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Induced pluripotent stem cell models of hematopoiesis in development and disease

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Cover illustration: Hand-drawn illustration for this thesis by Corinna Mayer, depicting the generation of hematopoietic cells from patient-derived iPSCs for disease modeling and drug discovery.

Induced pluripotent stem cell models of hematopoiesis in development and disease

Thesis for Doctoral Degree (Ph.D.)

By

Jonas Thier

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“There is no knowing for a fact. The only dependable things are humility and looking.”
— Richard Powers, *The Overstory*

Popular science summary of the thesis

Our bone marrow continually makes new blood cells, including oxygen-carrying red cells, infection-fighting immune cells, and platelets that prevent bleeding. This process, hematopoiesis, depends on stem and progenitor cells that must tightly balance self-renewal with differentiation. When these cells acquire disease-driving genomic changes, blood production can become unbalanced, abnormal clones can expand, and disorders such as myelodysplastic neoplasms (MDS) and acute myeloid leukemia (AML) can develop. Studying disease mechanisms directly in patients can prove difficult because bone marrow samples are limited, heterogeneous, and hard to maintain in long-term culture. This thesis therefore uses patient-derived induced pluripotent stem cells (iPSCs), which can be expanded indefinitely and differentiated into blood progenitors, to recreate disease-relevant models of hematopoietic development in a controlled system and to test whether specific genetic lesions create targetable weaknesses.

Study I investigated *SF3B1*-mutant MDS, where RNA splicing is altered. Using genetically matched *SF3B1*-mutant and control iPSCs, we identified an *SF3B1*-specific mis-splicing event in *UBA1*, detected in iPSC-derived progenitors, cell lines, and supported by MDS patient cohort data. While the mis-spliced *UBA1* RNA was stable, the resulting protein product was rapidly degraded, lowering total UBA1 protein levels. UBA1 is essential to maintain cellular protein balance and the reduced UBA1 reserve created a vulnerability. *SF3B1*-mutant cells showed greater sensitivity to the UBA1 inhibitor TAK-243 across cell models, iPSC-derived $CD34^+$ progenitors, and primary patient colony assays.

Study II focused on aggressive *KMT2A*-rearranged AML. Patient-derived AML iPSCs and isogenic controls were transcriptionally similar at the iPSC stage but diverged during hematopoietic specification, when AML-like progenitors showed abnormal repression of developmental and hematopoietic programs. Multiple analyses connected this state to a Polycomb (PRC2)-mediated epigenetic repression. Targeting PRC2 by pharmacologic EZH1/2 inhibition with UNC1999, especially in combination with 5-azacitidine, derepressed Polycomb-associated gene sets and preferentially impaired clonogenic output and replating capacity in *KMT2A*-rearranged models. Together, these studies show how iPSC-based disease modeling can connect defined genetic variants to tractable mechanisms and actionable vulnerabilities, supporting UBA1 targeting in *SF3B1*-mutant MDS and Polycomb-linked epigenetic dependency in *KMT2A*-rearranged AML.

Allgemeinverständliche Zusammenfassung

Unser Körper produziert ständig neue Blutzellen, darunter rote Blutkörperchen, Immunzellen und Blutplättchen. Dieser Prozess, die Hämatopoese, beruht auf Stamm- und Vorläuferzellen, die Selbsterneuerung und Differenzierung im Gleichgewicht halten müssen. Erwerben diese Zellen krankheitstreibende genetische Veränderungen, können sich abnorme Zellen ausbreiten und Erkrankungen wie myelodysplastische Neoplasien (MDS) und akute myeloische Leukämie (AML) entstehen. Krankheitsmechanismen lassen sich im Patientenmaterial oft nur schwer untersuchen, weil Proben begrenzt, heterogen und in Kultur schwer zu erhalten sind. Diese Arbeit nutzt daher induzierte pluripotente Stammzellen (iPSCs) von Patienten, um krankheitsrelevante Prozesse in Kultur kontrolliert nachzubilden und gezielt angreifbare Schwachstellen zu identifizieren.

Studie I untersucht *SF3B1*-mutiertes MDS, bei dem die RNA-Spleißung verändert ist. Mithilfe *SF3B1*-mutierter und Kontroll-iPSCs identifizierten wir ein SF3B1-spezifisches Fehl-Spleißen in *UBA1* (*UBA1*^{ms}), nachweisbar in iPSC-abgeleiteten Blutzellen und gestützt durch Patientenkohortendaten. Obwohl die fehlgespleißte *UBA1*-RNA stabil bleibt, war das entstehende Proteinprodukt instabil und wurde rasch abgebaut, wodurch die Gesamtmenge an UBA1-Protein sank. UBA1 ist ein Schlüsselenzym des Proteinhaushalts und die verringerte UBA1-Reserve stellte eine Verwundbarkeit dar. Entsprechend waren *SF3B1*-mutierte Zellen gegenüber dem UBA1-Inhibitor TAK-243 in Zellmodellen, iPSC-abgeleiteten CD34^{+}-Vorläuferzellen und primären Patientenzellen empfindlicher.

Studie II fokussiert auf aggressive *KMT2A*-rearrangierte AML. AML-iPSCs und isogene Kontrollen waren im iPSC-Stadium ähnlich, entfalteten jedoch während der Differenzierung unterschiedliche genetische Programme, in denen AML-Vorläuferzellen eine abnorme Repression entwicklungs- und hämatopoese-assoziierter Vorgänge zeigten. Mehrere Analysen verknüpften diesen Zustand mit Polycomb (PRC2). Die Hemmung mit UNC1999, insbesondere in Kombination mit 5-Azacitidin, stellte Polycomb-assoziierte Genprogramme teilweise wieder her und beeinträchtigte in *KMT2A*-rearrangierten Modellen die leukämische Kapazität.

Zusammen zeigen diese Studien, wie iPSC-basierte Krankheitsmodelle definierte genetische Veränderungen mit untersuchbaren Mechanismen und therapeutisch nutzbaren Verwundbarkeiten verknüpfen können. Sie stützen UBA1 als Zielstruktur bei *SF3B1*-mutiertem MDS und eine Polycomb-assoziierte epigenetische Abhängigkeit bei *KMT2A*-rearrangierter AML.

Abstract

Hematopoiesis is a tightly regulated process that sustains the production of blood cells. Disruption in hematopoietic stem and progenitor cells (HSPCs) can impair differentiation, promote clonal expansion, and lead to myeloid malignancies such as myelodysplastic neoplasms (MDS) and acute myeloid leukemia (AML). Mechanistic studies and drug discovery are often limited by the availability, heterogeneity, and limited ex vivo stability of primary patient material. In this thesis, patient-derived induced pluripotent stem cells (iPSCs), together with isogenic wild-type controls, were used to model hematopoietic differentiation and link recurrent disease-defining lesions to downstream mechanisms and therapeutic vulnerabilities.

In **Study I**, we investigated *SF3B1*-mutant MDS, a distinct subgroup characterized by RNA mis-splicing and erythroid dysplasia. Isogenic *SF3B1*^{K700E} and *SF3B1*^{WT} iPSCs from an MDS patient were differentiated into hematopoietic cells and analyzed by full-length RNA sequencing, uncovering mutated *SF3B1*-specific mis-splicing of *UBA1*, which encodes the major E1 enzyme at the apex of the ubiquitination cascade. While the mis-spliced *UBA1* transcript was stable, its protein product was rapidly degraded, lowering total UBA1 levels and rendering *SF3B1*-mutant cells particularly sensitive to the UBA1 inhibitor TAK-243. CD34^{+} RNA sequencing from an MDS patient cohort confirmed *UBA1* mis-splicing as a prevalent feature of MDS-*SF3B1*, absent in other spliceosome-mutant MDS cases and healthy controls. Functionally, TAK-243 selectively reduced *SF3B1*-mutant primary CD34^{+} cells and decreased mutant colony output, sparing wild-type hematopoietic progenitors.

In **Study II**, we addressed epigenetic and transcriptional deregulation in *KMT2A*-rearranged (*KMT2A*-r) AML using patient-derived iPSCs. Transcriptional analysis during iPSC-directed hematopoietic development identified key activators and repressors contributing to the altered regulatory landscape in *KMT2A*-r AML. Integration with chromatin immunoprecipitation sequencing analyses indicated that a substantial fraction of genes downregulated in AML iPSC-derived HSPCs were direct targets of Polycomb Repressive Complex 2 (PRC2). Pharmacologic inhibition PRC2 via EZH1/2 using UNC1999, in combination with 5-azacitidine, reactivated PRC2 target genes specifically in AML-HSPCs, shifting expression toward a more normal hematopoietic program and reducing leukemic properties in *KMT2A*-r cells. Together, these findings support targeting Polycomb-associated repression as a potential epigenetic strategy in *KMT2A*-rearranged AML.

List of scientific papers

I. ***SF3B1*-mutant models of RNA mis-splicing uncover *UBA1* as a therapeutic target in myelodysplastic neoplasms**
Thier J, Hofmann S, Kirchhof KM, Todisco G, Mortera-Blanco T, Barbosa I, Björklund AC, Deslauriers AG, Papaemmanuil E, Papapetrou EP, Hellström-Lindberg E, Moura PL, Lundin V.
Leukemia 2025; 39:2801-2811.
https://10.1038/s41375-025-02740-1.

II. **Targeting dysregulated epigenetic and transcription factor networks in *KMT2A*-rearranged AML using iPSC models**
Palau A#, Thier J#, Naughton A#, Tae-Jun Kwon A, Kaczkowski B, Cabrerizo Granados D, Hofmann S, Kaczkowski B, Zhong X, Lehmann S, Arner E, Lundin V*, Lennartsson A*.
#Equal contribution. *Shared last authorship.
Blood Neoplasia 2025; 3(1):100172.
https://10.1016/j.bneo.2025.100172.

Scientific papers not included in the thesis

I. **Targeting IMPDH to inhibit SAMHD1 in *KMT2A*-rearranged leukaemia**
Klootsema Y, Tsesmetzis N, Sharma S, Hofmann S, Thier J, Dirks C, Hormann FM, Yagüe-Capilla M, Bohlin A, Bengtzen S, Lehmann S, Chabes A, Jädersten M, Lundin V, Rudd SG, Lilienthal I*, Herold N*.
Cell Cycle 2025; 1-9.
https://doi.org/10.1080/15384101.2025.2601796.

II. **Human uterine NK cells express CD96/TACTILE under the regulation of IL-15 and TGFβ1**
Mayer C, Sun D, Thier J, Strunz B, Schott K, Kaipe H, Lundin V, Gidlöf S, Björkström NK, Ivarsson MA.
Journal of Reproductive Immunology 2025; 172:104647.
https://doi.org/10.1016/j.jri.2025.104647.

Contents

List of abbreviations

3D	three-dimensional
AGM	aorta–gonad–mesonephros
AML	acute myeloid leukemia
ANOVA	analysis of variance
ATM	ATM serine/threonine kinase
BFU-E	burst-forming unit–erythroid
BMP4	bone morphogenetic protein 4
CAGE	cap analysis of gene expression
CCUS	clonal cytopenia of undetermined significance
CD235a	glycophorin A
CD34	sialomucin
CD43	leukosialin
CD45	protein tyrosine phosphatase, receptor type, C
CD71	transferrin receptor protein 1
cDNA	complementary DNA
CFU	colony-forming unit
CFU-E	colony-forming unit–erythroid
CH	clonal hematopoiesis
CHIP	clonal hematopoiesis of indeterminate potential
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CO_2	carbon dioxide
ddPCR	droplet digital PCR
del(…)	deletion of chromosome …
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
ELN	European LeukemiaNet
EMP	erythro-myeloid progenitor
EPO	erythropoietin
FAB	French–American–British (classification)
FBS	fetal bovine serum
FDA	Food and Drug Administration
FISH	fluorescence in situ hybridization
FLT3-ITD	FMS-like tyrosine kinase 3-internal tandem duplication
G-CSF	granulocyte colony-stimulating factor
H3K27me3	histone H3 lysine 27 trimethylation
H3K4me3	histone H3 lysine 4 trimethylation
H3K79	histone H3 lysine 79
hESC	human embryonic stem cell
HOX	homeobox (gene family shorthand)
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HSPC	hematopoietic stem and progenitor cell
ICC	International Consensus Classification
IL	Interleukin
inv(…)	inversion of chromosome …
iPSC	induced pluripotent stem cell
IPSS	International Prognostic Scoring System
IRB	Institutional Review Board
KMT2A	lysine methyltransferase 2A (formerly MLL)
KMT2A-r	*KMT2A*-rearranged

LT-HSC	long-term hematopoietic stem cell
MARA	motif activity response analysis
MDS	myelodysplastic neoplasms
MDS-RS	MDS with ring sideroblasts
MDS-*SF3B1*	MDS with *SF3B1* mutation
MEP	megakaryocyte–erythroid progenitor
MG-132	proteasome inhibitor
MLLT3	Mixed-lineage leukemia translocated to 3 (AF9)
MPP	multipotent progenitor
mRNA	messenger RNA
NFYA	nuclear transcription factor Y subunit alpha
NMD	nonsense-mediated mRNA decay
P/S	penicillin–streptomycin
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PRC1/2	Polycomb repressive complex 1/2
RBC	red blood cell
RNA-seq	RNA sequencing
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT-PCR	reverse transcription PCR
RT-qPCR	reverse transcription quantitative PCR
SCF	stem cell factor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SF3B1	splicing factor 3B subunit 1
ST-HSC	short-term hematopoietic stem cell
STRING	Search Tool for the Retrieval of Interacting Genes
t-AML	therapy-related acute myeloid leukemia

t(…;…)	chromosomal translocation notation
TAK-243	selective UBA1 inhibitor
TGF-β	transforming growth factor beta
TPO	thrombopoietin
UBA1	ubiquitin-like modifier activating enzyme 1
UBA1^{ms}	UBA1 mis-spliced transcript/isoform
UNC1999	dual EZH1/2 inhibitor
VAF	variant allele frequency
VEGF	vascular endothelial growth factor
VEXAS	vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic syndrome
WHO	World Health Organization

Introduction

The Background section of this thesis sets the conceptual framework for the two studies that use patient-derived induced pluripotent stem cells (iPSCs) to model myeloid disease in a human, stage-specific hematopoietic context. It begins with an overview of normal hematopoiesis, spanning developmental and adult blood formation, to establish a physiological reference point. Since iPSC-based hematopoietic differentiation draws heavily on early developmental programs, a working understanding of developmental hematopoiesis is important for interpreting differentiation protocols and for recognizing the strengths and limitations of iPSC-derived models. The next section reviews myelodysplastic neoplasms (MDS), including diagnosis, mutations and risk stratification, and current therapeutic strategies, before focusing on *SF3B1*-mutant MDS/MDS-RS and the rationale for examining UBA1 in **Study I**. The chapter then turns to acute myeloid leukemia (AML), covering clinical features, classification, treatment, and the broader genetic landscape, followed by a focused presentation of *KMT2A*-rearranged AML, which provides the disease context for **Study II**. The final sections summarize commonly used experimental model systems for myeloid neoplasms and introduce iPSC approaches, including key principles of hematopoietic and erythroid differentiation and how iPSC-based platforms enable mechanistic interrogation and therapeutic testing in genetically defined settings.

1 Background

1.1 Hematopoiesis

Hematopoiesis is the process by which all cellular components of blood are generated and replenished throughout life. These cells perform essential functions, including oxygen transport, hemostasis and wound repair, and immune defense against pathogens and malignant transformation [1,2]. Although blood cells are highly specialized according to their function, their developmental programs are remarkably conserved among vertebrates. This has enabled the study of hematopoietic development and function using animal models, primarily mouse and zebrafish [3].

1.1.1 Developmental hematopoiesis

Given the essential functions of blood cells, the hematopoietic system is established early in embryogenesis, producing cells adapted to the demands of the developing conceptus [4]. In mammals, developmental hematopoiesis proceeds through three successive, spatially and temporally distinct waves (Figure 1) [5–8]. The extraembryonic yolk sac, a membranous structure outside the embryo, is the first site of hematopoietic development [4,9]. Here, mesoderm-derived blood islands predominantly generate large, nucleated primitive erythroblasts, along with primitive macrophages and megakaryocytes [10–12]. With the onset of cardiac activity, these primitive erythroblasts enter the circulation and supply oxygen to meet the demand for growth and organ development [13]. This wave is short-lived and followed by a second, yolk sac-derived, wave of erythro-myeloid progenitor cells and the first progenitors with lymphoid potential [7,14–16].

Definitive hematopoietic cells, capable of long-term multilineage reconstitution, originate from a third, intraembryonic wave within the aorta–gonad–mesonephros (AGM) region of the dorsal aorta [17,18]. Here, a specialized subset of $CD34^+$ hemogenic endothelial cells change identity through endothelial-to-hematopoietic transition, budding from the endothelium and ultimately generating the first hematopoietic stem cells (HSCs) [2,19–25]. The genesis and population size of AGM HSCs have been debated. Lineage-tracing experiments based on vascular endothelial cadherin expression, imaging of AGM explants in mouse and *in vivo* imaging of the AGM region in zebrafish embryos collectively support an endothelial origin of definitive HSCs [26–28]. Fluorescent reporter and

genetic barcoding approaches indicate that approximately 500 distinct clones arise from the AGM and go on to sustain adult hematopoiesis [29–31]. Detached from the aortic wall, this small pool of cells enters the bloodstream and migrates to the fetal liver to mature and expand further, before homing to the bone marrow to establish life-long niches and largely enter quiescence [32–35]. These AGM-derived HSCs are defined by long-term, multilineage reconstitution and self-renewal and support lifelong hematopoiesis [18,36].

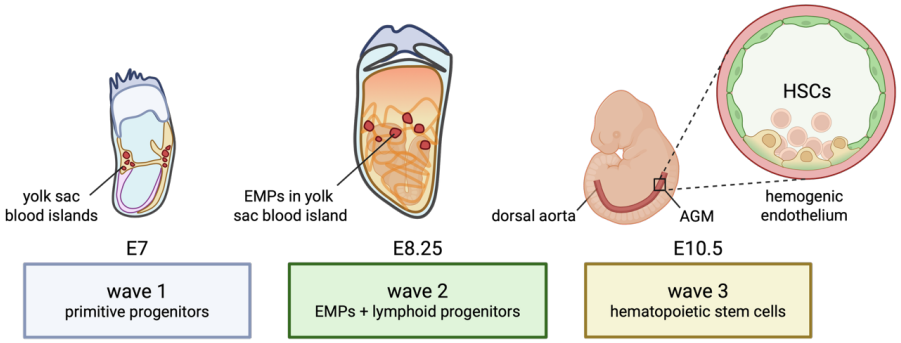

Figure 1: Sites of hematopoiesis during embryonic development adapted from Yoder (2014) [37]. E7, 8.5, and 10.5 correspond to mouse developmental stages. EMP, erythromyeloid progenitor; HSC, hematopoietic stem cell; AGM, aorta-gonad-mesonephros. Created with BioRender.com.

1.1.2 Adult hematopoiesis

The classical model for the generation of hematopoietic lineages has been described as a hierarchical (tree-like) structure with terminally differentiated cells arising from a small pool of self-renewing HSCs at the apex, generating distinct sets of progenitors that become progressively specialized and restricted to their respective lineages (Figure 2, left) [3,38,39]. The HSC compartment can be further subdivided by characteristics such as reconstitution capacity, quiescence, and lineage output into long- and short-term HSCs [38,40,41]. Long-term (LT) reconstituting HSCs persist over the lifetime, remaining largely quiescent but able to transition in and out of the cell cycle [42–44]. LT-HSCs give rise to short-term (ST) HSCs, which can still reconstitute all blood lineages but may exhaust their self-renewal capacity, as evidenced by failure to engraft secondary recipients in serial transplantation experiments [45]. ST-HSCs differentiate into multipotent progenitors (MPPs) that proliferate and give rise to progenitors with lymphoid and myeloid potential (often described as CLPs and CMPs in classical models).

Although this scheme of organized cell identities and stepwise lineage restriction is convenient for assigning markers and attributes, the classical model is increasingly challenged by advancements in the field. Advances in single-cell isolation and profiling, including single-cell RNA sequencing (RNA-seq), suggest that hematopoietic specification and cell fate restriction may occur along a continuum, with a more heterogeneous HSC and progenitor pool exhibiting plasticity and lineage biases (Figure 2, right) [46–53]. Investigating the properties of lineage biases within the HSC compartment and identifying new markers that distinguish specific populations have further demonstrated that cells collectively referred to as HSCs comprise multiple subsets of cells with distinct clonal contributions [54–58].

