



Deciphering the oncogenic network of PRC2 loss guided leukemogenesis

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Abstract

The polycomb repressive complex 2 (PRC2) and its catalytic subunit EZH2 are the crucial hub of epigenetic gene regulation during hematopoiesis by controlling stem cell fate and differentiation. Mutations causing loss of its activity - including -7/-7q deletions - frequently occur in adult AML, MDS, and pediatric ML-DS and JMML. Yet, leukemogenesis is orchestrated by the cooperation of multiple mutations and we therefore aimed to decipher the mutational and molecular context of PRC2 loss guided transformation.

Based on mutational complexity of AML genomes, we developed a two staged multiplexed CRISPR-Cas9 cooperation screening to test combinations of five recurrently mutated genes (1 gRNA / gene) for leukemic transformation properties in conjunction with the loss of *Ezh2*. The first stage, representing an *in vitro* setup, enabled the high throughput testing of 148 mutational combinations by exposing transduced LSK cells to differentiation promoting conditions. A selection of transformed clones with blocked differentiation, sustained self-renewal and enhanced proliferation was achieved in limiting dilutions.

Utilizing this strategy, we successfully identified two highly potent genetic configurations of five gRNAs (*Nf1*, *Bcor*, *Tet2*, *Stag2*, *Smc1a* and *Runx1*, *Bcor*, *Tet2*, *Stag2*, *Sh2b3*), presenting with a high transforming potential. Definitive dependency on the loss of *Ezh2* was highlighted by increased fitness of transformed cells lacking the *Ezh2* protein compared to wildtype counterparts. Next, targeted sequencing and reduced complexity screenings isolated a core transforming cooperation of *Ezh2*, *Bcor* and *Runx1* mutations.

In the second stage, the two highly transforming genetic combinations were tested in an *in vivo* setup of a syngeneic bone marrow transplantation model. The strong oncogenic capacity of the gRNA combinations, found in our first stage screening, was verified by inducing high penetrance (100%) and short latency (median survival 10 weeks) leukemia. Importantly, removal of the *Ezh2* gRNA from the mutational combinations caused a significantly delayed leukemia onset. Targeted sequencing based mutation analysis of leukemic samples stressed the cooperating effect between deletions of *Ezh2* and mutations in *Bcor* and *Runx1*. However, the *in vivo* stage expanded the mutational repertoire suggesting importance of *Tet2* and *Sh2b3* mutation. Reduced complexity screenings, utilizing only candidate gRNAs, verified and specified the cooperation between losses of *Runx1* and *Ezh2* in myeloid malignancy.

Towards their molecular mode of action, we performed global gene expression and histone modification profiling of *Ezh2* negative and positive samples that revealed distinct upregulation of fetal gene signatures, suggesting the *Ezh2* deletion to induce transformation by reactivation of an onco-fetal expression profile. To validate this hypothesis, we tested the transformative capacity of the *Ezh2* loss on *Runx1* negative fetal and adult hematopoietic cells *in vitro* and indeed additional loss

of *Ezh2* only promoted growth in adult cells stressing its role as an activator of fetal genes. Based on these findings, we aimed to replace the loss of *Ezh2* by overexpression of highly upregulated onco-fetal genes with the major candidates *Plag1*, *Igf2bp3*, *Ryk* and *Hif3a*. Actually, these genes effectively mimicked the *Ezh2* loss in syngeneic mouse models. To identify molecular synergies of the mutational cooperation of *Runx1* and *Ezh2*, we performed multi-comparison RNA-Seq of single and double mutated samples, including non-*Ezh2*-mutated AMLs. Thereby, the *Runx1* loss was demonstrated to repress lymphoid cell fate, and the interplay of deletions in *Ezh2* and *Runx1* activated myeloid and stem cell expression programs.

Overall, our study highlights the power of multiplexed CRISPR-Cas9 cooperation screenings to unravel cooperating effects of mutational combinations. Applying this principle for *Ezh2* depletion in leukemogenesis, we found strong synergy with mutations in *Runx1*. Functional analyses isolated the upregulation of fetal gene signatures by loss of *Ezh2* and the activation of myeloid and stem cell genes as a synergistic mode of both mutations to be the molecular mechanisms driving leukemia formation in AML. We thus gained essential insights into PRC2-loss AML biology that might pave the way for the identification of novel druggable targets opening novel therapeutic path for the future.

1. Introduction

1.1. Hematopoiesis

The hematopoietic system gives rise to all differentiated and functional cells of the blood, which need to fulfill multiple tasks including immune defense, gas transport, and clotting. As mature blood cells are short lived, the hematopoietic system needs to replenish mature cells of all lineages throughout life. In order to fulfill the changing demands of different lineages, while preventing the formation of cancer, this process is tightly regulated through a hierarchical structure. On top of this hierarchy sits the hematopoietic stem cell (Cheng, Zheng and Cheng, 2020) (Fig. 1). The existence of such a cell, being able to fully reconstitute the entire blood system, was proven by bone marrow transplantations into lethally irradiated mice already in the early 1960th (Till and McCulloch, 1961; Becker, McCulloch and Till, 1963). An hematopoietic stem cell is defined by two abilities that are multipotent differentiation and self-renewal (Orkin, 2000). If the production of novel mature cells is needed, mainly communicated by cytokine concentrations (Zhang and Lodish, 2008), the hematopoietic stem cell undergoes an asymmetric cell division: one of the daughter cells loses its self-renewing potential, starting the process of differentiation. Along the hierarchical tree, the differentiating cell minimizes multipotency, and gains characteristics and functions of mature blood cells (Orkin, 2000). Classically, HSC populations have been divided into two subpopulations that are LT-HSCs and ST-HSCs (Pietras *et al.*; Morrison *et al.*, 1997) (Fig. 1) based on their reconstitution capacity. However, single-cell studies demonstrated recently the diversification of the HSC population being more lineage-biased early on than initially believed (Wilson *et al.*, 2015; Nestorowa *et al.*, 2016). Still, ST-HSCs are believed to finally build multipotent progenitors (MPP) (Fig. 1) that do not show any self-renewing capacity anymore (Morrison *et al.*, 1997; Yang *et al.*, 2005). Also the MPP population has been sub-classified recently corresponding to several distinct features such as immunophenotype, lineage bias and bone marrow abundance (Pietras *et al.*, 2015). Classically, the first lineage commitment occurs upon the bifurcation into myeloid or lymphoid common progenitors (CMPs or CLPs), respectively (Fig. 1). CLPs give rise to all lymphoid cells such as B-, T- and NK-cells while CMPs develop either into granulocyte-monocyte or into megakaryocyte-erythrocyte progenitors (GMPs or MEPs), respectively. Subsequently, GMPs differentiate into the different types of granulocytes and monocytes while MEPs differentiate into megakaryocytes and erythrocytes (Akashi *et al.*, 2000; Kondo, Weissman and Akashi, 1997) (Fig. 1). However, myeloid progenitor populations are also discovered to be more heterogeneous than initially believed. Especially, the existence of MEPs is under debate proposing separated precursors for megakaryocytes and erythrocytes (Pronk *et al.*, 2007; Paul *et al.*, 2016).

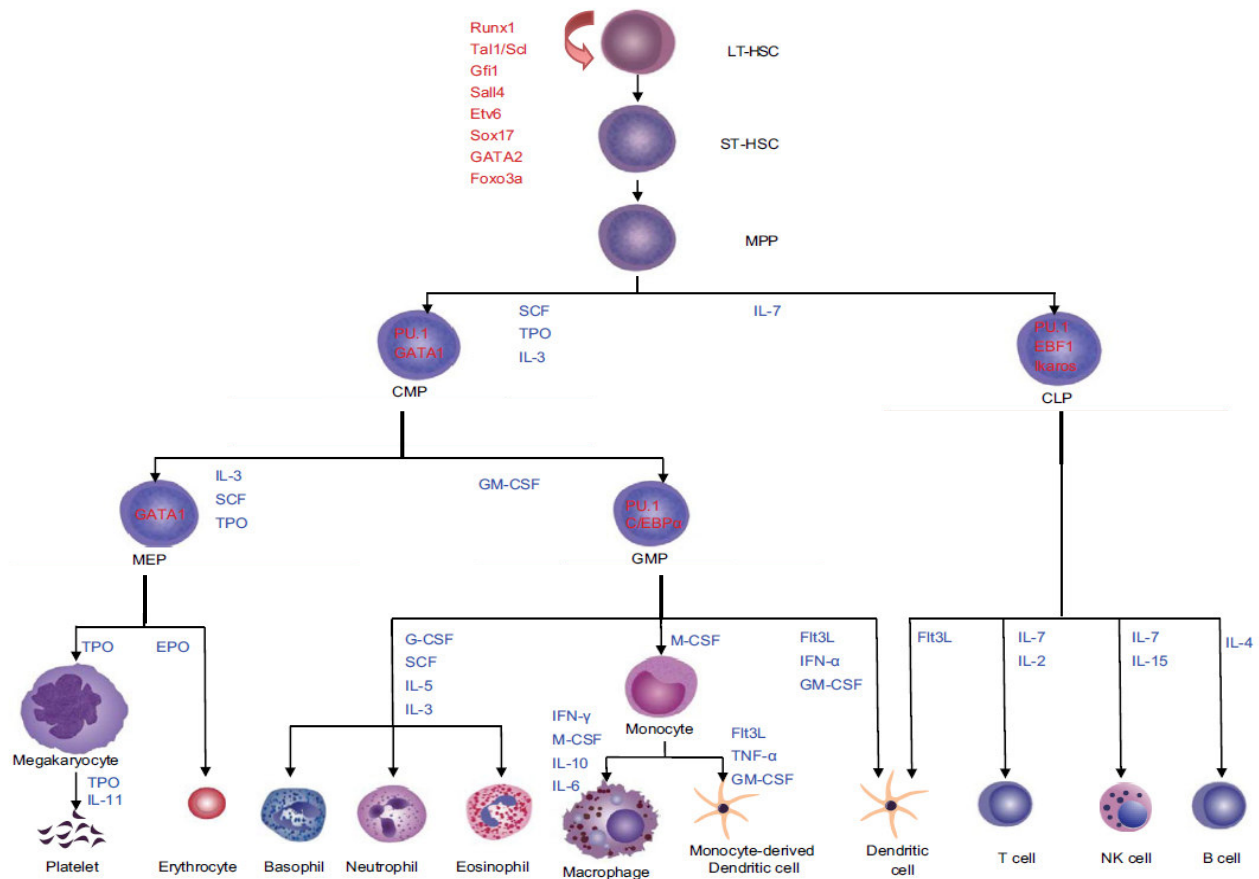


Figure 1. The classical hematopoietic tree. The diagram illustrates the process of hematopoiesis from HSCs to fully differentiated cells in a simplified manner. Important cytokines are highlighted in blue at the connection between two cell types and important transcription factors are highlighted in red in or next to the corresponding cell. Figure adapted from Cheng *et al.* 2019.

Overall, based on recent findings in single-cell studies, the whole process of hematopoiesis is much more continuous and biased than initially described (Buenrostro *et al.*, 2018; Velten *et al.*, 2017) meaning that HSCs acquire lineage biases very early on and differentiate continuously without passing through multiple defined progenitor stages.

All progresses during hematopoiesis, mainly enabled by changes in expression of functional factors, are tightly regulated by cytokines, transcription and epigenetic factors (Zhu and Emerson, 2002) (Fig. 1). For example, complex signaling of the WNT pathway as well as SCF signaling is involved in HSC generation and expansion. Additionally, SCF signaling is needed for HSC differentiation into myeloid progenitors (Zhang and Lodish, 2008). Examples for involved transcription factors are RUNX1, as a part of a complex transcriptional network, regulating HSC self-renewal, and the complex interplay between PU1, GATA1, and CEBP α that regulates myeloid differentiation (Zhu and Emerson, 2002). Epigenetic regulation might be especially important to enable early biased decisions in the new continuous model of hematopoiesis since it allows a very sophisticated and dynamic regulation

of transcription, and thus gene expression. For example, DNA-methylation was demonstrated to follow a program of changes during differentiation (Ji *et al.*, 2010) as promoters of genes, needed for cell specification, get de-methylated during differentiation and promoters, maintaining stem fate, get methylated. Similarly, a program for altered histone modifications during differentiation was identified underlining the importance of epigenetics during hematopoiesis (Cui *et al.*, 2009).

Nowadays, specific cells of all developmental stages can be isolated utilizing sophisticated, but well established immuno-phenotypic FACS protocols (Orkin and Zon, 2008). Murine HSCs were first characterized as lineage negative, Sca1 positive and cKit negative cells (LSK) (Spangrude, Heimfeld and Weissman, 1988). Even though many more exact developmental sub-classes were defined, considering many more markers, the isolation of LSKs remains a common method to enrich for HSCs. The tight regulation of adult hematopoietic system can be compared to the tight regulation of the ontogeny of hematopoiesis in the fetus. While taking place in the bone marrow of the adult, the primitive hematopoiesis is located in mesoderm-derived blood islands in the yolk sac (Mikkola and Orkin, 2006). Here, mainly erythrocytes are produced originating from a different subset of mesodermal cells than definitive HSCs. Definitive HSCs emerge by endothelial-to-hematopoietic-transition (EHT) from the endothelium of the aorta, relocate to the AGM (aorta-gonadmesonephros) region, finally colonizing the developing fetal liver (Kissa and Herbomel, 2010). The fetal liver is the main source for hematopoiesis until late fetal life when hematopoiesis moves to the bone marrow. In order to be able to constitute the whole hematopoietic system during embryogenesis, fetal HSCs cycle faster so that they have a much higher proliferative potential compared to adult HSCs (Bowie *et al.*, 2007; Popescu *et al.*, 2019). This is mainly enabled by the occurrence of symmetrical cell divisions (Lessard, Faubert and Sauvageau, 2004).

1.2. Acute myeloid leukemia

1.2.1. Pathophysiology, classification and therapy

Hematopoiesis is a highly dynamic process which needs to fulfill the large demands for functional mature blood cells lifelong by continuous proliferation and differentiation. With each cell division, errors in the genetic code accumulate (Welch *et al.*, 2012), which may eventually result in the disturbance of these processes and the development of a hematopoietic malignancy will be probable. One type of hematopoietic malignancy is acute myeloid leukemia (AML) originating from the myeloid side of the hematopoietic tree. AML is the most common leukemia in adults with an incidence of three to five cases per 100.000 increasing with age (Siegel, Miller and Jemal, 2015). It is characterized by the abnormal clonal proliferation and dedifferentiation of myeloid precursor cells (Bonnet and Dick, 1997) which develop into so-called leukemic blasts. The diagnosis of leukemia is given with at least 20% of cells in the bone marrow or the peripheral blood identified as leukemic

blasts (Döhner *et al.*, 2017). Patients present with anemia, disturbed blood clotting, and increased susceptibility to infections. However, AML is a very heterogeneous disease as many subtypes are described. Nowadays, the WHO defines six different classes of AML based on morphological, immune-phenotypical, genetic and clinical characteristics: AML with recurrent genetic abnormalities, AML with myelodysplasia-related features, therapy-related AML, AML not otherwise specified, myeloid sarcoma, and myeloid proliferation related to Down syndrome (Arber *et al.*, 2016). Within the group of AML with recurrent genetic abnormalities, several even more sophisticated subtypes are described corresponding to specific mutations. These are for example distinct chromosomal translocations, such as *RUNX1-RUNX1T1*, or recurrent mutations for example in the *NPM1* or *CEBPA* gene. In most cases, AML occurs as a *de novo* disease although secondary AML following previous hematologic disorders or therapies (irradiation or chemotherapy) is also common (De Kouchkovsky and Abdul-Hay, 2016).

AML is mainly treated by chemotherapy including cytarabine and anthracyclines following a two-phase regimen (7 days plus 3 days, respectively) (Estey, 2014). Only some cases were shown to profit from allogenic HSC transplantations. Even though many attempts have been made to optimize the therapy of AML, treatment schemes remained more or less unchanged for the past 40 years (Dombret and Gardin, 2016). Mainly due to relapse after first remission, AML stays a fatal disease in many cases especially in the elderly (up to 70%) (Meyers *et al.*, 2013) demonstrating the need for further research to guide novel therapies.

1.2.2. Mutational networks in AML

The need for a combination of multiple mutations, synergizing with each other, in order to induce development of a malignant cancer, has been established in most cases for a long time (Ashley, 1969; Tomasetti *et al.*, 2015; Dash *et al.*, 2019). Although leukemic cells present with much less mutations than solid tumors, most patients still harbor three to five recurrent driving mutations (Ley *et al.*, 2013). Recurrent mutations in *FLT3*, *NPM1*, *KIT*, *CEBPA* and *TET2* were discovered already by targeted sequencing while massively parallel sequencing additionally identified *DNMT3A* and *IDH1* mutations (Bacher, Schnittger and Haferlach, 2010; Yamashita *et al.*, 2010; Mardis *et al.*, 2009). Structured whole genome sequencing enabled the detection of many more occurring mutations proving the diversity of the disease (Ley *et al.*, 2013; Papaemmanuil *et al.*, 2016; Tyner *et al.*, 2018).

Many attempts have been made to categorize different mutational classes and to unravel the complex mutational demands and interplays needed by an HSC to develop into an AML cell. Hanahan and Weinberg foremost summarized six different classes of recurrent mutations in solid tumors already in 2000 adding even more categories ever since (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The initial six categories included sustained proliferation signaling, resistance to apoptosis, evasion from growth suppression, metastasis, replicative immortality and immune escape.

However, a much simplified model was suggested for AML specifying two classes of mutations only (Kelly and Gilliland, 2002) (Fig. 2). According to this model, class I of mutations conveys a proliferative advantage by affecting growth factor receptors and signaling pathways. Examples for this class are mutations in *K-* or *N-RAS*, activating mutations in *FLT3*, and fusion genes like *BCR/ABL*. Mutations in transcription factors like *RUNX1*, *CEBPα*, or fusion genes like *RUNX1/ETO* or *PML/RARα* impair hematopoietic differentiation and define class II of mutations leading to leukemia when combined with class I mutations. As many later described mutations did not fit into that model, a third group of initially unclassified mutations was established (Takahashi, 2011) (Fig. 2), mostly leading to a sustained self-renewal capacity and therapy resistance. To this additional mutational class, mutations in genes such as *DNMT3A*, *TET2* and *IDH1*, changing DNA methylation patterns, belong, as well as genetic alterations in *MLL1*, *ASXL1* and *EZH2* disturbing histone modifications, and cohesion complex mutations (*STAG2*, *SMC1A*) modifying 3D chromatin structure (Woods and Levine, 2015). Thus, the high occurrence of alterations in epigenetic modifiers in this class III of mutations is striking. However, the addition of even more mutational classes and subgroups to this model was proposed in recent years (Papaemmanuil *et al.*, 2016; Ley *et al.*, 2013) subdividing the 3rd group into histone and DNA modifying factors and splicing components, and adding a group of tumor suppressors.

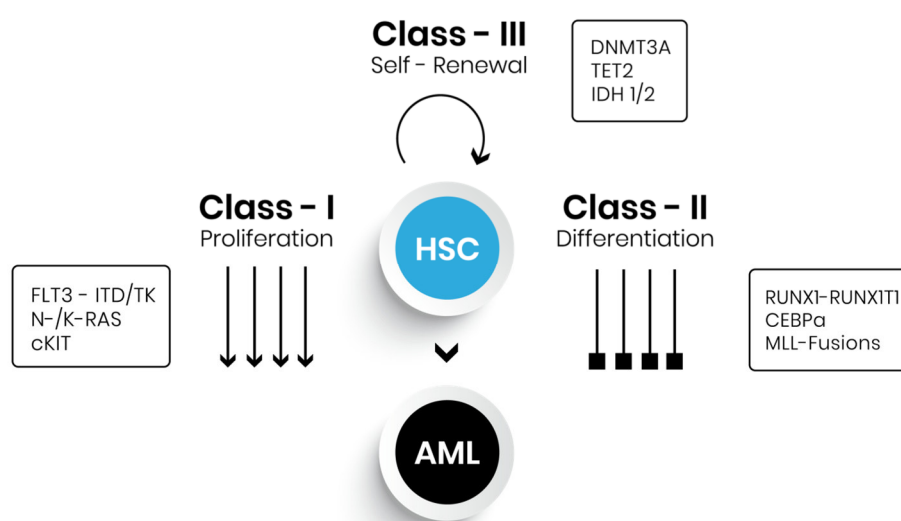


Figure 2. Mutational classes in AML. The diagram illustrates the established three classes of mutations in AML (Takahashi *et al.*, 2011). Class I of mutations increases proliferation (affecting growth factor receptors and signaling pathways), class II of mutations blocks differentiation (affecting transcription factors) and class III enhances self-renewal (affecting epigenetic factors). Three examples are indicated for each mutational class.

