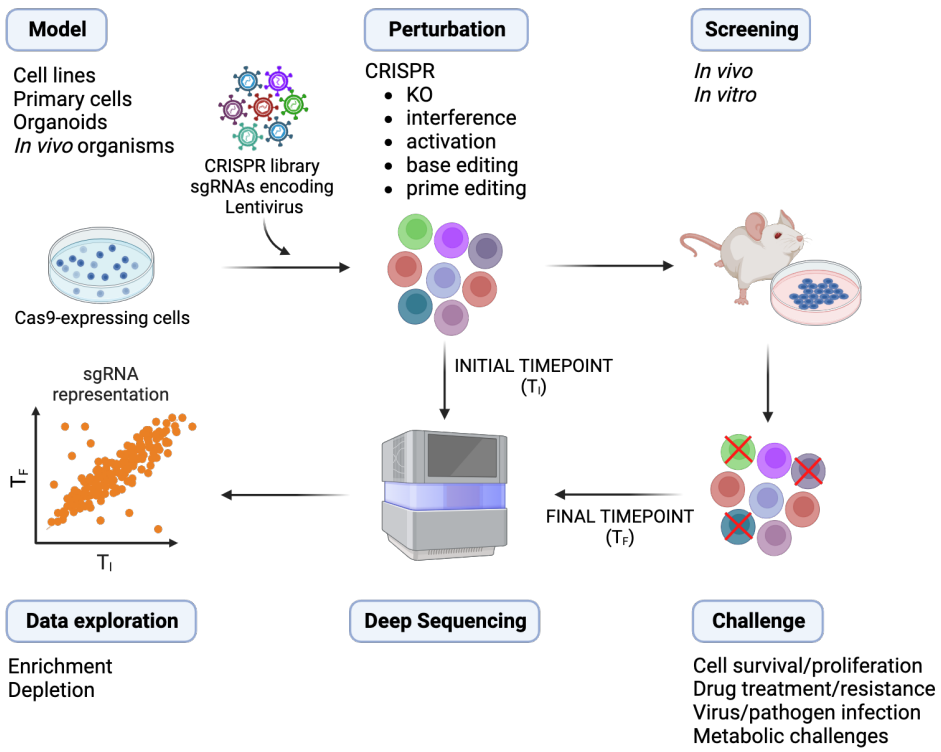

Figure 11| Experimental design for CRISPR/Cas9 screening. In a typical CRISPR/Cas9 screen, Cas9-expressing cells are perturbed through transduction of a sgRNA lentivus library and are challenged through selective pressures *in vitro* or *in vivo.* A read-out of the induced molecular or cellular defects is assessed through deep sequencing. Created using Biorender and adapted from Bock et al.[413]

Choosing an appropriate model system that effectively mirrors the relevant biological processes and is adaptable for genetic screening is the first step in a successful CRISPR screen.[413] Immortalized cell lines offer a cost-effective and manageable model for exploring biological processes in vitro. To address more physiological relevant dependencies, screening can be performed in primary cells, in tissue explants or in organoids.[413] Such screens can also be performed *in vivo*, albeit at a smaller scale compared to what is feasible with *in vitro* screens. When conducting screening in mouse models, there are two primary approaches. Cells can be edited *ex vivo* prior to transplantation, or the sgRNAs and Cas protein complex can be administered directly into the animal for *in vivo* editing.[413] *In vivo* screens provide invaluable insights on genetic dependencies in a biologically complex and context-dependent environment.

Screening in AML

High-throughput CRISPR screening plays an instrumental role in uncovering genetic susceptibilities linked to AML in this thesis (**Articles I-IV**). Genetic screening methods in AML have evolved over time, with early approaches primary relying on shRNA pooled libraries for use in RNA interference (RNAi) screens.[423] These shRNA libraries were employed to systematically silence the expression of individual target genes in cell and animal models, providing insights into the intricate molecular landscape of AML while identifying potential vulnerabilities. Notable work from the Ebert lab and others demonstrated the therapeutic relevance of these screening approaches, revealing unique dependencies specific to AML compared to normal hematopoietic cells, such as Integrin beta 3 and Csnk1a1 signaling.[220,424,425]

The limitations related to knockdown robustness and off-target effects in RNAi-based gene silencing were substantially overcome with the implementation of CRISPR/Cas9 screens in AML research. Notably, when conducted *in vivo*, these screens enabled the identification of essential genes specific to the physiological niche, which may have otherwise remained elusive if solely studied through *in vitro* screens.[426] In **Articles I** and **IV**, we conducted two distinct *in vivo* CRISPR screens, revealing two key regulators: CXCR4, which provides essential signaling support for AML development independently of its ligand CXCL12, and H2K1, which protects leukemia cells from NK cell-mediated immune surveillance. These regulatory mechanisms were not evident *in vitro*, demonstrating that the microenvironment can influence the essentiality of a target.[427]

The scalability of *in vivo* pooled libraries has also improved in the context of AML research, evolving from small libraries comprising of fewer than one hundred genes to

more recent approaches that assess thousands of genes.[220,424,428] It is advisable to incorporate at least four distinct sgRNAs for each target gene, along with positive and negative control genes for screen validation. As a result, enhancing scalability has become imperative to ensure sufficient sgRNA representation, yield reliable findings, and encompass a sufficiently large number of genes for comprehensive analysis.[413] Nevertheless, well-designed and validated sgRNA libraries with fewer sgRNAs per gene can still yield reliable results.[429] The extent of the scalability *in vivo* has even been shown to reach genome wide scale, where the RNA-binding protein STAUFEN2 was identified as a critical dependency for myeloid leukemia.[430] While these genome-wide *in vivo* screens offer added opportunities to identify novel regulators, they may also come with a trade-off in terms of sampling depth and reliability of potential discoveries.[413]