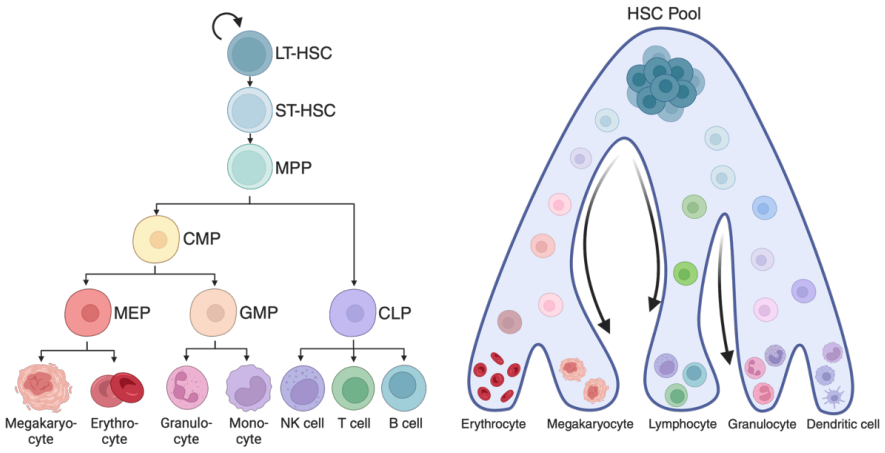

Figure 2: Hematopoiesis models as a "stepwise" process (left), compared to the "continuous" model (right), adapted from Laurenti and Göttgens (2018) [44]. LT, long-term; ST, short-term; MPP, multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor; CLP, common lymphoid progenitor. Created with BioRender.com.

1.1.3 Erythropoiesis

The remarkable generative capacity of the hematopoietic system is illustrated by the large numbers of red blood cells (RBCs) that must be replenished constantly to sustain gas exchange throughout the organism [59]. In healthy adults, erythropoiesis produces on the order of 2×10^{11} new RBCs per day [60], which corresponds to roughly two million erythrocytes every second, about one for every inhabitant of the Stockholm metropolitan area.

To meet this demand, RBCs are the product of a series of expansion and differentiation steps, along which hematopoietic progenitors become increasingly lineage-restricted (Figure 3) [59]. The earliest committed erythroid progenitors,

burst-forming unit–erythroid (BFU-E) and colony-forming unit–erythroid (CFU-E), arise from megakaryocyte–erythroid progenitors (MEPs) and are defined by their *in vitro* colony-forming capacity [61,62]. Along the trajectory from CFU-E to mature erythrocytes, erythroid precursors progressively decrease in size, accumulate hemoglobin, clear organelles, and condense their nuclei, culminating in enucleation [59,60,63]. Proerythroblasts progress through basophilic, polychromatic, and orthochromatic erythroblast stages, which can be distinguished morphologically or by surface expression of CD49d, CD71, CD105, CD233, and CD235a [64–67]. Nuclear extrusion generates reticulocytes, which complete terminal maturation by clearing residual organelles and entering the circulation, where they acquire the characteristic biconcave shape [60].

A primary regulator of erythroid expansion, differentiation, and survival is erythropoietin (EPO), produced by the kidneys in response to hypoxia [68]. Iron delivery via transferrin is crucial for heme synthesis and hemoglobinization of cells [69,70]. Additional regulators of erythroid development include insulin and insulin-like growth factors, interleukin-3 (IL-3) and IL-10, activin and other TGF-β family ligands, thrombopoietin (TPO), and angiotensin [71–76].

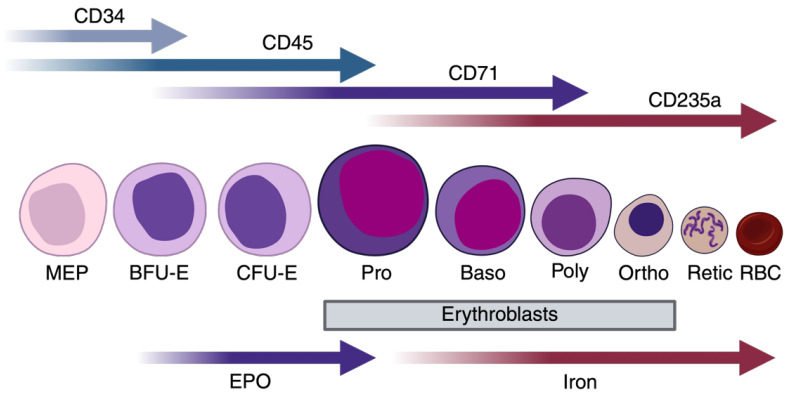

Figure 3: Key erythroid maturation stages, surface marker dynamics, and supplementation dependencies, adapted from Elvarsdóttir (2019). BFU-E, burst-forming unit–erythroid; CFU-E, colony-forming unit–erythroid; Pro, proerythroblasts; Baso, basophilic; Poly, polychromatic; Ortho, orthochromatic; Retic, reticulocyte; RBC, red blood cell. Created with BioRender.com.

1.1.4 The bone marrow niche

To maintain steady-state hematopoiesis while enabling rapid responses to demand, the hematopoietic system is subject to complex regulation from cell-intrinsic mechanisms and the external environment. This specialized bone marrow

microenvironment, composed of cellular and acellular components, is commonly referred to as the HSC niche [77,78].

The niche concept was proposed as early as the 1970s by Schofield [79]. Scadden later defined it as a specific anatomic location that integrates local and systemic signals to regulate stem cell self-renewal, differentiation, and protection from both exhaustion and uncontrolled proliferation [80]. The cellular compartment comprises a heterogeneous mixture of lineages, including endothelial, osteolineage, fibroblastic, stromal, neuronal, macrophage, and mesenchymal cells [32,81–86]. These cells regulate HSC quiescence, survival, proliferation, and differentiation in part by providing ligands and cytokines. A key cytokine for HSC maintenance is stem cell factor (SCF), which promotes anti-apoptotic signaling and can act systemically or proximally through soluble and membrane-bound forms [87]. Similarly, TPO, more commonly known for its role in thrombopoiesis, is also a critical factor in HSC maintenance [88].

In addition to cell–cell interactions and signaling through soluble factors, acellular factors of the niche, such as the extracellular matrix composition, mechanical properties, and oxygen tension, play important roles in the regulation of hematopoiesis [89,90]. Major components of the bone marrow extracellular matrix are collagens, proteoglycans, and glycoproteins, acting as scaffolding for cells and growth factors [91]. Biophysical properties (e.g., stiffness, topography, porosity) also influence stem cell behavior. This has been observed in various stem cell subtypes, such as neural stem cells, mesenchymal stem cells, muscle stem cells, and hematopoietic cells [92–97]. The role of the extracellular matrix in hematopoiesis has been reviewed in detail by Lee-Thedieck and colleagues [98–102].

Overall, the hematopoietic niche integrates signals that regulate HSC fate decisions and maintains hematopoietic homeostasis. The importance of this role becomes evident when dysregulation of HSCs or the niche occurs, which can eventually amount to hematopoietic malignancies [103,104].

1.1.5 Clonal hematopoiesis

Somatic variants arise continuously throughout life and are acquired across cell types, including stem cells. HSCs are therefore not spared; they accumulate somatic mutations over time (estimates are ~17 variants per year), leading to substantial accumulation over an individual's lifetime [105–108]. While most alterations change the nucleotide sequence, many do not affect the amino acid

sequence or measurably alter protein function. These variants remain largely inconsequential and are commonly referred to as passenger mutations.

By contrast, specific genetic abnormalities can confer a fitness advantage to the affected HSC, promoting clonal outgrowth of the mutant cell and its descendants through positive selection; these events are termed driver mutations. The process of expansion and increasing prevalence of such clones is known as clonal hematopoiesis (CH) [109,110]. Several studies have identified a recurring pattern of driver mutations in a small set of genes, often found in myeloid malignancies, in individuals without a diagnosis of hematological neoplasms [111–114]. These studies further reported that the prevalence of CH rises with age, reaching approximately 10–20% among individuals aged ≥70 years. Notably, germline genetic background can also shape CH dynamics by modifying the growth advantage of mutant clones. In a recent genome-wide association study, Agarwal et al. identified a protective noncoding regulatory variant (rs17834140-T) that downregulates expression of musashi RNA-binding protein 2 in HSCs and is associated with slower CH expansion and reduced risk of CHIP and myeloid malignancies[115]. Using next-generation sequencing approaches, Young et al. described CH-associated variants as ubiquitous in adults, detectable in a large fraction of individuals aged 50–60 when very low variant allele frequencies (VAFs) were included [116].

With advancements in our understanding of the underlying molecular mechanisms and their consequences, CH has been further subdivided. The presence of a CH clone with a VAF ≥2% in the absence of cytopenias is commonly termed clonal hematopoiesis of indeterminate potential (CHIP), whereas clonal cytopenia of undetermined significance (CCUS) refers to otherwise unexplained cytopenias with evidence of clonality [109,117,118]. While CH remains subclinical in most individuals, factors such as higher VAF, mutations in spliceosome components, and overall mutational burden increase the risk of disease progression [119–121].

1.2 Myelodysplastic neoplasms

Myelodysplastic neoplasms/syndromes (MDS) are a heterogeneous set of myeloid neoplasms arising from HSPCs and characterized by ineffective, dysregulated hematopoiesis. [122]. Key features of MDS include hematopoietic cell dysplasia, cytopenias—especially of the erythroid lineage—bone marrow failure, and an elevated risk of progression to AML [123–126]. MDS presents in

approximately 3–5 cases per 100,000 individuals in Sweden and the US. MDS is largely a disease of older individuals, with a median age at diagnosis >70 years. The incidence increases markedly with advancing age and is higher in men than in women, placing MDS among the most frequent hematologic malignancies in older populations [127–129].

Most MDS cases are de novo, arising without a clear precipitating cause. However, several risk factors have been identified. Prior exposure to cytotoxic chemotherapy and/or radiation is a well-established risk factor: therapy-related MDS (or therapy-related myeloid neoplasms) comprises ~10–20% of cases and is associated with substantially poorer outcomes than de novo disease [130–133]. Similarly, prolonged exposure to benzene and organic solvents has been connected to an increased risk of developing MDS [134]. Aside from acquired risk factors, there is growing recognition of hereditary predispositions to MDS. Together, these findings underscore that MDS pathogenesis is multifactorial, involving environmental exposures, genetic predispositions, and acquired somatic mutations, which will be described in a later section.

1.2.1 MDS diagnosis

MDS presents with a broad range of clinical features, reflecting the heterogeneous nature of the disease. Symptoms that raise suspicion of MDS are associated with an underlying cytopenia: anemia (fatigue, dyspnea, reduced exercise tolerance), neutropenia (recurrent infections), or thrombocytopenia (bruising, bleeding) [135–137]. Some patients are asymptomatic at diagnosis, with MDS first suspected due to abnormal routine blood counts. The initial clinical findings are not specific to MDS, and other causes of cytopenias—such as dietary deficiencies, chronic disease, medications, and other factors from the patient's history—must be ruled out. In clinical practice, older patients with persistent, unexplained cytopenias should be considered for bone marrow examination to evaluate for MDS [138]. Diagnosis and categorization are based on integrated clinicopathologic evaluation according to the 5th edition of the World Health Organization (WHO) classification of haematolymphoid tumours and the International Consensus Classification (ICC), incorporating morphologic, cytogenetic, and molecular genetic features [139,140].

1.2.1.1 Laboratory findings

Laboratory findings are not specific to MDS and are often linked to the underlying cytopenias. This can include elevated erythrocyte sedimentation rate and C-

reactive protein levels, low hemoglobin levels (<10 g/dL), and macrocytosis [136,141–143].

1.2.1.2 Bone marrow examination

The gold standard for the diagnosis of MDS is bone marrow examination via bone marrow aspirate and/or biopsy [135]. May-Grünwald–Giemsa staining enables assessment of cell identity and morphology, lineage dysplasia, and hypocellularity or hypercellularity [138]. Quantification of the percentage of nucleated bone marrow blasts is important for categorizing disease, predicting prognosis, and distinguishing higher-risk MDS from AML [139,140,144,145]. The 5th edition WHO distinguishes MDS from AML at a blast threshold <20%, whereas the 2022 ICC introduces the subgroup MDS/AML from 10-20% blasts. Additional iron staining with Prussian blue can detect ring sideroblasts [144]. While bone marrow aspirates remain essential to diagnosis, sampling error and subjective interpretation can limit reliability [135].

1.2.1.3 Cytogenetics

Cytogenetic analyses are performed using G-banding (karyotyping) and fluorescence in situ hybridization (FISH). Chromosomal abnormalities are present in around half of MDS cases; thus, their identification is essential to obtain a complete diagnosis. The most common aberrations in MDS involve partial deletion of large chromosomal segments [del(5q), del(7q), del(20q)], loss or gain of entire chromosomes (monosomy 7, trisomy 8), or an accumulation of multiple events referred to as a complex karyotype [146,147].

1.2.1.4 Targeted sequencing

Targeted next-generation sequencing (NGS) is a key component of the MDS diagnostic workup and is incorporated into the current classification frameworks. Both the 5th WHO classification of hematolymphoid tumors and the ICC include MDS subtypes defined by specific genetic variants, such as TP53 alterations or somatic mutations in *SF3B1* [139,140]. Because most MDS driver lesions occur in a core set of ~50 recurrently mutated genes, targeted panels enable sensitive detection of recurrent mutations that complement morphology, cytopenias, and cytogenetics by providing molecular evidence of clonality [148]. Diagnostic interpretation should account for both the mutational profile and clonal burden: the presence of multiple mutations and higher VAFs supports an underlying myeloid neoplasm, whereas the absence of detectable driver mutations has a high

negative predictive value but does not fully rule out MDS [148]. Testing can be done in peripheral blood and bone marrow which have been shown to be concordant for mutation detection [149]. NGS may also flag possible germline predisposition variants (often ~40–60% VAF), warranting confirmatory testing in non-hematopoietic tissues [150].

1.2.1.5 Clinical flow cytometry

Flow cytometric analysis of bone marrow cells can be a complementary tool to further refine diagnosis and classification, enabling analysis of antigen expression patterns across samples [151,152]. This is employed for assessment of lineage distribution, maturation patterns, and abnormal populations [153]. Consensus recommendations on sample preparation methods and staining panels can further improve reproducibility and interpretation of results [154].

1.2.2 Classification and prognosis

In summary, a confirmed MDS diagnosis involves correlating clinical, morphologic, and laboratory findings while excluding other conditions that can mimic MDS. Based on diagnostic findings, the underlying disease is then further categorized. Historically, classification systems mostly recognized morphological features and peripheral blood cytopenias [144]. However, cytogenetic and molecular events have gained importance in the most recent classification schemes of the WHO and ICC, both published in 2022. These include MDS subtypes defined by specific genetic lesions, such as TP53 alterations or somatic mutations in *SF3B1* [139,140]. Risk stratification for MDS patients has similarly evolved since implementation of the International Prognostic Scoring System (IPSS) in 1997, which was revised 15 years later (IPSS-R) [141,145]. In its latest iteration in 2022, referred to as IPSS-M, Bernard et al. proposed a molecularly informed scoring system based on 22 variables that assigns patients to one of six risk categories [155].

1.2.3 Treatment of MDS

Following diagnosis, therapeutic approaches focus on prolonging survival and, if possible, curing the patient; otherwise, improving the quality of life is the priority. The treatment approach for MDS patients depends on the specific risk score and generally distinguishes lower-risk from higher-risk MDS. This section summarizes the general treatment strategies, but a more detailed compilation can be found in a recent review series by Merz and Platzbecker, and Kröger [156,157].

Treating higher-risk MDS focuses on reducing disease burden and preventing progression to AML. Allogeneic hematopoietic stem cell transplantation (HSCT) remains the only curative treatment for MDS. Thus, eligibility should be considered following careful evaluation and, when appropriate, performed promptly to improve outcomes for higher-risk patients [158–160]. The hypomethylating agents azacitidine and decitabine are widely used disease-modifying therapies, either as a bridge to allogeneic HSCT or to delay progression in patients who are not transplant candidates.

Treatment strategies for lower-risk disease center on supportive care, focused on improving cytopenias. Erythropoiesis-stimulating agents are the standard-of-care first-line treatment to boost RBC counts and are administered alone or combined with granulocyte colony-stimulating factor (G-CSF). Thrombopoietin receptor agonists can improve platelet counts in some patients [161]. More recently, treatment of patients with MDS with ring sideroblasts (MDS-RS), which is described in more details in a later section, using luspatercept, a TGF-β superfamily ligand trap, has received Food and Drug Administration (FDA) approval [162–164]. For patients harboring del(5q), treatment with lenalidomide should be considered [165,166]. RBC transfusions are frequently administered to combat anemia, and transfusion dependency is common in patients (30–50% at diagnosis) [129,140]. While more liberal transfusion strategies may improve quality of life, transfusion dependency at diagnosis and within the first year is associated with worse outcomes. Thus, starting treatment with erythropoiesis-stimulating agents early and achieving transfusion independence can improve prognosis [167–169].

1.2.4 Genomic landscape of MDS

With the advent of reliable and widely available next-generation sequencing techniques over the past decades, it has become evident that genetic mutations are major drivers of malignant clonal evolution in many cancers, including MDS [135,170]. In 2013 and 2014, two landmark papers by Papaemmanuil et al. and Haferlach et al. provided detailed descriptions of the genomic landscape of MDS in large patient cohorts [171,172]. This was later complemented by an analysis of nearly 3,000 MDS patients from 24 centers by the International Working Group for Prognosis in MDS [155,173]. Within the cohort, 90% of patients harbored at least one oncogenic mutation (out of 9254 identified in total) distributed across 121 genes [155].

This diverse spectrum of recurrently mutated genes can be grouped into several functional groups, outlined in Table *1*. Somatic mutations in epigenetic regulators involved in DNA methylation and histone modification include *DNMT3A*, *TET2*, *ASXL1*, and *EZH2*, which are implicated in clonal expansion. Heterozygous spliceosome mutations involving *SF3B1*, *SRSF2*, *U2AF1*, or *ZRSR2* are associated with widespread RNA mis-splicing, often leading to nonsense-mediated mRNA decay (NMD) and reduced functional expression of some genes [174]. These effects can confer a fitness advantage, leading to clonal expansion, and/or impair progenitor maturation. A more detailed description of the role splicing factor mutations have in MDS is included in an upcoming section. Other somatic mutations involve transcription regulators, the DNA repair machinery and cohesion complex, as well as signaling pathways [173]. Mutation frequencies are not uniformly distributed across the mutational landscape. Instead, a small set of events is clearly overrepresented. This includes mutations in *TET2*, *ASXL1*, or *SF3B1* in >20% of patients, and *DNMT3A*, *SRSF2*, *RUNX1*, or *TP53* in approximately 10–20%.

Mutated genes	
Epigenetic regulators	*TET2* (>20% of patients) *ASXL1* (>20% of patients) *DNMT3A* (10–20% of patients) *EZH2*, *BCOR*, *IDH2*, *IDH1*, *PHF6*, *BCORL1*, *ZBTB33*, *EP300*, *KMT2D*
RNA splicing	*SF3B1* (>20% of patients) *SRSF2* (10–20% of pts) *U2AF1*, *ZRSR2*, *PRPF8*, *U2AF2*
Transcription regulation	*RUNX1* (10–20% of patients) *CUX1*, *MLL* (*KMT2A*), *ETV6*, *CEBPA*, *CTCF*, *WT1*, *ZBTB33*, *GATA2*, *NFE2*
DNA repair control	*TP53* (10-20% of patients) *PPM1D*, *BRCC3*
Cohesin complex	*STAG2*, *SMC1A*, *RAD21*
Signaling	*CBL*, *NRAS*, *KRAS*, *JAK2*, *MPL*, *SH2B3*, *PTPN11*, *GNB1*, *FLT3*
Miscellaneous	*SETBP1*, *DDX41*, *ETNK1*, *KMT2C*, *CSNK1A1*, *NPM1*, *GNAS*, *ARID2*
Cytogenetic alterations	
Alterations	del(5q) (10–20% of patients) complex karyotype +8, -Y, -7, del(7q), del(11q), -13, +21, del(4q), del(1p)

Table 1: Overview over recurrent (>1% of patients) gene mutations and cytogenetic alterations in patients with MDS [155]. Events used for IPSS-M prognostic calculations are underscored. Adapted from Cazzola and Malcovati (2025) [173]

Large-scale genomic studies and investigations of familial histories of hematologic malignancies have identified a group of germline mutations (e.g., *GATA2*, *RUNX1*, *DDX41*, *TP53*, *SAMD9*/*SAMD9L*) that confer inherited susceptibility to MDS/AML and collectively account for up to 15% of cases [173,175–181]. These germline predisposition syndromes can significantly influence therapeutic choices and the selection of suitable donors for transplantation.