The repeated co-occurrence of specific genetic alterations in patients suggests oncogenic synergy between several mutations, for instance *RUNX1* mutations combined with *MLL* rearrangements (Tang *et al.*, 2009), or concurrent mutations in *NPM1*, *FLT3* and *DNMT3A* (Ley *et al.*, 2013). It is widely accepted that at least two classes of mutations have to be affected simultaneously in order to

induce leukemogenesis as their effects need to cooperate (Takahashi, 2011). Still, the co-occurrence of multiple mutated epigenetic factors is rather common.

The biological functions of single mutations are widely understood today, mainly by utilizing single knockout mouse models, for example of *Tet2* (Moran-Crusio *et al.*, 2011), or by overexpression, for example of *NPM1* (Vassiliou *et al.*, 2011). However, mutational interplays and cooperation between multiple genetic aberrations are more complex to be studied. So far, mainly combinations of two different mutations have been examined: for instance *NPM1* and *FLT3ITD* mutations have been shown to interact and to jointly induce leukemogenesis in mice (Mupo *et al.*, 2013; Mallardo *et al.*, 2013). Especially, the combinatorial effect of alterations in epigenetic factors with class I or II mutations is hard to predict as epigenetic modifications convey global gene expression changes. Recent efforts enabled the analysis of some specific mutational combinations, including epigenetic factors. For instance the cooperation between *Dnmt3a* deletion and *FLT3IDT* mutation (Meyer *et al.*, 2016) was detected to transform MDS to aggressive AML. Still, comprehensive studies on mutational interaction of epigenetic factors, especially with more than one further aberration, are still missing. In order to target special mutational combinations as a novel therapeutic path, the in-depth analysis of biological effects driven by mutational interplays is crucial.

1.3. EZH2

1.3.1. Polycomb repressive complexes (PRC) and EZH2

EZH2 is the core subunit of the Polycomb repressive complex 2 (PRC2), catalyzing the mono-, di-, and trimethylation of H3K27 (Kuzmichev *et al.*, 2002), which functions as a repressive histone mark leading to condensation of chromatin and block in transcription (Fig. 3). The evolutionary conserved protein EZH2 comprises a C-terminal SET domain, which exhibits the enzymatic function (Joshi *et al.*, 2008). The PRC2 complex further consists of SUZ12 and EED both enabling the catalytic function of EZH2 (Cao and Zhang, 2004) (Fig. 3). The EZH2 subunit can be replaced by EZH1 executing the same catalytic function but presenting with a lower trimethylation activity and altered expression pattern (Margueron *et al.*, 2008). As their target genes are partly overlapping and partly exclusive, possible compensatory abilities are discussed (Xie *et al.*, 2014). While there are more proteins known to mono- and dimethylate H3K27 (like G9a and GLP), EZH proteins are the only ones catalyzing trimethylation (Pan *et al.*, 2018).

By which mechanisms the PRC2 is recruited to chromatin is not reliably elucidated today, but might be mediated by additional subunits such as RBBP4/7 and AEBP2 (Kim, Kang and Kim, 2009). Moreover, a number of lncRNAs were described to be responsible to guide EZH2 to its target genes (Brockdorff, 2013). Firstly, this was demonstrated for *XIST* in order to ensure X chromosomal inactivation (Plath *et al.*, 2003) and then transferred to other lncRNAs, like *HOTAIR*, in order to silence the *HOXC* locus (Rinn *et al.*, 2007). However, the PRC2 was described to bind many different

RNAs (including mRNAs) with a comparable affinity so that a general RNA binding activity of EZH2 is under debate (Davidovich *et al.*, 2013). Not only functions in chromatin recruitment, but also an EZH2 inhibiting effect of the RNA binding was proposed assuming a mutual exclusive binding of RNA and chromatin (Beltran *et al.*, 2016).

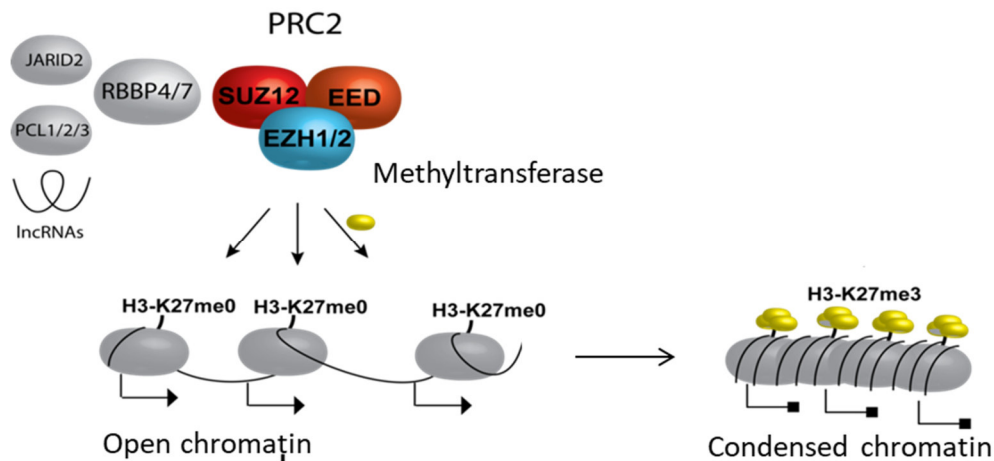


Figure 3. Graphical representation of the Polycomb repressive Complex 2. PRC2 consists of its core units EZH1 or 2, SUZ12, EED and is associated to JARID2, RBBP4/7, PCL1/2/3 and lncRNAs. EZH1/2 act as methyltransferases on H3K27 and lead to a condensation of chromatin and thus block in transcription.

Originally, the main molecular function of EZH2 activity was believed to be the chromatin recruitment of the PRC1 complex upon H3K27 trimethylation (Cao *et al.*, 2002). CBX proteins, which are part of canonical PRC1, bind H3K27me3 residues leading to complex recruitment (Cao, Tsukada and Zhang, 2005). Further PRC1 subunits are a RING protein, catalyzing the ubiquitination of histone 2A, and a PCGF protein (BMI1/ MEL18) supporting RING function. H2A ubiquitination, set by PRC1, leads to stabilization of nucleosomes and inhibition of RNA polymerase II thereby blocking transcription (Xiao *et al.*, 2020; Zhou *et al.*, 2008).

Nevertheless, the repressive effect of EZH2 and H3K27me3 was also described to occur independently of the PRC1 and H2A ubiquitination (Di Croce and Helin, 2013). Moreover, recruitment mechanisms have also been described to occur the other way around: PRC2 recruitment can occur upon H2A ubiquitination set by non-canonical PRC1 complexes (Blackledge *et al.*, 2014). Nowadays, there is a whole set of non-canonical PRC1 complexes described, which do not include CBX proteins so that they cannot bind H3K27me3 anymore. These complexes are numbered after the included PCGF protein (Gao *et al.*, 2012): for instance PRC1.1 consists, among others, of PCGF1, BCOR, RYBP and KDM2B, which could guide chromatin recruitment in this case (Wang *et al.*, 2018).

Originally, polycomb group proteins (PcG) were discovered based on their function to repress *Hox* gene expression (Lewis, 1978). However, there are many more target genes described today so that PcG proteins are recognized as global transcriptional repressors (Schwartz *et al.*, 2006). Well

established target genes of the PRC2 include genes involved in stem cell self-renewal, differentiation and tumor suppression (Simon and Lange, 2008). Polycomb activity is antagonized by proteins of the trithorax group, for example MLL1, that targets polycomb responsible elements (PREs) and activates transcription through trimethylating H3K4 (Schuettengruber *et al.*, 2007).

Nowadays, there are many non-canonical EZH2 functions described besides H3K27me3 and gene silencing. For example, non-histone proteins have been demonstrated to be posttranslationally modified by EZH2 such as the extranuclear regulatory protein talin (Gunawan *et al.*, 2015). Moreover, a methylation independent role of EZH2 in transcriptional activation has been discovered recently mediating the direct activation of transcription of the cyclin D1 and the androgen receptor (Kim *et al.*, 2018; Yan *et al.*, 2013).

Overall, the complex and diverse functions of EZH2 underline the difficulty to study the biology of global epigenetic factors.

1.3.2. The role of EZH2 in hematopoiesis

By silencing numerous target genes, EZH2 and the other polycomb proteins play a major role in the regulation of gene expression during hematopoiesis. Utilizing a sophisticated interplay with their activating counterparts of the Trithorax family, PRCs establish bivalent promoters to keep for example developmental genes in a poised status maintaining multipotency in HSCs (Weishaupt, Sigvardsson and Attama, 2010). Especially, PRC2 is part of the complex machinery controlling the balance between self-renewal and differentiation by repressing differentiation inhibitors, cell-cycle inhibitors, self-renewal enhancers, and pro-apoptotic genes (Xie *et al.*, 2014). Well established targeting of for example *CDKN2a*, *SOX7*, *NOXA* and *p21* prevents HSC from differentiation into mature blood cells and cell death (Xie *et al.*, 2014; Kamminga *et al.*, 2006).

Overexpression of *Ezh2* preserves HSCs from exhaustion by inhibiting senescence and increasing the pool of quiescent cells (Kamminga *et al.*, 2006). Furthermore, *Ezh2* knockin mice were shown to present a highly increased proliferation of HSCs and myeloid progenitor cells leading subsequently to a myeloproliferative disease (Herrera-Merchan *et al.*, 2012). Contradictory, *Ezh2* loss of function induces as well an increased proliferation of hematopoietic stem and progenitor cells in adult hematopoiesis (Majewski *et al.*, 2010). In fetal hematopoiesis, the *Ezh2* loss has a much more drastic effect since *Ezh2* deficient murine embryos die from anemia due to defects in HSC expansion and erythropoiesis (Mochizuki-Kashio *et al.*, 2011). *Ezh2* functions might be compensated by *Ezh1* in adult hematopoiesis but not in fetal. Still, a possible functional redundancy between *Ezh2* and *Ezh1* in hematopoiesis remains elusive although an organized expression pattern during differentiation and individual target genes were demonstrated (Xu *et al.*, 2015). *Ezh1* ablation in HSC was reported to have a different effect than the *Ezh2* loss inhibiting HSC proliferation and impairing HSC self-renewal capacity (Hidalgo *et al.*, 2012). The entire PRC2 complex has been demonstrated to be essential for

adult hematopoiesis as its complete loss, initiated by an *Eed* inactivation, leads to pancytopenia (Xie *et al.*, 2014).

1.3.3. EZH2 in cancer and AML

In chapter 1.2.2. it was noted that gain of self-renewal and block in differentiation is a key hallmark of leukemogenesis. With PRC2 being a main regulator of self-renewal and differentiation, its frequently detected deregulation in leukemia is thus not astonishing. However, EZH2 is a global regulator of gene expression, keeping poised states, so that the effects of changes in its expression or its disruption are hard to predict and to understand. These findings may be an approach to explain the diverse roles of *EZH2* mutations in the development and progression of cancer, which seem to be highly depending on the cellular context and factors yet to be discovered. More precisely, activating and inactivating mutations are equally detected in the very same subclasses of human cancers (Sashida and Iwama, 2017). For instance, *EZH2* overexpression is shown to support cancer progression and aggressiveness in melanoma, prostate and breast cancer (Melling *et al.*, 2015; Guo *et al.*, 2016; Zingg *et al.*, 2015), and its inhibition in cancer cell lines inhibited their proliferation (Eskander *et al.*, 2013). In lymphomas, *EZH2* overexpression has been described to confer increased proliferation, tumor cell aggressiveness and poor outcome (Abd Al Kader *et al.*, 2013; Yan *et al.*, 2013), and somatic gain-of-function mutations in *EZH2*, affecting the enzymatically active SET-domain, have been reported in germinal center diffuse large B-cell lymphoma (Morin *et al.*, 2010; McCabe *et al.*, 2012a; Béguelin *et al.*, 2013). This altered EZH2 protein targets H3K27me2 at a much higher probability raising global H3K27me3 levels. EZH2 might exert its oncogenic functions by mediating a block of differentiation in stem cells and keeping lineage-specifying genes in a poised state, especially in so called cancer stem cells (Ueda *et al.*, 2014; Xie *et al.*, 2016). Moreover, there are tumor suppressor genes described to be silenced by EZH2, such as *Ink4a/Arf* and *E-Cadherin* (Cao *et al.*, 2008; Bracken *et al.*, 2007).

In contrast, a down regulation of *EZH2* has also been connected to cancer initiation and progression. *EZH2* loss-of-function mutations are for example described in T-ALL (Ntziachristos *et al.*, 2012). In the case of MDS and AML the role of *EZH2* presents to be especially controversial. Besides some few reports, presenting *EZH2* as an oncogenic player (Grubach *et al.*, 2008; Xu *et al.*, 2011), more recent studies assign *EZH2* a tumor suppressor function in MDS and AML (Ernst *et al.*, 2010; Nikoloski *et al.*, 2010; Papaemmanuil *et al.*, 2016). Moreover, a recent study added additional complexity to the role of *Ezh2* in AML as a tumor suppressor role during AML induction and an oncogenic role during AML progression was reported (Basheer *et al.*, 2019). Loss of *EZH2* is also connected to pediatric myeloid disorders such as JMML and myeloid leukemia of the Down syndrome (Yoshida *et al.*, 2013; Caye *et al.*, 2015; Labuhn *et al.*, 2019). A very recent study observed *EZH2* mutations in 4% of analyzed AML patients, all of them harboring inactivating mutations, with only one exception (Stasik *et al.*, 2019).

As observed previously (Lindsley *et al.*, 2015), the loss of *EZH2* is especially frequent in secondary AML in this cohort.

Besides targeted inactivating mutations (frame-shift, premature stop-codon or point mutations), many different mechanisms, that lead to the inactivation of *EZH2*, are described. Firstly, the loss of chromosome 7q, where *EZH2* is located, and LOH are observed frequently (Jerez *et al.*, 2012). Moreover, incorrect splicing of the *EZH2* mRNA is detected after mutations in spliceosomal factors, like *SRSF2*, triggering its non-sense mediated decay in myeloid malignancies (Khan *et al.*, 2013; Kim *et al.*, 2015). Finally, mutations in *ASXL1* are reported to lead to a loss of *EZH2* mediated H3K27me3 levels phenocopying effects of *EZH2* mutations (Abdel-Wahab *et al.*, 2012).

The tumor suppressive effect might be mediated by the ability of *Ezh2* to suppress HSC proliferation and self-renewal (Sashida *et al.*, 2014; Majewski *et al.*, 2010) for example by targeting *HoxA9* (Khan *et al.*, 2013). *HOXA9* was also proven as an *EZH2* target gene in a recent study that demonstrated *EZH2* inactivation through a posttranslational modification leading to chemo-resistance (Göllner *et al.*, 2017). Drug insensitivity could be rescued by proteasomal inhibition, resulting in an increase of *EZH2* protein levels.

However, the *EZH2* inactivation alone – as true for most transformative events- is not able to convert an HSC into an AML cell even though most of the needed interaction partners remain elusive today. The interaction of mutations in *Ezh2* with mutations in *Tet2* and *Runx1* is described to induce MDS but not AML in mice (Sashida *et al.*, 2014; Muto *et al.*, 2013). Small CRISPR-Cas9 screenings also revealed a tumor suppressive effect of *Ezh2* in the context of additional mutations, especially *Nf1*, during AML development (Heckl *et al.*, 2014). Moreover, the cooperation of the *Ezh2* deletion with expression of the activating *NRAS* mutant G12D was detected recently to drive leukemic transformation by reprogramming BCAA metabolism (Gu *et al.*, 2019). The repeated co-occurrence of *EZH2* inactivation and mutations in *NRAS*, *CEBPA*, *RUNX1* and *ASXL1* in patients also point towards a potential synergistic effect (Stasik *et al.*, 2019) that remains to be studied mechanistically. It is thus conceivable that not only the cellular, but also the mutational contexts, decide on the function of *EZH2* as a tumor suppressor or as an oncogene.

In order to target oncogenic *EZH2* as a therapeutic option, *EZH2* inhibitors have been developed and demonstrated to inhibit proliferation of lymphoma, multiple myeloma and *EZH2* dependent leukemia (McCabe *et al.*, 2012b; Kim *et al.*, 2013; Zeng, Liu and Pan, 2017). Meanwhile, first clinical studies utilizing these inhibitors are running but reliable results are still receivable (Italiano *et al.*, 2018). In any case, such inhibitors should be used with the needed caution considering the tumor suppressive effect of *EZH2* in other contexts. Interfering therapeutically with the PRC2 is also under debate for *EZH2* loss scenarios. Given the much more drastic effect of a complete PRC2 loss, compared to an exclusive loss in *Ezh2* only (Xie *et al.*, 2014), and given the possible partial redundancy of *Ezh2* and 1,

targeting *Ezh1* in *Ezh2* loss AML might be a promising therapeutic path (Mochizuki-Kashio *et al.*, 2015). Following a similar idea a recent study utilized dual inhibition of EZH2 and EZH1 to eliminate leukemic stem cells in AML (Fujita *et al.*, 2018). Obviously, a more in-depth understanding of EZH2 biology in hematologic disorders will be needed for its successful exploitation in therapy.

1.4. Aim of the study

Genetic alterations in AML have been characterized in depth by NGS (Papaemmanuil *et al.*, 2016) and the effects of loss of function mutations on hematopoiesis and leukemogenesis have been extensively studied in simple monogenetic murine models and cell lines (Moran-Crusio *et al.*, 2011; Majewski *et al.*, 2010). However, the interplay between two, three or even more mutations has not been sufficiently analyzed so far even though the importance of such cooperation is widely recognized (Takahashi, 2011). Synergies of mutations that enhance proliferation and inhibit differentiation, thereby facilitating the induction of leukemia, need to be studied in detail. The PRC2 and its core enzymatic subunit EZH2 are major players in regulation of stemness and differentiation so that mutational and non-mutational inactivation in AML is a frequent event (Ernst *et al.*, 2010; Khan *et al.*, 2013). Nevertheless, the genetic context under which a loss of *EZH2* exerts a leukemogenic effect in AML is largely unknown today as only very few interacting mutations are described for MDS but not for AML (Muto *et al.*, 2013; Sashida *et al.*, 2014). The present study therefore aimed to decipher the mutational network around the *EZH2* loss in AML. The emergence of the CRISPR-Cas9 system enables easy multiplexing in genetic studies today (Cong *et al.*, 2013; Jinek *et al.*, 2012) providing a powerful tool for disease recapitulation and molecular analysis of AML (Labuhn *et al.*, 2019). In order to screen for a high number of transformative mutational combinations, we established a two-staged approach of an *in vitro* high throughput screening followed by extensive *in vivo* validation. Thereby we could identify a specific synergy between *Ezh2* and *Runx1* mutations.

Towards molecular understanding of events, guiding cellular transformation by loss of *Ezh2* and *Runx1*, we performed global gene expression analysis and ChIP-Seq experiments utilizing the cellular material generated during CRISPR-Cas9 assays. Firstly, we addressed the oncogenic effect of the *Ezh2* inactivation in the specific genetic context of identified mutational combinations and detected the upregulation of an onco-fetal gene signature. By subsequent comparison of *Ezh2* loss effects in fetal and adult hematopoietic cells and by overexpression of onco-fetal genes in murine transplants we aimed to establish fetal gene activation as a general oncogenic mechanism of the *Ezh2* loss in AML. Finally, we set out to isolate the molecular effects, guided by the *Runx1* inactivation, as well as the collaborative mechanisms mediated by the synergy between mutations in *Ezh2* and *Runx1*.