Advancements in model systems have further enhanced the usefulness of CRISPR/Cas9 screens. In particular, *in vivo* models employing PDXs have emerged as invaluable tools for guiding future research efforts, although challenging to use due to low engraftment efficacies [431] These models enable researchers to prioritize molecular components with the greatest relevance for therapeutic intervention, as they faithfully mirror the intricate interplay between genetic factors and the environment observed in AML patients.[413] Looking ahead, the insights gained from *in vivo* CRISPR/Cas9 screens will play a central role in shaping the development of targeted therapies, offering newfound hope for more effective and personalized treatments for individuals battling AML.

Present investigation

AML is a cancer resulting from the accumulation of immature myeloid progenitor cells unable to differentiate into mature blood cells. Therapeutic developments have focused on optimizing existing chemotherapeutics, which have substantially improved patient outcome and are effective at targeting leukemic blasts. However, similar to embers that can reignite a fire, quiescent LSCs are not extinguished from these therapies, often leading to disease relapse.[304] Thus, targeted approaches aimed at eradicating these disease-causing LSCs are urgently needed for sustained AML remissions. LSCs exhibit distinct molecular, metabolic, epigenetic, and immunological features, affording them a survival advantage and the ability to adapt to therapeutic pressures, ultimately leading to the development of treatment resistance. Identifying AML-specific dependencies reveals potential therapeutic vulnerabilities that can be leverage for the development of novel and highly effective targeted therapies.

Aims of the study

This thesis aimed at identifying cell surface molecules essential for AML cell survival and to elucidate the molecular networks driving leukemia progression, with the ultimate goal of developing novel therapeutic strategies. We employed a multifaceted approach integrating functional CRISPR/Cas9 screens, transcriptomics, metabolomics, and other molecular tools in primary patient material and in leukemia mouse models, pursuing three main objectives:

- Identify novel cell surface receptors critical for LSC progression *in vivo* and functionally characterize the most promising candidates (**Articles I** and **II**).
- Investigate the dependency of LSCs on the transferrin receptor TFR1 and assess the anti-leukemic potential of iron chelation in patient-derived AML cells (**Article III**).
- Identify and characterize the role of the MHC class-I molecule H2-K1 in immune surveillance escape (**Article IV**).

Summary of articles

Article I

CXCR4 signaling has a CXCL12-independent essential role in murine MLL-AF9-driven acute myeloid leukemia

AML is propagated by LSCs, which reside in the highly specialized microenvironmental niche where they are thought to be protected from genotoxic insults. Bidirectional interactions between leukemic cells and the microenvironment favour leukemic progression at the expense of healthy hematopoiesis.[83] These interactions are often mediated by molecules on the cell surface of LSCs, which have unique signaling functions that may be activating or inhibitory. Given their role in mediating cellular communication, combined with their utility as cellular markers and their extracellular accessibility, cell surface proteins represent optimal targets for pharmacological intervention.[432] In order to identify cell surface receptors essential for LSC activity in a physiologically relevant setting, we performed a pooled *in vivo* CRISPR/Cas9 drop out screen targeting 96 genes encoding cell surface proteins known to be upregulated in murine *MLL::AF9* (*KMT2A-MLLT3*) LSC-enriched (c-Kit$^+$) cells.[427] This strategy allowed probing of both cell-intrinsic programs essential for leukemia progression and microenvironment-responsive signals that are essential for LSC survival but may be missed in an in vitro setting.[413]

Results in short

- We performed an *in vivo* CRISPR/Cas9 screen in a murine model of *MLL::AF9* AML and identified CXCR4 as an essential regulator of LSCs.
- The expression of the CXCL12 ligand in the bone marrow was dispensable for AML development.
- Genetic disruption of CXCR4 induced oxidative stress and myeloid differentiation in LSCs.

We identified the chemokine receptor CXCR4 as the top-ranked regulator in the screen, as well as other known dependencies of AML, such as the macrophage checkpoint CD47 and the HOXA9 transcription factor, confirming the robusness of the screen. In normal hematopoiesis, CXCR4 plays an essential role in homing and retention of HSCs in the bone marrow niche.[42,433] CXCR4 is significantly elevated in patient-derived AML cells and correlates with poor prognosis, however, its functional role in AML has remained elusive.[434] We found that sgRNA-mediated disruption of *Cxcr4* profoundly depleted *MLL::AF9* LSCs in the bone marrow and spleen of

sublethally irradiated transplanted mice. This effect was not observed when culturing the cells in vitro, suggestive of a critical role of CXCR4 signalling mediating AML cell growth and survival *in vivo*. In contrast to the well established role of CXCR4 in HSC homing, it was unclear if these effects were also present in AML cells. In one study, homing of primary human AML cells to the bone marrow of NOD/ SCID mice was shown to be CXCR4-dependent, while another reported CXCR4 to be dispensable for the engraftment of human $CD34^+$ AML cells in NOD/ SCID mice.[103,435] We found that while CXCR4 was essential for leukemia development *in vivo*, it was dispensable for their homing to the bone marrow. Using RNA-seq, we found *Cxcr4* disruption resulted in a distinct gene expression signature which, upon gene set enrichment analysis (GSEA), revealed enrichment of pathways associated to oxidative stress and differentiation. In agreement with the transcriptome analysis, loss of Cxcr4 led to an accumulation of cellular ROS, activation of the p38-MAPK known to be induced upon oxidative stress, and upregulation of the myeloid differention marker GR-1.[436]