1.2.5 *SF3B1*-mutant MDS and MDS-RS

Next-generation sequencing studies of large patient cohorts established that MDS is frequently driven by mutations in spliceosome components, with *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2* among the most commonly mutated splicing factor genes [155,171,172]. Among these, *SF3B1* mutations uniquely stand out as they are tightly linked to the disease phenotype of MDS-RS [182]. MDS-RS is a distinct subset of MDS which was originally described in the 1950s and later recognized as a separate entity in the French–American–British (FAB) and WHO classifications [144,183,184]. The defining morphological feature is the presence of ring sideroblasts in the bone marrow of patients (Figure 4). These aberrant erythroblasts contain iron-laden mitochondria forming a perinuclear ring, which becomes visible after iron staining [124,185].

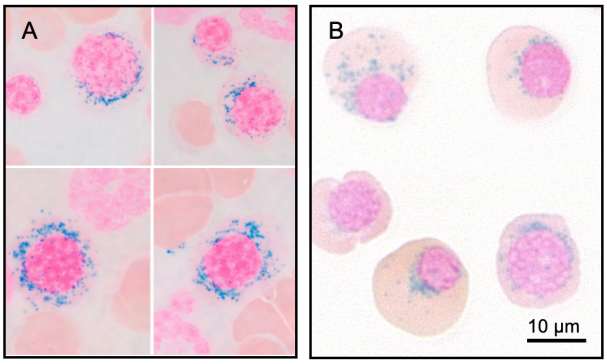

Figure 4: Ring sideroblasts stained with Perls' Prussian blue stain. (A) RS from MDS-RS patient bone marrow samples. Image adapted from Lours et al. (2022) [186], licensed under CC BY 4.0. Modifications include removal of labels and image sharpening. (B) Isolated RS from MDS-*SF3B1* patient-derived iPSCs from **Study II**.

The SF3B1 protein encodes a core component of the U2 small nuclear ribonucleoprotein complex that contributes to 3′ splice site recognition during spliceosome assembly [187–189]. *SF3B1* mutations in MDS typically constitute heterozygous missense substitutions, clustering in the HEAT repeat domain with K700 as a common hotspot [190–193]. Mechanistically, *SF3B1* mutations drive

misrecognition of 3′ splice sites on pre-mRNAs, resulting in widespread cryptic splicing (Figure 5) [194,195]. Aberrantly spliced transcripts are frequently targeted for degradation by NMD, reducing functional protein levels. *In vitro* studies connected mis-splicing of key erythroid genes, including the mitochondrial iron transporter *ABCB7* and genes involved in heme biosynthesis (*ALAS2*, *TMEM14C*, *PPOX*, *MAP3K7*), to impaired heme production, mitochondrial iron accumulation, and RS generation [185,196–201]. Further, RNA mis-splicing increases during erythroid differentiation, causing cells to engage pathways that downregulate oxidative stress and NMD, which promotes cell survival and may contribute to clonal expansion [202].

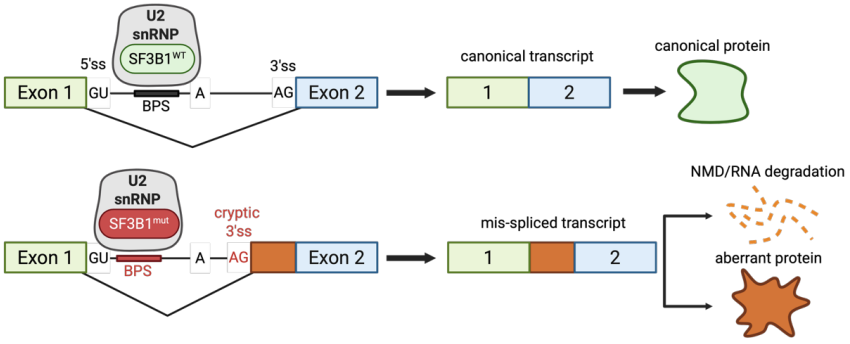

Figure 5: Mechanism-of-action and downstream consequences of wild-type SF3B1 ($SF3B1^{WT}$)- and mutant SF3B1 ($SF3B1^{mut}$)-mediated mRNA splicing, adapted from Zhou et al. [203]. U2 snRNP, U2 small nuclear ribonucleoprotein; ss, splice site; BPS, branchpoint sequence; NMD, nonsense-mediated mRNA decay. Created with BioRender.com.

Across patients, *SF3B1* mutations occur in most MDS-RS cases, have a strong positive predictive value, and have been incorporated into diagnostic frameworks as a defining feature of the MDS-RS entity [126,139,140]. From an evolutionary perspective, *SF3B1* mutation is considered an early event in MDS-RS, often represented in the dominant clone and present at higher VAFs than other genetic lesions [171,204]. Clinically, MDS-RS is relatively indolent compared to higher-risk disease and has one of the most favorable outcomes across MDS subtypes [182,205]. Subsequent studies demonstrated that *SF3B1*-mutant MDS-RS represents a relatively homogeneous subgroup characterized by erythroid dysplasia and abnormal erythroid maturation. Accordingly, treatment typically focuses on alleviating anemia through erythropoiesis-stimulating agents and establishing transfusion independence [206]. However, it is increasingly understood that favorable prognosis is not uniformly distributed but depends on co-mutations. Isolated *SF3B1* mutations or a "simple" co-mutation pattern

involving epigenetic regulators retains favorable prognosis, whereas del(5q) or mutations in *BCOR*, *NRAS*, *RUNX1*, and others are associated with worse outcome [155,207,208].

1.2.6 *UBA1* mutations

Despite major advances in identifying genetic alterations and integrating them into modern classification, treatment, and risk-stratification systems, 5–10% of patients still lack an identifiable disease-defining mutation [139,140,155,172,209]. In addition, a sizable fraction of patients (approximately 10–30%) develop inflammatory manifestations without an obvious etiology, which can complicate both diagnosis and management and has been associated with higher-risk disease features [209–213]. A major advance in this area came in 2020, when Beck et al. identified somatic *UBA1* mutations as the cause of a subset of these unexplained inflammatory phenotypes and introduced the entity VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic) syndrome [214]. *UBA1* encodes the principal ubiquitin-activating E1 enzyme, which is essential for initiating protein ubiquitination and thereby influences protein homeostasis and diverse downstream cellular processes (Figure 6, left). Pathogenic variants in VEXAS commonly disrupt expression of the cytosolic UBA1b isoform, frequently by affecting translation initiation (Figure 6, right) [214–217]. Clinically, the overlap with myeloid disease is notable: MDS is reported in roughly 25–55% of individuals with VEXAS, and in a large, representative diagnostic MDS cohort, about 1% of patients carried likely pathogenic *UBA1* variants [209,218,219]. Collectively, these data support considering *UBA1* mutation testing in the diagnostic work-up of MDS when inflammatory features are prominent and particularly in male, given the X-linked nature of UBA1 and the marked male predominance of VEXAS syndrome.

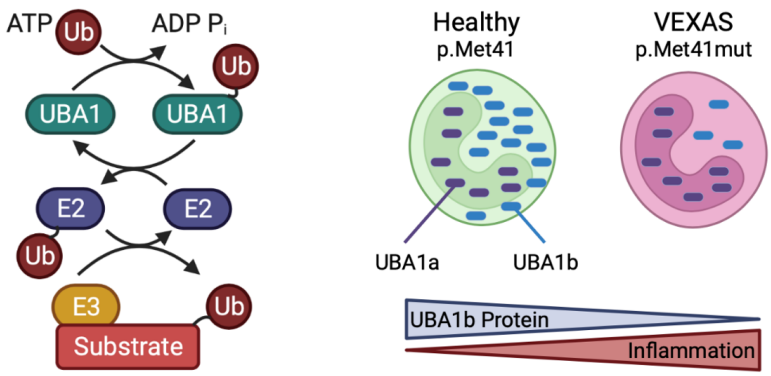

Figure 6: UBA1 mechanisms of action (left), and role in VEXAS (right), adapted from Ferrada et al. (2022) [220].

1.3 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a myeloid neoplasm closely related to MDS and can arise from MDS when additional genetic and/or cytogenetic events drive leukemic transformation. AML is defined by excessive proliferation of leukemic blasts (poorly differentiated hematopoietic progenitors) that infiltrate the bone marrow, blood, and other tissues [221]. The resulting disruption of normal hematopoiesis causes severe cytopenias, and AML remains a life-threatening diagnosis. Outcomes have nevertheless improved over recent decades. Whereas AML was historically associated with very poor long-term survival, 5-year survival rates now stand at 62% for patients diagnosed before age 50, 37% for those aged 50–64, and 9.4% for patients 65 years and older [222]. A Swedish registry study reported that survival gains over the past 20 years were most pronounced among middle-aged men [223]. While AML often presents sporadically, an increased risk of development has been associated with the same factors as in MDS, including exposure to certain chemicals, cytotoxic therapies, and germline predispositions [126].

1.3.1 Presentation and diagnosis

AML is a medical emergency that requires urgent evaluation and management. Similar to MDS, symptoms are often non-specific but typically reflect suppression of normal hematopoiesis and may raise suspicion of a hematological malignancy. This includes severe cytopenias (anemia, thrombocytopenia, and/or neutropenia) and, in some patients, abnormally high white blood cell counts (leukocytosis). Patients may present with fatigue, dyspnea, bleeding, infections, or headaches.

Historically, AML diagnosis required a minimum of 30% myeloid blasts in the bone marrow or peripheral blood according to the FAB system, later revised to ≥20% in WHO classifications. In the most recent frameworks, AML may be diagnosed below the 20% blast threshold for specific genetically defined entities. A comprehensive diagnostic work-up includes assessment of morphology, immunophenotype, cytogenetics, and molecular genetics, while the patient is closely monitored and acute complications are managed [140,224,225].

1.3.2 Classification and prognosis

The classification of AML has evolved from the morphology-based FAB system to frameworks that increasingly emphasize cytogenetic and molecular features, as reflected in the 2022 5th edition WHO and ICC systems. Both prioritize genetic

abnormalities over morphology in defining AML subtypes by recurrent gene mutations and chromosomal rearrangements [139,140,144]. Despite minor differences, both systems broadly agree on genetically defined entities, but they apply blast thresholds differently. WHO 2022 permits diagnosis of many AML entities with defining genetic abnormalities even when blasts are <20%, while maintaining a ≥20% blast requirement for selected entities, including *BCR::ABL1* fusion AML and AML with *CEBPA* mutation [139]. The ICC defines many genetically recurrent AML categories with a blast threshold of ≥10%, while maintaining a ≥20% blast requirement for patients with the *BCR::ABL1* fusion to limit overlap with chronic myeloid leukemia [140]. An additional change in the ICC is the introduction of "MDS/AML" for cases with 10–19% blasts in settings that do not otherwise meet criteria for a genetically defined AML entity, reflecting the biologic and clinical continuum and potentially expanding access to therapies and trials [226,227]. The ICC also recognizes "AML with mutated *TP53*" as a distinct, high-risk category with particularly poor prognosis. In contrast to WHO, the ICC does not retain therapy-related, secondary, or germline-associated myeloid neoplasms as separate AML entities; instead, these features are used as diagnostic qualifiers alongside the genetically defined diagnosis.

Prognosis is informed by biological and clinical factors at diagnosis. Besides age, performance status, comorbidities, and prior history, prognosis is largely determined by the genetic background of the leukemia [223,228–231]. The current European LeukemiaNet (ELN) guidelines stratify AML patients undergoing intensive chemotherapy into favorable, intermediate, and adverse risk groups based on cytogenetic events and genetic events [232]. The core-binding factor leukemias, *NPM1* mutations without *FLT3*-ITD, and AML with in-frame *CEBPA* bZIP mutations comprise the favorable risk group. These subtypes are generally more chemotherapy-sensitive and are characterized by higher remission rates and improved survival. Conversely, the adverse-risk category is linked to poorer response, with fewer patients achieving complete remission, and a propensity for relapse. This group includes *TP53* mutations, specific adverse cytogenetic abnormalities (including monosomies such as -5 and -7, inv(3)), complex karyotype, and myelodysplasia-related gene mutations. The remaining ~40% of cases fall within the intermediate risk group, including many patients with normal cytogenetics and, as of the 2022 ELN update, *FLT3*-ITD–mutated cases in the absence of favorable or adverse defining features [225]. Recently, the ELN also proposed risk models tailored to patients receiving less-intensive therapies [233].

1.3.3 AML therapy

Recent years have seen advances in AML treatment options, moving beyond conventional chemotherapy to incorporate more targeted approaches. Treatment is generally divided into an induction phase (to achieve complete remission, defined by <5% bone marrow blasts with peripheral blood count recovery), followed by consolidation to eliminate residual malignant cells and prevent relapse [234]. An upfront evaluation of patient fitness guides treatment decisions, as intensive chemotherapy is not advised for patients of higher age or with significant comorbidities [235–237]. While there is no universal tool to assess fitness, the Ferrara criteria are often used to identify patients unfit for intensive chemotherapy [238].

The standard intensive approach is the "7+3" induction regimen, consisting of 7 days of continuous cytarabine plus 3 days of an anthracycline (daunorubicin or idarubicin) [239]. In Sweden, a modified "5+3" regimen with higher-dose cytarabine is commonly used; reported remission rates in younger adults are ~60–85% following this intensive induction strategy [239,240]. Several additions to 7+3 have improved outcomes in selected molecular and clinical subgroups [241]. Incorporation of FLT3 inhibitors (midostaurin, quizartinib) for *FLT3*-mutated AML has been associated with improved remission rates and long-term survival [242,243]. Secondary AML and therapy-related AML (t-AML) benefit from CPX-351, a liposomal daunorubicin–cytarabine formulation, compared to conventional 7+3 in selected settings [244,245]. Immunotherapy approaches, including antibody-based therapies, cancer vaccines, immune-checkpoint inhibitors, and adoptive T-cell therapies, are under active investigation to address primary and acquired resistance [246,247]. Following induction, patients in remission receive consolidation therapy. Allogeneic HSCT is recommended for intermediate- and adverse-risk AML in first remission, whereas favorable-risk patients frequently undergo intensive post-remission chemotherapy [234].

In patients who are ineligible for, or elect not to receive, transplant, remission can be prolonged through maintenance therapy, including oral 5-azacitidine (CC-486) [248]. Patients evaluated as unfit for intensive chemotherapy are generally treated with a low-intensity regimen, often combining hypomethylating agents with the BCL-2 inhibitor venetoclax [249,250]. In addition, targeted inhibitors (e.g., IDH1/IDH2 or FLT3-directed agents) have shown promising results in molecularly defined subsets [251–253]. Prognosis remains unfavorable in relapsed or refractory disease, and only a small proportion of patients attain a second

remission with salvage therapy. [237,254]. When remission is achieved, salvage allogeneic HSCT remains the best option for durable cure [255–257].

1.3.4 Genomic landscape of AML

Large sequencing studies report that ~97% of AML patients harbor at least one recurrent somatic mutation [258]. These variants frequently co-occur, and most patients harbor multiple mutations at diagnosis. Recurrent AML mutations can be clustered into functional groups including signaling/kinase pathways, *NPM1*, epigenetic modifiers, transcription factors, tumor suppressors, spliceosome genes, and cohesin complex genes (Table 2) [221,259,260]. Together, these alterations illustrate how AML pathogenesis involves combinations of proliferative signaling lesions, differentiation blockades, epigenetic dysregulation, and loss of tumor suppression. The following section provides an overview of these categories, including example genes and disease-contributing mechanisms [258,261].

Functional Group	**Example mutations**
Signaling/Kinase pathway	*FLT3*, *KRAS*, *NRAS*, *KIT*, *PTPN11*, *NF1*
Nucleophosmin	*NPM1*
Epigenetic regulators	*DNMT3A*, *IDH1*, *IDH2*, *TET2*, *ASXL1*, *EZH2*, *MLL/KMT2A*
Transcription factors	*CEBPA*, *RUNX1*, *GATA2*
Tumor suppressors	*TP53*
RNA splicing	*SRSF2*, *U2AF1*, *SF3B1*, *ZRSR2*
Cohesin complex	*RAD21*, *STAG1*, *STAG2*, *SMC1A*, *SMC3*

Table 2: Overview of recurrent genetic lesions in AML sorted by functional groups. Adapted from Döhner et al. (2015) and DiNardo and Cortes (2016) [221,259]

1.3.4.1 Signaling and kinase pathways mutations

Found in ~60–70% of patients, this is the most frequently mutated functional group. Frequently mutated genes include *FLT3*, *NRAS*/*KRAS*, *KIT*, *PTPN11*, and *NF1*. *FLT3* is mutated in nearly one third of patients, often resulting in ligand-independent FLT3 tyrosine kinase signaling [262]. Similarly, RAS pathway genes are mutated in ~10–15% of cases, driving aberrant activation of MAPK signaling. *KIT* mutations are comparatively rare overall but are enriched in core-binding factor AML [263]. Collectively, this group confers a proliferative advantage to the malignant clone through hyperactivation of cell growth and survival pathways.

1.3.4.2 Nucleophosmin (NPM1) mutations

NPM1 mutations are among the most frequent genetic lesions in AML, found in ~25–30% of cases and enriched in patients with normal karyotype. *NPM1* mutations disrupt the nuclear localization of the NPM1 shuttle protein, leading to aberrant accumulation of NPM1 and its binding partners in the cytoplasm. Cytoplasmic mislocalization of NPM1 is a hallmark of this subtype and interferes with normal nucleolar functions, including regulation of p53 and HOX gene expression programs [264].

1.3.4.3 Epigenetic modifier mutations

Epigenetic regulators—affecting DNA methylation and chromatin modification—are recurrent mutations in >50% of AML cases and include *DNMT3A*, *TET2*, *IDH1*, *IDH2*, *ASXL1*, and *EZH2*. Mutations in *DNMT3A* are among the most common events in AML (~20% of de novo AML) and are connected to changed DNA methylation patterns, increased self-renewal, and impaired differentiation [265,266]. Conversely, *TET2* mutations (~10–20% of AML) disrupt 5-methylcytosine demethylation, resulting in accumulation of DNA methylation marks and impaired myeloid differentiation. Neomorphic *IDH1*/*IDH2* mutations (~20% of AML) produce 2-hydroxyglutarate, which can inhibit TET enzymes and certain histone demethylases, promoting an aberrant hypermethylation state and contributing to a differentiation block [267]. Truncating mutations in *ASXL1* can reduce the stability and function of PRC2, leading to loss of repressive histone methylation marks on lysine 27 (H3K27me3), derepression of normally silenced programs, and aggressive disease biology [268].

1.3.4.4 Transcription factor mutations

Somatic mutations in transcription factors (e.g., *RUNX1*, *CEBPA*, *GATA2*) and fusion genes generated by chromosomal rearrangements can disrupt transcriptional programs and impair differentiation.

1.3.4.5 Tumor suppressor mutations

TP53 is mutated in ~5–15% of AML cases, is enriched in older patients, and is associated with complex karyotype as well as secondary and therapy-related AML. Mutations in *TP53* disrupt the canonical function of p53 in mediating responses such as cell-cycle arrest and apoptosis, contributing to cell survival and genomic instability [269]. As a consequence, TP53 mutations represent a particularly high-risk lesion associated with poor prognosis.

1.3.4.6 Spliceosome complex mutations

Spliceosome gene mutations are also observed in AML, particularly in secondary AML in older patients; for mechanistic background, see the MDS section.

1.3.4.7 Cohesin complex mutations

The cohesin complex mediates sister chromatid cohesion and is important for proper chromosome segregation during mitosis and 3D genome organization [270]. Mutations in cohesin members (e.g., *STAG2*, *RAD21*, *SMC1A*, *SMC3*) may contribute to genome dysregulation and altered expression of differentiation-associated gene programs.

1.3.4.8 Cytogenetics

Over half of AML patients present with cytogenetic abnormalities, frequently resulting in chromosomal rearrangements and gene fusions, and ~10–12% have complex karyotype (often defined as ≥3 abnormalities) [271–273]. Recurrent AML-defining rearrangements include the core-binding factor events t(8;21) and inv(16)/t(16;16), generating the *RUNX1::RUNX1T1* and *CBFB::MYH11* fusions, respectively [139,140,274]. Translocation t(15;17) generates the *PML::RARA* fusion, encoding the PML–RARA oncoprotein that functions as a transcriptional repressor, blocks myeloid differentiation, and promotes aberrant survival signaling [275,276]. Less frequent events include *DEK::NUP214* and *MECOM* rearrangements.