2. Material and methods

2.1. List of equipment and materials

2.1.1. Technical devices

Autoclaves:

Systec Dx-150 (Omnilab)

LVSA 50/70 (Zirbus Technology)

Balances:

Sartorius CPA64; (Sartorius)

Sartorius Extend; (Sartorius)

Cell culture laminar flow hoods:

Safe 2020, Biological Safety Cabinet; (Thermo Scientific)

Centrifuges and Rotors:

Beckman L-70 Ultra Centrifuge; (Beckman Coulter)

Eppendorf 5424; (Eppendorf)

Eppendorf 5418; (Eppendorf)

Heraeus Fresco 17; (Thermo Scientific)

Heraeus Megafuge 1.0R; (Thermo Scientific)

Heraeus Megafuge 16; (Thermo Scientific)

Heraeus Multifuge X3; (Thermo Scientific)

Sorvall RC6+ Superspeed; (Thermo Scientific)

Cytospin 2, Shandon, Pittsburgh)

Sprout® Mini-Centrifuge; (Heathrow Scientific)

FiberLite® F14-6x250y; (Thermo Scientific)

FiberLite® F21S-8x50y; (Thermo Scientific)

FiberLite® F10-6x500y; (Thermo Scientific)

50.2 Ti; (Beckman Coulter)

Chemiluminescence system:

ChemiDoc XRS; (BIO-RAD Hercules)

Counting chamber:

Neubauer improved; (Marienfeld-Superior)

Flow cytometers:

BD FACSCanto (BD Biosciences)

BD FACSAria (BD Biosciences)

Gel and Blot chamber for WB:

Mini Protean Tetra Electrophoresis System; (BIO-RAD Hercules)

Gel chambers for agarose gels:

Mini-SUB® Cell GT; (BIO-RAD Hercules)

Wide Mini-SUB® Cell GT; (BIO-RAD Hercules)

Gel documentation system:

Gel Doc XR+; (BIO-RAD Hercules, CA, USA)

Irradiator:

GammaCell2000 including a Cs-137 radiation source (Theratronics)

Incubators:

Binder incubator; (Binder GmbH)

Multitron Standard; (Infors HAT)

BBD 6220 CO2 incubator; (Thermo Scientific)

CO2 incubator C200; (Labotect)

Microscopes:

Axiovert 40CFL (Carl Zeiss)

B2-9000 Bioevo (Keyence)

Olympus CKX14 (Olympus)

Microwave:

Sharp Inverter Microwave (Sharp)

pH-meter:

SevenEasy pH-meter (Mettler Toledo)

Power supplies:

PowerPac Basic Power Supply (BIO-RAD Hercules)

Real-time PCR system:

StepOnePlus Real-Time PCR system (Thermo Scientific)

Refrigerators:

Liebherr Premium NoFrost, 4°C and -20°C (Liebherr)

-86°C Freezer MDF-U73V (Sanyo)

Shaker:

Polymax 1040 (Heidolph)

RM5 (Karl Hecht "Assistent" GmbH)

Sonicator:

Branson Sonifier 250 (Emerson)

Thermocycler:

96 Universal Gradient peqSTAR (Peqlab)

Thermomixer:

Thermomixer comfort (Eppendorf)

UV-Nanospectrometer:

NanoDrop 2000 Spectrophotometer (Thermo Scientific)

UV-Table:

TFX-20-ML (Peqlab)

Vortexer:

Lab Dancer (IKA, Staufen)

MS 3 basic (IKA, Staufen)

Reax 2000 (Heidolph)

Vortex Genie 2 (Scientific industries)

Water bath:

GFL (Omnilab)

Western Blot equipment:

Mini Protean Tetra Electrophoresis System (BIO-RAD Hercules)

2.1.2. Plastic ware

All plastic ware was obtained from Sarstedt or Greiner Bio-One.

2.1.3. Bacteria and bacterial media

Bacteria: XL-1 blue competent bacteria

Luria-Bertani-Medium (LB): 10 g/L Trypton (Applichem No.A1553), 5 g/L yeast extract (Applichem No.A1552), 10 g/L NaCl (Applichem No.A3597)

LB-Agar plates: LB medium plus 15 g/L Agar bacterial grade (Applichem No. A0949.1000)

Super-Optimal_Broth_Medium (SOB): 20 g/L Trypton (Applichem No.A1553), 5 g/L yeast extract (Applichem No.A1552), 0.58 g/L NaCl (Applichem No.A3597), 0.186 g/L KCl (Applichem No. A2939.0500)

SOC-Medium: 10 mL SOB-medium, add 200 µL 1M glucose (Applichem No. A1422.1000), add 100 µL 1 M MgCl₂ (Applichem No. 131396.1210) and 100 µL 1 M MgSO₄ (Applichem No. 212486.1211)

2.1.4. Antibiotics

Ampicilline: final concentration 100 µg/mL (Applichem No.69-52-3)

Blasticidine: final concentration 10 µg/mL (InVivogen No. ant-bl-1)

Puromycin: final concentration 1 µg/mL (Sigma Aldrich No. P8833)

Ciprofloxacin: final concentration 50 µg/mL (Fresenius Kabi No. 15LF506R1)

2.1.5. Buffers, Enzymes and Kits for the work with DNA, RNA and proteins

Buffers and reagents for the work with DNA:

6x DNA loading dye (Thermo Fisher Scientific No. R0611)

GeneRuler DNA ladder mix, ready-to-use (Thermo Fisher Scientific No. SM0331)

Tris-Acetate-EDTA-Buffer, 50x (Thermo Fisher Scientific No. B49)

Ultrapure DNase/RNase free distilled water (Thermo Fisher Scientific No. 10977035)

Ethanol absolut (Baker No.8025.2500)

Tris-EDTA buffer (TE): 10 mM Tris-HCl (pH 7.5) (Sigma Aldrich No. T6066), 1 mM EDTA (pH 8.0) (Sigma Aldrich No. E5134)

Buffers and reagents for the work with proteins:

RIPA Lysis Buffer (Thermo Fisher Scientific No. 89900)

Towbin Buffer for Western Blotting, 10x concentrated (Serva No. 42558.02)

Tween 20 (Panreac No. A4974)

Nonfat dried milk powder (Applichem No. A0830)

Amersham ECL Prime Blotting Detection Reagent (GE Lifescience No. RPN2232)

Methanol (Carl Roth No. 8388.2)

Protease inhibitor cocktail (Sigma Aldrich No. P8340)

10xTBS (BioRad No. 170-6435)

Stacking gel buffer for SDS-PAGE (BioRad No. 1610799)

Resolving gel buffer for SDS-PAGE (BioRad No. 1610798)

Rotiphorese® 10x SDS-PAGE buffer (Carl-Roth, No. 3060.2)

Acrylamide 4K solution (30 %) - Mix 37.5 : 1 (AppliChem, No. A1672.0500)

Buffers and reagents for ChIP-Seq:

RIPA 0: 0.1% SDS (Sigma Aldrich No. L6026), 1% Triton X-100 (Sigma Aldrich No. T8787), 10mM Tris-HCl (pH 7.4) (Sigma Aldrich No. T6066), 1 mM EDTA (pH 8.0) (Sigma Aldrich No. E5134), 0.1% NaDOC (Sigma Aldrich No. D6750)

RIPA 0.3: 0.1% SDS (Sigma Aldrich No. L6026), 1% Triton X-100 (Sigma-Aldrich No. T8787), 10 mM Tris-HCl (pH 7.4) (Sigma Aldrich No. T6066), 1 mM EDTA (pH 8.0) (Sigma Aldrich No. E5134), 0.1 % NaDOC (Sigma Aldrich No. D6750), 0.3 M NaCl (Sigma Aldrich No. S5886)

LiCl buffer: 250 mL LiCl (Sigma Aldrich No. L9650), 0.5% NP-40 (Sigma Aldrich No. L8896), 0.5% NaDOC (Sigma Aldrich No. D6750), 1mM EDTA (pH 8.0) (Sigma Aldrich No. E5134), 10 mM Tris-HCl (pH 8.1) (Sigma Aldrich No. T6066)

Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Sigma Aldrich No. P3803)

Dynabeads Protein A (Novex by Life technologies No. 10001D)

N-Lauroylsarcosine sodium salt solution 20% (Sigma Aldrich No. 7414)

DL-Dithiothreitol (DTT) (Sigma Aldrich No. D9779)

Kits:

GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific No. K0503)

GeneJET PCR purification Kit (Thermo Fisher Scientific No. K0702)

GeneJET Gel Extraction Kit (Thermo Scientific, No. K0692)

Quick RNA MicroPrep (Zymo Research No. R1050)

QiaAmp DNA Blood Mini Kit (Qiagen No. 51104)

High capacity cDNA reverse Transcription Kit (Thermo Fisher Scientific No. 4368814)

Taqman Fast advanced Master Mix (Thermo Fisher Scientific No.4444556)

SYBR Select MasterMix (Thermo Fisher Scientific No. 4472908)

Qiagen Plasmid Maxi Kit (Qiagen No. 12163)

Qia MinElute PCR Purification Kit (Qiagen No. 28004)

NEBNext High Fidelity 2x Master Mix (New England Biolabs No. M0541L)

Phusion HotStart II High Fidelity DNA-Polymerase (Thermo Fisher Scientific No. F549L)

HiSeq SR Rapid Cluster Kit v2 (Illumina No. 6D_402_4002)

Enzymes:

T4 DNA Ligase (Thermo Fisher Scientific No. EL0011)

Fast AP alkaline phosphatase (Thermo Fisher Scientific No. EF0651)

T4 Polynucleotide Kinase (Thermo Fisher Scientific No. EK0031)

Proteinase K solution (20mg/mL) (Ambion by Life technologies No. AM2546)

Rnase, Dnase and Protease free (Thermo Scientific No. EN0531)

Restriction Enzymes:

AgeI HF, (New England Biolabs No. R3552L)

MluI HF (New England Biolabs No. R3198S)

BsmBI (New England Biolabs No. R0580L)

2.1.6. Oligonucleotides

All oligonucleotides were synthesized by IDT (Integrated DNA Technologies) and purchased dried and standard desalted. All oligonucleotides were dissolved in water at 100 μ M.

Oligonucleotides for CRISPR-Cas9 gRNA cloning:

mCbl	Cbl.s1*	CACCGCCTGTCGAAAGCTCTTCCAC
	Cbl.as1*	AAACGTGGAAGAGCTTTTCGACAGGC
mNf1	Nf1_P2_s*	CACCGCAGATGAGCCGCCACATCGA
	Nf1_P2_as*	AAAC TCGATGTGGCGGCTCATCTGC
mStag2	Stag2_P1_s*	CACC GTCTCTACAATATTTCGGATC
	Stag2_P1as*	AAACGATCCGAAATATTGTAGAGAC
mSh2b3	Sh2b3.s2*	CACCGCGCGGTAGTCGTGACCCGG
	Sh2b3.as2*	AAACCCGGGTCACGACTACCGCGC
mRunx1	Runx1_P1_s*	CACC GCACTGGGGGACGTCCCGGA
	Runx1_P1_as*	AAAC TCCGGGACGTCCCCCAGTGC
mTet2	Tet2_P4_s*	CACC GAACAAGCTCTACATCCCGT
	Tet2_P4_as*	AAAC ACGGGATGTAGAGCTTGTTTC
mAsxl1	Asxl1_P6_s*	CACC GAAAATGTCCGTCCCGTTGAC
	Asxl1_P6_as*	AAAC GTCAACGGGACGGACATTTTC
mSmc1a	Smc1a.s.1*	CACC GGCCTCTCGAGCATAGATAC
	Smc1a.as.1*	AAAC GTATCTATGCTCGAGAGGCC
mBcor	Bcor_P2s*	CACC GATCACAGGCGACTACTATG
	Bcor_P2as*	AAAC CATAGTAGTCGCTGTGATC
mEzh2	Ezh2_P3s*	CACCGAAGACACCACCTAAACGCCC
	Ezh2_P3as*	AAAC GGGCGTTTAGGTGGTGTCTTC
mWt1	Wt1_P1_s*	CACC GAACTGCGCCGCGTGATGCG
	Wt1_P1_as*	AAAC CGCATCACGCGGCGCAGTTC
mZrsr2	Zrsr2_2ex2_s	CACC GGCGCTCGCTCGGCTCAGAG
	Zrsr2_2ex2_as	AAAC CTCTGAGCCGAGCGAGCGCC
mKmt2b	Kmt2b.s2*	CACCGCTGAGGCGGGGTACATCTTC
	Kmt2b.as2*	AAACGAAGATGTACCCCGCCTCAGC
mGata2	Gata2_1_s	CACC GCTGCGCATTCAATACGGCG
	Gata2_1_as	AAAC CGCCGTATTGAATGCGCAGC

Table 1: Single stranded oligonucleotides to be cloned into SGL40C (see 2.1.8.). Overhangs for BsmBI cloning are included (CACC/ AAAC). * published in (Heckl *et al.*, 2014)

Primers for genotyping by NGS:

Cbl_fw	TTGTGGAAAGGACGAAACACCTGGTAGTGTCTGCAGTTGCC
Cbl_rev	ATCTCGTATGCCGTCTTCTGCTTGCAATAGTGGACTTCAGAGCC
Nf1_fw	TTGTGGAAAGGACGAAACACCGACTTGCTTATCCCCAACTC
Nf1_rev	ATCTCGTATGCCGTCTTCTGCTTGTGATGAGCAGCCCATGTGTG
Sh2b3_fw	TTGTGGAAAGGACGAAACACCACCTGGTGTGCTGCAGTTTC
Sh2b3_rev	ATCTCGTATGCCGTCTTCTGCTTGAGGTGCTGCAGGGCACAGGC
Runx1_fw	TTGTGGAAAGGACGAAACACCCATCAGCGCAATTGCCAGAC
Runx1_rev	ATCTCGTATGCCGTCTTCTGCTTGGCTACCTGGTTCTTCATGGC
Tet2_fw	TTGTGGAAAGGACGAAACACCCACCAATTCGAGCAACTTAC
Tet2_rev	ATCTCGTATGCCGTCTTCTGCTTGTCTGAAAGCACCAGGTGAAC
Stag2_fw	TTGTGGAAAGGACGAAACACCGAGCTATATCCTTAGCCTTAC
Stag2_rev	ATCTCGTATGCCGTCTTCTGCTTGAAGATGGTGAACCTTTCG
Bcor_fw	TTGTGGAAAGGACGAAACACCGACAACATCGAGAAGCCATC
Bcor_rev	ATCTCGTATGCCGTCTTCTGCTTGGAGTGCAGTCCAGGAAGATG
Smc1a_fw	TTGTGGAAAGGACGAAACACCCCCAAGCACCATTTCATCTC
Smc1a_rev	ATCTCGTATGCCGTCTTCTGCTTGAGCAATCCGCTGAAGCACAC

Ezh2_fw	TTGTGGAAAGGACGAAACACCAGAACTTTACTCTGATGCCTC
Ezh2_rev	ATCTCGTATGCCGTCTTCTGCTTGTCTGTATCCTTTGACTCCAGC
BC_FW1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT nnnnnnATATCTTGTGGAAAGGACGAAACACC

Table 2: Primers for the analysis of CRISPR-Cas9 induced mutations. Overhangs for hU6 and P7 are needed for Next Generation Illumina Sequencing. For secondary PCR a barcoding fw-Primer (BC_FW1) including a P5 overhang was used.

Primers for cDNA cloning:

Plag1co_S_fw	CTCACCGGTGGATCCGCCACCATGTTCCACCGGAAGGACCACC
Plag1co_rev	GATACGCGTTCACTGGAAGGCCTGGTGAATC
BCOR_fw_AgeI	GATCACCGGTGCCACCATGCTCTCAGCAACC
BCOR_rev1_afterBamHI	GGTCACATCCACACTTGGTG
BCOR_fw2_beforAcc65I	CGTAAGCAAGGACAGTGTTCC
BCOR_rev2_MluI	GTCAAACGCGTTCACCAGTAGTTG

Table 3: Primers for *Plag1s* and *BCOR* cDNAs cloning. Restriction sites for AgeI and MluI are underlined.

Sequencing Primer:

DH077_SFFV_fwd_Seq	ATCAGCCTGCTTGTGCTTC
U6int_fw	AGTACAAAATACGTGACGTAG
Ryk_Seq_fw	TTCTGGTGGCTATCATCCTGG
Ryk_Seq_fw2	AAGCTGGTCGAGGCTAACAAC
Ryk_Seq_fw3	TGCTGGAACCTGGGTCTTCC
Igf2bp3_Seq_fw	TGGCTCACAACAACCTTCGTGG
Igf2bp3_Seq_fw2	AGCTATCATTGGCAAGCAGGG
Hif3a_Seq_fw	ACATCCACGCTCTGGACTCTG
Hif3a_Seq_fw2	ACACTGGACCTGGAATGCTG
BCOR_Seq_fw1	CCTCTGTTGGAGAAGCAGAC
BCOR_Seq_fw2	GCAAAGTCTCAGGTGACAGC
coEZH2_Seq1_fw	GTACAAAGAGCTGACCGAGC
coEZH2_Seq2_fw	CCGCTCACTGCAGAAAGATC

Table 4: Primers for Sanger sequencing of cloned cDNAs and gRNAs.

RT-qPCR Primers:

Igf2b3_qPCR_fw1_ex1	GAAAGTGTCTTCAAGGACGC
Igf2b3_qPCR_rev1_ex2/3	GAAGTTTACGAATCCTCTGCC
Hif3a_qPCR_fw1_ex2	AATCAGCTACCTGCGCATGC
Hif3a_qPCR_rev1_ex3/4	TCCAATGAGCTCCAGCTGAC
Ryk_qPCR_fw1_ex1	AGCCTCTACCTGAGCGAGGAC
Ryk_qPCR_rev1_ex3	ATATTGACCTGGGGCATGCC
Plag1_fw2	ACCTTTGGGAATGACATGCC
Plag1_rev2	TCTGAGGCTCTAACCCTAGC
mB2m_qRT_fw	TCCACCCGCCTCACATTGAAATC
mB2m_qRT_rev	GGCCATACTGGCATGCTTAAGTCT
PTBP2_fw	TCTCCATTCCCTATGTTTATGC
PTBP2_rev	GTTCCCGCAGAATGGTGAGGT

Table 5: Primers for expression analysis by RT-qPCR. *mB2m* or *PTBP2* primers were used for normalization.

Primers for Taqman assays:

SFFV_tm_fw	ATCGATACCGTCGACGGATC
Igf2b3_tm_rev	GGATCTTAGCGTCCTTGAACAC
Hif3a_tm_rev	TCTCTGGGATCTTCTGCTTCT
Ryk_tm_rev	GAGGAGGAGGTGCTCTAAGT
Plag1_rev3	AGCCAGGTGTCTCTTGAAGC
Igf2bp3_probe	ATCCGCCACCATGAACAAGCTGTA
Hif3a_probe	CGGAGCAACACCGAGCTGAGAAA
Ryk_probe	ATGAGAGCTGGAAGAGGCGGAGTT
Plag1s_probe	ACCACCTGAAGAACCATCTGCACA

Table 6: Primers for detection of cDNA vector integration. SFFV_tm_fw binds to the promotor of DHV020 (see 2.1.8). All probes carry a 6-FAM/ZEN dye.