CXCL12 is the main ligand for CXCR4 and is a membrane bound or soluble homeostatic chemokine mainly produced by BMSCs. CXCL12 signalling mediates support and retention of HSPCs in the niche, confirmed in a study showing that disruption of *Cxcl12* in endothelial cells depleted HSPCs from the bone marrow.[42] We used transgenic mouse models that were either globally devoid of CXCL12 expression in all tissues, or with *Cxcl12* knocked out specifically in endothelial cells or mesenchymal progenitor cells. Interestingly, we found that in contrast to normal HSPCs, CXCL12 expression is despensible for *MLL::AF9* AML development, suggesting that leukemia cells are less dependent on these niches for growth support and maintenance.

To further characterize the role of how CXCR4 signalling promotes AML development, we generated two mutated retroviral *Cxcr4* variants with amino acid substitutions in codons with critical functional relevance. $CXCR4^{D99G}$, harbouring a mutation in the extracellular domain of the receptor leading to an inability to bind to CXCL12, and $CXCR4^{L251P}$, with a mutation in the transmembrane signaling domain, resulting in a signaling-dead receptor. Using a wild-type Cxcr4 cDNA construct as reference, we found that while $Cxcr4^{D99G}$ rescued depletion of leukemia cells *in vivo*, the signaling-dead variant $CXCR4^{L251P}$ failed to do so. These findings demonstrated that CXCR4 signalling but not CXCL12 binding is essential for *MLL::AF9* AML development. Besides CXCL12, extracellular UBIQUITIN and macrophage migration inhibitory factor (MIF) have two other known CXCR4 ligands.[437,438] Culture media supplementation with these ligands failed to significantly stimulate growth and survival of LSCs in a CXCR4-dependent manner. Our data thus suggests that CXCR4 provides baseline signalling to promote and sustain leukemia cell survival *in vivo* independent of ligand stimulation.

In conclusion, this study highlights the use of *in vivo* CRISPR screening as an effective explorative platform for defining novel dependencies of AML progression. We identify a critical role of CXCR4 in maintaining LSC activity independent of CXCL12 stimulation by protecting them from oxidative stress and differentiation.

Article II

Combined GLUT1 and OXPHOS inhibition eliminates acute myeloid leukemia cells by restraining their metabolic plasticity

The inability to fully eradicate LSCs with conventional treatments remains one of the most important challenges in AML therapy. Based on the potential benefits of LSC targeting as a mean of eliminating chemoresistant populations and the source of relapse, multiple LSC-directed strategies have been employed. Targeting energy metabolism has recently emerged as an effective therapeutic strategy that can eradicate LSCs in AML patients.[302] Metabolic targeting strategies involve specifically targeting transforming metabolic perturbations, as is the case with inhibitors of *IDH* mutations, or targeting metabolic states by leveraging the distinct metabolic profiles exhibited between AML cells and normal hematopoietic cells, as with the BCL-2 inhibitor venetoclax.[193,240,295] In this study, we scale up our previous screen and perform an *in vivo* CRISPR/Cas9 screen using a sgRNA library targeting almost one thousand genes encoding cell surface proteins expressed in murine *MLL::AF9*-driven AML cells.[427] We identified GLUT1 as the strongest-scoring dependency of LSCs in the screen, highlighting a potentially actionable metabolic reliance of AML cells *in vivo*. We systematically assess the vulnerability of patient-derived AML cells and murine LSCs to GLUT1 inhibition, while using transcriptomic and metabolomic analyses to identify the molecular basis underlying GLUT1 dependencies.

Results in short

- GLUT1 is an essential regulator of energy metabolism driving *MLL::AF9* LSC growth and survival both *ex vivo* and in the bone marrow niche.
- The inhibition of GLUT1 suppresses bioenergetics and induces autophagy as a metabolic adaptation.
- A dual inhibition of GLUT1 and OXPHOS eliminates human AML cells.

AML cells take up glucose through GLUT1, a specialised transmembrane transporter that facilitates the transport of glucose across the plasma membrane. Overexpression of GLUT1 is an important hallmark in cancer, and is associated with increased glycolytic rates and resistance to chemotherapy in AML, constituting a promising treatment strategy.[323,333,334] Here, we showed that sgRNA-mediated *Glut1* knockdown in

MLL::AF9 LSCs suppressed leukemia development *in vivo*, validating the findings from in the original screen. The strong anti-leukemic effect also translated ex vivo, indicating that besides potential extrinsic signals regulating GLUT1 from the microenviroment at play, there are also cell autonomous GLUT1-mediated mechanisms driving leukemia. By performing molecular, morphological and transcriptomic analyses, we found that *Glut1* ablation arrested AML progression by inducing late-stage apoptosis accompanied by activation of apoptotic markers PARP and Caspase-3, and myeloid differentiation.