1.3.5 *KMT2A*-rearranged AML

Chromosomal rearrangements involving the *KMT2A* gene (*KMT2A*-r; formerly *MLL*), located on chromosome 11q23, constitute a recurring group of cytogenetic abnormalities present in roughly 5–10% of acute leukemias [277]. These rearrangements are particularly frequent in infant leukemias, where 70–80% of cases harbor *KMT2A* fusions [278]. In AML, *KMT2A*-r comprise about 20% of pediatric cases compared to 5–10% of adult cases and are generally a dismal prognostic factor, including higher relapse rates and resistance to intensive chemotherapy [279]. *KMT2A*-r are heterogeneous, and over 100 fusion partners have been identified in acute leukemias. However, specific fusion partners are overrepresented, with a small set accounting for the majority of cases [280]. Fusion partners often influence disease phenotype. For example, t(4;11)(q21;q23), encoding *KMT2A::AFF1* (historically MLL–AF4), is most common in ALL. In contrast, t(9;11)(p21;q23), encoding *KMT2A::MLLT3* (historically MLL–AF9), is the most common fusion in *KMT2A*-rearranged AML [280,281]. Overall, *KMT2A::MLLT3* is

among the most prevalent *KMT2A*-r and accounts for a substantial fraction of cases across acute leukemias [282].

1.3.5.1 Canonical roles of wild-type KMT2A

KMT2A is a crucial epigenetic regulator of hematopoiesis and development. It encodes a large protein with histone methyltransferase activity that is proteolytically cleaved into two subunits [283–285]. In the healthy setting, the KMT2A protein regulates expression of key developmental genes, including *HOX* clusters and the cofactor *MEIS1*. Specifically, KMT2A has been shown to sustain expression of *HOXA9* and *MEIS1* in the earliest HSC and MPP populations, supporting expansion and self-renewal [286–289]. Structurally, the KMT2A protein contains several functional domains, including N-terminal DNA-binding motifs and a C-terminal SET domain that trimethylates histone H3 lysine 4 (H3K4me3) in association with multiple core cofactors (Figure 7, left) [290–292]. Through this, KMT2A deposits active histone marks and supports an open chromatin state at *HOX* loci. KMT2A also harbors a transactivation domain that recruits histone acetyltransferases, reinforcing active chromatin and transcription [293,294]. KMT2A functions within a multi-protein complex including Menin, LEDGF, and PAFc, which recruit KMT2A to target promoters and gene loci [295–297].

1.3.5.2 Consequences of the KMT2A::MLLT3 fusion

KMT2A protein fusions retain the N-terminal domain, maintaining the DNA- and Menin-binding functions, but lose the C-terminal SET domain. In its place, the fusion protein gains interaction motifs contributed by the partner. Many *KMT2A* fusion partners encode components of the super elongation complex (SEC) machinery e.g., *MLLT3* (AF9), *AFF1* (AF4), *MLLT1* (ENL), and *ELL* [298,299]. Through this, the fusion proteins aberrantly recruit SEC to KMT2A target genes, driving sustained transcriptional activation (Figure 7, right) [300]. A hallmark molecular consequence is enforced expression of *HOX* genes (especially *HOXA9*) and *MEIS1*, promoting an early arrest of myeloid differentiation while maintaining a self-renewing state that drives uncontrolled proliferation and leukemogenesis [301,302]. AF9 additionally contributes to epigenetic activation through interactions with the DOT1L methyltransferase complex, which mediates activating H3K79 methylation at *HOXA*/*MEIS1* loci and sustains expression [303]. Activation of *HOX*/*MEIS1* programs, Menin dependence, and recruitment of DOT1L are shared features across many *KMT2A* fusions, creating convergent therapeutic vulnerabilities.

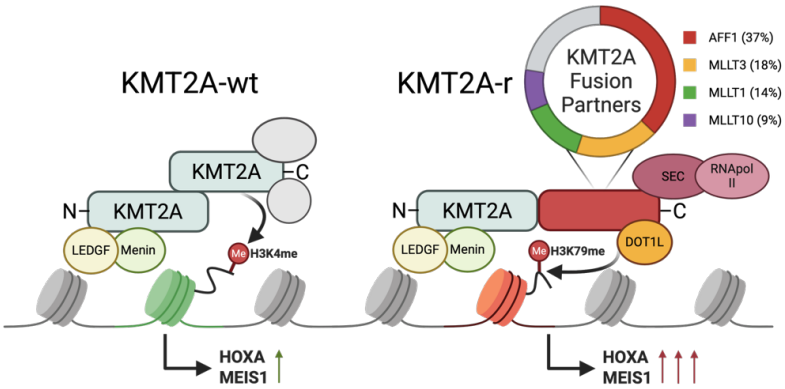

Figure 7: Molecular function of wild-type KMT2A (KMT2A-wt, left) and rearranged KMT2A (KMT2A-r, right) in regulating histone methylation marks and downstream consequences adapted from Mercher and Schwaller (2019) [304]. Recurrence of KMT2A fusion partners is adapted from Meyer et al. (2023) [280]. H3K4me/H3K79me, histone 3 lysine 4/79 methylation; SEC, super elongation complex; DOT1L, H3K79 histone methyltransferase. Created with BioRender.com.

1.3.5.3 Targeted treatments for KMT2A-r leukemias

Menin acts as a scaffolding protein that regulates gene expression by bridging DNA-bound factors and chromatin-associated complexes and is an essential cofactor in recruiting KMT2A fusion proteins to target loci [305–307]. This dependency has been demonstrated in studies where loss or inhibition of Menin abrogated oncogenic activity of KMT2A fusions, causing downregulation of HOX/*MEIS1* expression and reversal of the leukemic phenotype [308–311]. Mouse studies using small-molecule Menin inhibitors further reinforced the therapeutic potential of this strategy [312,313]. Several Menin–KMT2A inhibitors, including SNDX-5613 (revumenib) and KO-539 (ziftomenib), have entered clinical testing [314]. Targeting DOT1L using pinometostat (EPZ-5676) reduces H3K79 methylation and has shown modest activity in subsets of advanced KMT2A-r leukemia patients [315]. Combination strategies—especially with venetoclax-based regimens and hypomethylating agents —are being explored to improve depth and durability of response and to overcome resistance to Menin inhibition [316–320].

1.4 Experimental models of myeloid neoplasms

Preclinical models are pivotal for understanding MDS/AML pathogenesis and for testing therapies under controlled conditions. Most commonly, cell lines, primary patient material, patient-derived xenografts, and genetically engineered mouse

models have been employed [195,201,321–324]. This section briefly summarizes how these systems have advanced the field.

Immortalized cell lines have been particularly useful for studying AML biology and for evaluating efficacy and toxicity profiles of candidate drugs. With hundreds of characterized leukemic cell lines (e.g., KG-1, MOLM-13, Kasumi-1, HL-60, U937, and THP-1), these models are generally easy to maintain and modify, inexpensive, and scalable, but they have limited fidelity relative to primary disease [325,326]. A key limitation of cancer cell lines is that they can adapt to *in vitro* conditions through clonal selection and genetic drift. This may lead to the acquisition or enrichment of additional genetic alteration and downstream functional changes that diverge from the original patient background [327]. As a result, the genomic profile of the same cell line and drug responses can vary substantially between labs and findings from cell line-based screens may not reliably predict therapeutic activity in primary patient samples [328]. In contrast to the abundance of immortalized cell line models of AML, MDS cell lines are notoriously scarce, often fail to represent the disease phenotype, and are limited by poor proliferation and overall performance *in vitro* [329].

Genetically engineered mouse models that carry mutations designed to model key oncogenic events in humans have been widely used to dissect AML mechanisms and therapeutic responses [330]. For example, mouse models have been central to understanding the role of the *KMT2A::MLLT3* fusion in leukemogenesis and the regulation of *HOX* gene programs in hematopoiesis [287]. Similar mouse models have been generated for recurrent MDS genetic events, including heterozygous mutations in *SF3B1*, *SRSF2*, and *U2AF1* [331–334]. However, modeling low-risk MDS in mice is challenging, as current models often recapitulate only partial disease phenotypes and may fail to produce overt disease [335]. For example, in *SF3B1*^{K700E} models of MDS-*SF3B1*, mice develop anemia but typically lack defining features such as bone marrow dysplasia and ring sideroblasts and do not establish clear MDS [333]. More broadly, murine models with single-gene perturbations often miss the genetic complexity of primary MDS/AML. Encouragingly, ongoing advances in gene-editing approaches increasingly enable multi-lesion models that better reflect the heterogeneous nature of human disease [336]. Patient-derived xenograft models are widely used for studying leukemic complexity *in vivo* and are generated by transplanting primary patient cells into immunodeficient mice [337]. These systems have been successful in many AML contexts, including enabling identification of leukemic stem cells and

associated gene signatures, as well as supporting preclinical testing of novel treatments [338–340]. For MDS, results have been more mixed due to limited engraftment potential, particularly in lower-risk disease, which continues to hamper drug development [341]. Several strategies have improved engraftment in some settings by "humanizing" the niche, such as mice engineered to express human cytokines, as in MISTRG and NSGS mice, or through co-transplantation of human stromal components [342–345].

Because these systems incompletely recapitulate MDS (especially lower-risk disease), primary patient-derived cells have remained instrumental for advancing mechanistic understanding. For example, gene expression analyses of bone marrow $CD34^+$ cells from *SF3B1*-mutant patients identified key mis-splicing events and downstream pathways that shape MDS biology and drive ring sideroblast development [190,200,201,321–323,346,347]. At the same time, work with primary patient material has practical constraints, including limited availability, invasive sampling procedures, ethical considerations, short *in vitro* viability, and restricted experimental tractability. Induced pluripotent stem cells (iPSCs) have increasingly emerged as a tool to help bridge these gaps and are the focus of the following sections.

1.5 Induced pluripotent stem cells

In 2006, Takahashi and Yamanaka first described reprogramming mouse fibroblasts into iPSCs by expressing four transcription factors, OCT4, SOX2, KLF4, and c-MYC (OSKM; the "Yamanaka factors") [348]. Soon thereafter, iPSCs were generated from human cells using similar transcription factor combinations (Figure 8) [349,350]. In 2012, John B. Gurdon and Shinya Yamanaka were jointly awarded the Nobel Prize in Physiology or Medicine for the discovery that mature cells can be reprogrammed to pluripotency [351]. Like embryonic stem cells, iPSCs are capable of virtually unlimited self-renewal and can generate derivatives of all three germ layers [348,352].

While the original protocols used integrating retroviral vectors to deliver reprogramming factors, methods have evolved toward non-integrating systems (Sendai virus, mRNA, episomal DNA) to avoid genomic integration [353]. Further optimization of reprogramming cocktails has improved efficiency, and the range of somatic cell sources has expanded substantially [354]. The reprogramming process comprises two phases. In an initial stochastic phase, somatic lineage programs are progressively shut down while pluripotency-associated loci

become activated through epigenetic remodeling. In a subsequent hierarchical phase, an autoregulatory network consolidates and stabilizes self-sustaining pluripotency [355]. Once iPSC clones are established, iPSC quality is typically assessed by pluripotency marker expression, tri-lineage differentiation capacity, and genomic integrity [349,352]. Although iPSCs can be cultured for extended periods, it is recommended to routinely screen for acquired chromosomal abnormalities [356].

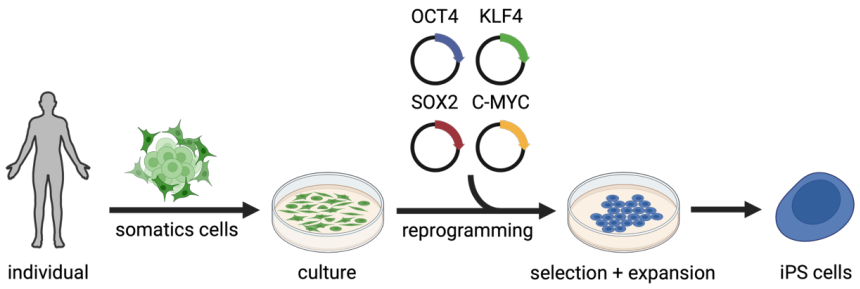

Figure 8: Generation of induced pluripotent stem cells. iPSC, induced pluripotent stem cell. Created with BioRender.com.

Today, iPSC culture is commonly performed under feeder-free and serum-free conditions (and in some cases xeno-free), enabling improved reproducibility while maintaining pluripotency. Cells are maintained in defined media on extracellular matrix components such as Matrigel, fibronectin, vitronectin, or laminins [357].

1.5.1 Hematopoietic differentiation protocols

iPSCs have been transformative for *in vitro* research and hold strong potential in disease modeling and regenerative medicine [358]. Beyond their expansion capacity and amenability to genetic manipulation, iPSCs can differentiate into a broad range of somatic cell types [353,359,360]. Generating bona fide HSCs from iPSCs has been a longstanding goal in regenerative medicine, with the promise of reducing donor dependence and immune rejection. As a result, multiple strategies to generate hematopoietic cells from iPSCs have been developed over the past decades [361].

Most differentiation protocols attempt to mimic aspects of *in vivo* hematopoietic development, although they differ in media composition, cytokine combinations, and timing [362]. In general, iPSCs are differentiated either as three-dimensional (3D) aggregates (embryoid bodies and related formats) or as a monolayer.

Mesoderm induction is followed by a hematopoietic specification stage that promotes development of hemogenic endothelium and endothelial-to-hematopoietic transition, producing $CD34^+$ HSPCs (Figure 9). Established protocols use sequential growth factors and morphogens, such as BMP4, VEGF, SCF, IL-3, and TPO, applied with staged timing to guide each developmental step [362–368].

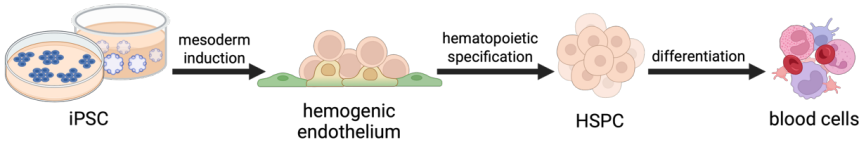

Figure 9: Critical steps and cell populations for the generation of hematopoietic cells from iPSCs, adapted from Rao et al. (2022) [369]. HSPC, hematopoietic stem and progenitor cell. Created with BioRender.com.

Despite advances, generating functional HSCs with robust long-term engraftment from iPSC cultures has proven challenging. iPSC-derived hematopoietic outputs often resembled yolk sac-like programs rather than AGM-like definitive hematopoiesis [370,371]. Multiple studies have shown that *HOXA* expression distinguishes yolk sac from AGM-like progenitors, and that adding the Wnt agonist/GSK3 inhibitor CHIR99021 and/or the ALK inhibitor SB431542 during mesoderm differentiation can promote a $HOXA^+$, AGM-like state [371–376]. Transient overexpression of *HOXA5* and/or *HOXA9* during endothelial-to-hematopoietic transition or in myeloid precursors has been reported to enhance repopulating capacity of iPSC-derived progenitors, but these approaches rely on genetic modification [377,378]. More recently, Ng et al. reported the generation of iPSC-derived HSCs capable of long-term multilineage engraftment in approximately half of recipient mice across multiple iPSC lines. This was achieved through precise timing and dosing of Wnt agonists, retinoic acid derivatives, and VEGF in a fully defined culture medium [379]. This development supports progress toward clinical translation and increases the relevance of iPSC-derived hematopoiesis for modeling adult hematopoietic malignancies.

1.5.2 Erythroid differentiation of iPSCs

In parallel to efforts to generate definitive HSCs, the production of functional erythroid cells from iPSCs holds promise for therapeutic applications and disease modeling. In transfusion medicine, iPSC-derived RBCs are attractive because they

could enable scalable production, expand access to rare blood types, and support donor-independent inventories that reduce immunological risks [380,381].

Differentiation of iPSC-derived hematopoietic progenitors toward erythroid cells is well described and typically involves multi-week protocols supplying factors such as EPO, IL-3, IL-6, TPO, and SCF, along with iron sources [382]. However, two major hurdles remain: yield and maturation. Current protocols fall short of producing the number of RBCs required for a single transfusion unit (on the order of 10^{12} cells) at a cost-effective scale [365,383]. To address this, multiple efforts to scale cultures using bioreactors, microcarriers, and agitation have been explored, alongside cost-reduction strategies such as simplified media formulations and reduced cytokine or iron supplementation [362,363,384–389].

The second hurdle is maturation. Key features of definitive RBCs include efficient enucleation and expression of β-globin, a component of the adult hemoglobin HbA. In contrast, iPSC-derived erythroid cells often remain partially nucleated and show incomplete switching from embryonic and fetal globin programs [390]. Strategies that better recapitulate physiological environments, including dynamic culture, 3D systems incorporating niche matrix components, or co-culture with macrophages or stromal elements, can improve maturation. In addition, transplantation studies suggest that iPSC-derived erythroid cells can complete maturation more effectively *in vivo*, supporting the possibility that clinically relevant RBC production may become feasible [391–393].

1.5.3 iPSCs as models of hematopoietic malignancies

Whereas therapeutic applications of iPSC-derived hematopoiesis still face major hurdles, iPSC systems have been used successfully to model a broad range of hematological diseases [394]. iPSCs can provide patient-specific, genetically defined platforms that capture the mutational landscape of the originating clone. They enable clonal expansion in culture and can be differentiated into desired target cell types, helping to overcome the scarcity and fragility of primary patient cells. Importantly, isogenic controls enable direct genotype-phenotype comparisons and can be generated either by reprogramming wild-type cells or by CRISPR-based editing of iPSC lines [353,395]. iPSCs also enable human-specific drug screening and mechanistic studies at scale (Figure 10) [396,397].

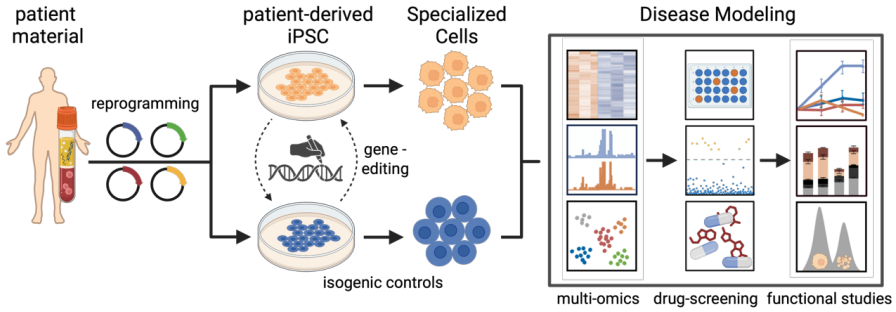

Figure 10: Schematic workflow for the generation and application of patient-derived iPSCs for disease modeling, mechanistic studies, and drug discovery. Created with BioRender.com.

Until recently, iPSCs were primarily applied to monogenic disorders with clear genotype-phenotype relationships, including inherited bone marrow failure syndromes [398,399]. Examples include Fanconi anemia, Diamond-Blackfan anemia, congenital neutropenia, familial platelet disorder, and others [400–404]. These diseases are often rare, patient material is limited, and a monogenic etiology simplifies generation of disease lines and corresponding isogenic controls [399]. More recently, improvements in gene editing and clonal isolation have expanded iPSC modeling to more genetically complex myeloid diseases, including MDS and AML.

In 2015, Kotini et al. generated MDS patient-derived iPSCs with del(7q) and established isogenic controls with normal karyotype [405]. In culture, del(7q) iPSC-derived hematopoietic progenitors showed impaired myeloid differentiation, consistent with features observed in primary patient samples [406]. Mechanistically, engineering heterozygous loss of defined chromosome 7 regions in normal iPSCs helped identify cooperating haploinsufficient genes, including *EZH2* and additional loci, as disease-relevant events [405]. These findings were later reproduced across panels of isogenic iPSC lines, where del(7q) induced a severe differentiation block in hematopoietic progenitor cells, consistent with its association with higher-risk MDS and disease progression. A large-scale drug screen using iPSC-derived hematopoietic progenitor cells from multiple del(7q) lines identified niflumic acid as a compound that selectively inhibited growth of del(7q) cells while sparing isogenic controls. This effect was also validated in primary samples from MDS and secondary AML patients with del(7q) or monosomy 7, illustrating how iPSC models can support discovery of targetable vulnerabilities [407].