2.1.7. cDNAs

BCOR: MGC Fully Sequenced Human BCOR cDNA, purchased from Dharmacon, #MHS6278-202757783, clone ID: 4384724

RUNX1C:

5' GCTTCAGACAGCATATTTGAGTCATTTCTTCGTACCCACAGTGCTTCATGAGAGAATGCATACTTGAATGAATCCTTCTAGGGACGTCCACGATGCCAGCAGAGCCGCCGTTCACGCCGCTTCCACCCGCTGAGCCAGGCAAGATGAGCGAGGCGTTGCCGCTGGCGCCCCGGACGCCGCGCTGCCCTGGCCGGCAAGCTGAGGAGCGCGACCCGACGATGTGTGGAGGTGCTGGCGCACCCGGGCGAGCTGGTGCGCACCGACAGCCCCAACTTCTCTGCTCCGTGCTGCTACGCACTGGCGCTGCAACAAGACCTGCCATCGCTTCAAGGTGGTGGCCCTAGGGGATGTTCAGATGGCACTCTGGTCACTGTGATGGTGCAATGATGAAACTACTCGGCTGAGCTGAGAAATGTACCGCAGCCATGAAGAACCAGGTGCAAGATTTAATGACCTCAGGTTTGTGGTGAAGTGAAGAGGAAAAAGCTTCACTCTGACCATCTGTTTCAAAACCCACCGCAAGTCGCCACCTACCAAGAGCCATCAAAATCAGATGGATGGGCCCCGAGAACCTCGAAGACATCGGCAGAACTAGATGATCAGACCAAGCCCCGGAGCTTGAGCTTCAGCGAGAGACTGAGTGAATGGAGCAGTGGCGGCACAGCCATGAGGCTCAGCCACACCCAGCCCCACGCCAACCTCTGCTCCCTGAACCACTCCATGCTTTAACCTCAGCCTCAGAGTCAGATGCAGGATACAAGGCAGATCCAACATCTCTCCCTGGAGCTATGACCACTCTACCAATACCTGGGAAGCATTGCTCTCTTCTGTGACCCAGCAACGCCCATTTACCTGGACGTGCCAGCGGATGACAACCTCTCTGAGAACTTTCAGTCGACTCTAACGGCACCCGACCTGACAGCGTTGAGCGACCCGCGCAGTTCCCCGCGCTGCCCTCCATCTCCGACCCCCGATGCACTATCCAGGCGCTTCACTACTCCCCGACGGGTCACTCGGGCATCGGCATCGGCATGTGCGGCATGGGCTCGGCCACGCGCTACCACTACCTACCTCGCCGCCCTACCCCCGGTCTGCGAAGCGCAGGGAGGCCGTTCAAGCCAGCTCGCCCTCTACCACTGTACTACGGCGCTCGGCCGCTCTACCACTTCTCATGGTGGGCGGCAGCGCTCGCGCGCGCATCTCTGCGCCCTGCACCAACGCCCTCCACCGCTCGCGCTGCTCAACCCAGCTCCCGAACAGAGCGACGTGGTGGAGGCCGAGGGCAGCCACAGCAACTCCCCACCAACATGGCGCCTCCGCGCGCTGGAGGAGCCGTGTGGAGGCCCTACTG-3'

coEZH2:

5' TCCCTCCGAGCCACCATGGACTACAAGACCACGACGGCGATTATAAGGATCACGACATCGACTACAAAGACGACGATGACAAGGGCCAGACCGCAAGAAGTCTGAGAAGGCCCTGTGTGCTGGCGGAAGAGAGTGAAGTCCGAGTACATCGCGCTCGCGCAGCTGAAGAGATTGAGAAGGGCCGACGAAGTCAAGAGCATGTTTCAGCAGCAACCGGCAGAAATCCTGGAACGGACCGAGATCTGTAATCAAGAGTGAAGCAGCGGAGAATCCAGCCTGTGCACATCTGACAAGCGTGTCCAGCCTGAGAGGCCACAGAGAATGTAGCGTGAACAGCGACCTGGACTCCCTACACAAGTGATCCCTCTGAAAACCTGAACGCCGTGGCCAGCGTGCCCATCATGTATTCTTGAGCCCTCTGCAGCAGAACTCATGTTGGAAGATGAGACAGTGTGCACAACATCCCTACATGGGCGACGAGGTGCTGGACAGGATGACACCTTTATCGAGGAATGATCAAGAACTACGACGGCAAGGTGACGGCGACAGAGATGCGGCTTCATCAACGACGAGATCTCTGGAAGTGTCAACGCCCTGGGCCAGTACAACGATGACGATGATGACGACGAGGGGACGACCCCGAGGAAAGAGAGGAAAAAGCAGAAGGACCTGGAAGATACCGGGACGACAAGAGAGCCGGCTCTAGAAAGTTCCCCAGCGATAAGATCTTCGAGGCCATCAGCAGCATGTTCCCCGATAAGGGCACCGCCGAAGAACTGAAAGAGAAGTACAAGAGCTGACCGAGCAGCAGTGCCTGGTCTTTCCTCTGAGTGACCCCTAACATCGACGGCCCTAATGCCAAGAGCGTGACAGAGAGCAGAGCCTGCACAGCTTTCACACCTGTTCTGCGCGCGTCTTAAGTACGACTGCTTCTGACCCGGAAGTGCAACTACAGCTTCCACGCCACCTAACACCTACAAGCGGAAGAACCCGAGACAGCCCTGGACAACAAGCTTGGCGCCCTCAGTGTATCAGCACTGGAAGGCGCCAAAGAGTTTGGCGTCTCTGACCGCGAGCGGATTAAGACCCCTCTAAAGACCTGGCGGACAGCGAGAGGACAGTGCCTAACATAGCAGCAGACCCACCTCAACGTGCTGGAAGCAAGGACACCGACAGCGATAGAGAGGCCGGAACAGAGACAGGCGCGAGAACAATGACAAGAGGAAGAAGAGAAGAAGGACGAAACCAGCAGCTCCAGCGAGGCCAACTCCAGATGCCAGACCTATCAAGATGAAGCCCAACATCGAGCCTCAGAGAAGCTGAGGGAGCGGAGCCGAGGCCAGCATGTTTAGTAGTGCTGATCGAACCTACTACGACAATCTGCGCCATTTGCCAGACTGATCGGCACCAAGACCTGCAGACAGGTGTACGAGTTGAGAGTGAAGAGAGCAGCATATTGCCCTGTCTCCAGCCGAGGATGTGGACACCCCTCCACGGAAGAAGAAGCGGAAGCACAGACTGTGGGCGGCTCACTGCAGAAAGATCCAGCTGAAAAAGGACGGCAGCTCAACCACTGTACAACCTACAGCCTTGGCATCACCCAGGACGCCCTGTGATAGCAGCTGTCTTGTGTGATCGCCAGAAATTTCTGCGAGAAGTTCTGTGAGTGACGAGCGAGTGCCAGAAGACAGATTCCCTGGTGTCAGATGCAAGGCCAGTGCAACACCAAGCAGTGCCCTTGTACCTGGCCGTGCGCGAGTGTGATCTGTGCTGACATGCGGAGCCCGCATCACTGGGACAGCAAGAATGTGCTGCAAGAACTGCAGCATCCAGCGGGGACAGCAAGAAGCCGATAGAAGAGGCCAAAGGTACGACAAGTACATGTGACGCTTCTGTTCACCTGAACAACGACTTCGTGGTGGACGCCACAGAAAGGGCAACAAGATCAGATTGCGCAACC

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GTACAGCCAGGCCGACGCTCTGAAATACGTGGGCATCGAGAGAGAGATGGAATCCCTGAACGCGT-3'

Hif3a_iso1_co

5' CTGTAACCGGTGGATCCGCCACCATGGCTCTGGGACTGCAGAGAGTGGGAGCAACACCGAGCTGAGAAAAGAGAAGTCTAGGGACGCCGCCAGAAGCAGAAGATCCCAAG
AGACAGAGGTGCTGTACCACTGGCCCCACACTGCCTTTTCCAGGGGAGTGTCTGCCACCTGGACAAGGCCAGCATCATGAGACTGACCATCAGCTACCTGAGAATGCACAG
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GGCCGCCACATGGAAGGTGCTGCACTGTAGCGGACACATGAGAGCCTACAAGCTCCAGCTCAGACAAGCCCTGCTGGCAGCCCTAGATCTGAACCTCCTCTGCAGTGCCTGGTG
CTGATCTGCGAGGCTATCCCTCATCTGCCAGCCTGGAACCACTCTTGGCAGAGGCGCTTCTGAGCAGACACAGCCTGGACATGAAGTTCACCTACTGCGACGAGAGAATCG
CCGAGGTGGCGGCTACTCTCTGATGATCTGATCGGCTGTAGCGCTACGAGTACATCCAGCTCTGGACTCTGATGCCGTGTCCAGATCCACACTCTGCTGTCTAAAGGC
CAGGCCGTGACCGCCAGTACAGATTCCTTGTAGAACCAGGCGCTACCTGTGGACACAGACAGGCTACAGTGGTGTCTGGCGGCAGAGGACCTCAGAGCGAGTCTATTATC
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AGTGGGACTCGAGAAGCCACAGACTGAGCACAGCCAGAAGAACAAGCCGTCGAGACAGACCTGGATATCGCTCAGGACCCGACACACTGGACCTGGAATGCTGGCCC
CTTACATCAGCATGGACGACGACTTCAGCTGAACGACGCGAGCAGCTGCCAAAGTGATAGACGGCCACCAAGAGTGGCCAGACGGCCTAGAGCTAGAAGCTTCCACGGAC
TGAGCCCTCAATTCTGAGCCTAGCCTGCTGCCTAGATGGGGCTCTGACCAAGACTGAACTGCAGCAGCCCTTCAGGGGCGATAGACCTACAGCTTCCCTGATGCTGCGCACC
AGAAAGAGAGCCCTGGCTCAGTCTAGCGAGGACAAGGCTGGAACCTGCTCGAGACAAGCCCTCAAGAGAAGCCCGAGCTGGAACCCGGCAGTTTTTGTGCTCCTCTG
AGCCTGAGCTTCTGCTCAAGGACAGACAGTGCCTGGAACCCAGCAGGACCTAGAGCACCCTGCTGCACTCTCACGAACCTCTCGGACTGGCTCCATCTCTGCTGAGCCTGT
GCCAGCACGAGGAACCGTGACGCTTAGAAACAGATTCCCTCTGCTGCTGGCTGGACAGACACATTGAACGCGTTCTAGT-3'

Ryk_201_co

5' CTGTAACCGGTGGATCCGCCACCATGAGAGCTGGAAGAGGCGAGTCTCTGGATCTGGCGACTTAGAGCACCTCCTCCTCACTGCTTCTGCTGCTGGCTATGCTCCAG
CCGCCGCTCTAGATCTCTGCTCTTGTCTGAGCTCCTGCTGCCCTTCTGTGTCTGTACTGTAGCGAGGACGAAGTGGGAGACTGCTGGGACTGATGCCGAGCTGTACTAC
GTGCGGAACGACCTGATCAGCCACTACGCCCTGAGCTTCAACCTGCTGGTGGCCAGCGAGACAACTTCTGCACTTACCTGGCAGCCGAAGAGCAAGGTCGAGTACAAGCTGG
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AGGTGGACAGCGAAGTGTATCTGATGACGCTGAACCTGACCGTGAACAGCAGCAAGAATTCACCGTGTGAACCTCAAGCGGCGGAAGATGTGTACAAGAAGCTCGAG
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GCCGTGATCTTCTGGTGGCTATCATCTGGCCGTGCTGCATCTGCACAGCATGAAGAAGTTCGAGCTGGACGACAGCATCAGCGCCAGCAGTTCCTCAGGCGCTGAGCCAGC
CTAGCACACAGACCACAGTACCTGAGAGCGGACACCCCAACAACGCGCACACCTATCACAAGTACCCACACTGAGGATCGAGAAGAAGCATCTGAGAAGCGTGACCTGTCT
GGAAGCCAAGGCCAAAGTGAAGGATATCGCCATCAGCAGAGAGCGGATCACCTGAAGGACGTGTGCAAGAGGGCACTTCGGCAGAATCTTCCAGCGATCCTGGTGGACG
AGAAGGACCCCAACAAGAAAAGCAGACCTTCTGAAGACCGTGAAGGACGAGCCAGCGAGGTGCAAGTACCATGATGCTGACCGAGAGCTGAAGCTGAGAGGCGTGCAC
CACGAAACCTGTGCTATCACTACGTGTGCATGAAGAGGGGAGAGAAGCCATGGTGGTGTGCTGCCATACAGTGGGGCAACCTGAAGCTGTTCTGAGACAGTGAAG
CTGGTCGAGGCTAACACCTCAGGCCATCTCTCAGCAGGACCTGGTGACATGGCCATCCAGATCGCTGTGGCATGAGCTACCTGGCCAGACGGGAAGTATCCACAGAGATC
TGGCCCGCAGAACTGCGTATCGACGACACCTGCAAGTGAAGATACCGACAACGCGCTGTCCAGGGATCTGTTCCCATGGACTACCATGCTGGGCGACAACGAGAACA
GACCTGTGATGGATGGCCCTGGAAGCCTGGTCAACAACGAGTTGACGAGCGCTCTGACGTGTGGGCTTTTGGCGTGACACTGTGGGAGCTGATGACCTGGGACAGACCC
CTTACGTGGACATCGACCTTTCTGAGATGGCCGCTACCTGAAGGATGGCTACAGAATCGCCAGCCTATCAACTGCCCCAGCAGCTGTTTGGCGTATGGCCTGTTGTTGGGC
CTCGATCTGAGGAAAGACCAAGTTCCAGCAGCTGGTGACGTGCTGACAGAGTTCATGCTGCTGTGGCGCCTACGTGTAACGCGTTCTAGT-3'

Igf2bp3_201_co

5' CTGTAACCGGTGGATCCGCCACCATGAACAAGCTGTACATCGGCAACCTGAGCGACCAAGCTGGACCTGCCGATCTGGAAGCGTGTTCAGGACGCTAAGATCCCCGTGGCT
GGCCATTCTGCTCAAGACAGGCTACGCCTTCTGGACTGTCTGATGAAGGTGGGCCCTGAAGGCTATCGAGGCTCTGTCTGGCAAGATGGAAGTGCACGGCAAGCCCATG
GAAGTGAACACAGCGTGCCCAAGAGACAGAGAATCAGAAAGCTGCAGATCCGGAACATCCCTCCACATCTGCAAGTGGGAAGTGTGACTCTCTGCTGCTGAGTACGGCGTG
GTGGAAGCTGCGAGCAAGTGAACACCGACAGCGAGACAGCGTGGTCAACGTGACCTACAGCAGCAAGGATCAGGCCAGACAGGCCCTGGATAAGCTGAACGCTTTCAGCT
GGAAAATTCACCTGAAGGTGGCTACATTTCCGACGAGACAGCTGCCAGCAGAACCATCTCCACAGCTGAGAGGCAGAAGAGGCCAGGCCAGAGGAAGCTCTAGAC
AGGCTTCTCTGGCAGCGTGTCCAAGCAGAAGCCTTGCATCTGCCCTGAGACTGCTGGTCCCTACACAGTTTGTGGCGCCATCATCGGCAAGAGGGCGCCACCATCAGAAA
CATCACAAGCAGACCCAGAGCAAGATCGAGTGCACAGAAAAGAGAACCAGGCGCTGCCGAGAAGTCCATCACCATCTGTCTACCCCTGAGGGCAGATCTGCCGCTGCAA
GAGCATCTGGAATCATGCACAAAGAGGCTCAGGACATCAAGTTTACCAGAGAAATCCCGTGAAGATCCTGGCTCACAACAATCTGTGGGCGAGACTGATCGGAAAAGAGG
CCGGAACCTGAAGAAGATCGAGCAGGACACCGACACCAAGATCACAATAGCCCTCTGCAAGAAGTACCTGTACAACCCGAGAGAACCATACCGTGAAGGGCAGCGTGA
AACCTGTGCCAAGGCCGAAGAGAAATATGAAGAAAATCCGCGAGAGCTACGAGAAGCATATCGCCAGCATGAACCTGCAGGCCATCTGATCCCTGGACTGAACCTGAACGC
CCTGGGCTGTTCTCTCATCTGGCATGCTCCACTACAAGCGGCCCTCATCTACACTGACCCCTCATATCTCTAGTTCGAGCAGAGCGAGACTGAGACAGTGCACCTGTT
CATCCCGCTCTGAGCGTGGGAGCTATCATTGGCAAGCAGGGCCAGCACATCAAGCAGCTGAGCAGATTCTGCTGGCGCTCCATCAAGATCGCTCAGCTGAGGCCCTGATGCC
AAAGTGGGATGGTATCATCAGAGGCCACCTGAGGCTCAGTTCAAGGCTCAGGGCAGAATCTACGGCAAGATCAAAGAAGAGAATCTGTCAGCCCCAAGAGGAAGTCAAG
CTGGAAGCCACATCAGAGTGCCAGCTTCTGCTGCTGGAAGAGTGATTGGCAAAGGCGGCAAGACCGTGAACGAGCTGAGTCTCTGCTAGCGCTGAGGTGGTGGTGGCCAGA

GATCAGACACCCGATGAGAACGACAGGTGGTCGTGAAGATCACCGGCCACTTCTACGCCTGTCAAGTGGCCAGAGAAAGATTCAAGAGATCTGACACAAGTGAAGCAGCAT
CAGCAGCAGAAGGCCCTCGAGTCTGACCTCTCAGAGCAGACGAAAGTGAACGCGTTCTAGT-3

***Plag1s*_201_co**

5' ATGGCCACAGTGATCCCTGGCGACCTGTCTGAAGTGCGGGACACAGAAAGCTCCAGCGGCAAGAGAAAGAGAGGCGAGTCCAAGCCTAGAAAGAACTTCCCTGCCAGC
TGTGCGACAAGGCCTTCAACAGCGTGGAAGCTGAAGGTGCACAGCTTCAGCCACACCGCGAGAGGCCTTACAAGTGTACCCACCAAGGACTGCACAAAGGCCTTCGTGTCCA
AGTACAAGCTGCAGAGACACATGGCCACACAGCCCGAAAAGACCCACAAGTGAACCTACTGCGAGAAGATGTTCCACCGGAAGGACCACTGAAGAACCATCTGCACACAC
ACGACCCCAACAAAGAACCTTCAAGTGCAGAGGAATGCGGCAAGTCTACAACCAAGCTGGGCTTCAAGAGACACCTGGCTCTGCACGCTGCCACAAGCGGAGATCTGACAT
GCAAAGTGTGCTGCAGAACTTCGAGAGCACCGCGTGTCTGTGGAACACCTGAAGTCTCACGCCGCAAAAGCTTGGCGGCGTGAAGAAAAGAAACACCAAGTGCAGAC
TGCGAGAGAAGGTTCTACACGAGAAAGGACGTGCGGAGGCACATGGTGGTGCATACCGGCAGAAAGGATTCTGTGCCAGTACTGCGCCAGAGATTCCGAAGAAAGGATCA
CCTGACCAAGGCACATGAAGAAGTCCACAATCAAGAGCTGCTGAAAGTCAAGACCGAGCCTGTGGACTTTCTGGACCCCTTACCTGTAACATGAGCGTGCCCATCAAGGACGAG
CTGTGCCCCGTATGAGCTGCCTTAGCGAACTGCTGAGCAAGCCCTCACCAACACTGCAGCTGAACCTGTACAACACCCCATTCAGAGCATGCAGTCTAGCGGCTCTGC
CCACCAGATGATCACCACTGCCTCTGGGCATGACCTGTCTATCGACATGGACGCGTGCATCTTCTACCACCTGGCTTCAAGTGCCTTTCAGCAGCACCAGCTACGCCAT
CAGCATCCCCGAGAAAGAGCAGCTCTGAAGGGCGAGATCGAGAGCTACCTGATGGAAGTGAAGCGCGCTCTAGCAGCAGCAAGATTCTCTGCCAGCAGCAGCAAGCT
GGGACTCGAACTCAGAGCGGCTCTCTGATGATGGTCCGCGATCTGAGCTGAGCAAGAGCAGCATCTCCATCAGCAGCCCTCTGAGCACCCTGCTTGGACTTCAGCCAG
CTGTTCAACTTATCCCTCTGAACGGCCCTCCATACAACCACTGAGCGTGGGAAGCCTGGGCATGTCTACTCTCAAGAAGAGGCCACAGCTCCGTGTCTCAGCTGCCTACACA
GACACAGGACCTGCAGGACCTGCTAACACAGTGGGACTGAGCAGCTGCACTCTGTCTGCGCCTTACAAGCAGCTGTCCAGCTCTACAACCTGCCTAGATTCCACCAGG
CCTTCAGTGA-3'

cDNA sequences of *Ezh2*, *Runx1*, *Hif3a*, *Igf2bp3*, *Ryk* and *Plag1s* were designed based on the NCBI genome browser sequences and codon optimized. Gene fragments were ordered as GBlock fragments from IDT and purchased dried and standard desalted. AgeI, AccIII and MluI cloning sites are underlined and were used for immediate cloning. Only *Plag1s* cDNA was amplified by PCR using Primers with AgeI and MluI overhangs (see 2.1.6.).

2.1.8. Vectors

SGL40C-NL-SL-EFS-dTomato/E2Chrimson/BFP (Reimer *et al.*, 2017):

This vector encodes the invariable part of the gRNA under control of a hU6 promotor and a reporter dye under the control of an EFS promotor. The variable part of the gRNA sequence is cloned into the gRNA backbone as a double stranded oligonucleotide (see 2.1.6.) by a BsmBI digestion.

pRRL.PPT.SFFV.dTomato/GFP:

This vector is a cDNA overexpression vector. cDNAs encoding for *EZH2*, *BCOR*, *RUNX1*, *Igf2bp3*, *Plag1s*, *Hif3a* and *Ryk* (see 2.1.7.) were cloned behind the SFFV promotor by an AgeI/ MluI double digestion.

pMD2.G (Addgene #12259):

This vector encodes the lentiviral envelope.

psPAX2 (Addgene #12260):

This vector is the lentiviral packaging plasmid.