Transcriptional profiling revealed that a disruption of *Glut1* associated with significant downregulation of genes involved in OXPHOS and the TCA cycle. Importantly, we correlated RNA-seq data with metabolomics, the latter of which has recently emerged as an important tool for characterizing the enzymatic activity and byproducts of metabolic pathways in AML.[439] *Glut1*-mediated glucose uptake disruption led to an overall suppression of multiple metabolic pathways in LSCs, reflected by a significant reduction in the level of metabolites belonging to glycolysis, PPP, and the TCA cycle. As an adaptation to glucose limiting conditions, *MLL::AF9* AML cells were found to metabolically reprogram to elevate amino acid levels through an induction in autophagy, a catabolic process acting to sustain core metabolic functions under nutrient starvation. The role of autophagy as a pro-survival mechanism to augment the import and/or synthesis of amino acids was confirmed by the synergistic anti-leukemic effect of combining GLUT1 and autophagy inhibition. LSCs isolated from *de novo* AML patients have recently been reported to be uniquely reliant on amino acids metabolism to drive OXPHOS pathways and survival. This dependency was less relevant for the survival of HSPCs, thus pharmacological inhibition of amino acid metabolism was found to selectively target LSCs in AML patients.[298] Our findings thus suggest a potential for using GLUT1 inhibition as a way of sensitizing LSCs to targeting with inhibitors of amino acid metabolism.

To assess the feasibility of targeting GLUT1 in a therapeutic context, we used the small molecule BAY-876 which, benchmarked against other reported GLUT inhibitors, has been shown to be highly potent and selective for GLUT1 over other glucose transporters.[440] Consistent with the effects seen upon sgRNA-mediated *Glut1* knockdown, BAY-876 treatment of *MLL::AF9* leukemia has selective growth inhibitory effects ex vivo and reduced leukemia burden in transplanted recipients following a daily 10 day treatment. The anti-leukemic effects of BAY-876 also translated into prolonged survival, highlighting the therapeutic efficacy of pharmacological targeting of GLUT1 in the *MLL::AF9* leukemia mouse model.

Given the robust anti-leukemic effect of GLUT1 inhibition in murine LSCs, we evaluated whether human AML cells isolated from the bone marrow were dependent on GLUT1. Interestingly, treatment with BAY-876 alone resulted in no obvious effect

on growth inhibition in human AML cell lines nor in patient-derived AML cells. We thus speculated whether, in response to limited glucose conditions, leukemia cells were metabolically rewiring to alternative metabolic pathways to acquire alternative non-carbohydrate sources of fuel to sustain their high metabolic needs.[332] Indeed, restraining their metabolic plasticity using cotreatments with BAY-876 and the OXPHOS inhibitor IACS- 010759 led to a strong synergistic dose-dependent reduced viability in human AML cells. Notably, we could stratify patient samples into responders (7 of 12) and non-responders (5 of 12) based on their response to the dual treatment. Genomic examination revealed that all 4 samples belonging to the *RUNX1*-mutated subtype clustered in the responder group and exhibited stronger sensitivity to the combination treatment compared to the effects observed in $CD34^+$ normal bone marrow cells. By performing seahorse-based bioenergetic analyses in *RUNX1*-mutated AML, we confirmed suppression of glycolysis and OXPHOS by BAY-876 and IACS-010759 treatment, respectively.

In light of the treatment-induced changes in the metabolic profile in AML cells, we interrogated the TCGA AML cohort for differential transcriptomic signatures between *RUNX1*-mutated and non-*RUNX1*-mutated AML that could predict responsiveness to the combination treatment. We observed that the *RUNX-1* mutated signature exhibited negative enrichment of OXPHOS-related pathways, which correlated with markedly higher expression of pyruvate dehydrogenase kinase 1 (*PDK1*) and retinoblastoma tumor suppressor (*RB1*). PDK1 and RB1 have been described to be metabolic gatekeepers and determinants of metabolic states in multiple cancer types. Specifically, a loss of these regulators mediates a metabolic switch from a glycolytic to an OXPHOS state and increases mitochondrial respiration to allow for the use of alternative fuels.[343,441,442] Notably, RB1 levels strongly correlated with the degree of sensitivity to genetic or BAY-876-mediated inhibition of GLUT1 in triple negative breast cancer, highlighting a targetable RB1-GLUT1 metabolic axis.[442] Based on these studies, we could hypothesize that *RUNX1*-mutated AML exhibit a glycolysis-biased metabolism, a feature that has been attributed to loss of function *RUNX1* mutations, rendering this subtype particularly susceptible to GLUT1-directed therapies.[443]

Collectively, this work identifies and characterizes the metabolic dependency of murine *MLL::AF9* LSCs on GLUT1 for sustained leukemia survival. We reveal a pro-survival metabolic adaption in leukemia cells following *Glut1* disruption involving an increase in amino acid supply as a result of autophagic activation. In human AML cells, dual inhibition of GLUT1 and OXPHOS act synergistically to counteract metabolic reprogramming, suggesting a strong rationale for combinatorial targeting of multiple metabolic pathways to achieve superior therapeutic benefits for patients with AML.

Article III

TFR1 inhibition has p53-dependent therapeutic efficacy in acute myeloid leukemia

Cellular metabolism relies on iron due to its catalytic capabilities, enabling the activity of enzymes involved in essential cellular processes such as energy production, DNA synthesis and antioxidant defense.[369] Cancer cells exhibit perturbations in iron acquisition, efflux, storage, and regulatory pathways, underlying a pivotal role for iron metabolism in driving tumorigenesis.[412] In light of the increased iron demand of leukemia cells to sustain high proliferation, several therapeutic strategies modulating iron metabolism are being explored in the management of hematological malignancies. These approaches range from modulation of iron regulatory proteins, to iron deprivation or overload.[369,373–375] One promising therapeutic target in AML is TFR1, given its heightened expression across multiple leukemia types and its role as a major bottleneck in iron uptake.[375,380] In a recent *in vivo* CRISPR/Cas9 screen, we found that TFR1 was required for the survival of mouse *MLL::AF9* leukemia cells enriched for LSCs.[310] In this study, we sought to explore the undefined role of TFR1 in AML, with a particular focus on investigating the molecular determinants by which a disruption in iron availability induces anti-leukemic effects.