Hsu et al. reported the generation of iPSC lines from *SF3B1*-mutant MDS using episomal reprogramming and showed that iPSC-derived hematopoietic progenitors could differentiate into erythroid cells with mitochondrial defects, recapitulating a key feature of *SF3B1*-mutant MDS [204]. Through integration of splicing and gene expression analyses, Asimomitis et al. identified a mis-splicing signature shared between iPSC-derived progenitors carrying *SF3B1*^{K700E} and primary patient cells. The same study also used chromatin accessibility analyses to implicate TEA domain transcription factor as a transcriptional regulator associated with the mutant state [408]. In a complementary study, Singh et al. reported that *SF3B1*-mutant cells derived from patient iPSCs, cell lines, and patient CD34^{+} cells accumulate R-loops (RNA–DNA hybrids). This was tied to increased DNA damage and activation of the ATR–Chk1 pathway, which could be mitigated by RNase H1-mediated R-loop resolution. Notably, *SF3B1*-mutant cells were selectively sensitive to ATR or Chk1 inhibition, and this vulnerability was enhanced by the splicing modulator sudemycin D6, suggesting a potential therapeutic strategy [409].

As an additional disease-relevant readout, Clough et al. generated ring sideroblasts in *in vitro* differentiated erythroid cells from *SF3B1*^{K700E} iPSCs. RNA-seq confirmed mis-splicing events and downregulation of genes involved in iron metabolism and heme synthesis (*ABCB7*, *MAP3K7*, *PPOX*, *TMEM14C*), consistent with observations in primary cells. Restoring expression of *ABCB7* (and to some extent *PPOX*) reduced ring sideroblast formation, supporting a functional link between reduced *ABCB7* and this phenotype [410]. Together, these studies reinforce mechanistic connections between spliceosome dysfunction and MDS pathophysiology and suggest downstream vulnerabilities for therapeutic targeting [394].

Modeling disease progression from MDS to AML and dissecting the contributions of individual mutations is challenging, given the complexity of clonal evolution. To address this, Kotini et al. generated iPSC panels from four patients reflecting preleukemia, lower-risk MDS, higher-risk MDS, and secondary AML. Hematopoietic progenitors derived from these lines captured stage-specific phenotypes and transcriptional programs associated with disease progression. Modeling transitions by correcting variants or sequentially introducing mutations through gene editing enabled either reversal of disease severity or stepwise progression from a near-normal phenotype toward transplantable AML [411]. Collectively, this work provided a framework for how stage transitions across myeloid malignancies

can be driven by combinations of cooperating lesions [412]. Using a similar approach, Wang et al. modeled progression from a healthy state through CH and MDS to AML by serially introducing mutations in *ASXL1*, *SRSF2*, and *NRAS*. Stage transitions were associated with transcriptomic and chromatin accessibility signatures that mirrored primary human MDS/AML. Importantly, inflammatory signaling dysregulation emerged as an early and persistent feature of leukemogenesis, suggesting a potential target for early intervention [413].

RAS pathway mutations are often late events, acquired upon progression from MDS or in relapsed/refractory AML [171,258,414–416]. The mechanistic basis for their timing has been unclear. Sango et al. generated CRISPR-edited iPSC models and reported that NRAS mutations alone were insufficient to establish leukemia, instead requiring preceding cooperating lesions to transform granulocyte-monocyte progenitors [417]. Acquisition of *RAS* mutations drove aberrant expression of BCL2 family genes, promoted a monocytic phenotype, coupled with a resistance against BCL-2 inhibition by venetoclax, offering a mechanistic explanation for poorer therapeutic responses in these settings [418,419]. A recurring theme across these studies is that disease phenotypes often emerge only during hematopoietic differentiation. Moreover, iPSC-derived hematopoietic progenitors corresponding to overt AML states have been most successful at serial engraftment and disease propagation in mice, whereas modeling pre-malignant states and lower-risk disease remains more challenging.

These dynamics were also observed in *KMT2A*::*MLLT3*-rearranged AML. Chao et al. derived iPSC lines from two individuals with these rearrangements and found that iPSCs originating from malignant and non-malignant clones were highly similar in the pluripotent state, including comparable transcriptional and epigenetic profiles and tri-lineage differentiation potential. In contrast, hematopoietic specification re-established leukemic molecular and cellular features, underscoring the requirement for a specific cellular context for disease manifestation [420]. This model provides a platform to study KMT2A-driven leukemogenesis in a human setting and enables testing of targeted therapies for this subtype.

Collectively, iPSC-based studies have uncovered genotype–phenotype relationships for specific lesions and enabled controlled modeling of disease evolution, improving understanding of clonal hierarchies and therapeutic vulnerabilities across myeloid neoplasms. At the same time, these studies

highlight important limitations: some clones, particularly those with complex genetic backgrounds, reprogram inefficiently and may be underrepresented during iPSC generation even when they constitute dominant clones in the patient [395,421]. In addition, limited engraftment of pre-malignant or lower-risk states remains a barrier. Ongoing improvements in gene editing, reprogramming, differentiation, and transplantation protocols are beginning to address these limitations, enabling more faithful modeling of complex clonal architectures and earlier disease states [379,413,417,422–424].

2 Research aims

STUDY I: *SF3B1*-MUTANT MODELS OF RNA MIS-SPLICING UNCOVER *UBA1* AS A THERAPEUTIC TARGET IN MYELODYSPLASTIC NEOPLASMS

- To evaluate previously established patient-derived iPSC lines and isogenic wild-type cells as models of *SF3B1*-mutant MDS biology by confirming key *SF3B1*^{K700E}-associated features, including established splicing abnormalities and erythroid phenotypes.
- To discover novel *SF3B1*^{K700E}-associated mis-splicing events.
- To assess these across iPSC-derived hematopoietic cell types, additional *SF3B1*-mutant model systems, and an MDS patient cohort.
- To define the molecular consequences of novel mis-splicing events by evaluating RNA fate, ribosome association/translation efficiency, and the stability and/or function of the resulting protein products.
- To test whether these splicing-driven molecular consequences create therapeutic vulnerabilities by assessing differential sensitivity of *SF3B1*-mutant versus controls, with the goal of selectively impacting mutant cells

STUDY II: TARGETING DYSREGULATED EPIGENETIC AND TRANSCRIPTION FACTOR NETWORKS IN *KMT2A*-REARRANGED AML USING IPSC MODELS

- To leverage patient-derived AML iPSCs and isogenic controls to model hematopoietic dysregulation in *KMT2A*-rearranged AML by differentiating iPSCs into hematopoietic progenitor populations suitable for mechanistic and functional analyses.
- To define when and how transcriptional dysregulation emerges during hematopoietic development in *KMT2A::MLLT3* AML via time-course transcriptomic profiling of iPSC differentiation.
- To identify transcription factor programs and epigenetic regulatory mechanisms associated with the AML-like HSPC transcriptional state by integrating promoter activity with motif enrichment and regulatory network inference.
- To test therapeutic actionability of these inferred dependencies by perturbing selected epigenetic regulators and assessing effects on AML-associated gene programs and hematopoietic output.

3 Materials and Methods

This section provides an overview of the relevant methods used in this thesis; detailed descriptions are provided in the Methods sections of the studies.

Ethical considerations and patient material

All studies involving human-derived material were conducted in accordance with ethical principles for medical research, including the Declaration of Helsinki, with written informed consent obtained from all donors or patients prior to sample collection and research use. The original iPSC lines used in **Study I** were generated from bone marrow samples obtained from three patients with MDS-RS. Bone marrow samples for primary $CD34^+$ CFU assays were obtained from three patients with MDS-*SF3B1* and two healthy donors at Karolinska University Hospital, Huddinge, Sweden. The study was approved by the Ethics Research Committee at Karolinska Institutet (2017/1090-31/4, 2022-03406-02 and 2024-03119-02). The iPSC lines used in **Study II** were previously generated from AML patient samples obtained under Institutional Review Board-approved protocols at Stanford University (Stanford IRB 18329 and 6453), following informed consent, and reprogramming of AML samples was conducted under Stanford IRB 28197.

iPSC culture

Patient-derived iPSC lines were central to all projects of this thesis, and their detailed properties are summarized in Table 3. In **Study I**, we used iPSC lines from a female MDS patient with ring sideroblasts harboring an isolated *SF3B1*^{K700E} mutation, which were generated by Asimomitis et al. [408]. iPSC lines used in **Study II** were previously generated by Chao et al. and are derived from two female AML patients harboring *KMT2A* rearrangements [420]. iPSCs were generated by transducing bone marrow mononuclear cells, primary AML cells, and T cells using the CytoTune-iPSC 2.0 Sendai reprogramming kit. Specific details for the reprogramming conditions are provided in the original publications.

All iPSC lines were cultured in feeder-free conditions on Matrigel hESC-Qualified Matrix. Matrigel is a basement membrane extracellular matrix preparation isolated from Engelbreth-Holm-Swarm mouse sarcomas, rich in extracellular matrix proteins that support iPSC attachment and growth. iPSCs were maintained in mTeSR Plus with 1% penicillin–streptomycin (P/S), clump-passaged with EZ-LiFT Stem Cell Passaging Reagent.

Study	Background	Original ID	Study Name	Cytogenetics	Co-Mutations
Study I	Female 65 Years MDS-RS	N-22.45	*SF3B1*^{WT} iPSC	46, XX, +mar	
		MDS-22.45	*SF3B1*^{K700E} iPSC	46, XX, +mar	*SF3B1*-K700E
Study II	Female 20 Years Relapsed AML	SU223-T3	Normal iPSC	46, XX	
		SU223-B3	AML iPSC 1.1	46, XX t(9;11)(p22;q23)	*FLT3-ITD* *NRAS*-G12D *SEMA4A*-Y589H
		SU223-B5	AML iPSC 1.2	(9;11)(p22;q23)	*FLT3-ITD* *NRAS*-G12D *SEMA4A*-Y589H
	Female 61 Years De novo AML	SU042-3	AML iPSC 2.1	46, XX t(10;11)(p11.2~12; q23)	*ARID1A*-P1326 *ATM*-V2193I *DNMT3A*- S837Stop *SMG1*-L250V *SPEN*-T1673S

Table 3: iPSC lines used for this thesis including mutational and cytogenetic features.

Cell culture

Leukemic cell lines were used in both studies to assess whether findings from the iPSCs were consistent across *in vitro* models. **Study I** employed the widely used K562 erythroleukemia cells, including an engineered line harboring an *SF3B1*^{K700E} mutation. To investigate whether PRC2 inhibition selectively acts in *KMT2A*-rearranged cells in **Study II**, we compared responses in HL-60 and OCI-AML-3 (*KMT2A* wild-type) to THP-1 and MONO-MAC-6 (*KMT2A*::*MLLT3*) cells. K562, HL-60, OCI-AML-3, and THP-1 cells were maintained in RPMI 1640 with glutamine, 10% heat-inactivated fetal bovine serum (FBS), and P/S. MONO-MAC-6 cells were further supplemented with non-essential amino acids, sodium pyruvate, and insulin. Leukemic lines were maintained at densities between 0.1–1.0 × 10^6 cells/mL as suspension cultures. HEK-293T cells were cultured in DMEM supplemented with 10% newborn calf serum and P/S. All cells were cryopreserved in 50% culture medium, 40% heat-inactivated FBS, and 10% DMSO using controlled-rate freezing. Cell cultures were maintained in a humidified 37°C incubator under standard tissue-culture conditions (5% CO_2, normoxia) and regularly confirmed to be mycoplasma-negative.

Hematopoietic differentiation

The generation of hematopoietic cells from iPSCs was central to the studies included in this thesis, as HSPCs enabled experimental modeling of disease-associated phenotypes and responses to treatment. Over the course of the PhD projects, multiple differentiation approaches and protocol modifications were evaluated. Changes were made based on advances in the field as well as practical considerations such as reagent availability, cost, and reproducibility. The following section provides an overview of the hematopoietic differentiation workflows applied in **Study I** and **Study II**, and outlines the current protocol implemented in the group for ongoing projects (Figure 11). As described in the background section, the generation of hematopoietic cells from iPSCs generally follows a stepwise protocol designed to recapitulate key aspects of embryonic hematopoietic development *in vitro*. This involves sequential media changes and timed cytokine additions to guide lineage progression from early mesoderm-like states to hemogenic endothelial cells, from which HSPCs emerge and accumulate in the non-adherent/supernatant fraction.

For **Study I**, HSPCs were generated using the commercially available and widely used STEMdiff Hematopoietic Kit. This kit is based on two media stages that promote mesoderm-like induction, followed by hematopoietic specification. In our hands, hematopoietic progenitors emerged from ~day 10, and cells were harvested on day 13 for downstream experiments. This protocol was reproducible, but it is comparatively cost-intensive and based on proprietary formulations, which limits control over individual media components.

For **Study II**, we implemented a protocol adapted from Matsubara et al., based on the Stemline II Hematopoietic Stem Cell Expansion Medium formulation [425]. This 13-day protocol comprises four media stages, supported by the addition of recombinant cytokines and morphogens. Compared with the kit-based protocol, this approach provided greater flexibility and allowed for adjustments; however, varying availability and incomplete disclosure of the base media formulation remained a challenge.

Finally, recent work has marked a major milestone in the field with the development of differentiation protocols capable of generating iPSC-derived hematopoietic cells with multilineage engraftment potential, achieved without introducing leukemic driver mutations or relying on artificial transcription factor overexpression [378,379,411]. While this protocol was not used in the studies

comprising this thesis, we have adapted it in our research group and it currently serves as the standard for ongoing projects. Besides the biological relevance of the generated cells, a practical advantage of this workflow is the use of fully defined, serum-free conditions, which support long-term reproducibility and simplify standardization across experiments.

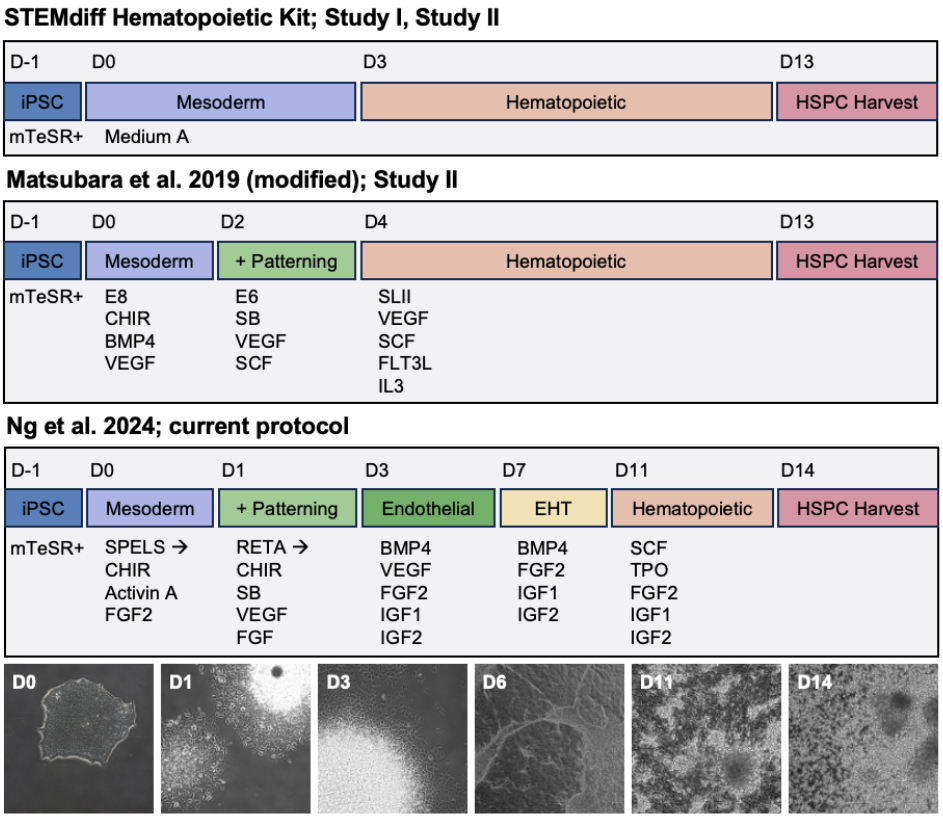

Figure 11: Overview of the hematopoietic differentiation protocols used in the Study I and Study II as well as the current approach adapted by our research group. Arrows indicate additions that are continued from that timepoint onward. Bottom: Representative images of differentiating cultures from normal iPSC. E8, Essential 8 medium; E6, Essential 6 medium; SPELS, SPELS medium; CHIR, CHIR99021; SB, SB-431542; RETA, retinyl acetate; EHT, endothelial-to-hematopoietic transition.

In **Study I**, iPSC-derived hematopoietic progenitors were further directed toward the erythroid lineage by changing to media conditions that promote erythroid maturation and expansion. This included supplementing cells with erythropoietin and transferrin throughout the protocol, as well as SCF and IL3 during the first week. CD235a^{+} erythroid cells were enriched by magnetic separation for downstream analyses.

Cell transfection experiments

Transfection-based approaches were used in **Study I** to manipulate *UBA1* expression and to validate sensitivity to targeted inhibitors. Plasmid transfection introduces an expression vector into cells to drive ectopic production of a protein of interest, whereas siRNA-mediated knockdown delivers short double-stranded RNAs that promote sequence-specific degradation of the target mRNA, thereby reducing endogenous protein levels. For overexpression experiments, plasmids encoding wild-type *UBA1* and the mis-spliced *UBA1* variant (including the additional 135 bp sequence) were introduced into HEK-293T cells using cationic lipid-mediated transfection. To reduce endogenous *UBA1* expression, *UBA1*-targeting siRNAs were delivered into K562 cells by electroporation. Following transfection, cells were harvested at defined time points for downstream RNA and protein analyses.

PCR-based assays

PCR-based assays were used in **Study I** to quantify individual gene expression levels and to assess specific transcript isoforms, with a particular focus on *UBA1* splice forms. For splice variant quantification, primer design was adapted from a previously published strategy [426] to generate primer pairs spanning the canonical splice junction, priming within the mis-spliced (variant) sequence, or amplifying an external control region upstream of the splice site (Figure 12). Primers were designed using various online tools and selected based on predicted specificity against human transcript databases. Nucleic acids were isolated using spin column–based kits, and input amounts were quantified prior to downstream analysis. For reverse transcription quantitative PCR (RT-qPCR), RNA was extracted, quantified, and reverse-transcribed to cDNA prior to SYBR Green–based qPCR. Expression changes were reported as fold change after normalization to *18S* rRNA and, where applicable, to the upstream control signal. Comparisons between groups were made using the ΔΔCt method. Conventional PCR was used to detect splice products using exon-spanning primers, with amplicons resolved by gel electrophoresis to visualize product sizes. In addition, droplet digital PCR (ddPCR) was used in **Study I** to determine *SF3B1*^{K700E} status per colony.

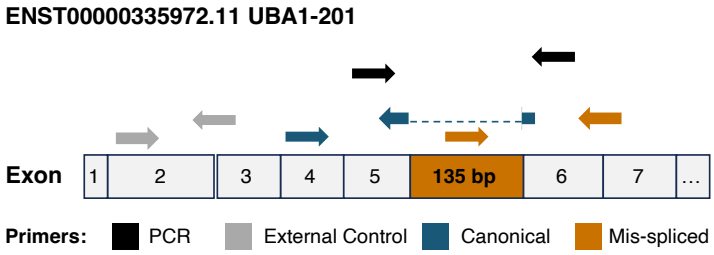

Figure 12: Primer design strategy for the detection *UBA1* splice forms by PCR amplification of the *UBA1*^{ms} region (black) and quantification of *UBA1* splice forms by RT-qPCR (gray, blue, orange).

Immunoblotting

Immunoblotting was used to assess protein abundance and post-translational marks in response to experimental perturbations. In brief, proteins were extracted from cell pellets, separated by SDS–PAGE (where proteins are denatured and resolved according to molecular weight), and then transferred to nitrocellulose membranes. Proteins were detected using specific primary antibodies followed by HRP-conjugated secondary antibodies. Signal was generated by addition of a chemiluminescent HRP substrate, which produces light in an enzyme-catalyzed reaction at sites where the target protein is bound, enabling band detection and quantification. In **Study I**, immunoblotting was performed on whole-cell lysates to confirm UBA1 isoform expression across different experimental settings and cell models. The method was also used to evaluate protein-level responses to treatment, including global ubiquitination and apoptosis-associated readouts such as PARP1 and caspase-3 cleavage. In **Study II**, immunoblotting was used to quantify changes in H3K27me3 following treatment and to guide dose selection based on target engagement. Across both studies, protein concentrations were determined prior to loading, signals were acquired on an Odyssey FC system, and band intensities were quantified in ImageStudio with normalization to housekeeping or reference proteins.

mRNA and protein stability assays

In **Study I**, mRNA and protein stability assays were used to determine how the mis-spliced *UBA1* transcript and its encoded protein are regulated post-transcriptionally. Transcript stability was assessed in a time-course experiment by blocking de novo transcription with actinomycin D, which intercalates into DNA and prevents RNA polymerase progression [427], followed by RT-qPCR using splice form–specific primers. In parallel, we evaluated sensitivity to NMD, a pathway that promotes the degradation of transcripts containing premature stop

codons, by inhibiting translation with cycloheximide and assessing splice variant presence by RT–PCR [198]. At the protein level, UBA1 isoform stability was measured using cycloheximide chase experiments in cells transfected with expression-tagged UBA1 constructs, with time-dependent changes in protein abundance quantified by immunoblotting to estimate protein half-life. To evaluate proteasome-mediated degradation, transfected cells were co-treated with the proteasome inhibitor MG-132 and protein levels were assessed by immunoblotting. Throughout these experiments, short-lived transcripts and proteins, as well as established NMD target transcripts, were included as controls.