2.1.9. Media and reagents for cell culture

293T medium: DMEM (Biochrom, No. F0435), 10% FBS advanced (Capricorn No.FBS-11A), 1% Penicillin/ streptomycin (Biochrom No. A2212), 1% Sodium pyruvate (Gibco No. 11360070), 1% L-Glutamine (Biochrom No. K0282), 1% MEM-NEAA (Gibco No. 11140050)

Medium for suspension cell lines:

RPMI 1640 (Capricorn No. RPMI-A), 10% FBS (Gibco No. 16000044), 1% Penicillin/streptomycin (Biochrom No. A2212), 1% Sodium pyruvate (Gibco No. 11360070)

Medium for primary derived murine cells:

IMDM (Lonza No. BE12-722F), 10% FBS (Gibco No. 16000044), 1% Penicillin/streptomycin (Biochrom No. A2212), 1% Sodium pyruvate (Gibco No. 11360070)

Medium for murine fetal liver cells:

DMEM (Biochrom, No. F0435), 10% FBS (Capricorn No.FBS-11A) 1% Penicillin/streptomycin (Biochrom No. A2212), 1% Sodium pyruvate (Gibco No. 11360070), 1% L-Glutamine (Biochrom No. K0282), 1% MEM-NEAA (Gibco No. 11140050)

Medium for LSK cells:

StemSpan SFEM (Stemcell technologies No. 09650), 1% Penicillin/ streptomycin (Biochrom No. A2212)

Phosphate buffered saline: Dulbecco's PBS (Capricorn No.PBS-1A)

Cell dissociation buffer (Gibco No. 13151014)

FACS Buffer: PBS (Capricorn No.PBS-1A), 2% FBS (Gibco No. 16000044), 1 mM EDTA (Sigma Aldrich No. E5134)

DAPI (Sigma Aldrich No. D9542-5MG)

RetroNectin® (Takara Bio Inc., Japan)

Polyethylenimine (PEI) (Polysciences No. 23966)

Easy Sep Mouse Strepavidine RapidSpheres Isolation Kit (Stemcell Technologies No. 19860)

Formaldehyde (Millipore No.344198)

Trypsin/EDTA solution (10x) (Biochrom No.L2153)

Opti-MEM (Gibco by Life technologies No. 31985062)

D+ Sucrose (Applichem No. 2211)

Polybrene: Hexadimithrine bromide (Sigma No. 107689)

Erythrocyte-lysis buffer: 8.2 g NH₄Cl (Merck No. 1.011.45.0500), 1 g KHCO₃ (Riedel-de Haen No. 31259) in 1 l H₂O, 2mM EDTA (Sigma Aldrich No. E5134)

2.1.10. Cytokines

hGM-CSF (Peprotech No. 300-03)

hFLT3L (Peprotech No. 300-19)

mlI3 (Peprotech No. 213-13)

mlI6 (Peprotech No. 216-16)

mScf (Peprotech No. 250-03)

mThpo (Peprotech 315-14)

2.1.11. Antibodies

Antigen	Flouorochrome	Species and clone	Company No.
mCd11b	PE-Cy7	Rat M1/70	BD No. 552850
mGr1 (Ly-6G/C)	PerCp-Cy5.5	Rat RB6-8C5	BD No. 552093
mCd3e	PE-Cy7	Hamster 145-2C11	BD No. 552774
mB220	APC-Cy7	Rat RA3-6B2	BD No. 552094
mCd117	APC	Rat 2B8	BD No. 553356
mCd117	APC-H7	Rat 2B8	BD No. 560185
mSca1 (Ly-6A/E)	PE	Rat D7	eBioscience No. 12-5981-82
Mouse Lineage cocktail (Lin-1)	Pacific blue (Pb)	Rat	BioLegend No. 133310
mTer119	Biotin	Rat Ter-119	Stemcell Biotechnologies No.60033BT

Table 7: Antibodies used for flow cytometry.

Antigen	Species and clone	Company No.
Ezh2	Rabbit D2C9	Cell Signaling No. 5246S
H3K27me3	Rabbit	Millipore No. 07-449
H3K4me3	Rabbit MC315	Millipore No. 04-745
H3K27ac	Rabbit	Abcam No. ab4729
Beta-Actin	Mouse C4	Santa Cruz Biotechnology No. sc-47778
Anti rabbit IgG HRP	goat	Santa Cruz Biotechnology sc-2004
Anti mouse IgG HRP	rabbit	Santa Cruz Biotechnology sc-358917

Table 8: Antibodies used for WesternBlot and ChIP.

2.1.12. Cell lines

HEK 293T (DSMZ No. ACC 635): cultured in 293T media (see 2.1.9.)

HEL (DSMZ No. ACC 11): cultured in media for suspension cell lines (see 2.1.9.)

2.1.13. Mice

C57Bl6/J (Janvier)

B6J.129(Cg)-Gt(ROSA)26Sortm1.1(CAG-cas9*,-EGFP)Fezh/J (*Cas9* knockin mice on C57BL/6J background)

2.1.14. Primary cells

LSK cells: Lineage negative, Sca1 positive, cKit positive cells isolated from adult bone marrow from heterozygous *Cas9* knockin mice (see 2.1.13.). Cells were cultured in medium for primary cells (see 2.1.9.). Cytokine concentrations differed based on the assay used (see 2.2.1.9. and 2.2.1.11.).

Fetal liver cells: Ter119 depleted fetal liver cells isolated from d13.5 heterozygous *Cas9* knockin mouse embryos (see 2.1.13.). Cells were cultured in medium for primary fetal cells (see 2.1.9.). Cytokine concentrations differed based on the assay used (see 2.2.1.11.).

2.1.15. Software

FlowJo (BD)

BD FACSDIVA™ Software (BD)

StepOne™ software (Life Technologies, Thermo Fisher)

GSEA (Broad Institute) (Subramanian *et al.*, 2005)

Clone Manager (Sci-Ed software)

Lasergene (DNASTAR Software solutions)

GraphPad Prism 6

IGV Genome Browser (Broad institute) (Robinson *et al.*, 2011)

2.2. Methods

2.2.1. Cell biology

2.2.1.1. Maintaining cell lines and primary cells

Cells were cultured at 37°C in 5% CO₂ in their corresponding appropriate medium (see 2.1.9.) and optimal density. Suspension cells were split by medium change every two to three days to maintain optimal cell density. Adherent 293T cells were split by aspirating the medium, washing the cells with PBS and trypsinization for 5 min at RT. Detached cells were seeded in an appropriate amount of medium.

2.2.1.2. Freezing and thawing of cells

For long-term storage cells were frozen in FCS supplemented with 10% DMSO in cryotubes in 1°C containers at -80°C and then stored in liquid nitrogen. For reuse of the cells they were quickly thawed in a 37°C water bath, washed in medium and resuspended at an appropriate density.

2.2.1.3. Transfection

For the production of viral supernatants 293T cells were transfected with the viral vectors for packaging combined with the vectors to be used for transduction (see 2.1.8.) by the PEI method. Cells were seeded at a density of 4 Mio. cells per 10 cm plate or of 0.75 Mio. cells per 6 well 24 h before transfection. Directly before transfection, the medium was changed, the PEI-DNA mixture prepared and incubated for 5-10 min:

Amount per 10 cm plate	Amount per 6 well	Plasmid
12 µg	2 µg	psPax2
2 µg	0,15 µg	pMD2.G
6 µg	1 µg	Specific vector
Ad 220 µL	Ad 25 µL	Opti-MEM
220 µL	25 µL	PEI (0,3 mg/mL)

Table 9: Amounts of plasmids used for transfection.

440 μ L or 25 μ L respectively were added dropwise to the medium that was changed after 6-8 h. Viral supernatants were collected 36 h after transfection and either used directly for transduction or concentrated by the ultra-centrifuge method.

2.2.1.4. Virus concentration by ultra-centrifuge

Viral supernatant was collected, centrifuged at 2000 rpm for 5 min and filtered. Afterward the supernatant was overlaid on 5 mL of 20% sucrose in centrifugation tubes (32 mL/ tube) and ultra-centrifuged over night at 12000 rpm or for 4 h at 18000 rpm. Viral pellets were resuspended in 150 μ L media appropriate to the downstream assay. Concentrated virus was immediately used for transduction or stored at -80°C . The MOI of the produced virus was determined by titrating on HEL cells and measuring transduction efficiency by flow cytometry.

2.2.1.5. Transduction

HEL cells were counted and seeded on 12 or 24 well plates (2 Mio cells in 500 μ L or 1 Mio. cells in 250 μ L respectively). Unconcentrated viral supernatant from transfected 293T cells was added at a MOI of 2-5 together with 5 μ g/mL polybrene. Six hours after transduction 1 mL of medium was added.

LSK cells were seeded at a density of 1×10^5 cells in 150 μ L or 2×10^5 cells in 300 μ L on retronectin coated 96 or 48 well plates, respectively. Concentrated virus was added at a MOI of 60 to 100 together with 2 μ g/mL polybrene.

Fetal liver cells, *in vitro* derived cells from CRISPR-Cas9 cooperation assays, and bone marrow cells were seeded at a density of 1×10^6 cells in 0.5 mL or 3×10^6 cells in 1 mL on 12 well or 6 well plates, respectively. Concentrated virus was added at a MOI of 10 to 20 together with 2 μ g/mL polybrene. Six hours after transduction two times the transduction volume was added to the cells. Transduction efficiency was determined two days after transduction by measuring the signal of cotransduced fluorescent reporter proteins by flow cytometry.

2.2.1.6. Flow cytometry and FACS

For measuring the expression of fluorescent reporter genes, suspension cells were centrifuged at 1000 rpm for 5 min, resuspended in an appropriate volume of FACS buffer and the corresponding fluorescent signal was measured in the FACS Canto.

For measuring the expression of surface markers, cells were stained by centrifugation and resuspension of 1×10^6 cells in 100 μ L of FACS buffer and addition of 1 μ L antibody. Cells were incubated with the antibody for 30 min at 4°C and their fluorescent signal was measured in the FACS Canto (BD). The same procedure was used for sorting specific cell populations by reporter gene or surface marker expression. Prior to sorting cells were filtered and then sorted in a FACS Aria (BD).

2.2.1.7. Preparation of LSK cells

LSK cells were gained from the bone marrow of femur, tibia, pelvic bones, and spine of donor heterozygous *Cas9* knockin mice. Bone marrow tissue was filtered and erythrocytes were removed by lysis. Cells were stained at a density of 2×10^7 cells / 100 μ L FACS buffer and 10 μ L Lineage Cocktail Pb, 1.25 μ L Sca1-PE and 1.25 μ L cKit-APC antibodies were added for the LSK staining. Pb negative, PE positive and APC positive cells were sorted on a FACS Aria (BD). Sorted cells were seeded at an appropriate density in medium for LSK cells (see 2.1.9.) and cytokine concentrations were adjusted to the downstream assay.

2.2.1.8. Preparation of fetal liver cells

Fetal liver cells were gained from heterozygous *Cas9* knockin 13.5 d embryos. To produce a single cell solution, fetal livers were pressed eight times through a 23G needle. Cells were counted, centrifuged at 1000 rpm for 5 min and resuspended at a density of 1×10^8 cells in 1 mL FACS buffer. Ter119 depletion was performed using the Easy Sep Mouse Strepavidine RapidSpheres Isolation Kit according to the kit's protocol. Cells were again centrifuged and seeded at a density of 4-5 1×10^6 cells per 6 well in 3 mL fetal liver medium supplied with 25 ng/mL Thpo and 25 ng/mL Scf.

2.2.1.9. CRISPR-Cas9 cooperation assay *in vitro*

LSK cells were gained from heterozygous *Cas9* knockin mice (see 2.1.13.) and seeded in LSK medium supplied with 100 ng/mL FLT3L, 50 ng/mL Scf and 10 ng/mL Il3. 48 h after preparation cells were transduced with the needed gRNA combination and expanded for 14 days keeping them at a density of 5×10^5 per 1 mL in a 12 well (medium is changed to IMDM after two days). Afterwards, cells were seeded in a limiting dilution of ten cells per 96 well in 150 μ L (one complete 96 well plate per transduction). The number of proliferating clones was determined under the microscope after two weeks in culture. Proliferating clones were subsequently expanded in a low cytokine medium (10 ng/mL Scf and 5 ng/mL Il3) and checked for further proliferating capacity under the microscope another two weeks later.

2.2.1.10. Rescue experiments

Rescue experiments were performed in cultured proliferating clones from the CRISPR-Cas9 cooperation assay *in vitro*. Cells were transduced with the cDNA expression vectors and expression of the dTomato reporter gene was analyzed by flow cytometry every second day.

2.2.1.11. Proliferation assays

To compare fetal and adult cell growth proliferation, assays, using adult LSK cells and fetal liver cells, were performed. Both cell types were transduced with the needed gRNA combination (adult cells in LSK medium supplemented with 50 ng/mL Scf and 50 ng/mL Thpo and fetal cells in fetal liver medium

supplemented with 25 ng/mL Scf and 25 ng/mL Thpo) two days after isolation. 24 h after transduction cells were seeded in a low cytokine medium (adult 4×10^4 cells per 48 well: 5 ng/mL Scf, 2 ng/mL Il3; fetal 4×10^5 cells per 12 well: 5 ng/mL Scf, 2 ng/mL Il3, 1 ng/mL Thpo). Cells were counted every second day and analyzed for dTomato and E2Chrimson reporter gene expression once a week.

2.2.2. Mouse work

All studies, involving mice, were approved by the Hannover Medical School Institute for laboratory animal science and local authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) and performed in accordance with the relevant protocols. Mice were maintained in a specific pathogen free environment.

2.2.2.1. Bone marrow transplantation

Bl6 mice were irradiated in a GammaCell 2000 with 7.5 Gy (primary transplant) or 7 Gy (secondary transplant) one day before transplantation. Simultaneously, oral antibiotics treatment with ciprofloxacin was started and given for three weeks to prevent infections. Subsequently, transduced LSK cells or whole bone marrow, resuspended in IMDM supplied with 2% FCS (150 μ L/ mouse), were applied intravenously. Transplanted mice were checked every day for the first five days and afterwards three times a week for vitality. 100 μ L of blood was drawn every 4 weeks to analyze blood cells for the expression of the cotransduced fluorescent reporter genes by flow cytometry after erythrocyte lysis. As soon as the mice presented with signs of leukemia in the blood or in vitality, but latest after six month, mice were euthanized and analyzed.

2.2.2.2. CRISPR-Cas9 cooperation assay *in vivo*

LSK cells ($1-4 \times 10^4$ / mouse) were gained from heterozygous *Cas9* knockin mice (see 2.1.13.) and seeded in LSK medium supplied with 50 ng/ μ L Scf and 50 ng/ μ L Thpo. 24 h after preparation, cells were transduced with the needed gRNA or cDNA combination. One day after transduction, cells were harvested using cell dissociation buffer, and transplanted into irradiated Bl6 mice.

2.2.2.3. Secondary transplantation

Whole bone marrow from leukemic mice was either thawed or prepared freshly (1×10^7 cells/ mouse) and immediately used for transplantation, or first transduced following a quick transduction protocol: In this case, cells were seeded on a 6 well (5×10^6 cells/ well) in LSK medium (see 2.1.9.) supplied with 50 ng/mL Scf, 5 ng/mL Il3, 10 ng/mL Il6, 50 ng/mL FLT3L and 0.1 mM β -mercaptoethanol and transduced with the needed gRNA combination at an MOI of ~ 5 . Four hours after transduction, cells were harvested using cell dissociation buffer and transplanted into irradiated Bl6 mice.

2.2.2.4. Analysis of leukemic mice

Parts of the liver, spleen and sternum were taken and stored in 4% formaldehyde in PBS for later histopathologic analysis. Preparation of histologic samples and HE staining was performed by the central animal facility of the Hannover Medical School. Tissue from bone marrow of femur, tibia, pelvic bones and from the spleen was isolated and filtered; erythrocytes were removed by lysis. Aliquots of cells were pelleted and used for DNA/ RNA extraction and flow cytometry, respectively. Flow cytometry samples were stained using a myeloid (Cd11b-PECy7, Gr1-PerCp-Cy5.5, cKit-APCCy7) and a lymphoid panel (Cd3e-PE, B220-APCCy7). Left over bone marrow cells were frozen alive and possibly used for secondary transplantation.

2.2.3. Molecular biology and biochemistry

2.2.3.1. Cloning

For gRNA cloning, all spacer sequences were designed using CCTop and CRISPRater online tools (Labuhn *et al.*, 2018; Stemmer *et al.*, 2015) and were ordered as single stranded oligonucleotides containing the overhangs for subsequent cloning (see 2.1.6.). Oligonucleotides were hybridized, phosphorylated and ligated into the BsmBI digested and dephosphorylated SGL40C vector.

For cDNA cloning cDNA sequences were excised from a G-Block, plasmid or PCR product by an AgeI/MluI digestion and ligated into the AgeI/MluI digested and dephosphorylated RRL vector.

Ligation mixtures were transformed into competent XL-1 blue bacteria by a heat shock of 45 s at 42°C and subsequent incubation in SOC media at 37°C and 200 rpm for 30 min. Bacteria were then suspended on LB-agar plates supplemented with ampicillin and incubated over night at 37°C. Growing colonies were picked, expanded and used for plasmid-DNA preparation. Plasmid-DNA was analyzed by a control digestion and subsequent Sanger sequencing by SeqLab Microsynth.

2.2.3.2. PCR for cDNA cloning

The BCOR and the Plag1s cDNA were cloned by PCR amplification from a plasmid (purchased from Dharmacon) or a G-Block (purchased from IDT) using the Phusion polymerase from Thermo fisher and the following protocol. All PCR products were gel purified and used for subsequent digestion.

98°C 2 min

98°C 10 s	
56°C 30 s	35 cycles
72°C 30 s	

72°C 5 min

2.2.3.3. Sequencing targeted loci

All loci, targeted by gRNAs in the CRISPR-Cas9 cooperation assays, were genotyped by a next generation sequencing approach using murine bone marrow and proliferating *in vitro* clones. Genomic DNA was isolated using the Qiagen DNA Blood Mini Kit and used for PCR. By utilizing specific primers, binding ~100 bp upstream and ~200 bp downstream of the protospacer sequence, the targeted locus was amplified in a primary PCR using the NEB Next polymerase. In a secondary PCR a specific barcode sequence (one barcode / sample) was added by using a universal barcoding fw primer (binding the U6 overhang of the fw primer in the primary PCR).

Primary PCR:		Secondary PCR:	
98°C 2 min		98°C 2 min	
98°C 10 s	25 cycles	98°C 10 s	15 cycles
56°C 30 s		56°C 30 s	
72°C 30 s		72°C 30 s	
72°C 5 min		72°C 5 min	

PCR products were gel purified, pooled and sequenced. Sequencing and raw data analysis was performed by the cooperating laboratory of M.L. Yaspo at the MPI for vertebrate genomics in Berlin using an Illumina HiSeq and the HiSeq SR Rapid Cluster Kit. All reads were quality and adapter trimmed using the BBduk tool (BBMap-Bushnell B.) and aligned to the mm9 mouse genome utilizing BWA software (Li and Durbin, 2009). Variant detection and functional annotation at the region of interest was performed using a custom python script with the pysam module (<https://github.com/pysam-developers/pysam>) and the annovar tool (Wang, Li and Hakonarson, 2010).

2.2.3.4. Taqman assay

In order to detect the genomic incorporation of specific cDNA vectors, taqman qPCR based assays were used. The fw primer was designed to bind the SFFV promotor in the vector, the probe and the reverse primer to bind the specific cDNA sequence. gDNA was isolated from murine bone marrow using the DNA Blood Mini kit and 100 ng were used for the taqman assay using the Taqman Fast advanced Master Mix. For normalization of the DNA amount, Ct values of a *PTBP2* PCR were used. For normalization of the vector copy number, single HEL clones, initially transduced at very low rates (~5%), were utilized.