Results in short

- TFR1-mediated iron regulation is essential for LSC survival, and its disruption suppresses leukemia development in a p53-dependent manner.
- *Trfc* knockdown leads to a p53-mediated transcriptional repression of antioxidant defense and mitochondrial respiration pathways.
- Iron chelation has selective cytotoxicity against patient-derived AML cells.

Cellular uptake of iron primary occurs through TFR1 in a Tf-bound form, which is internalized as a complex by receptor-mediated endocytosis. Through endo-lysosomal acidification mediated by the proton-pump v-ATPase, iron is internalized into the cytosol where it makes up the LIP for use in numerous cellular processes.[376] Interestingly, besides TFR1, a catalytic subunit of the multi-subunit v-ATPase, ATP6V1A, also scored among the strongest dependencies in the screen. Through sgRNA-mediated disruption of *Tfrc* and *Atp6v1a*, the genes encoding TFR1 and ATP6V1A, respectively, we could show that *MLL::AF9* leukemia cells were rapidly depleted both under culturing conditions and in transplanted recipient mice, thus validating the findings in the screen. We also confirmed that the disruption of these iron regulators profoundly depleted the LIP, essentially compromising the crossroad of cellular iron traffic. Consistent with our findings, in two independent studies performing CRISPR/Cas9 screens, it was revealed that inhibition of v-ATPase depleted

intracellular iron, in turn leading to activation of HIF1α.[444,445] The iron depleting abilities of v-ATPase inhibition have been attributed to a disruption in TFR1 recycling as a result of impaired Tf/TFR1 internalization and retention in the membrane, shown to have therapeutic potential in breast cancer.[446] In line with this, *Atp6v1a* disruption or pharmacological inhibition of v-ATPase resulted in an accumulation of TFR1 on the cell surface of *MLL::AF9* LSCs, suggesting iron metabolism may be impaired as a result of receptor recycling disruption.

Through pathway enrichment analysis of RNA-seq data, we found that *Tfrc* disruption associates with significant enrichment of apoptosis and p53 signatures. The bid-directional regulation of p53 and iron regulators is instrumental in maintaining iron homeostasis, a crosstalk which is commonly deregulated in cancers.[447] We thus set out to examine whether and how iron deprivation mediated by disruption of *Trfc* might affect p53 signaling and thus contribute to an arrest in leukemia development. We found that disruption of *Tfrc* in *MLL::AF9* leukemia in a $Trp53^{-/-}$ background ($p53^{-/-}$) exhibited a significant delay in disease progression compared to their p53 wt leukemia counterparts ($p53^{wt}$). This is in line with a study that showed that iron deprivation suppresses tumor growth in a p53-dependent manner through direct heme-p53 interaction, suggesting that the presence of endogenous $p53^{wt}$ underlies at least in part the tumor-suppressing effects of iron deprivation.[448]

Despite the protective effects of a $p53^{-/-}$ background under iron deprived conditions, the anti-leukemic effect was not completely rescued in $p53^{-/-}$ LSCs, indicating that other p53-independent factors may be at play. To discern which pathways under oncogenic control of TFR1 are influenced by p53 and which are not, we conducted a comparative transcriptomic profiling analysis of leukemia cells either with or without p53. By interrogating iron metabolism-related gene sets, we found that loss of *Tfrc* induced a downregulation of iron-sulfur (Fe-S) cluster binding signatures exclusively in $p53^{wt}$ LSCs. As iron enters the cytosol, it forms Fe-S clusters in the LIP and subsequently serves as cofactors for various iron-requiring proteins with functions spanning oxidative stress regulation, ribosome maturation, DNA replication and repair, and TCA cycle control.[449] Notably, we observed significant transcriptional repression of numerous genes associated with the harbouring, synthesis, assembly, or donation of Fe-S clusters, including those involved in antioxidant defence. This repression correlated with an increase in ROS accumulation in $p53^{wt}$ LSCs. We propose that the anti-leukemic effects resulting from *Tfrc* disruption may be attributed to p53-mediated impairment of Fe-S cluster assembly, potentially compromising the capacity of AML cells to counteract free radical generation. This notion is supported by prior studies that demonstrated the p53-mediated transcriptional regulation of genes involved in Fe-S cluster biogenesis in hepatocellular carcinogenesis.[450]

Distinct metabolic profiles in response to *Trfc* silencing could also be inferred from the transcriptomic data based on p53 status. While the presence of p53 associated with suppression of genes encoding key enzymes catalyzing rate-controlling steps in the TCA cycle, the absence of it did not affect mitochondrial-related pathways; instead, it metabolically adapted by upregulating the transcriptional expression of key glycolytic enzymes. The role of iron in metabolic reprogramming has recently been reported to be involved in cancer stem cell maintenance and plasticity.[411] Our findings suggest a potential role of p53 in modulating metabolism under iron deprived conditions with implications for LSC survival.