RNA sequencing analysis

RNA-seq was used in **Study I** and **Study II** to profile the transcriptome, analyze splicing patterns, and quantify promoter activity. In both studies, RNA from bulk or sorted cell populations was isolated using column-based kits, quality-controlled prior to library preparation, sequenced, and analyzed using workflows that follow the same overall logic: read pre-processing, alignment/mapping, quantification, and statistical or functional interpretation.

In **Study I**, full-length bulk RNA-seq libraries were prepared from total RNA using SMARTer Stranded Total RNA-Seq Kits v2 with enzymatic ribosomal depletion and sequenced using an Illumina NovaSeq 6000 S4 as paired-end 150 bp reads. Reads were adapter- and quality-trimmed with TrimGalore and Cutadapt prior to two-pass alignment to the human reference genome (GRCh38) using STAR, and gene-level counts were generated from uniquely mapped reads using featureCounts. Differential expression testing was performed with DESeq2, with p-values adjusted using the Benjamini–Hochberg method. Differential splicing analysis between splicing factor–mutant and normal samples was performed using rMATS, with p-values calculated using a likelihood-ratio test and adjusted using the Benjamini–Hochberg correction. Selected splicing events were visualized using sashimi plots generated with ggsashimi.

In **Study II**, RNA-seq and cap analysis of gene expression (CAGE) were used to quantify transcript abundance and promoter activity, respectively. RNA quality was assessed using Agilent TapeStation prior to library construction following Illumina stranded messenger RNA prep ligation sample preparation protocols. Sequencing was performed on an Illumina NextSeq platform. Raw sequencing data were processed using the MOIRAI pipeline to obtain uniquely mapped reads. For CAGE analyses, mapped reads were overlapped with the FANTOM5 robust

promoter set to quantify promoter activity and compare promoter usage between conditions. In addition, motif activity response analysis (MARA) was performed to assess the promoter-proximal region (-300 bp to +100 bp) surrounding representative CAGE peaks and infer transcription factor motif activity. Protein interaction context for selected candidates was explored using STRING network analysis (v12.0) with default parameters.

Flow cytometry

Flow cytometry was used throughout this thesis to benchmark the output of hematopoietic differentiation protocols and to assess the cellular composition of the generated progenitor populations. In **Study I** and **Study II**, iPSC-derived hematopoietic progenitors were characterized using CD34, CD43, and CD45, together with a viability dye to exclude dead cells. In **Study I**, erythroid differentiation was additionally evaluated using CD71 and CD235a. Cells were stained on ice and fixed prior to acquisition and analyzed on a BD LSRFortessa at the MedH Flow Cytometry Core Facility, which receives funding from the Infrastructure Board at Karolinska Institutet. Flow cytometry was further used for viability assays, which are described in a separate section.

CFU assays

The colony-forming unit (CFU) assay is a standard functional readout for hematopoietic progenitors and is used to assess clonogenic capacity and lineage output. Cells are seeded at low density in a semi-solid, methylcellulose-based matrix supplemented with cytokines, where single progenitors proliferate and differentiate into discrete colonies that can be counted and scored. In this thesis, CFU assays served as a functional readout in both studies and were performed using iPSC-derived HSPCs, primary $CD34^+$ bone marrow cells, and leukemic cell lines.

In **Study II**, HSPCs derived from control and AML iPSCs were plated in CFU medium containing combinations of the EZH1/2 inhibitor UNC1999, the DNA methyltransferase inhibitor 5-azacitidine, or vehicle, to assess the impact of these compounds on the clonogenic capacity of *KMT2A*-rearranged cells. Colonies were scored after 14 days. To assess self-renewal following treatment, replating assays were performed by collecting colonies from co-treated and untreated AML cultures and re-seeding cells in fresh CFU medium without drugs; secondary colonies were scored after 10 days. To validate the findings obtained in iPSC-derived cells, the same experimental setup was applied to an AML cell line panel

(HL-60, OCI-AML-3, THP-1, and MONO-MAC-6), with adjustments to seeding density and culture duration.

In **Study I**, CFU assays were performed using *SF3B1*^{WT} and *SF3B1*^{K700E} K562 cells to examine the effect of *UBA1* inhibition with TAK-243 (or vehicle) on colony-forming potential. CFU assays were also performed using primary CD34$^+$ cells from *SF3B1*-mutated MDS patients and healthy donors. CD34$^+$ cells were enriched from bone marrow mononuclear cells by magnetic separation and plated under TAK-243 or vehicle conditions. After 14 days, colonies were scored and individual colonies were picked for DNA isolation followed by droplet digital PCR to determine the contribution of *SF3B1*-mutant versus residual wild-type progenitors. To reduce bias and improve reproducibility, colony scoring and colony picking were performed in a blinded manner.

Viability assays

Viability assays were used in **Study I** to determine how different compounds affected *SF3B1*-mutant versus wild-type cells and to generate dose–response curves across the model systems. Cells were treated for 24–72 hours and, depending on the assay format, viability was assessed by either flow cytometry–based staining or a luminescence-based readout. For flow cytometry, treated cells were stained with ApoTracker Green and the Aqua LIVE/DEAD viability dye, and live cells were defined as Aqua$^-$/ApoTracker$^-$ singlets. For the luminescence-based approach, CellTiter-Glo was used to quantify intracellular ATP as a proxy for metabolically active cells. Following treatment, CellTiter-Glo reagent was added directly to the cultures and luminescence was recorded on a plate reader. Dose–response curves were fitted to determine IC_{50} values where applicable.

Data analysis

Flow cytometry data were analyzed using FlowJo v10. Statistical analyses were primarily performed in GraphPad Prism v10 and RStudio, and data are presented as mean ± standard error of the mean (SEM) unless stated otherwise. Statistical comparisons were performed using unpaired t-tests, one-way ANOVA, or two-way ANOVA, with multiple comparisons controlled using Šidák, Holm–Šidák, Tukey, or Dunnett post hoc tests, as indicated in the figure legends. Nonlinear regression was used to fit dose–response relationships (four-parameter logistic curves for IC_{50} estimation) and decay kinetics (one-phase decay).

4 Results and Discussion

4.1 Study I

Study rationale

MDS with *SF3B1* mutation (MDS-*SF3B1*) is a clinically and biologically distinct subtype of myelodysplastic neoplasms, characterized by ring sideroblasts and predominantly erythroid cytopenias. *SF3B1* mutations alter splice site recognition, causing widespread cryptic 3′ splice site usage, and resulting in mis-splicing of genes implicated in hematopoietic and erythroid differentiation. Progress in identifying disease-relevant therapeutic targets has been slowed by the fact that splicing patterns are cell context-dependent and are not consistently captured across commonly used systems (including *SF3B1* mouse models, limited primary material, and unrepresentative cell line models). To address this in **Study I**, we used genetically matched *SF3B1*^{K700E} and *SF3B1*^{WT} patient-derived iPSCs as a scalable human platform. We applied full-length RNA-seq with unsupervised splicing profiling during hematopoietic differentiation to uncover *SF3B1*-linked mis-splicing events with tractable downstream consequences, ultimately highlighting *UBA1* mis-splicing as a potential therapeutic vulnerability.

Results

Patient-derived, isogenic *SF3B1*^{K700E} iPSCs were differentiated toward hematopoietic and erythroid lineages and analyzed by full-length RNA-seq. This revealed a previously unreported RNA mis-splicing event in *UBA1* (*UBA1*^{ms}), created through the retention of an intronic sequence between *UBA1* exons 5–6 **(Figure 13A; Study I, Figure 1C)**. RT-qPCR and RT-PCR detected *UBA1*^{ms} in both *SF3B1*^{K700E} iPSC-derived erythroid cells and CD34^{+} HSPCs, but not in *SF3B1*^{WT} controls and these results were reflected in K562 cells and primary material **(Figure 13B; Study I, Figure 1D–F)**. The same event was present in the patient used for iPSC reprogramming and, in cohort CD34^{+} RNA-seq data, appeared exclusively in *SF3B1*-mutated cases, with no detection in other splice factor-mutated or wild-type MDS or in healthy donors **(Figure 13C; Study I, Figure 5C–F)**.

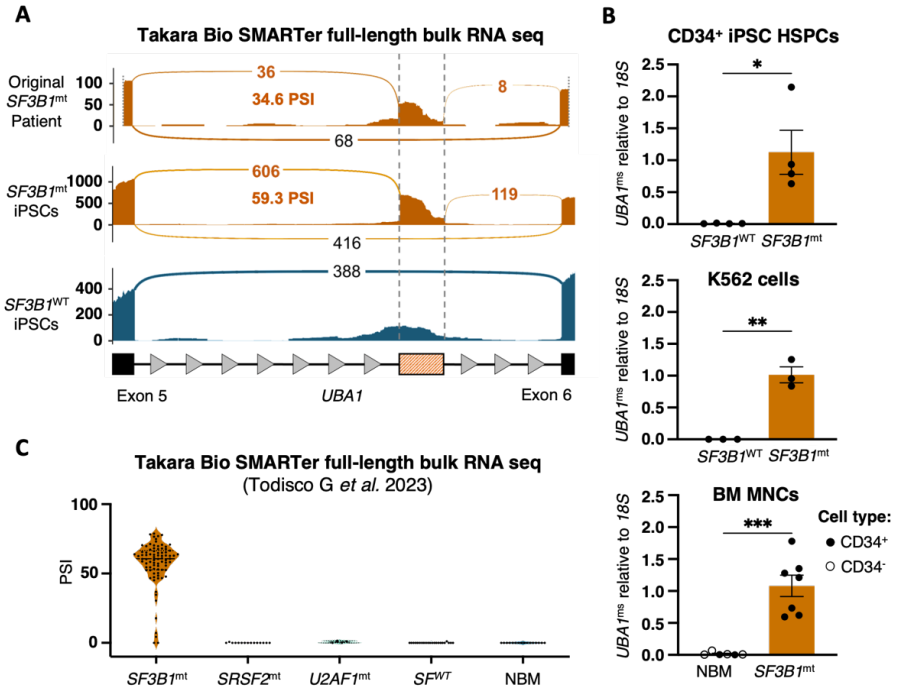

Figure 13: *UBA1* mis-splicing in MDS-*SF3B1*. (A) Sashimi plots of the mis-spliced region of *UBA1* in *SF3B1*^{WT} and *SF3B1*^{K700E} from total RNA sequencing of iPSC-derived CD235a^{+} erythroblasts, and primary CD34^{+} BM MNCs from the original MDS-*SF3B1* patient (n = 1). Black, canonical splice junction counts; orange, mis-spliced junction counts. y-axis, absolute read counts. (B) qPCR analysis of *UBA1*^{ms} relative to *18S* in iPSC-derived CD34^{+} HSPCs (n = 4), K562 cells (n = 3), and CD34^{+} (filled circles) or CD34^{-} (empty circles) cells from primary BM MNCs of healthy donors (NBM; n = 6) and *SF3B1*-mutated MDS patients (*SF3B1*^{mt}; n = 7). Mean ± SEM relative expression. Unpaired *t*-test. (C) Violin plots of *UBA1* intron 5 mis-splicing PSI from total RNA sequencing of CD34^{+} BM MNCs from our previously published data [208], organized by splicing factor mutation. *SF3B1*^{mt}, *SF3B1*-mutated; PSI, percent spliced-in; BM MNC, bone marrow mononuclear cells; NBM, normal bone marrow from healthy donors. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Because *UBA1* encodes an E1 enzyme essential for initiating the ubiquitination cascade, we asked whether *UBA1*^{ms} was accompanied by altered protein abundance. Indeed, *SF3B1*^{K700E} iPSC-derived hematopoietic cells showed lower total UBA1 protein than *SF3B1*^{WT} cells **(Figure 14A; Study I, Figure 1G–H)**. An orthogonal *SF3B1*^{K700E} K562 model recapitulated both *UBA1*^{ms} and reduced UBA1 protein, indicating that this association was not limited to iPSC-derived cells **(Figure 14B-C; Study I, Figure 2A–B, 2H–I)**.

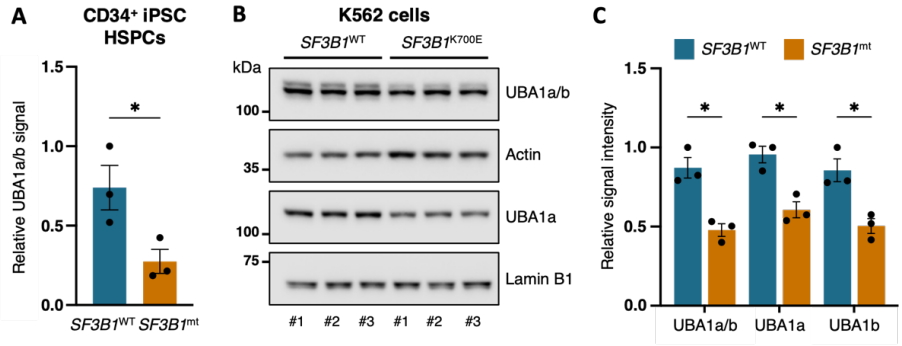

Figure 14: Models of MDS-*SF3B1* feature reduced UBA1 protein levels. (A) Quantification of UBA1a/b protein levels in whole-cell lysates of *SF3B1*^{WT} and *SF3B1*^{K700E} iPSC-derived CD34^{+} cells by immunoblotting analysis (n = 4). Actin was used as a loading control, and relative signals were normalized by lane normalization factor. Mean ± SEM relative UBA1 signal intensity. Unpaired *t*-test with Holm-Šídák's multiple comparisons test. (B) Immunoblot analysis and (C) quantification of UBA1 isoforms in whole cell lysates from *SF3B1*^{WT} and *SF3B1*^{K700E} K562 cells (n = 3). Actin was used as a loading control for total UBA1 and UBA1b; Lamin B1 was used as a loading control for nuclear UBA1a, and relative signals were normalized by lane normalization. Mean ± SEM relative signal intensity. Unpaired *t*-test with Holm-Šídák's multiple comparisons test. *, $P \leq 0.05$.

Because many *SF3B1*-associated mis-splicing events are degraded through NMD, reducing levels of functional transcript, we tested whether altered RNA stability could explain reduced UBA1 protein. *UBA1*^{ms} does not introduce a premature stop codon, and after transcriptional shutoff, *UBA1*^{ms} and canonically spliced *UBA1* transcripts displayed similar stability **(Figure 15A; Study I, Figure 2C)**. In parallel, inhibiting NMD did not increase *UBA1*^{ms} abundance; in contrast, *ABCB7*, a known MDS-*SF3B1* mis-spliced NMD target accumulated under the same conditions **(Study I, Figure 2D-E)**. Polysome profiling further showed enrichment of *UBA1* transcripts in polysome fractions irrespective of splice form, suggesting that impaired ribosome engagement is not the primary driver of reduced protein **(Study I, Figure 2F–G)**. This shifted our focus toward post-translational mechanisms. When expressed in HEK-293T cells, FLAG-tagged UBA1^{MS} protein accumulated to much lower levels than UBA1^{WT} despite comparable transcript levels **(Study I, Figure 3A–E)**. Cycloheximide chase experiments showed rapid decay of UBA1^{MS} protein compared with UBA1^{WT}, and proteasome inhibition partially rescued UBA1^{MS} abundance, consistent with proteasome-dependent clearance of an unstable protein **(Figure 15B; Study I, Figure 3F–I)**.

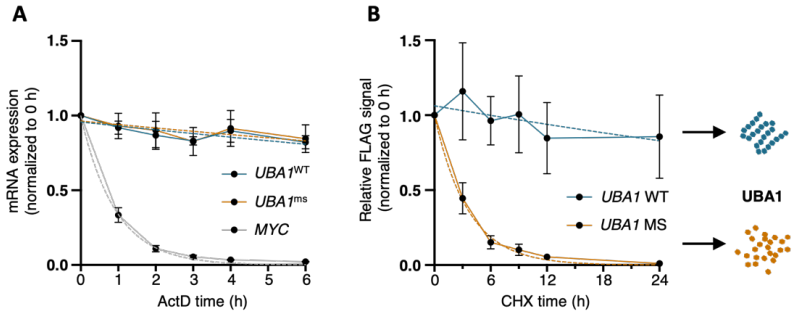

Figure 15: UBA1 stability analysis. (A) qPCR analysis of *UBA1*^{WT} and *UBA1*^{ms} transcript levels in *SF3B1*^{K700E} K562 cells after treatment with actinomycin D (ActD) for the indicated time points (n = 3). Results were normalized to 0 hours, and *MYC* was included as a fast-degrading transcript control. Mean ± SEM relative expression, One-phase decay nonlinear curve fit (dotted line). (B) quantification of FLAG-tagged protein levels in HEK293T cells 72 hours post-transfection with *UBA1* WT, *UBA1* MS or control plasmids and treatment with 50 µg/ml cycloheximide (CHX) for the indicated time points (n = 3). Actin was used as a loading control, and signals were normalized to relative signals at 0 hours for each group. Mean ± SEM relative signal intensity, interpolation of a one-phase decay non-linear regression curve (dotted line).

On this basis, we tested whether *SF3B1*^{K700E} cells are more sensitive to pharmacologic UBA1 inhibition. A previous study identified TAK-243 as a potent, selective, small-molecule inhibitor that blocks catalytic activity of UBA1, leading to a depletion of cellular ubiquitin conjugates [428]. TAK-243 reduced viability to a greater extent in *SF3B1*^{K700E} than in *SF3B1*^{WT} K562 cells **(Study I, Figure 4A–B)**. We observed a similar effect in iPSC-derived CD34^{+} HSPCs, where *SF3B1*^{K700E} cells were more sensitive than isogenic controls **(Study I, Figure 5A–B)**. Sensitivity also correlated with UBA1 abundance: siRNA-mediated *UBA1* knockdown increased TAK-243 sensitivity **(Study I, Figure 4C–F)**. Functionally, TAK-243 shifted clonal composition in WT:mutant co-cultures and reduced clonogenic output in colony assays, with mutant progenitors markedly reduced while WT clonogenicity was relatively preserved **(Study I, Figure 4G–I)**. Finally, in primary CD34^{+}-enriched bone marrow mononuclear CFU assays, TAK-243 reduced colony output more in MDS-*SF3B1* samples than in healthy controls, and single-colony genotyping indicated that this reduction was largely driven by loss of *SF3B1*-mutant colonies, with relative preservation of WT colonies from residual healthy clones **(Figure 16; Study I, Figure 5G–I)**.

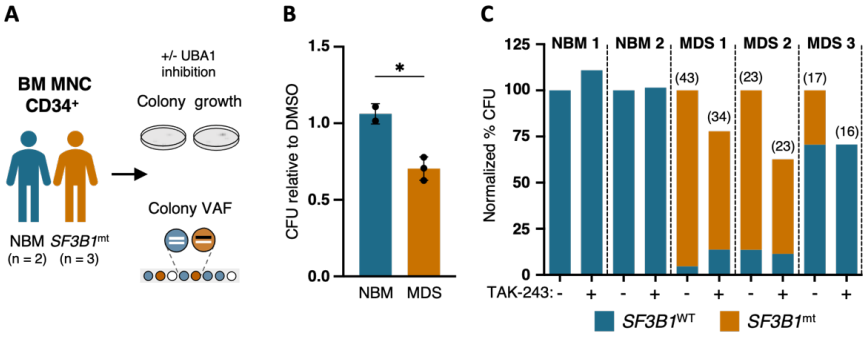

Figure 16: *UBA1*^{ms} in MDS-*SF3B1* patients confers sensitivity to UBA1 inhibition. (A) Experimental strategy to assess the effect of UBA1 inhibition on colony growth and composition in CD34^{+}-enriched bone marrow MNCs from MDS-*SF3B1* patients and healthy controls. (B) Effect of UBA1 inhibition on CFU counts relative to DMSO and (C) frequency of *SF3B1*^{WT} and *SF3B1*^{mt} colonies relative to total CFU counts from MDS patient (n = 3) or healthy control (n = 2) cells treated with 32 nM TAK-243 or DMSO for 14 days. Numbers within brackets indicate colonies assessed by ddPCR. Mean ± SEM. Unpaired *t*-test. *, $P \leq 0.05$; ns, not significant. MDS-RS, MDS with ring sideroblasts.