2.2.3.5. Working with RNA for expression analysis

RNA was isolated from murine bone marrow or cultured cells using the Zymo RNA Micro kit. RNA quality was assessed on an Agilent 2100 bioanalyzer in the genomics core unit of the Hannover Medical School and was either used for Microarray, RNA Seq or RT-qPCR.

Microarrays: Microarrays were performed by the HZI in Braunschweig using Affymetrix AfyGE:ClariomS Pico Assay and raw data analysis was performed by Adrian Schwarzer (Experimental hematology, MHH) using R 3.5.0 (Gentleman *et al.*, 2004) and Bioconductor (Huber *et al.*, 2015). Raw files (.cel) were read in using the function “read.celfiles” from the R package “oligo”. Quality control was performed by inspection of the RLE (Relative Log Expression) plots and NUSE (normalized unscaled standard error) plots after fitting a Probe Level Model to the raw intensity data by using the function “fitProbeLevelModel” from the package “oligo”. The Raw data was background corrected, quantile-normalized, and log₂-transformed using the function “rma” from the “oligo” package. Correct preprocessing was verified by visualization and inspection of probe intensity distributions before and after “rma”. Probes interrogating the same gene were automatically collapsed by the “rma” function using the pd.clariom.s.mouse annotation file. For Annotation of the probe sets the package “AffyCoreTools” was used. Differential expression analysis between the experimental groups were computed on the quantile normalized expression matrix of 22,203 annotated probes using the moderated t-test from “limma” with Benjamini-Hochberg adjustment for multiple testing (Ritchie *et al.*, 2015).

RNA-Seq: RNA-Seq was performed and analyzed by Novogene using Illumina HiSeq technology. Sequencing reads were aligned to the mouse genome using the HISAT2 tool (Kim *et al.*, 2019). Gene expression was measured by transcript abundance, normalized by the FPKM method, utilizing HTSeq software (Anders, Pyl and Huber, 2015).

Gene set enrichment analysis: GSEA was performed utilizing the Broad GSEA software with the permutation type set to Gene_set (1000 permutations). Gene sets related to hematopoiesis and leukemia (Schwarzer *et al.*, 2017), based on published gene sets, were queried first. In addition, gene sets related to fetal expression were generated from unpublished and published data and tested for enrichment.

RT-qPCR: Microarray results were validated by RT-qPCR. Reverse transcription was performed using 250 ng of RNA and the reverse transcription kit from Thermo fisher. 1 µL of the cDNA was utilized in subsequent qPCR using the SYBR Select master mix by Thermo fisher and cDNA specific, exon spanning primers. Ct values were normalized to Ct values from a *B2m* qPCR.

2.2.3.6. Western Blotting

RIPA lysates were prepared from cultured cells or murine bone marrow according to the manufacturer's protocol (Thermo fisher), mixed in 2x Laemmli buffer and denatured at 95°C for 5 min. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane and detected with an Ezh2 antibody (Cell signaling technology) or a β -Actin antibody (Santa Cruz). Detection and image acquisition was done using Amersham ECL Prime Blotting reagent and a ChemiDoc XRS machine (Biorad).

2.2.3.7. CHIP-Seq

ChIP experiments were performed based on a protocol published by Xu *et al.*, 2010. Murine bone marrow cells were crosslinked directly after isolation using 1% formaldehyde for 5 min. Crosslinking was stopped by using 125 mM glycine. Cells were spun down and washed twice in PBS. Crosslinked cells were resuspended in RIPA0 supplemented with 0.25% sarkosyl, 1mM DTT and protease inhibitor (1:200) and sonicated for 15 min at a duty cycle of 20% and output 3,5 in an ice bath. Fragment size was determined after decrosslinking by boiling for 5 min in 1% SDS and phenol chloroform extraction on a 1.5% agarose gel. If DNA fragments were 200 to 300 bp long, chromatin would be used for downstream ChIP experiments. Insoluble cellular parts were spun down at 13000 rpm for 10 min at 4°C and the supernatant was supplemented with the needed antibody (1-1.5 μ g antibody/ 2×10^6 cells/ IP) and 0.3 M NaCl. 1% of the chromatin was kept as an input control. The antibody chromatin mixture was incubated over night at 4°C head over tail. After washing, protein A magnetic beads three times in RIPA0.3 20 μ L were added to the reaction mix and incubated another 3 h at 4°C head over tail. Subsequently, the beads were washed two times in each of the following buffers: RIPA0, RIPA0.3, LiCl buffer, TE buffer. Finally, the beads were resuspended in 200 μ L TE buffer and RNA digested. Crosslinking was removed by supplementing 0.75% SDS and 3 μ L protease for overnight incubation at 65°C. DNA was eluted by using the Qia MinElute PCR extraction kit. Eluted DNA was used for library preparation using the NEB Next Ultra DNA library kit for Illumina. Sequencing on an Illumina HiSeq and raw data analysis was performed by the cooperating laboratory of M.L. Yaspo at the MPI for vertebrate genomics in Berlin. Sequencing reads were aligned to the murine mm9 genome using BWA software (Li and Durbin, 2009) and peak calling was done utilizing MACS2 software (Zhang *et al.*, 2008). Peak visualization was performed using the IGV genome browser tool (Robinson *et al.*, 2011).

3. Results

3.1. CRISPR-Cas9 cooperation screenings *in vitro*

3.1.1. Pooled CRISPR-Cas9 cooperation screenings *in vitro* can quantify the transformative potential of mutational combinations

Aiming to identify cooperating mutations of the *Ezh2* loss in AML we screened simultaneously for multiple mutations frequently occurring in AML (Papaemmanuil *et al.*, 2016) in this study (see 1.2.3.). To reach our goal of defining the mutational network guiding cellular transformation in *Ezh2* deficient AML, the establishment of methods with sufficiently high throughput was strictly required, since the overall number of potential candidates and resulting mutational combinations summed up to hundreds. In order to model one to five jointly occurring mutations -the average load of driver mutations in adult AML-, we utilized a pooled CRISPR-Cas9 approach allowing easy multiplexing (Cong *et al.*, 2013; Heckl *et al.*, 2014). By transducing HSCs with a pool of five different gRNAs at a medium transduction rate of around 30 to 60%, a random distribution of one to five mutations per cell can be achieved. Subsequent clonal selection mechanisms isolate mutational combinations that give a proliferation advantage. Such assays thereby mimic the clonal evolution of malignant diseases. The functionality of this approach for the definition of cooperation has already been demonstrated *in vivo* (Heckl *et al.*, 2014; Labuhn *et al.*, 2019).

In order to test more mutational mutations in shorter time, an assay with higher throughput than mouse bone marrow (BM) transplantation (Tx) was required. Screening the potential of existing *in vitro* approaches, we evaluated a formerly published *in vitro* setup for viral vector safety highly as suitable (Modlich *et al.*, 2006) due to its efficient quantification and implementation: In short, murine LSK cells are transduced with a pool of five different gRNAs and the *Ezh2* gRNA at a total MOI of ~60 (as determined on HEL cells), reaching a transduction rate of 30 to 60%, and then expanded under differentiation promoting conditions for two weeks. Subsequent seeding in limiting dilution (10 cells per well) eventually isolates proliferating clones after additional two weeks of culture and selects for transformative mutational combinations thereby (see Fig. 4A and 2.2.1.9.). To cover the total mutational interactome of *Ezh2*, we compiled six gRNA pools (5 gRNAs each) that were used in the *in vitro* assay in combination with the *Ezh2* gRNA (Figure 3B). Pool compositions were defined with the directive to include at least one target gene from every described class of mutations in AML (Takahashi, 2011) (see 1.2.3.). *Cbl*, *Nf1* and *Sh2b3* belong to class I of mutations, *Runx1*, *Gata2* and *Wt1* belong to class II, and class III of mutations include *Asx1*, *Stag2*, *Smc1a*, *Kmt2b*, *Zrsr2*, *Bcor* and *Tet2*. These six pools of 5 gRNAs enable the analysis of 148 mutational combinations if calculating for all possible combinations of single gRNAs or two to five combinations of gRNAs, respectively.

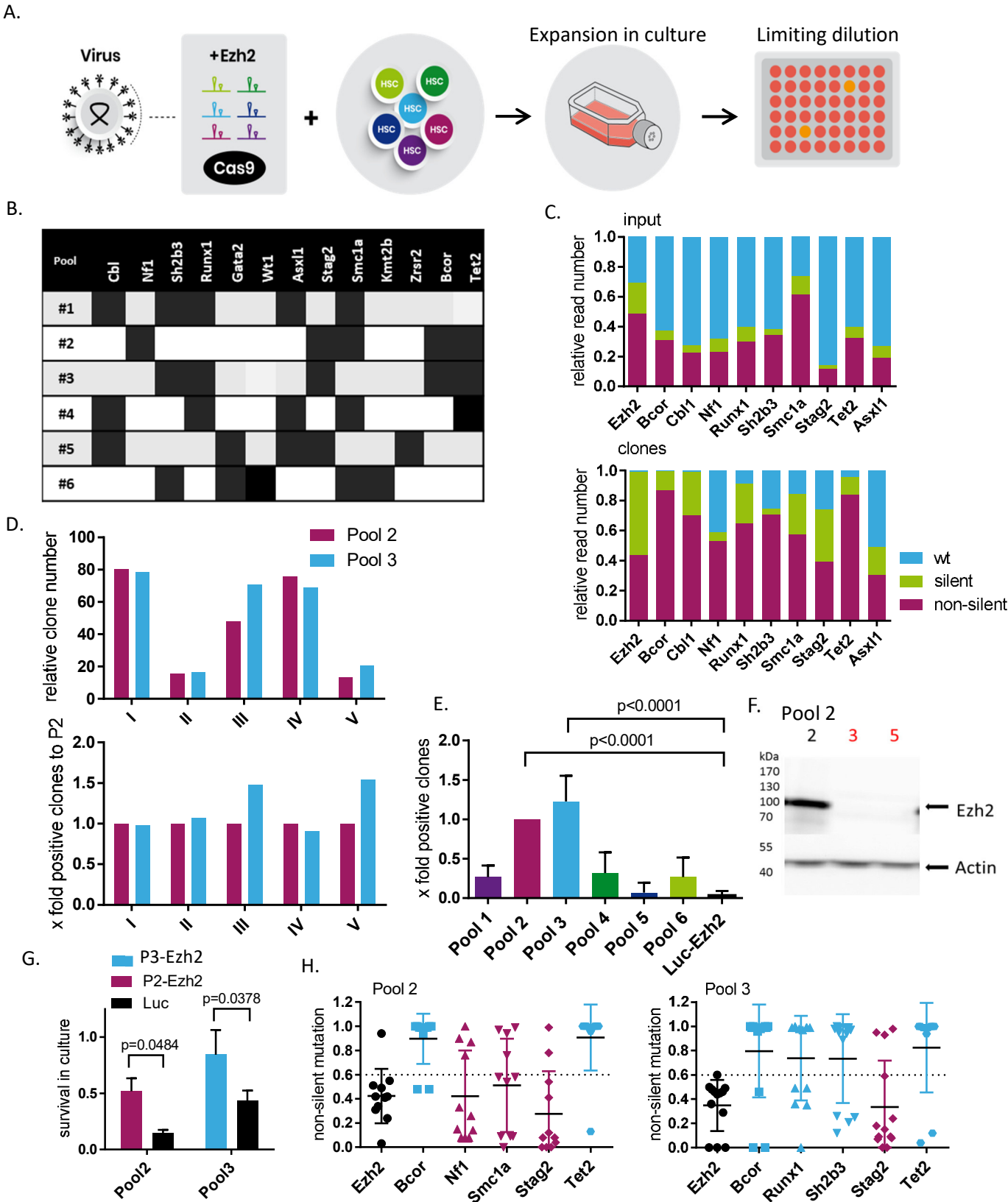


Figure 4. Pooled CRISPR-Cas9 cooperation screenings *in vitro* demonstrate a high oncogenic potential of Pool2 and 3 in combination with the *Ezh2* gRNA. A. Experimental setup of the pooled CRISPR cooperation screening *in vitro*. Murine LSK cells are transduced with the gRNA pool, expanded in liquid culture in differentiation promoting medium and seeded in limiting dilution. B. Gene/ gRNA combinations of pools 1 to 6. Black boxes indicate the inclusion of a target gene. C. Relative distribution of reads on wt sequence, silent or non-silent mutations in NGS for all targeted loci in input samples or clones derived from the CRISPR screening. The average of ≥ 2 replicates in the input and of ≥ 7 clones in the CRISPR assay is shown. D. Comparison of primary and normalized readout of replicates I to V of the CRISPR assay after two weeks in limiting dilution for pool 2 and 3. The upper panel illustrates relative proliferating clone numbers and the lower panel illustrates normalized clones numbers (relative to pool 2). E. Normalized relative clone numbers for pool 1 to 6 as average of three replicates are shown, av $\pm$ SD, Anova. F. α -EZH2 and α - β Actin Western Blot. Two *Ezh2* neg clones indicated by red numbers and one *Ezh2* pos clone derived from the CRISPR screening using pool 2 are shown. G. Relative numbers of proliferating clones during two weeks of expansion after two weeks in limiting dilution are shown for the CRISPR cooperation screenings, using pool2 and 3 combined with the *Ezh2* or a *Luc* gRNA, n=2, av $\pm$ SD, t-test. H. Relative numbers of reads presenting non-silent mutations in NGS for all targeted loci in pool 2 and 3. Each dot represents a clone, av $\pm$ SD. Blue dots highlight all genes where more than 60% of the clones presented with more than 60% non-silent mutations.

To note, all employed gRNAs were designed for minimal off-target effects using the cctop online tool (Stemmer *et al.*, 2015) and pretested in a special reporter system for their cutting efficiency (Labuhn *et al.*, 2018). Beyond pre-testing, cutting was confirmed by targeted next generation sequencing of all targeted loci in an input sample taken three days after transduction and in proliferating clones after two weeks in limiting dilution (Fig. 4C). Based on the limited transduction efficiency at the employed MOI and the random distribution of gRNAs, the rate for non-silent mutations varied between 15 to 60% for all used gRNAs in the input samples conferring an approximately equal gRNA efficacy. The comparison of the mutational spectrum and distribution of in-frame and out-of-frame mutations in the input samples to the proliferating clones after the assay demonstrated the increase of mutations during expansion and limiting dilution (Fig. 4C). Our sequencing thereby illustrates the efficient introduction of transformative mutations that undergo clonal selection mechanisms.

To measure the transformative potential of a given gRNA combination the number of proliferating clones was counted after two weeks of limiting dilution and normalized to a fixed condition (pool 2 for the screening). This normalization was necessary as different efficiencies were detected in various replicates despite constant relative differences between the conditions (Fig. 4D). Figure 4E presents the relative clone number for the six tested gRNA pools and a non-targeting *Luc* control, always in conjunction with the *Ezh2* gRNA and normalized to pool 2. Only pool 2 and 3 (*Nf1*, *Stag2*, *Smc1a*, *Bcor*, *Tet2* and *Sh2b3*, *Runx1*, *Stag2*, *Bcor*, *Tet2*) showed a significantly increased relative number of clones compared to the *Luc* control whereas all other pools showed a relatively low transforming

capacity reaching 32% of pool 2 at maximum. This clear result demonstrates that the newly established CRISPR-Cas9 cooperation assay *in vitro* reveals a potent measurement for the transformative potential of a given gRNA combination. Beyond sequencing genomic DNA, the loss of the *Ezh2* protein was confirmed by a α *Ezh2* Western blot in two of three shown clones from pool 2 (Fig. 4F). With pool compositions of recurrently mutated tumor suppressor genes only, lacking the *Ezh2* gRNA, we further validated cooperation with loss of *Ezh2*. Although initial numbers of clones at two weeks post seeding of the limiting dilutions did not differ, clonal cultures, being defective for *Ezh2*, showed a markedly increased self-renewal visible in maintained long term cultures (Fig. 4G). Four times more clones in pool 2 and two times more clones in pool 3 could survive and proliferate during long-term culture when the *Ezh2* gRNA was included compared to the inclusion of a *Luc* gRNA. Since the inclusion of a gRNA in a pool does not automatically lead to the selection of a mutation, we were interested in the actually present mutations in the clones of the highly transforming pools 2 and 3 after limiting dilution. Therefore, we analyzed the NGS sequencing results for all targeted loci of pool 2 and 3 (Fig. 4H). Interestingly, heterozygous *Ezh2* mutations were sufficient to convey the proliferation advantage, shown in Fig. 4F, as most clones presented non-silent mutations in around 50% of the reads. For pool 2, sequences of *Bcor* and *Tet2* revealed homozygous mutations for 83% of the clones (> 90% of the reads) while sequences for *Nf1* and *Smc1a* were only mutated in half of the clones. *Stag2* mutations seem to be dispensable as they only occurred in a minority of clones (36% in pool 2 and 30% in pool 3). For pool 3, all other included gRNAs, *Bcor*, *Runx1*, *Sh2b3*, and *Tet2*, induced homozygous mutations in most of the proliferating clones (more than 60%). Strikingly, the recurrent detection of *Bcor* and *Tet2* mutations in both used gRNA pools suggests the cooperation with *Ezh2* mutations. Moreover, the especially high co-occurrence of *Sh2b3* and *Runx1* mutations with the heterozygous *Ezh2* loss in pool 3 hints towards a cooperative effect.

3.1.2. Single gRNA combinations highlight the oncogenicity of combined losses in *Ezh2*, *Bcor*, *Tet2*, and *Runx1*

Our pooled CRISPR-Cas9 cooperation screenings stressed gRNA combinations of pool 2 and 3 as especially potent in order to induce transformation. Moreover, sequencing of all targeted loci in proliferating clones narrowed down the possible mutational cooperation partners of the *Ezh2* loss to inactivation of *Bcor*, *Tet2*, *Runx1* or *Sh2b3*. We thus decided to focus on these specific mutational combinations and their interplay conferring the transformational power. Hypothesizing that our candidates still contained drivers and passengers, we tested combinations of only three gRNAs to eventually isolate the minimally needed mutations. Based on the finding that *Bcor* and *Tet2* mutations occurred in both powerful gRNA pools, we decided to test their combination with the *Ezh2* loss in a reduced complexity setup first. As a baseline, the effect of the single gene knockouts was

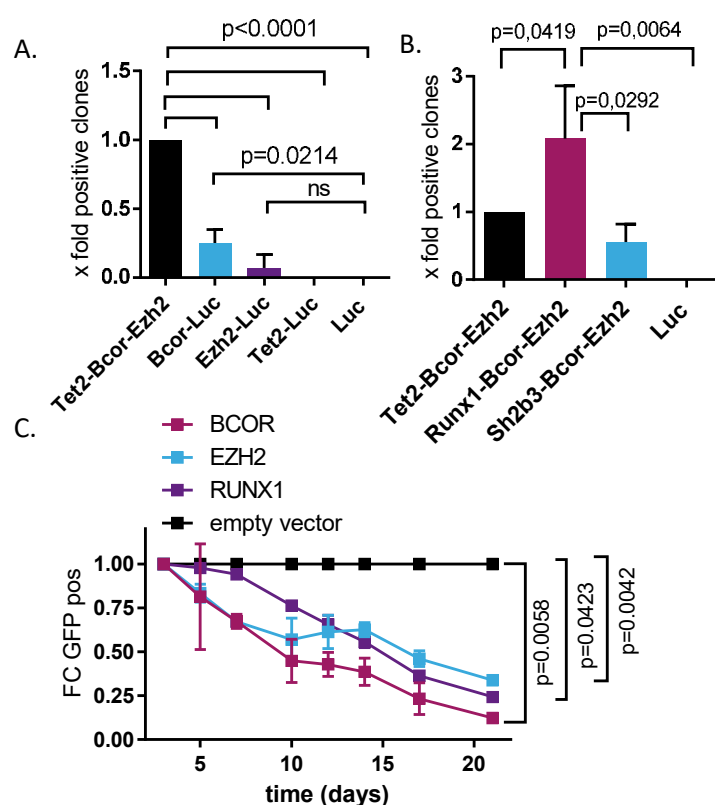


Figure 5. Reduced complexity screenings *in vitro* isolate a potent cooperation between losses in *Ezh2*, *Runx1* and *Bcor*. A and B. Readout of CRISPR-Cas9 cooperation assays *in vitro* using combinations of gRNAs against *Ezh2*, *Bcor*, *Tet2*, *Sh2b3* and *Runx1*. Relative numbers of proliferating clones after two weeks in limiting dilution normalized to the number of clones of *Tet2-Bcor-Ezh2* ko are shown, $n \geq 2$, av $\pm$ SEM, Anova. C. Rescue experiments for liquid cultures of an *in vitro* derived clone of Pool 3- *Ezh2* ko, reexpressing the indicated cDNAs. Relative numbers of GFP pos cells over time normalized to d3 and the empty vector control are shown. d0 marks the time point of transduction with the cDNA, $n=2$, av $\pm$ SD, Anova.

assessed and compared to the triple combination of *Ezh2*, *Bcor* and *Tet2* gRNAs (Fig. 5A) that presented with a highly significantly increased number of clones (by more than three times) in relation to all single gRNAs emphasizing the cooperating effect between these mutations. Of note, the single gRNAs against *Ezh2* and *Tet2* gave no or a very low number of clones (Fig. 5A) while only the gRNA against *Bcor* presented some significant transforming capacity on its own. Overall, oncogenic effects of these gRNAs are only displayed after their combination. After proofing the transforming capacity of the first triple combination, we analyzed the possible transforming effect of other triple candidates identified in the sequencing: *Runx1* and *Sh2b3*, respectively. The triple combination of *Ezh2*, *Bcor* and *Tet2* gRNAs served as a positive control for normalization in this setup (Fig. 5B). Surprisingly, the triple combination of gRNAs against *Runx1* with *Bcor* and *Ezh2* presented with a two times higher transformative potential than the triple combination with *Tet2* (Fig. 5B). The number of proliferating clones transduced with this gRNA combination was significantly increased compared to both other triple combinations (with *Tet2* and *Sh2b3* gRNAs) highlighting its transformative effect. The enforced transforming activity after combining the *Ezh2* gRNA with the gRNAs against *Bcor* and *Runx1* suggests a high mutational cooperating effect.