We confirmed the proposed connection between p53 and selective efficacy of iron deprivation through the treatment of *MLL::AF9* LSCs with two iron chelators, DFO and Dp44mT, the former approved for managing iron overload arising from recurrent blood transfusions in leukemia patients.[374] Strikingly, iron chelator treatment exhibited strong and selective cytotoxicity against AML patient samples compared to normal bone marrow cells isolated from healthy individuals. While our cohort size may not have been large enough for robust statistical conclusions, we did not observe a correlation between Dp44mT response and p53 status, implying the involvement of p53-independent mechanisms in driving the anti-leukemic effects. Our findings are in agreement with a study reporting that sensitivity of tumor cells to DpT chelators, including Dp44mT, is independent of p53 status.[451] Additionally, cancers with heterogenous genetic backgrounds within patient populations, as is the case in leukemias, are expected to respond differently to iron chelation based on the functionality of their individual p53 signalling networks.[448]

This study demonstrates the therapeutic potential of iron deprivation in AML through *Trfc*-targeting or iron chelation. Understanding the mechanistic interplay between disrupted iron metabolism and p53-signalling is instrumental in the development of optimal iron deprivation-based therapies for the treatment of AML.

Article IV

H2-K1 protects murine *MLL::AF9* leukemia cells from NK cell-mediated immune surveillance

Immune surveillance mechanisms comprising of adaptive and innate immune responses have evolved to fight infections but can also counteract cancer development. However, leukemia cells apply a number of immune evasion mechanisms to escape host immune responses in order to survive and progress. Escape strategies involve the direct adaptation of leukemia cells to hide from immune recognition, or leukemia-cell-mediated modifications of immune effector cells such as T cells, NK cells, and DCs.[252]

We recently identified a MHC class-I molecule, H2-K1, among the strongest leukemia dependencies in an *in vivo* CRISPR/Cas9 screen using an LSC-enriched (c-kit$^+$) *MLL::AF9* mouse model of AML.[310] MHC class I molecules serve as cell surface recognition elements on host cells to signal their physiological state to immune effector. Given that modulation of MHC class I antigen presentation has been reported as an immune evasion strategy in many cancer types, we set out to examine how H2-K1, the murine ortholog of human HLA-A, regulates the survival of LSCs.[452]

Results in short

- H2-K1 is essential for the survival of *MLL::AF9* leukemia cells *in vivo* through evasion of NK cell-mediated immune surveillance.
- H2-K1 suppresses the activation and maturation of NK cells in the niche.
- Disruption of *H2-k1* alone is sufficient to restore NK-cell mediated surveillance against AML cells.

We determined that the regulatory effects of H2-K1 in driving AML progression were predominantly microenvironment dependent, as we observed that genetic disruption of *H2-k1* did not confer any anti-leukemic effects under cultured conditions. Consistently, recipients transplanted with *H2-k1*-disrupted c-Kit$^+$ AML cells exhibited reduced leukemia burden in the bone marrow and spleen, as well as succumbing to disease at a significantly longer disease latency. We thus hypothesized that H2-K1 may have a role in suppressing innate immune cells as a mechanism of immune evasion to drive leukemogenesis. We found that depletion of NK cells but not macrophages rescued the leukemia phenotype, demonstrating that *H2-k1* facilitates immune evasion of *MLL::AF9* leukemia cells by inhibiting NK cells in this model.

In patients, AML cells have previously been shown to have a negative impact on NK cell function, which have been associated with adverse clinical outcomes in AML.[263,453] In order to assess if the presence of *MLL::AF9* leukemia affects NK cell function, we characterized the bone marrow NK cell compartments of leukemic and healthy mice. We assessed the surface density of CD27 and CD11b, reported to address NK cell maturation state based on approaches assessing the kinetics of appearance, gene expression profile, rate of proliferation, and potential to give rise to the other subsets *in vivo*.[454] Consistent with a suppression of NK cell function, leukemia development correlated with an increase of immature M1 (CD27$^+$CD11b$^-$) NK cells and a decrease in the more cytotoxic M2 (CD27$^+$CD11b$^+$) and M3 (CD27$^-$CD11b$^+$) populations. We demonstrate that functional impairment of NK cells is thus associated with a disruption in the maturation process and a consequent exhaustion of cytotoxic NK cells. A maturation block has also been recently reported through pseudotime analyses with scRNA-seq and mass cytometry-based phenotyping data of the NK cell compartment

in the bone marrow of AML patients.[263,453] The emergence of these phenotypically and functionally impaired NK cells may be an important feature of immune escape from innate immunity during AML progression.

Additional evidence supporting the role of *MLL::AF9* leukemia cells was obtained from the decreased expression of the activating receptor NKG2D across all NK cell populations. Notably, loss of *H2-k1* partially rescued the leukemia-induced changes in the M1-M3 subpopulations and restored NKG2D expression to levels comparable to those observed in healthy mice. These findings demonstrate a direct implication of H2-K1 in regulating NKG2D expression and NK cell maturation. Supporting our work, previous studies have exemplified the pivotal role of NKG2D signaling with its ligand NKG2DL to avoid NK-mediated clearance in AML. Epigenetic regulation of NKG2D ligands and AML-mediated release of a soluble form of NKG2DL are among the mechanisms reported to evade NKG2D-mediated immunosurveillance.[260,261] The role of the NKG2D/NKG2DL axis in immune evasion has recently been linked to LSCs. Interestingly, NKG2DL-expressing AML cells were cleared by NK cells, while NKG2DL-negative leukemic cells evaded NK-mediated killing, the latter of which was characterized for having an immature morphology, stemness characteristics, and chemoresistance.[262]

Taken together, this study identifies H2-K1 as a suppressor of the activation and maturation of NK cells in the *MLL::AF9* mouse model. Considering that deletion of *H2-k1* restored NK cell-mediated immune surveillance against murine LSCs, it leads us to speculate that uncovering analogous mechanisms in human AML might suggest new strategies for overcoming MHC Class I-mediated immune evasion.