Discussion

The main finding of **Study I** is the identification of *UBA1* mis-splicing as a mutant *SF3B1*-linked event that lowers the available UBA1 protein pool and preferentially sensitized mutant cells to pharmacologic UBA1 inhibition across model systems. Advances in sequencing and integrative analyses continue to refine our view of aberrant splicing events in MDS-*SF3B1* and help connect individual splicing events to their molecular consequences, which is important for improving mechanistic understanding and therapeutic approaches [429]. Using 5´-based full-length RNA-seq with unsupervised splicing analysis in iPSC-derived hematopoietic cells, we identified *UBA1*^{ms} as a reproducible, *SF3B1* mutation-specific event detected across progenitor and erythroid stages. Because 5′-based RNA-seq generates reads across the transcript body and captures intron-exon junctions in both nascent pre-mRNA and mature transcripts, it provides better splice junction coverage for identifying novel splice variants than 3′ chemistry, which is biased toward the 3′ end and can miss upstream splicing events.

We supported these findings with primary material and a patient cohort analysis in which *UBA1*^{ms} was observed in *SF3B1*-mutant cases but not in MDS with mutations in other members of the splicing machinery, or in healthy donors. More broadly, these results illustrate how iPSC-based differentiation can provide a controlled, disease-relevant human system to discover and validate specific molecular events and then test their consequences across complementary models.

Mechanistically, *UBA1*^{ms} differs from many previously described *SF3B1*-linked mis-splicing events that introduce premature termination codons and reduce gene output through NMD [198,199]. Here, *UBA1*^{ms} behaves as a stable transcript that is not subject to NMD and remains engaged with the translation machinery, directing attention to post-translational mechanisms. The mis-spliced product translates to a sequence of 45 amino acids, inserted into the inactive adenylation domain, and protein stability assays demonstrated a markedly reduced half-life with proteasome-dependent degradation of the UBA1^{MS} protein. While we could not assess whether the UBA1^{MS} protein retains enzymatic function, rapid turnover is consistent with reduced total UBA1 protein in *SF3B1*-mutant cells and offers a practical reason why detecting an endogenous UBA1^{MS}-specific protein species is challenging when degradation is fast. A prior study observed reduced UBA1 RNA and protein levels in splicing-factor–mutant MDS, but did not attribute this to UBA1 mis-splicing [430]. In contrast, our data show reduced UBA1 protein in MDS-*SF3B1* despite unchanged *UBA1* transcript levels, consistent with protein-level loss driven by the unstable mis-spliced variant.

Because UBA1 catalyzes the initiating step of ubiquitin activation and is essential for cellular viability [431], a reduced UBA1 protein pool would be expected to impair proteostasis capacity. Notably, partial UBA1 loss has been described to trigger adaptive stress responses, which is compatible with the idea that *SF3B1*-mutant hematopoietic cells can persist despite a reduced UBA1 pool [432]. Prior work has also described stage-specific mis-splicing and survival-associated programs in *SF3B1*-mutant cells [202,433], providing more context for how this deficit may be tolerated. Finally, *UBA1*^{ms} also connects conceptually to VEXAS, where somatic *UBA1* mutations cause loss of cytosolic UBA1b expression and accumulation of catalytically impaired isoforms [214]. In MDS-*SF3B1*, SF3B1-driven mis-splicing is associated with reduced total UBA1 protein, irrespective of isoform, without complete loss of function. The clinical context differs as well, with a prominent inflammatory phenotype in VEXAS [219] versus a comparatively lower inflammatory profile reported for MDS-*SF3B1* relative to other low-risk subgroups [434].

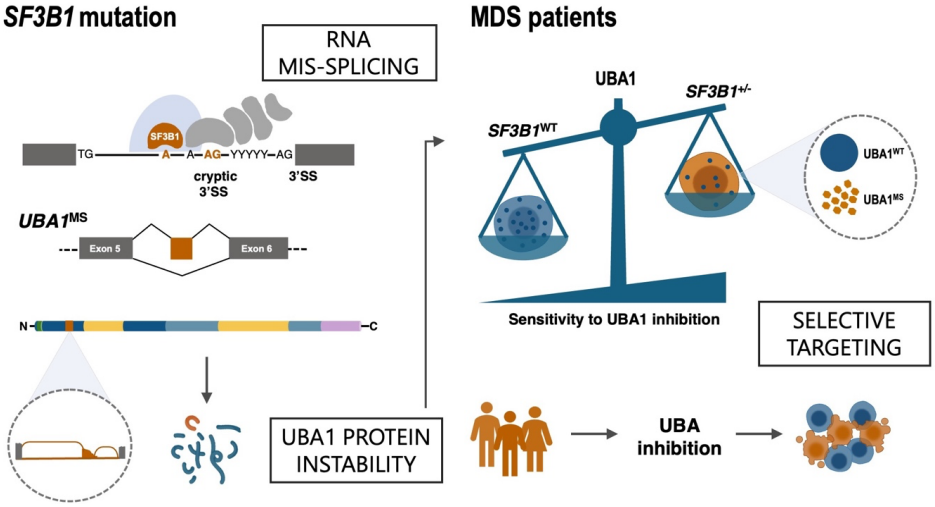

Figure 17: Visual Abstract of **Study I**.

4.2 Study II

Rationale

Acute myeloid leukemia (AML) with *KMT2A* rearrangements (*KMT2A*-r) is an aggressive subtype that occurs in both adults and children but is particularly enriched in younger patients. *KMT2A* encodes a histone H3K4 methyltransferase that helps maintain transcriptionally active chromatin at promoters and enhancers. Rearrangements convert this epigenetic regulator into oncogenic fusion proteins with many partners, frequently involving elongation machinery components such as AF9, encoded by *MLLT3*. *KMT2A*-rearranged AML is generally associated with poor prognosis, with resistance to chemotherapy and high relapse rates. Disruption of epigenetic and transcriptional regulation often represents early, disease-initiating lesions in HSPCs, making epigenetic dependencies promising therapeutic avenues. **Study II** aims to address a lack of physiologically relevant, human model systems by using HSPCs from patient-derived *KMT2A*-r AML-iPSCs and isogenic wild-type controls to capture disease-associated regulatory mechanisms. The goal was to define the transcription factor and epigenetic networks that underlie the gene expression program in AML development and test whether they can be exploited for treatment.

Results

To generate disease-relevant hematopoietic cells from patient-derived *KMT2A*-r AML-iPSCs and isogenic wild-type controls, we adapted a differentiation protocol that robustly produced hematopoietic progenitors **(Figure 18A; Study II, Figure 1A–B)**. Flow cytometry analysis showed that while both cell lines generated viable hematopoietic populations, AML-derived cultures retained an earlier progenitor-like surface phenotype at the sampled time point, consistent with delayed maturation **(Figure 18B; Study II, Figure 1C–D)**. RNA-seq analysis separated the two lines by developmental stage: At the iPSC stage, AML and control cells clustered closely but diverged after hematopoietic specification, indicating that the *KMT2A::MLLT3*-associated transcriptional program becomes apparent during differentiation **(Figure 18C; Study II, Figure 1E)**. Comparing differentiation-associated gene expression changes, AML-HSPCs included gene sets that failed to activate normally, genes that were inappropriately repressed, and genes that were uniquely induced in AML **(Study II, Figure 1F)**. Together, these findings show that *KMT2A::MLLT3* is associated with stage-dependent transcriptional dysregulation that emerges as cells enter the progenitor state.

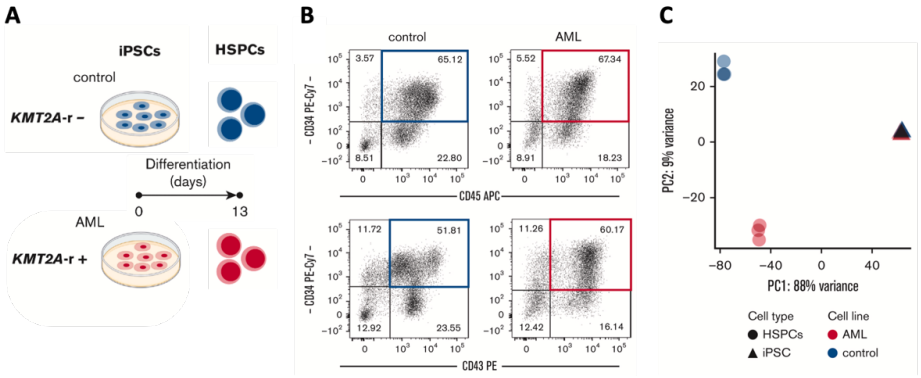

Figure 18: Hematopoietic specification of KMT2A-r iPSCs induces a transcriptionally distinct profile. (A) Schematic depicting the generation of HSPCs from AML and control-iPSC lines. (B) Representative flow cytometry diagrams of hematopoietic cell populations from control- and AML-HSPCs after 13 days of differentiation. (C) Principal Component Analysis plot from RNA-seq of iPSCs (triangles) and day 13 HSPCs (circles) from AML and control lines, showing the first two principal components (n = 3).

To connect these changes to upstream regulatory mechanisms, we performed CAGE profiling across differentiation time points and observed time-dependent deregulation of gene expression in AML, including a prominent subset of genes showing aberrant repression during hematopoietic specification **(Figure 19A; Study II, Figure 2A–B)**. Motif activity response analysis (MARA) showed broad divergence as differentiation progressed, with AML cultures displaying altered motif activity, including motifs that remained upregulated and others that failed to activate appropriately **(Figure 19B; Study II, Figure 2C–D)**. ChIP-signature analyses associated the repressed gene set with Polycomb complex-connected regulators, and DNMT-associated signatures also emerged among variable regulators **(Figure 19C, Study II, Figure 2F)**. Network analysis further connected transcription factors with altered motif activity to Polycomb Repressive Complex 1/2 (PRC1/2) components, supporting a coupled transcription factor–Polycomb network that contributes to transcriptional repression in AML-HSPCs **(Study II, Figure 2G)**.

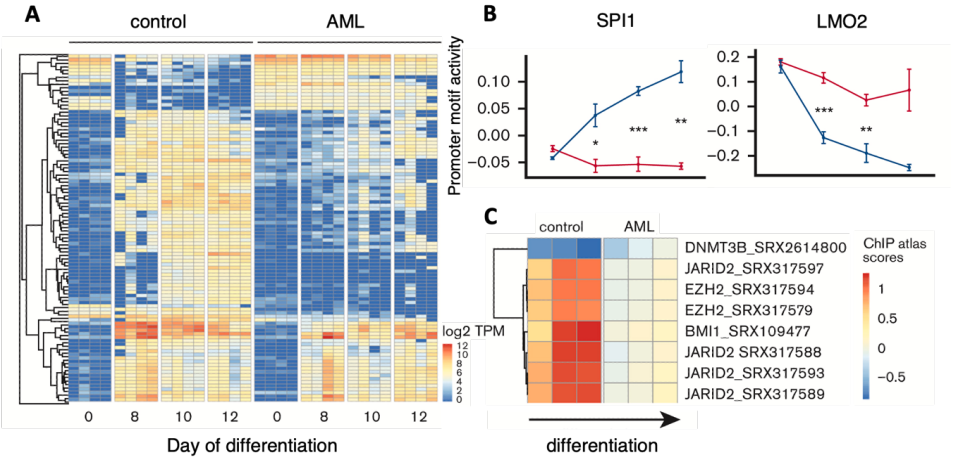

Figure 19: PRC2 members associate with repressed genes in AML-HSPCs. (A) Heatmap showing unsupervised clustering of the 100 most variable genes in control- and AML-iPSC during hematopoietic specification with cells harvested at the indicated time points (n = 4). (B) Individual motif activity profiles of *SPI1* and *LMO2* promoters between control and AML differentiation as inferred from CAGE data using MARA. Mean ± SEM. Unpaired *t*-test for each time point. (C) Heatmap showing unsupervised clustering of candidate ChIP-seq signatures from ChIP-Atlas, highlighting differential ChIP-seq signatures between control- and AML-iPSC over hematopoietic differentiation. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

This motivated us to functionally test Polycomb dependency in *KMT2A*-r cells. EZH1 and EZH2 are core components of PRC2, catalyzing repressive H3K27me3 marks. Treatment of iPSC-derived HSPCs with the dual EZH1/2 inhibitor UNC1999, alone or combined with 5-azacitidine, reduced global H3K27me3, consistent with on-target PRC2 inhibition, while short-term expansion was not detectably altered **(Study II, Figure 3A–D)**. In contrast, colony assays showed that PRC2 inhibition impaired clonogenic output across independent AML-HSPC clones, while control-derived HSPCs were less affected under the same conditions; prior exposure also reduced replating capacity, consistent with diminished progenitor function and self-renewal potential **(Figure 20A; Study II, Figure 3E–F)**. The dependence of this effect on *KMT2A*-r was supported in leukemia cell lines, where clonogenic suppression corresponded to *KMT2A*::*MLLT3* status **(Figure 20B; Study II, Figure 3G)**.

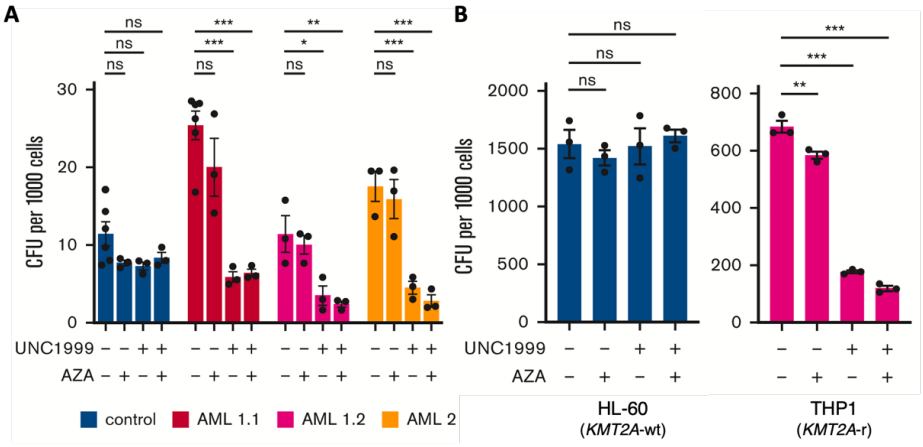

Figure 20: Epigenetic targeting selectively impairs clonogenicity in *KMT2A-r* AML cells. (A) CFU counts per 1000 seeded HSPCs treated with 2 µM UNC1999, 1 µM AZA, 2 µM UNC1999 + 1 µM AZA, or DMSO for 14 days (n = 6 for DMSO in AML 1.1 and control, n = 3 for others). Mean ± SEM. Two-way ANOVA with Dunnett's multiple comparisons test. (B) CFU counts per 1000 seeded cells from leukemic cell lines treated with 2 µM UNC1999, 1 µM AZA, 2 µM UNC1999 + 1 µM AZA, or DMSO for 10 days (n = 3). Mean ± SEM. One-way ANOVA with Dunnett's multiple comparisons test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.

To define upstream transcriptional changes, RNA-seq after treatment showed a markedly stronger response in AML-HSPCs than in controls, with enrichment for Polycomb targets and gene sets consistent with derepression of developmentally regulated (bivalent) loci. The combination treatment with 5-azacitidine enhanced gene derepression relative to UNC1999 alone **(Figure 21A; Study II, Figure 4A–C)**. Collectively, these results tie Polycomb-associated repression in *KMT2A*-r AML progenitors to a functional dependency that can be targeted pharmacologically, partially shifting gene expression toward a more typical hematopoietic program and reducing clonogenic capacity **(Figure 21B; Study II, Figure 4E)**.

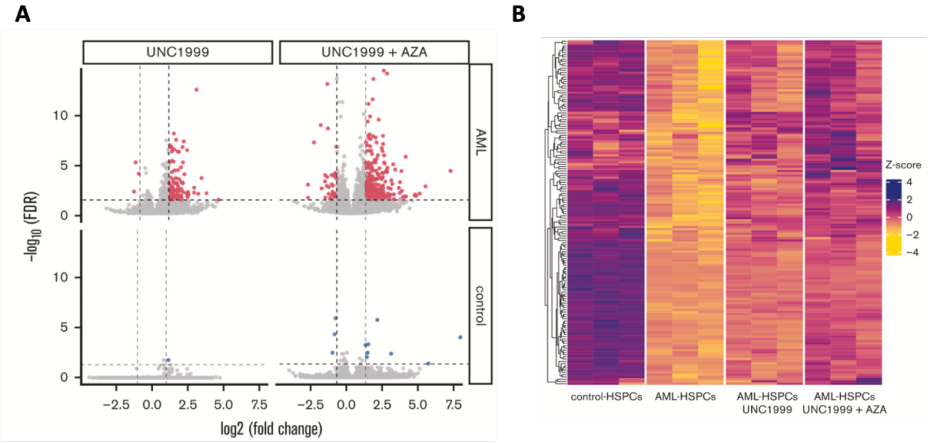

Figure 21: PRC2 inhibition derepresses transcription of Polycomb target genes that are downregulated in AML-HSPCs. (A) Volcano plot displaying differentially expressed genes identified using DESeq2 in AML (rose) and control (blue) HSPCs, following 72 h of treatment with 2 µM UNC1999 (left) or 2 µM UNC1999 and 1 µM AZA (right) compared to DMSO (n = 3). Dashed lines denote cutoffs for the significance threshold (FDR = 0.05, horizontal; $|\log_2(\text{fold change})| = 1$, vertical). (B) Heatmap showing row-wise z-scores of $\log_2$(CPM) expression values of 154 upregulated genes in AML-HSPCs treated with 2 µM UNC1999 or 2 µM UNC1999 + 1 µM AZA that overlap with genes that are downregulated in DMSO-treated control-HSPCs.

Discussion

In **Study II**, we used patient-derived *KMT2A*-rearranged AML-iPSCs to show that hematopoietic specification is accompanied by transcriptional rewiring of HSPCs cells with prominent Polycomb-associated repression. We show that PRC2 inhibition can partially relieve this repressive program and reduce leukemic-associated phenotypes, most clearly reflected by reduced clonogenic capacity. Because iPSCs retain the genetic background of the patient [353], while reprogramming broadly resets epigenetic state [435], this system provides a tractable way to test whether disease-linked regulatory states re-emerge specifically during hematopoietic lineage development [420]. In line with this, AML and control cells showed highly similar transcriptional profiles at the pluripotent stage but diverged as they transitioned into HSPC-like states, supporting the view that the leukemic program becomes evident during specification rather than being present in iPSCs. A plausible cause for this is disruption of transcription factor–epigenetic control mechanisms that shape AML initiation and maintenance [436]. Consistent with this, MARA network analysis identified changes in motif activity for several transcription factors overlapping previously described regulatory networks in *NPM1*-mutated AML [437,438]. Among the transcription factors with reduced motif activity, SPI1 (PU.1) is a driver of myeloid specification and is active in THP-1 cells [439]. In addition, dysregulated genes

were enriched for PRC2 targets and bivalent genes, supporting a model in which altered transcription factor activity and chromatin regulation together constrain normal hematopoietic programs.

The Polycomb axis provides a mechanistic bridge between these transcriptional patterns and progenitor cell function. Polycomb regulation is central to balancing self-renewal and differentiation in hematopoiesis [440,441], and aberrant Polycomb activity has been implicated across hematologic malignancies [442]. In *KMT2A*-rearranged AML, PRC2 members have been connected to disease maintenance and progression, arguing that PRC2 activity may contribute to disease maintenance rather than reflecting only the leukemic state [442]. In our study, PRC2 inhibition with UNC1999, alone or combined with 5-azacitidine, preferentially reduced clonogenic output and replating potential in *KMT2A*-rearranged models, while the corresponding effect was weaker in control HSPC-like cells and in *KMT2A*-wild-type leukemia cell lines. This supports the interpretation that sensitivity is most consistent with ties to the *KMT2A*-r background rather than co-occurring mutations. Prior mouse studies are consistent with this, showing that *KMT2A*-r AML cells are dependent on PRC2 activity [443]. Finally, bivalent loci provide a connection between Polycomb activity and developmental regulation. Bivalency is frequently perturbed in cancer, and AML is often associated with increased DNA methylation and transcriptional repression [444,445]. Here, we showed reduced expression of bivalent genes in *KMT2A*::*AF9* AML-HSPCs, particularly those related to hematopoietic fate. Combined PRC2 and DNMT inhibition preferentially reactivated these genes in AML-HSPCs compared with controls. Collectively, these results suggest that Polycomb-associated repression contributes to a constrained developmental state in *KMT2A*-rearranged hematopoietic progenitors and can be partially reversed by epigenetic inhibition.