To further validate the involvement of all three protein losses in transformative events, we performed rescue experiments for *Ezh2*, *Bcor* and *Runx1*, respectively, in transformed clones. Therefore, we transduced the cultured clones with cDNAs, co-expressing a fluorescent marker (GFP), and tracked the fluorescent signal over time as a measure of relative cell proliferation compared to untransduced cells (Fig. 5C). All three cDNAs, *RUNX1*, *EZH2*, and *BCOR*, depleted the proliferation of

the pool 3- *Ezh2* clone significantly compared to an empty vector control as transduced cells were outcompeted by non-transduced ones. The reversion of transformation by re-expression of a single protein even in the condition of mutations of the complete pool 3 emphasizes the need for the interplay of all three mutations *in vitro*.

3.2. Pooled CRISPR-Cas9 cooperation assays *in vivo*

3.2.1. Loss of *Ezh2* fosters leukemic aggressiveness in multiple genetic contexts

By using *in vitro* CRISPR-Cas9 cooperation assays, we efficiently screened for mutations cooperating with loss of function mutations in *Ezh2*. Initially, we analyzed the transformative effect of 6 different pools containing five gRNAs each combined with the *Ezh2* gRNA. gRNA combinations of pool 2 and 3 (*Nf1*, *Stag2*, *Smc1a*, *Bcor*, *Tet2* and *Sh2b3*, *Runx1*, *Stag2*, *Bcor*, *Tet2*) proofed to be potent initiators of transformation by producing a high number of proliferating clones in the *in vitro* setup. Since *in vitro* transformation assays did not represent the stage of overt leukemia and may lack effects conferred by the microenvironment, we decided to validate our findings on the oncogenic effects *in vivo* by testing the leukemogenic potential of pools 2 and 3 in murine BM-Tx experiments. Following the same transduction strategy as in the *in vitro* setup, we transduced murine *Cas9* knockin LSK cells with the gRNA pool and the *Ezh2* gRNA or a non-targeting Luc control. 24 h after transduction, LSKs were transplanted into irradiated C57Bl/6 mice. Tracking of the cells by flow analysis of peripheral blood mononuclear cells (MNCs) allowed monitoring of leukemia induction (Fig. 6A and section 2.2.1.9., compare Heckl *et al.*, 2014). Comparable to the limiting dilution step in the *in vitro* setup, clonal evolution and selection of the most powerful mutational combinations did now take place in the murine bone marrow *in vivo* resembling a biologically more relevant situation.

Both gRNA combinations induced the development of leukemia robustly with a short latency (median survival of 10 weeks for pool 3) and full penetrance (Fig. 6B). Strikingly, the removal of the *Ezh2* gRNA from the gRNA pools significantly delayed the onset of leukemia from 11 to 13 weeks median survival in pool 2 and from 8 to 22 weeks median survival in pool 3. This effect was especially pronounced in pool 3 revealing a strong cooperating effect of the *Ezh2* loss with the other gRNAs included in pool 3. The presence of a fully developed leukemia in transplanted mice was confirmed by phenotyping in flow-cytometry (Fig. 6E), the detection of hematological infiltrates in histological staining of liver tissue (Fig. 6D) and secondary transplants that presented with a shorter survival (median survival between 5 and 9 weeks) than the primary counterparts (Fig. 6C). Fully established leukemias, used for secondary transplantations, did not lead to any significant differences in survival between *Ezh2* gRNA harboring and not harboring samples for pool 2 (Fig. 6C). In contrast to that, the genetic conditions of pool 3 led to a survival difference in secondary transplants indicating only a leukemia initiating role in pool 2 but an additional tumor promoting role in the context of pool 3.

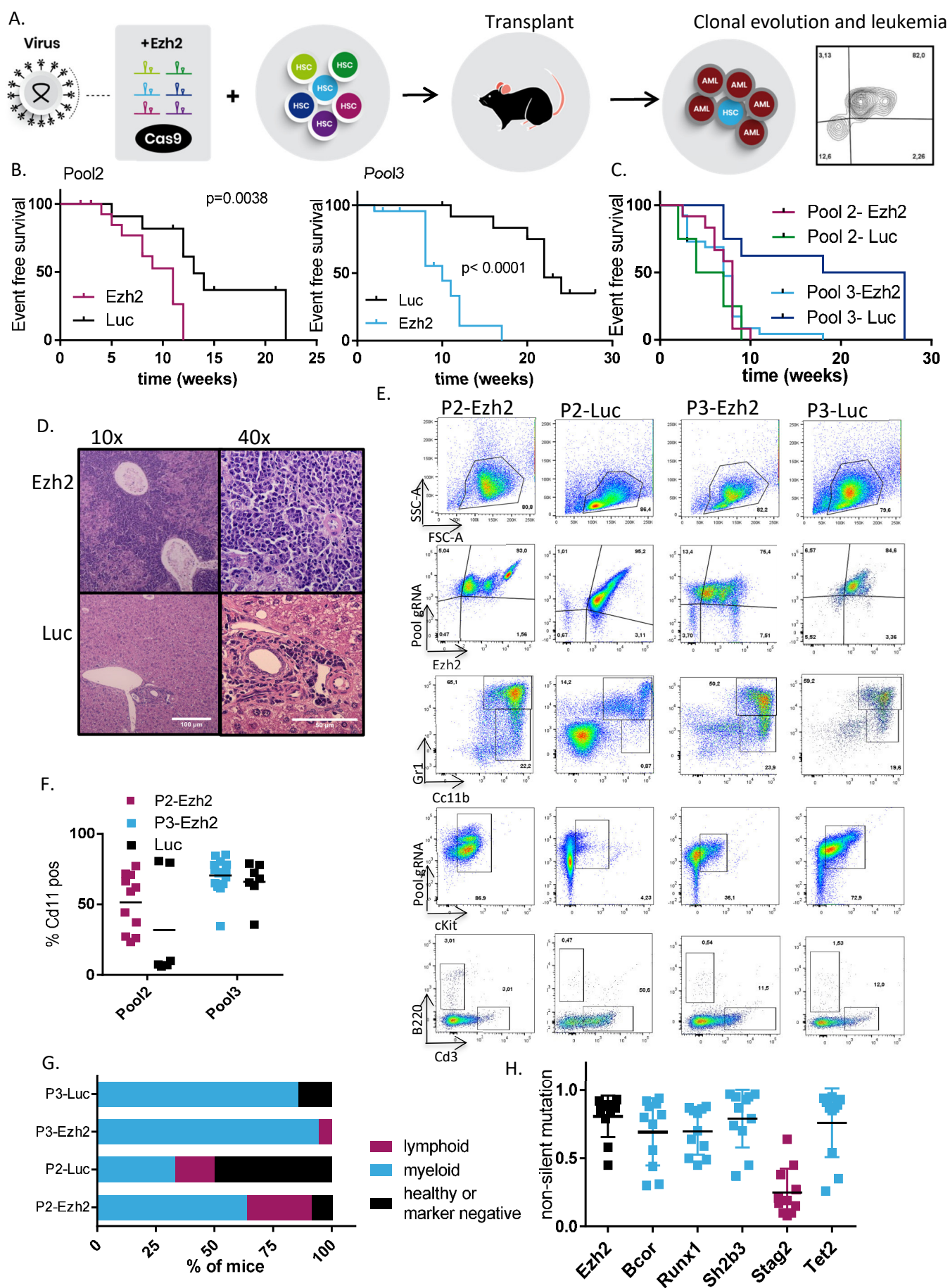


Figure 6. Loss of *Ezh2* enhances leukemogenicity of different mutational combinations. A. Experimental setup of the pooled CRISPR cooperation assay *in vivo*. Murine LSK cells are transduced with the gRNA pool, transplanted into irradiated C57Bl6 mice that were observed for the induction of leukemia. B. Kaplan-Meier survival curves of primary recipient mice showing leukemia free survival over time. Lines in black indicate Pool + Luc gRNA, colored lines indicate Pool + *Ezh2* gRNA, $n \geq 13$, Mantel-Cox. C. Kaplan-Meier survival curves for secondary transplants generated from the transplants in B, $n \geq 4$. D. Representative HE stainings of liver tissue from transplanted mice of indicated groups. 10x and a 40x magnification are shown. E. Representative dot plots from flow cytometry analysis of leukemic cells from the bone marrow of transplanted mice at the onset of disease. The axis represents SSC or FSC, reporter gene expression or immunophenotypic staining. F. Relative Cd11b expression in leukemic bone marrow cells of primary transplanted mice measured by flow cytometry after Cd11b-PECy7 staining. Every dot represents a mouse, black lines indicate the average. Black dots indicate pool + *Luc* gRNA, colored dots indicate pool + *Ezh2* gRNA. G. Phenotypic distribution of occurring leukemias based on myeloid and lymphoid marker expression measured in flow cytometry. Myeloid leukemia is defined by > 60% Cd11b pos cells, lymphoid leukemia is defined by > 50% B220 or Cd3e pos cells. H. Relative number of reads presenting non-silent mutations in NGS for all targeted loci in pool 3 + *Ezh2*. Each dot represents a mouse, av +/- SD. Black dots indicate *Ezh2* reads, blue dots highlight all genes where more than 60% of the mice present with more than 60% non-silent mutations and purple dots highlight all genes where more than 60% of the mice present with less than 30% non-silent mutations.

Flow cytometry analyses in Fig. 6E show representative bone marrow samples from leukemic mice of all experimental groups at the onset of disease. In all conditions, transgene expression of the gRNAs (determined by measuring E2Chrimson and dTom, cotransduced with the gRNA vector) reached almost 100% and the FSC/SSC blots demonstrate the accumulation of leukemic blasts (high FSC, low SSC signal) and granulocytic cells (high FSC, high SSC signal) except for Pool 2- *Luc* leukemia that harbored less granulocytic cells. This accumulation of myeloid cells was also seen in the staining for CD11b and Gr1 (granulocytes: Cd11b pos and Gr1 pos, monocytes Cd11b pos and Gr1 neg) (Fig 5E and F). Interestingly, mice lacking the *Ezh2* gRNA in pool2 did not show this pronounced myeloid effect as average Cd11b expression dropped from 51% to 32% (Fig. 6F). cKit expression, as a hallmark of leukemic blasts, was elevated in all gRNA conditions but was decreased when lacking the *Ezh2* gRNA in pool 2 samples. Due to the detection of the phenotypical differences between the leukemic cells in different mutational conditions, we distinguished myeloid and lymphoid leukemia based on the marker gene expression in our analysis (Cd11b pos >60% as myeloid, Cd3 and/or B220 >50% as lymphoid) (Fig. 6E and F). As a result, pool 3 induced an AML like disease in the majority of mice independent of the presence of the *Ezh2* gRNA. In contrast, pool 2 could induce myeloid and lymphoid leukemia, as well as a leukemic disease negative for all stained markers but shifting the balance towards the myeloid side after deletion of the *Ezh2* protein.

Overall, transplantation experiments demonstrated that gRNA combinations of pool 2 and 3 in combination with the *Ezh2* gRNA are indeed powerful to induce leukemogenesis confirming the findings of the *in vitro* setup (section 3.1.1.). However, in contrast to the *in vitro* results, an obvious difference between pool 2 and 3 is detectable since pool 3 triggers a greater and more significant difference after removing the *Ezh2* gRNA from the pool even in secondary Tx. Moreover, only pool 3 induces a myeloid phenotype on a reliable basis. As we were especially interested in *Ezh2* loss mediated effects in myeloid disorders, we decided to focus on mutations in genes included in the gRNA combinations of pool 3 for further analysis. Paralleling our approaches in the *in vitro* setup, not all included gRNAs need to be selected during clonal evolution in the mice. Thus, we sequenced all targeted loci in a NGS approach using whole bone marrow to detect all occurring mutations in the leukemic cells. Almost all of the analyzed mice presented with homozygous *Ezh2* mutations (Fig. 6H) underlining its importance for leukemogenesis. *Stag2* mutations seem to be dispensable as they occurred at a very low frequency (less than 30% non-silent mutations in more than 60% of the mice) while all other mutations in *Bcor*, *Runx1*, *Sh2b3* and *Tet2* were detected at comparable high frequencies (more than 60% non-silent mutations in more than 60% of the mice). These sequencing findings exactly resemble the *in vitro* results presenting all four candidate genes (*Bcor*, *Runx1*, *Sh2b3* and *Tet2*) as potential interactors of the *Ezh2* loss during leukemogenesis.

3.2.2. Reduced complexity screenings isolate LOF in *Ezh2*, *Bcor* and *Runx1* as major players in myeloid leukemia formation

In order to precisely map the mutations cooperating with the loss of *Ezh2* in AML, we performed small CRISPR-Cas9 cooperation screenings *in vitro* and *in vivo* that delivered an especially strong gRNA combination (Pool 3: *Sh2b3*, *Runx1*, *Stag2*, *Bcor* and *Tet2*) (section 3.1.1. and 3.2.1.). The targeted NGS approach both in *in vitro* and *in vivo* clones highlighted inactivating mutations of *Bcor*, *Runx1*, *Sh2b3* and *Tet2* as potential cooperators for the *Ezh2* loss guided induction of leukemogenesis. The absence of *Stag2* mutations proved their dispensability; in contrast the detection of the other mutations does not proof their driving potential as possibly being passenger mutations. As applying reduced complexity was already successful in *in vitro* studies, we further tested triple combinations of gRNAs in murine BM Tx. Firstly, we utilized the combination of *Ezh2*, *Bcor* and *Runx1* gRNAs, which already proofed to be potent in order to induce transformation *in vitro*. However, due to the full-pool results of our *in vivo* screening we also shuffled the third gRNA to *Tet2* or *Sh2b3* gRNAs, respectively: All triple gRNA combinations (*Ezh2*, *Bcor*, *Runx1* and *Ezh2*, *Bcor*, *Tet2* and *Ezh2*, *Bcor*, *Sh2b3*) induced leukemia with comparable penetrance but delayed onset of leukemia compared to the full pool 3 with a median survival of around 20 weeks (Fig. 7B) (Pool3-*Ezh2*: 10 weeks compared to *Ezh2*-*Bcor*-*Runx1*: 19 weeks; $p < 0.001$). As the non-targeting *Luc* control

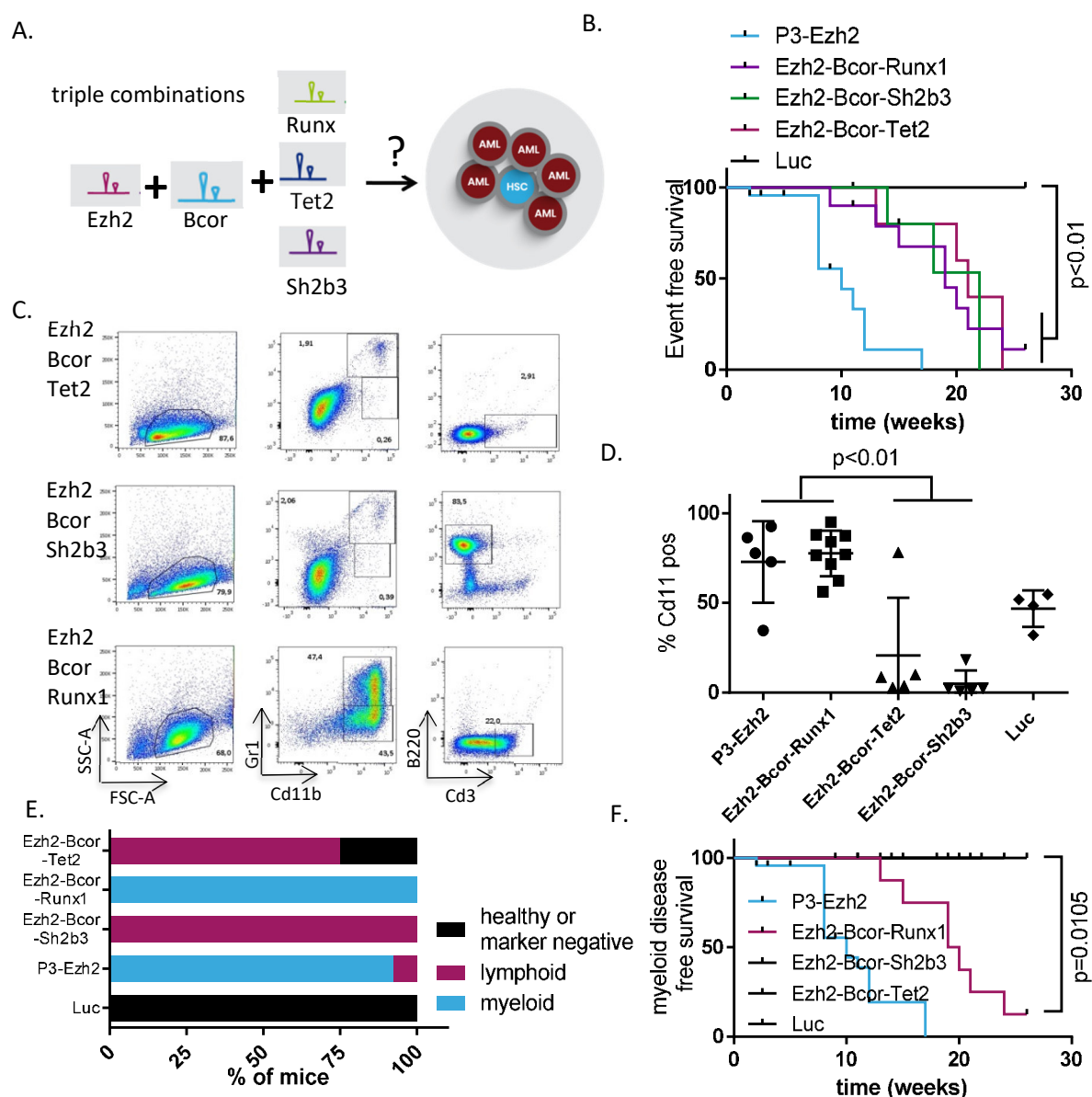


Figure 7. The triple combination of mutations in *Ezh2*, *Runx1* and *Bcor* induces myeloid leukemia in reduced complexity screenings *in vivo*. A. Experimental setup for reduced complexity screening *in vivo* using triple gRNA combinations of *Runx1*, *Tet2* or *Sh2b3*, respectively, in addition to *Ezh2* and *Bcor* for transduction into LSK cells and subsequent transplantation. B. Kaplan-Meier survival curves for primary transplanted BL6 mice showing disease free survival over time. Experimental groups as indicated, n≥5, Mantel cox (statistical comparisons to Luc control). C. Representative dot plots from flow cytometry analysis of leukemic cells from the bone marrow of transplanted mice at the onset of disease. The axis represents SSC and FSC or immunophenotypic staining. D. Relative Cd11b expression in leukemic bone marrow cells of primary transplanted mice measured by flow cytometry after Cd11b-PECy7 staining. Every dot represents a mouse, black lines indicate the average, n≥4, av +/- SD, Anova. E. Relative amount of mice, presenting with myeloid or lymphoid malignancies, analyzed based on myeloid and lymphoid marker expression measured in bone marrow by flow cytometry. Myeloid leukemia is defined by > 60% Cd11b pos cells, lymphoid leukemia is defined by > 50% B220 or Cd3e pos cells. F. Kaplan-Meier survival curves for primary transplanted BL6 mice showing myeloid disease free survival over time, n≥5, Mantel cox (statistical comparisons to Luc control).

alone did not cause any leukemic growth, it is still evident that leukemia occurred due to the introduced mutations of the triple combinations. Even though additional gRNAs from the full pool might foster leukemic aggressiveness, the interplay of the induced mutations was in all three genetic conditions powerful enough to induce leukemogenesis. Surprisingly, only the combined loss of *Ezh2*, *Bcor* and *Runx1* produced a myeloid disease as demonstrated by immune-phenotyping in flow cytometry (Fig. 7C and D). Detailed analysis revealed an expansion of the granulocytic and monocytic cell compartment (seen in FSC/ SSC and Cd11b/Gr1 blots in 6C) in the combination, harboring the *Runx1* gRNA, while in particular the combination with the *Sh2b3* gRNA presented with an expansion of the lymphoid compartment (B220 or Cd3e pos). Combinations with the *Tet2* gRNA presented either negative for all markers or positive for lymphoid markers (see Fig. 7C and D). In detail, the amount of myeloid cells was significantly increased from 5% and 21% in the triple combinations including the *Sh2b3* or *Tet2* gRNA to 72% or 77% in the *Runx1* gRNA containing samples (whole pool 3 and *Ezh2*, *Bcor*, *Runx1*). *Tet2* and *Sh2b3* gRNAs even induced a depletion of myeloid cells compared to the Luc control (Fig. 7D). Based on these findings, we reevaluated the survival curves for the occurrence of a myeloid disease as we intended to find collaborations of losses in *Ezh2* in this context (see Fig 6E and F). Only the triple combination of *Ezh2*, *Bcor* and *Runx1* gRNAs was able to recapitulate myeloid leukemia induction of pool 3 with a median survival of 19.5 weeks. Contrasting to that, all other combinations did not provoke the development of any myeloid malignancy. Thereby, our studies highlight the role of a *Runx1* loss as an important factor to shift the leukemic phenotype towards the myeloid side. The robust induction of myeloid leukemia as well as the capacity to transform LSKs *in vitro* (see section 3.1.2.) highlight the oncogenic cooperation of the genetic inactivation in *Ezh2*, *Bcor* and *Runx1*.