Concluding remarks and future perspectives

Recent advancements in understanding the pathophysiology of AML have led to the approval of targeted therapies, reshaping the therapeutic landscape over the past five years. Even with these advances, many challenges and unresolved questions remain. Perhaps the biggest challenge is the resilience of LSCs to eradication due to their scarcity, pronounced similarity to healthy HSCs, molecular and metabolic plasticity, and their inherent and adaptive chemoresistance. Consequently, therapy-resistant LSCs that persist following conventional treatments act as a reservoir for AML relapse. Successful AML treatment requires the elimination of LSCs, necessitating the identification and therapeutic exploitation of novel vulnerabilities specific to LSCs. This thesis was dedicated to the identification and functional characterization of AML

vulnerabilities, with a particular focus on LSCs, using CRISPR/Cas9 screens as a robust tool for biological discovery.

We uncovered a previously unknown critical role for CXCR4 signaling in *MLL::AF9* LSCs *in vivo*, which, as opposed to the relevance in normal HSC-microenvironment interaction, it is independent of CXCL12 stimulus (**Article I**). We thus propose that anti-leukemic therapeutic strategies should be aimed at inhibiting CXCR4 signaling rather than blocking the dispensable CXCR4-CXCL12 interaction.[427] Further work is needed to validate the translation of these findings beyond the *MLL::AF9* AML mouse model, both in other AML subtypes and in the setting of human disease. A central question that remains is the identity of the unknown potential interacting ligand(s) mediating their effect through CXCR4. This warrants the use of large-scale explorative methodologies such as high-throughput screening of compound libraries, proteomics and mass spectrometry, and phage display libraries for ligand identification. If proven successful, these findings could offer new avenues for therapeutic intervention.

We also identified the key metabolic regulator GLUT1, which constitutes the first rate-limiting step to glucose uptake in mammalian cells (**Article II**). GLUT1 overexpression is considered a hallmark in many cancers, and in AML, its targeting has been explored in the context of overcoming resistance to chemotherapy using AML cell lines. While the genetic and pharmacological inhibition of GLUT1 rapidly eliminated murine *MLL::AF9* c-Kit^{+} leukemia cells by suppressing bioenergetic pathways, this effect was not observed in human cells. Transcriptional exploration revealed that while murine AML cells predominantly express *Glut1*, human AML cells exhibit comparably high levels of both GLUT1 and GLUT3. Even though out findings argue against a redundancy between GLUT1 and GLUT3 in AML cells, they underscore potential metabolic distinctions between murine and human AML. These inter-species discrepancies, coupled with the overall complexity of human systems, cannot be underestimated and emphasize the importance of employing humanized disease models in research.

Metabolic targeting has transformed the therapeutic landscape of hematological malignancies as a result of the introduction of the BCL-2 inhibitor venetoclax to frontline treatments in AML. The remarkable 70% response rate in AML when used in combination with azacytidine has been attributed to the selective effect of venetoclax on LSCs through metabolic perturbations that disrupt OXPHOS and the TCA cycle. Despite these excellent outcomes, challenges persist in the form of upfront resistance and post-remission relapse, driven by the ability of AML cells to undergo metabolic reprogramming to acquire alternative sources of fuel.[304] An increased degree of metabolic plasticity in both intra- and inter-patient settings has been reported for therapy-refractory LSCs, ultimately becoming the drivers of relapse.[65] **Article II**

introduces a novel approach of restraining the metabolic plasticity and inducing synergistic anti-leukemic effects in human AML cells through the dual inhibition of GLUT1 using BAY-876 and OXPHOS using IACS-010759. Metabolic rewiring is an underlying factor causing resistance to BCL-2 inhibitor, and LSCs that arise in relapsed disease have been reported to become reliant on glucose as a fuel to synthesize TCA cycle intermediates. We thus propose the potential benefits in combining glycolysis or GLUT1 inhibition with venetoclax, particularly in relapsed AML. However, these ideas remain speculative and require further exploration, including the ongoing investigation through over 60 trials evaluating the therapeutic efficacy of venetoclax-based regimens in AML. Notably, patients with *RUNX1*-mutated AML demonstrate heightened susceptibility to the combination of GLUT1 and OXPHOS inhibition, likely due to their glycolysis-centric metabolism driven by the upregulation of key glycolytic regulators PDK1 and RB1. These findings underscore the importance of identifying biomarkers to optimize patient outcomes while minimizing treatment-related toxicities.

IACS-010759 has shown a limited therapeutic range, with dose-limiting toxicities observed in two phase I trials involving patients with R/R AML and solid tumors.[346] These toxicities involved a compensatory increase in glycolysis as evidenced by elevated lactate levels.[346] This metabolic adaptation suggests a potential strategy to ameliorate the reported neurotoxic effects by combining IACS-010759 with agents that suppress lactate production, such as GLUT1 inhibitors or other glycolysis-targeting drugs.