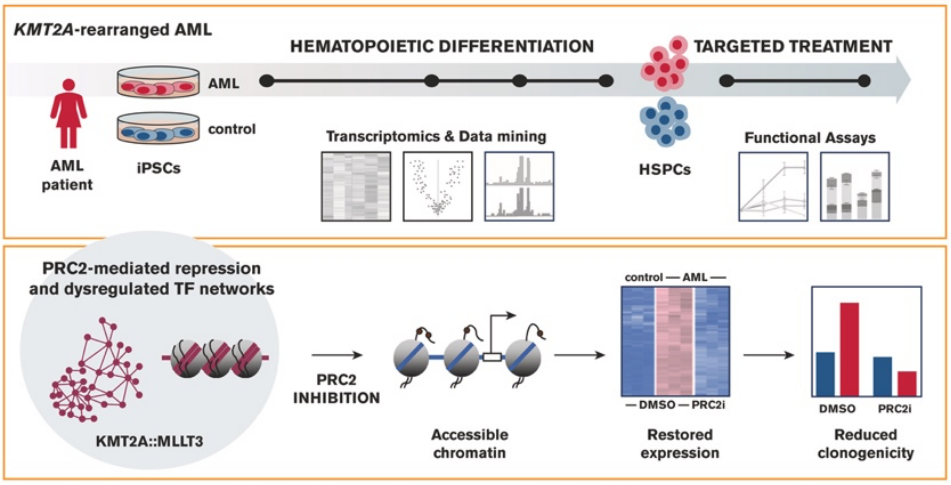

Figure 22: Visual abstract of **Study II**.

5 Conclusions

Both **Study I** and **Study II** used patient-derived iPSCs to model myeloid neoplasms and to connect defined genetic lesions to molecular mechanisms that emerge during hematopoietic specification. In both studies, the iPSC platform supports functional testing of these mechanisms, including whether lesion-associated molecular changes create targetable vulnerabilities. Key observations from the iPSC-derived systems were then validated in complementary cell models and primary material to demonstrate that they are not confined to a single experimental setting. Together, these studies underscore the value of iPSC-derived hematopoietic cells as disease-relevant models and as a practical bridge between unbiased molecular discovery and experimentally testable mechanisms in myeloid malignancies.

Study I: *UBA1* as an *SF3B1*-linked vulnerability in MDS

- *SF3B1* splice factor mutations are associated with a specific *UBA1* mis-splicing event (*UBA1*^{ms}) in MDS, identified in iPSC-derived hematopoietic progenitors and corroborated in cell lines and primary patient material.
- While the *UBA1*^{ms} transcript is stable and translation-engaged, the resulting protein product is rapidly degraded, consistent with reduced UBA1 levels in *SF3B1*-mutant cells.
- A reduced UBA1 "buffer/capacity" in *SF3B1*-mutant cells is associated with increased susceptibility to UBA1 inhibition across model systems, supporting UBA1 targeting as a potential treatment strategy in MDS-*SF3B1*.

Study II: Targeting Polycomb in *KMT2A*-rearranged AML

- *KMT2A*-rearranged AML iPSCs diverge from isogenic controls during hematopoietic specification, establishing a distinct transcriptional program, characterized in part by aberrant repression of developmental genes.
- Promoter- and network-level analyses indicate disrupted transcription factor–epigenetic regulation enriched for Polycomb targets and bivalent genes, consistent with a constrained developmental program in AML-HSPCs.
- PRC2 inhibition, alone or in combination with DNMT inhibition, partially relieves this repressive program and reduces leukemic features in a *KMT2A*-rearranged context.

6 Points of perspective

Over the past decade, patient-derived iPSCs have enabled mechanistic dissection of how defined genetic events in HSPCs reshape differentiation programs and create targetable therapeutic vulnerabilities [411,413]. Reprogramming captures patient mutational backgrounds in clonal lines, while genome editing enables introduction or correction of mutations in an isogenic setting, helping to delineate the contribution of individual lesions to disease phenotypes [446].

Both **Study I** and **Study II** leverage iPSC lines derived from primary patient material, converting finite clinical specimens into a renewable and expandable experimental resource. This helps overcome practical constraints that often limit work with primary hematopoietic samples, including restricted material availability, variable viability, and differences between sampling time points, and it also reduces the vulnerability of a project to irreversible loss of scarce primary specimens. In **Study I**, access to biobanked, clinically well-annotated MDS patient material at our center enabled the establishment and long-term use of disease-relevant iPSC models, underscoring how well-managed biobanks can transform one-time collections into reusable experimental platforms. In **Study II**, patient-derived AML iPSC lines generated in a different laboratory were shared and expanded for downstream analyses in this thesis. This is particularly valuable in AML, where aggressive and/or relapsed disease can make repeated collection of high-quality primary material difficult, or not feasible.

A central advantage of iPSC-based modeling is the availability of clonal lines that can be expanded and differentiated repeatedly, improving experimental control and strengthening causal connections between genotype and phenotype relative to heterogeneous primary samples. A particular strength is the possibility of generating isogenic control cells that help isolate lesion-associated effects from patient-to-patient variability. In **Study I**, paired *SF3B1*^{K700E} and *SF3B1*^{WT} iPSC lines from the same patient enabled a close comparison of mutant and wild-type hematopoiesis. In **Study II**, control iPSCs were generated from patient-derived T cells that did not harbor the *KMT2A* rearrangement present in the leukemic sample, providing a genetically matched non-malignant reference, albeit also lacking the other genetic variants of the mutant clone. iPSC reprogramming largely resets the epigenetic landscape, and it has been shown that many disease-associated regulatory programs are re-established only upon lineage

commitment and maturation [447]. This allows for the study of disease mechanisms and treatment responses in a cell-type–specific manner. **Study II** illustrates how iPSC differentiation can be used to resolve the developmental timing and cellular contexts in which lesion-linked transcriptional and epigenetic programs emerge during hematopoietic specification, rather than relying solely on analyses of differentiated populations. However, this also necessitates robust protocols for the generation of disease-relevant cells.

Despite these strengths, important limitations remain for modeling MDS and AML *in vitro*. It has become evident that some variants are difficult to reprogram and that reprogramming is a selective bottleneck rather than a neutral snapshot of the patient's clonal architecture. Consistent with this, iPSC derivation in the MDS-*SF3B1* setting can be skewed toward normal clones even when the starting material has a high *SF3B1*^{K700E} variant allele fraction. While the *KMT2A*-r was successfully captured in the cells used for **Study II**, prior reports suggest that certain cytogenetic events and mutations can be selected against during reprogramming [411,448,449]. This may reflect a requirement for intact epigenetic regulators to establish pluripotency and/or activation of stress checkpoints such as p53 in highly aneuploid or mutation-burdened cells [450]. Consequently, not all leukemic genotypes are likely to be directly amenable to derivation of stable pluripotent lines. Encouragingly, refined protocols have improved reprogramming efficiency in AML, and genome editing has enabled introduction of lesions after reprogramming, together supporting the development of more representative iPSC panels [204,423,424].

The study of diseases originating at the HSC level *in vitro* remains challenging due to the rarity of these cells and difficulties in maintaining this multipotent cell state in culture [451]. Differentiation efficiencies of iPSCs into HSPCs can vary by genotype, and often yield progenitors with restricted expansion capacity, limiting scalability and reproducibility [452]. More fundamentally, generating bona fide long-term repopulating HSCs from iPSCs has proven difficult. The hematopoietic differentiation protocols used in **Studies I** and **II** generated a heterogeneous population of hematopoietic progenitor cells that were characterized by flow cytometry using common HSPC markers. However, we did not specifically isolate cell populations resembling phenotypic HSCs nor confirm HSC properties such as self-renewal and multipotency through transplantation. Accordingly, our data do not establish whether the iPSC-derived cells include functionally defined HSCs, and their engraftment capacity remains uncertain, given that iPSC-derived HSPCs

frequently lack definitive hematopoietic potential [453]. Recent fully defined differentiation protocols yielding multilineage-engrafting hematopoietic cells therefore represent a notable advance toward the generation of higher-fidelity models, and it will be interesting to see how these developments shape the robustness and interpretability of *in vitro* disease modeling systems in the coming years [379,394,422]. Another more general limitation of conventional *in vitro* culture systems is the omission of bone marrow niche cues that influence clonal fitness, inflammation, differentiation, and treatment resistance. This has motivated the incorporation of microenvironmental components through co-culture, 3D systems, and engineered niche approaches to enhance physiological relevance while balancing experimental convenience [454–456].

Importantly, these limitations do not preclude translational impact of iPSC platforms, as evidenced by progress in other iPSC-derived lineages. Although iPSC-derived hematopoietic models are most often used for mechanistic studies and hypothesis-driven testing, translational iPSC workflows are currently more mature in several other areas [457]. In neurodegenerative disease, patient iPSC-derived neurons and neural organoid models have supported phenotypic screening and drug-repurposing efforts that have progressed into clinical studies, including iPSC-informed trials in ALS and familial Alzheimer's disease [458]. In parallel, iPSC-derived cell types are increasingly used to capture human-relevant drug liabilities, for example through nephrotoxicity testing in iPSC-derived podocytes and cardiotoxicity testing in iPSC-derived 3D cardiac tissues [457]. Finally, iPSC technology is also contributing directly to therapeutic development via cell-based products, with clinical trials spanning multiple indications, including iPSC-derived dopaminergic progenitors in Parkinson's disease and allogeneic iPSC-derived immune cell products such as NK and CAR-NK cells [459,460].

Another consideration is that many studies using iPSCs include relatively few patient lines, limiting generalization given the heterogeneity of MDS/AML. Similarly, the studies in this thesis are largely based on iPSC lines derived from a single individual per genotype, and while key experiments were complemented with additional iPSC lines/clones, orthogonal *in vitro* models, and interrogation of available datasets, this limited number of patient-derived lines remains an important limitation. The generation, maintenance, and differentiation of iPSCs are time-consuming, costly, and at times inefficient. However, continued advances in reprogramming, gene editing, and differentiation protocols are expected to facilitate the generation of larger, genetically diverse iPSC panels. Such resources,

particularly when paired with matched isogenic controls, should enable more systematic interrogation of lesion-specific mechanisms, co-mutation effects, and therapeutic responses [421].

While MDS-*SF3B1* typically presents as a lower-risk disease dominated by symptomatic anemia, standard therapy remains largely supportive and focused on improving erythropoiesis. Allogeneic HSCT is the only potentially curative option; however, it is often precluded by advanced age and comorbidities. Our identification of *UBA1* mis-splicing as an *SF3B1*-linked vulnerability in **Study I** suggests a potential strategy to preferentially reduce the mutant clone while preserving residual wild-type hematopoiesis. Consistent with this, TAK-243, which blocks UBA1-mediated ubiquitin activation, preferentially reduced *SF3B1*-mutant cells while sparing wild-type HSPCs at lower concentrations. Although treatment did not fully eradicate mutant cells and TAK-243 also impacted normal cells at higher concentrations, our data support the possibility of a lower-intensity, disease-modifying approach aimed at reducing mutational burden and potentially delaying the need for transplantation.

To advance this concept toward clinical translation in MDS-*SF3B1*, a next priority is to strengthen the evidence for mutant-selective activity of TAK-243 in primary hematopoiesis. Although we observed increased sensitivity of *SF3B1*-mutant patient cells compared with normal bone marrow controls in CFU assays, the number of primary samples analyzed in **Study I** was limited, and validation in a larger cohort is warranted. While MDS-*SF3B1* represents a relatively molecularly defined lower-risk subtype, expanding the analysis across additional patients would help capture clinical and genetic variability and better define the robustness of the effect. Beyond comparisons to healthy donors, lower-risk MDS samples lacking *UBA1*^{ms} would represent a particularly informative control group, enabling a direct test of whether TAK-243 sensitivity is driven by *SF3B1*-associated *UBA1*^{ms} biology. Because clonal fitness in MDS is shaped by competition and microenvironmental cues, it will also be important to assess whether mutant selectivity is preserved in niche-supported settings. In this regard, a previously developed 3D scaffold culture model for MDS-RS may be useful, as it supports prolonged culture of primary bone marrow–derived populations and maintenance of the mutant clone, providing a tractable system to study clonal dynamics under more physiological conditions [347]. More broadly, incorporation of iPSC-derived stromal support and other microenvironmental components into advanced 3D culture systems should further improve

assessment of drug effects in settings that better approximate the marrow niche [457].

A recent study in a human VEXAS model suggested that impaired UBA1 function can create a compensatory dependency on the alternative ubiquitin E1 enzyme UBA6. Genetic loss of UBA6 or pharmacologic inhibition with the allosteric compound inositol hexaphosphate (IP6) reduced growth and colony formation of *UBA1*-mutant cells [461]. Although IP6 inhibits UBA6 only at high (millimolar) concentrations, highlighting the need for more potent and selective agents, these findings raise the question of whether similar UBA6-dependent compensation occurs in *SF3B1*-mutant cells and could be therapeutically exploited.

Beyond E1 inhibition alone, another angle not addressed in **Study I** is the essential role of UBA1 in DNA damage responses that resolve replication stress and double-strand breaks [462]. Interestingly, increased R-loop formation and DNA damage have been associated with *SF3B1* and other splice factor mutations in MDS, and mutant cells were preferentially sensitive to targeting of the downstream ATR–Chk1 pathway [409,463,464]. Similarly, Bland et al. showed that *SF3B1*-mutant cells were unable to resolve replication stress induced by PARP inhibition, leading to selective killing of mutant cells [465,466]. Future work will be important to assess rational combination strategies that leverage *SF3B1*-associated vulnerabilities to deepen responses and improve selectivity for mutant clones [429].

Finally, while **Study I** demonstrates that UBA1 inhibition can selectively suppress *SF3B1*-mutant progenitors *in vitro*, and TAK-243 has shown activity across multiple preclinical models of hematologic malignancies, clinical efficacy and tolerability remain to be established [467–469]. An ongoing trial evaluating TAK-243 in intermediate-2 or high-risk refractory MDS and leukemias (NCT03816319) may be informative to define the therapeutic window and dosing, and to inform whether UBA1-targeted strategies could be extended to MDS-*SF3B1*.

In **Study II**, we used patient-derived iPSC models of *KMT2A*-rearranged AML to map transcriptional and epigenetic dysregulation during hematopoietic differentiation and to test whether targeted epigenetic therapy could partially counteract these abnormalities. Transcriptional profiling and regulatory network analyses implicated Polycomb-associated repression as a prominent feature of AML iPSC-derived HSPCs, and pharmacologic inhibition of EZH1/2 using UNC1999, in combination with 5-azacitidine, reactivated a subset of repressed genes and reduced leukemic phenotypes in *KMT2A*-r cells. Through this analysis, we

identified NFYA as a candidate target with evidence of fusion occupancy at the *NFYA* promoter, consistent with elevated *NFYA* expression and increased NFY motif activity in AML-HSPCs. NF-Y is a CCAAT-binding transcription factor complex in which NFYA confers sequence-specific DNA recognition [470]. Across cancer types, NF-Y has been implicated in maintaining pro-growth transcriptional programs, including cell-cycle regulation and metabolism, and it emerges as a proliferation-linked node also in *KMT2A*-r AML [439,471]. Consistently, NFYA or broader NF-Y complex loss-of-function suppresses proliferation and can trigger apoptosis. Transcription factor-focused CRISPR screens have placed all three NF-Y subunits among a small set of transcription factors broadly required for cancer cell proliferation, including in *KMT2A*-r contexts [471,472]. Conversely, multiple studies support oncogenic behavior upon *NFYA* upregulation, suggesting that increased NFYA activity can reinforce malignant growth programs [471]. Collectively, these data support a model in which KMT2A fusion-dependent binding at the NFYA promoter is associated with elevated *NFYA* expression and sustains transcriptional programs that favor leukemic growth and self-renewal. While our analysis indicates a potential role for NFYA in the *KMT2A*-r gene expression program, we did not test this mechanistically. Follow-up work should therefore focus on directly perturbing NFYA or the NF-Y complex in *KMT2A*-r AML models to establish its functional requirement for the leukemic state and to evaluate whether NFY-dependent circuitry represents a therapeutic vulnerability.

Targeting epigenetic dependencies in *KMT2A*-r AML is the focus of ongoing therapeutic development. As outlined in the introduction, Menin, together with cofactors such as LEDGF, coordinate chromatin binding and the activation of target genes by KMT2A fusion complexes, making disruption of the Menin–KMT2A interaction a strategy to inhibit *HOXA9* and *MEIS1* gene expression [297,311]. Clinically, the oral Menin inhibitor revumenib has shown meaningful activity in heavily pretreated *KMT2A*-r/*NPM1*-mutant AML and has received FDA approval in the relapsed/refractory *KMT2A*-r acute leukemia setting [473,474]. Additional Menin inhibitors, including ziftomenib and newer agents such as bleximenib and enzomenib, have also shown encouraging efficacy signals and are now being advanced into combination regimens with intensive chemotherapy or 5-azacitidine/venetoclax in early-phase studies [475]. In parallel, DOT1L is aberrantly recruited by KMT2A fusion proteins to deposit H3K79 methylation and maintain *HOXA* gene expression [303]. While the DOT1L inhibitor pinometostat demonstrated only modest single-agent efficacy, this has shifted clinical

emphasis toward combination strategies [315]. Although UNC1999 and 5-azacitidine in **Study II** reactivated PRC2 target genes and reduced leukemic properties, we did not observe downregulation of *HOX*/*MEIS1* expression, suggesting that this regimen alone may not directly disrupt the core KMT2A-fusion transcriptional program. This supports a rationale to explore combination strategies that pair PRC2-directed therapy with agents that more directly target KMT2A-fusion function, such as Menin or DOT1L inhibitors, to more comprehensively attenuate leukemogenic gene expression and phenotype.

Taken together, **Study I** and **Study II** illustrate how patient-derived iPSC hematopoiesis can bridge mechanistic discovery and therapeutic hypothesis testing in myeloid malignancies. In MDS-*SF3B1*, this approach tied a mutation-specific splicing event (*UBA1*^{ms}) to a defined molecular consequence and a pharmacologically addressable vulnerability, supported across iPSC-derived progenitors, complementary models, and primary cells. In *KMT2A*-r AML, stage-resolved profiling in iPSC-derived hematopoiesis mapped when leukemic regulatory programs emerge during differentiation and identified a PRC2-controlled repressive profile that can be partially reversed pharmacologically, underscoring the value of developmental context for interpreting malignant cell states and drug responses.

Through these studies, I also came to appreciate the current boundaries of iPSC-based hematopoietic modeling. Key challenges include generating cells that faithfully capture bona fide HSC biology, incorporating the instructive complexity of the niche and microenvironment, modeling clonal competition in genetically diverse settings, and translating selective *in vitro* effects into durable clinical benefit. Looking forward, I see integration as the central direction for the field: coupling higher-fidelity stem and progenitor differentiation with engineered microenvironments, expanding genetically diverse patient-derived and isogenic panels, and applying systematic therapeutic testing, including rational combinations, to exploit convergent dependencies in stress responses, proteostasis, and epigenetic regulation while preserving normal hematopoiesis.

7 Acknowledgements

As I close this PhD chapter, I keep coming back to how lucky I have been in the people around me. Over these years, I was supported by individuals who are not only brilliant and hardworking, but also remarkably generous and warm. This thesis is the result of that shared effort, and I am sincerely grateful to each and every one of you. The journey may be ending, but my gratitude certainly is not.

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8 Declaration about the use of generative AI

I declare that I have used AI-assisted tools in preparing the comprehensive summary ("kappa") of this thesis. Specifically, ChatGPT 5.2 Thinking (OpenAI) was used for language editing and consistency checks, including proofreading (grammar, spelling, and tone), and assisting with internal consistency (e.g., identifying duplicated references, checking that citations and figure/table callouts were consistently formatted, and flagging possible inconsistencies for my manual review).

The Images function was used for figure preparation purposes, limited to resolution upscaling and removal of non-essential labels/annotations as described in the figure legends, without altering the underlying scientific content of the images.

I confirm that this does not infringe on the originality of this work and that I take full responsibility for the content of the "kappa"/comprehensive summary of the thesis.

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