We also reveal a susceptibility to disruptions in iron metabolism through the inhibition of TFR1 or iron chelation (DFO and Dp44mT) in both mouse and human AML models (**Article III**). This finding holds significant implications, particularly in light of recent research indicating cancer stem cells are particularly vulnerable to iron metabolism disruptions. While various anti-TFR1 strategies have emerged to block the "cellular iron gate" as anticancer agents, there are concerns regarding their safety in clinical applications.[380,381] These concerns stem from the essential role of TFR1 in normal HSC development and hematopoiesis. However, TFR1 primarily functions in iron uptake rather than signal transduction in hematopoiesis, especially during HSPC differentiation in mid-gestation. A thorough understanding of these mechanistic distinctions between normal and malignant hematopoiesis through further research may facilitate the identification of a therapeutic window for iron deprivation-based therapies.[392] While one clinical trial has reported no toxicity of human TFR1 antibodies in healthy volunteers, future studies should assess their safety and efficacy in leukemia patients.

We observed a correlation between the response to iron deprivation and the presence of wt p53 using the murine *MLL::AF9* AML model. However, due to limitations in

patient cohort size and availability of p53-mutated AML samples, we were unable to confirm this correlation in patient-derived AML. Nonetheless, p53-dependent and -independent effects have been reported for DFO and Dp44mT chelators, respectively.[448] Therefore, it is essential to consider various factors, including the distinct mechanisms of action, binding properties, treatment duration, and concentration, which have all been shown to influence treatment response. Notably, p53 abnormalities are observed in approximately 5% to 10% of de novo AML patients, with higher frequencies in older individuals, those with therapy-related myeloid malignancies, and patients with specific karyotype abnormalities.[181] These high frequencies underscores the importance of unraveling the molecular interplay between p53 signaling and specific iron chelator treatments to better identify patients who may benefit from these therapies. With the field moving towards tailored patient-specific treatment approaches, the impact of co-mutations in genes such as TET2, SF3B1, ASXL1, and DNMT3A on treatment outcomes should also be taken into account.[177]

The treatment paradigm for AML is shifting from more traditional cytotoxic and HMA-based therapies to innovative targeted strategies. Among these novel strategies are immunotherapies, designed to leverage and enhance the immune system of the patient for precise targeting of leukemia cells while preserving healthy tissue. In **Article IV**, we uncover the role of H2-K1, an MHC class I molecule analogous to human HLA, in facilitating the development of *MLL::AF9* AML within the bone marrow niche by evading NK cell-mediated immune surveillance. While immune evasion through MHC class I antigen presentation has been previously reported in cancer, it is influenced by various factors, including effector cell types, intrinsic signaling pathways, and external signals from the tumor microenvironment, such as interferon signaling and the hypoxic conditions of the niche.[452] Notably, loss of MHC class I avoids elimination by $CD8^+$ T cells, while we find that it is the upregulation of these molecules that mediates evasion of NK cells.[452] Understanding these immune evasion mechanisms is essential for the development of safe and improved immunotherapies. Several NK cell-based immunotherapies are showing promise in preclinical and early-phase clinical trials for AML. These approaches include enhancing the immunogenicity of leukemia cells by targeting NKG2D ligands, employing chimeric proteins to expand NK cell populations, utilizing immune checkpoint inhibitors, and using compounds that indirectly boost the expression of immune-stimulatory proteins in LSCs.[281] Overall, NK cell-based therapies offer potential advantages, including improved safety profiles compared to T cell therapies, with reduced risks of cytokine storms and neurotoxicity

Recent advancements in methodology have enabled the identification of complex and diverse cell populations at single-cell resolution, revealing unprecedented inter- and intra-patient heterogeneity. Some advanced multi-omic single-cell approaches now allow profiling of surface proteins (CITEseq), chromatin accessibility (ATAC-seq),

mutational profiling and clonal dynamics (Muta-Seq and TARGET-seq). The challenge now lies in integrating these high-throughput approaches to comprehensively understand the clinical, cellular, and molecular aspects of AML, ultimately paving the way for personalized therapeutic strategies. While these methodologies have already proven to effectively trace the drivers of relapse, including LSCs, further exploration is needed to decipher the molecular mechanisms of treatment resistance. Furthermore, it is becoming evident that the conceptual framework for LSC target validation must consider not only cell-intrinsic signaling, but also the systemic influences of the microenvironment, which play pivotal roles in AML progression and maintenance.[455]

In summary, this thesis highlights the value of CRISPR/Cas9 screens for the unbiased identification of critical dependencies in AML. We provide new biological insights into potentially targetable LSC vulnerabilities, findings that could inform the integration of much-needed LSC-targeting agents into first-line regimens in AML.

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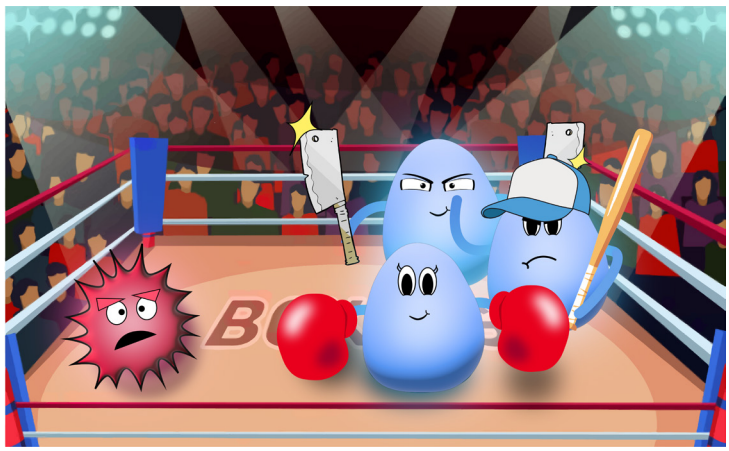

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