Next, we asked if the collaboration of all three mutations (*Ezh2*, *Bcor* and *Runx1*) is strictly required for the development of a myeloid malignancy or if even less mutations may be sufficient. Therefore, we further reduced the transduction complexity of the *in vivo* setup and transduced all possible double gRNA combinations targeting either two or one gene of the three (*Bcor*, *Runx1*, *Ezh2*) (Fig. 8A). Based on the prior findings of phenotypically significantly differing leukemias depending on their genetic configuration (Fig. 6C and E), we again categorized all occurring leukemias based on their marker expression in myeloid or lymphoid classes (Fig. 8B). Strikingly, in 80% or 100% of the animals, the gRNA combinations *Ezh2* and *Runx1* or *Bcor* and *Runx1*, respectively, induced the development of a myeloid leukemia while the double combination of *Ezh2* and *Bcor* gRNAs yielded lymphoid leukemia in 40% of the mice. Single mutations rarely caused a leukemic disease (one diseased mouse in the *Bcor*-Luc group). Survival curves, based on the appearance of myeloid diseases, illustrate that exclusively *Ezh2*^{-/-} *Runx1*^{-/-} and *Bcor*^{-/-} *Runx1*^{-/-} genotypes led to a robust induction of AML with full penetrance and a median survival of 21 weeks significantly differing from

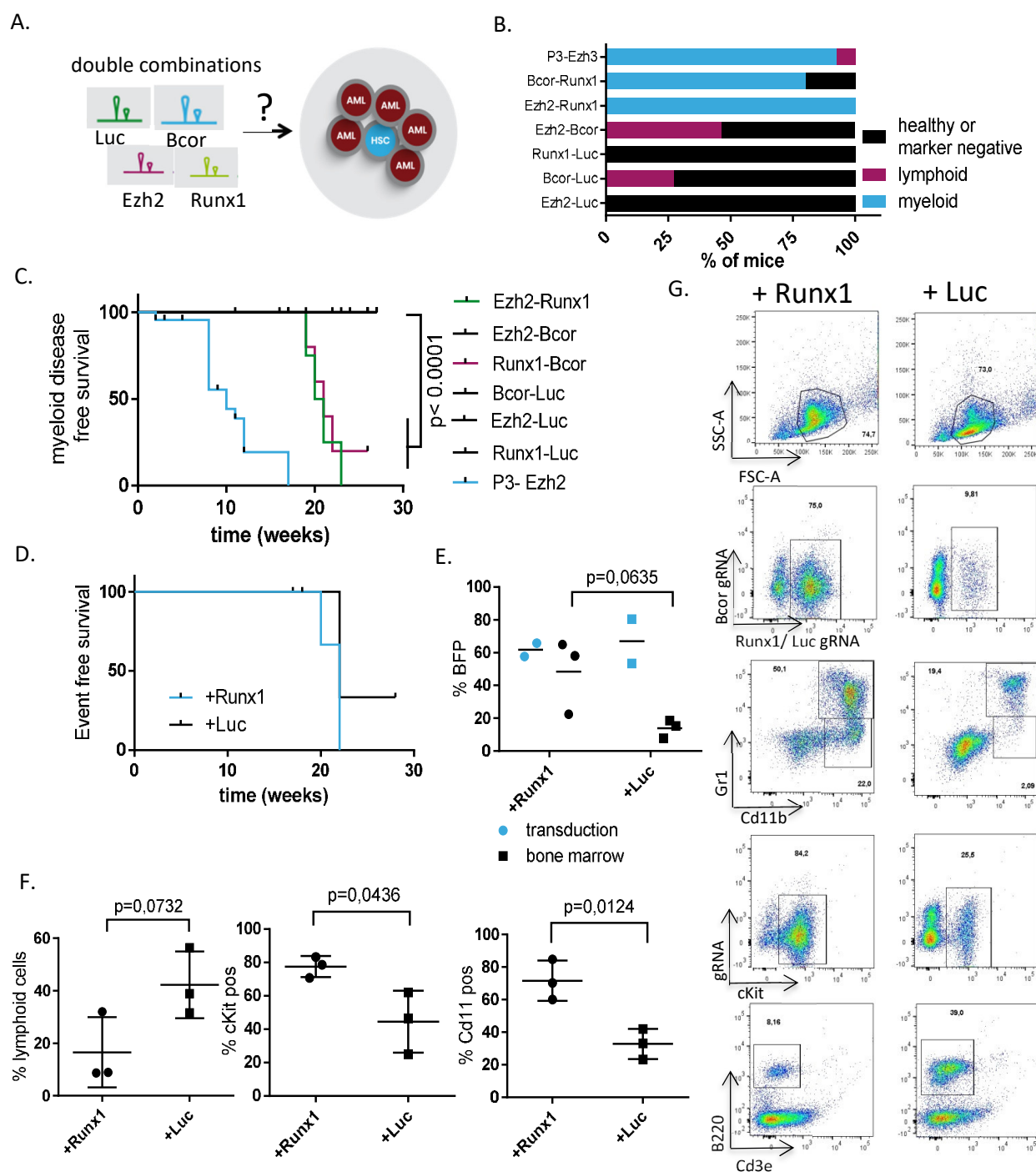


Figure 8. Cooperation of the *Ezh2* loss and the *Runx1* loss is sufficient to induce myeloid leukemia in mice.

A. Experimental setup for reduced complexity screening *in vivo* using double gRNA combinations of or single gRNAs against *Runx1*, *Ezh2*, *Bcor* for transduction into LSK cells and subsequent transplantation. B. Relative amount of mice presenting with myeloid or lymphoid malignancies analyzed based on myeloid and lymphoid marker expression measured in bone marrow by flow cytometry. Myeloid leukemia is defined by > 60% Gr1 pos cells, lymphoid leukemia is defined by > 50% B220 or Cd3e pos cells. C. Kaplan-Meier survival curves for primary transplanted BL6 mice showing myeloid disease free survival over time. Experimental groups as indicated, $n \geq 5$, Mantel cox (statistical comparisons to *Ezh2-Luc* control). D-G. Whole bone marrow from *Ezh2-Bcor* gRNA primary transplanted mice was transduced with a *Runx1* or *Luc* gRNA, respectively, and secondarily transplanted into irradiated BL6 mice. D. Kaplan-Meier survival curves showing leukemia free survival over time. Experimental groups as indicated, $n=3$. E. Relative amount of transduced cells at transduction and the onset of disease.

Every dot represents a mouse, black lines indicate the average, $n=3$, $av \pm SD$, t-test. F. Cd11b expression, B220/ Cd3e or cKit expression in bone marrow measured by flow cytometry after Cd11b- PECy7, B220-APC-Cy7, Cd3e-PE-Cy7 staining at the onset of disease. Every dot represents a mouse, black lines indicate the average, $n=3$, $av \pm SD$, t-test. G. Representative dot plots from flow cytometry analysis of leukemic cells. The axis represents SSC and FSC, reporter gene expression or immunophenotypic staining. Experimental groups as indicated.

single mutations and the *Ezh2*^{-/-} *Bcor*^{-/-} genotype. These results reveal the sufficiency of the cooperation between the *Ezh2* loss and *Runx1* mutations to induce myeloid leukemogenesis. The addition of a *Bcor* loss of function is obviously dispensable leading only to a minor decrease of leukemia onset latency (from 21 to 19 weeks median survival) (see Fig. 7F and 8C). Only the addition of more gRNAs of the complete pool 3 led to a notable earlier onset of leukemia with a median survival of ten weeks (compare section 3.2.1. and Fig. 8C). Of note, the cooperation of *Bcor* and *Runx1* mutations seems to exert a comparably strong interaction.

So far, all genetic combinations, harboring *Runx1* mutations, induced myeloid leukemia development, while all other combinations also favored lymphoid phenotypes. To further underline the importance of *Runx1* depletion in developing a myeloid phenotype we designed an experimental setup of lympho-myeloid leukemia fate switching: Mutational combinations (*Bcor-Ezh2*) with strong propensity for lymphoid bias and lack of transformation in primary BM-Tx (Fig. 8B) were subjected to secondary Tx and prior addition of a further *Runx1* or control gRNA. Leukemia onset presented with a long latency (22 weeks), and did not differ between the *Runx1* and the *Luc* gRNA group (Fig. 8D). However, in line with our hypothesis the leukemic phenotype differed: A significant expansion of the myeloid cell compartment (two times more Cd11b pos cells) and a depletion of the lymphoid cells (two times less B220 or CD3 pos cells) were observed in the *Runx1* transduced mice compared to the *Luc* control (see Fig. 8F and G). *Runx1* gRNA transduced cells also presented with a significantly higher amount of cKit positive cells demonstrating a more stem cell like phenotype. Moreover, reporter gene expression (cotransduced BFP gene) was increased from 17% in the *Luc* transduced cells to 42% in the *Runx1* transduced cells emphasizing the growth advantage conveyed by the *Runx1* mutation during myeloid malignant evolution (Fig. 8E). Overall, the *Runx1* loss seems to be necessary in the genetic context of *Ezh2* mutated leukemia in order to induce a myeloid leukemia development.

3.3. Loss of *Ezh2* mediates leukemogenesis by reactivating a fetal gene signature

3.3.1. The oncogenic effect of the loss of *Ezh2* is mediated by the overexpression of onco-fetal genes and restricted to adult hematopoiesis

In small CRISPR-Cas9 cooperation screenings we could demonstrate that the loss of *Ezh2* acts as a potent driver of leukemogenesis when combined with five gRNAs targeting *Runx1*, *Sh2b3*, *Tet2*, *Stag2* and *Bcor* (=Pool 3) (see Fig. 6). Replacing the *Ezh2* gRNA by a non-targeting control, we

provided compelling evidence for synergistic cooperation of the *Ezh2* deletion and the named driver mutations. However, the molecular mechanisms mediating these leukemic effects of *Ezh2* inactivation remained elusive so far. As *Ezh2* functions are highly diverse, the effects of the *Ezh2* loss may be influenced by the particular genetic configuration in our experiments. We therefore set out to analyze the molecular changes induced by the *Ezh2* inactivation in the genetic context of the mutations induced by pool 3. Being an epigenetic modifier, *Ezh2* acts as a regulator of transcription, and the interrogation of transcriptional changes was therefore the most obvious question. Our BM-Tx experiments, employing the Pool 3 with and without the *Ezh2* gRNA, provided rich cellular resources that displayed a strong biological effect of the *Ezh2* loss. Thus, the analysis of global gene expression by microarray and of the histone signatures by ChIP-Seq was promising. Murine whole bone marrow from leukemic primary transplants was FACS sorted for leukemic cells (marked by gRNA vector expression (dTomato and e2chromson)) (Fig. 6 and section 3.2.1.), and utilized for RNA preparation and subsequent cDNA microarray. Unsupervised clustering of the results revealed a striking difference in expression pattern of the two genetic conditions (Pool 3 +/- *Ezh2* gRNA) (Fig. 9A). In order to understand the altered molecular biology, we performed gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005) (Fig. 9B). The upregulation of a gene set from Wong and colleagues (Wong *et al.*, 2008), summarizing the expression programs in embryonic stem cells, was the first interesting finding. Subsequently, we generated more gene sets that enabled us to test in detail for the activation of hematopoietic fetal gene programs: Two gene sets were generated from gene expression data comparing murine fetal and adult HSCs populations (LT-HSC: Cd150 pos, Cd48 neg LSK; ST-HSC: Cd150 neg, Cd48 neg LSK) (conducted by Maurice Labuhn, unpublished) ("Upregulated in fetal HSC" and "Upregulated in FL vs BM"). These gene sets were clearly enriched in the *Ezh2* ko samples, indicated by a high NES (normalized enrichment score) of 1.86, demonstrating the activation of fetal programs in leukemic cells after *Ezh2* loss. Moreover, we generated two more gene sets from published data by Oshima and colleagues that compared gene expression in fetal liver LSKs and *Ezh2* ko LSKs ("Expressed in FL and EZH2ko"), and that defined *Ezh2* target genes expressed in fetal liver by ChIP-Seq analysis ("BM targets, expressed in FL") (Oshima *et al.*, 2016). The enrichment of these gene sets in the *Ezh2*^{-/-} samples was especially pronounced (NES=2.1) confirming the similarity of the *Ezh2* inactivation in adult cells and fetal hematopoietic cells that was already observed by Oshima *et al.*

After demonstrating the activation of fetal gene signatures after *Ezh2* deletion, we argued that transformation of fetal cells would not necessarily require loss of *Ezh2*. Fetal genes are activated in *Ezh2*^{-/-} adult cells, and potentially drive leukemogenesis, but are endogenously active in fetal cells. Consequently, the *Ezh2* loss would be dispensable in the latter. In order to test this hypothesis, we utilized a proliferation assay *in vitro* comparing the growth behavior of fetal and adult cells.

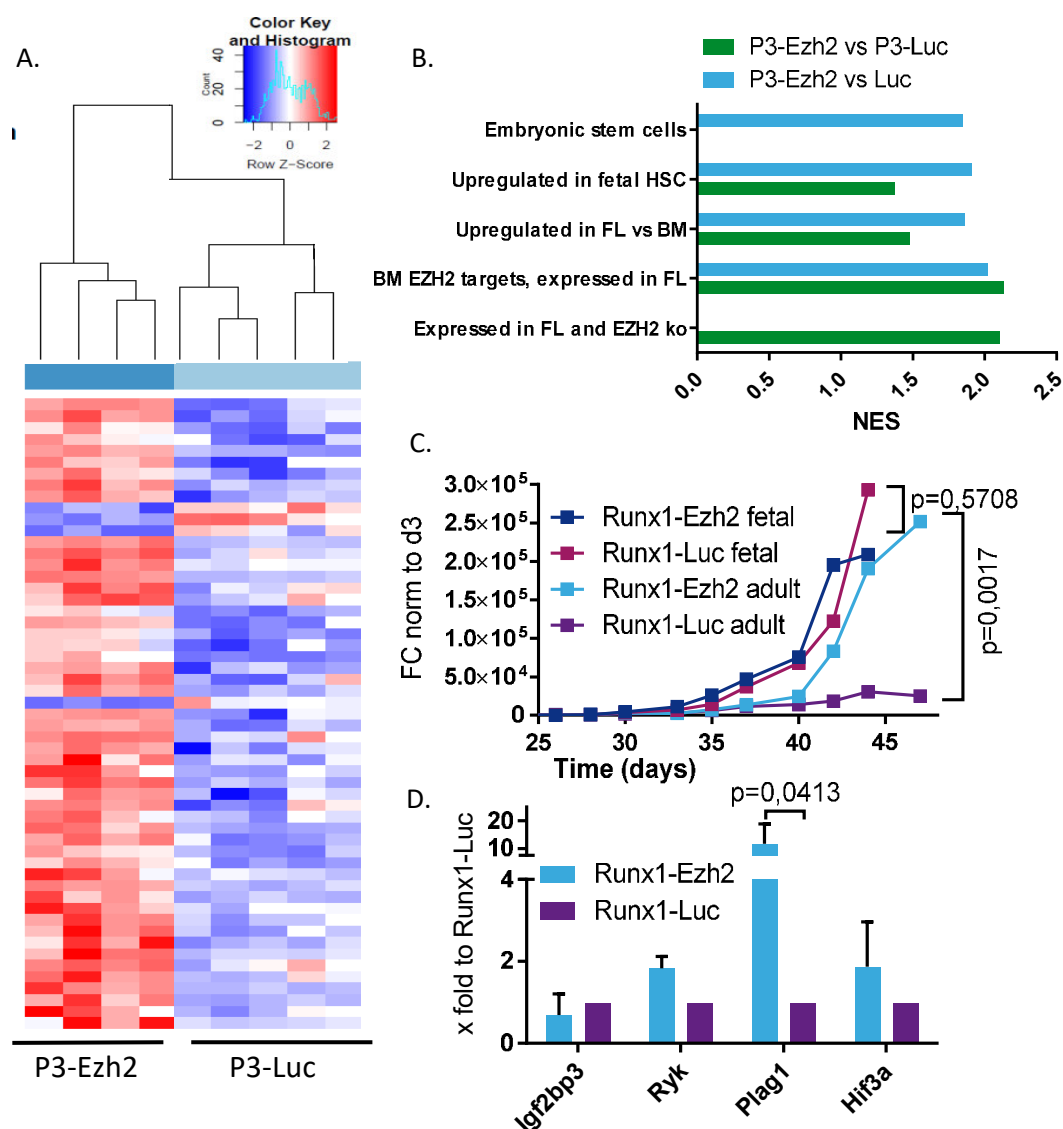


Figure 9. The *Ezh2* loss reactivates a fetal gene signature in adult cells. A. Heat map illustrating global gene expression measured by cDNA microarray comparing *Ezh2* mutated and non-mutated samples in conjunction with Pool 3 using whole bone marrow from primary transplanted mice, n=8. All genes being regulated with a logFC>1 and adjP<0.05 are illustrated. B. Gene set enrichment analysis summarizing data from A using P3-*Ezh2 ko*, P3-*Luc* leukemic and *Luc* transduced healthy bone marrow. Gene sets are indicated on the y-axis, NES on the x-axis, FDR<0.25. C. Growth curves from proliferation assays in liquid culture using adult LSK cells or Ter119 depleted fetal liver cells transduced with the *Runx1* gRNA and the *Ezh2* or *Luc* gRNA, respectively. The cell count of the reporter gene positive population (dTomato/E2Chrimson) normalized to day 3 is shown over time. d0 marks the time point of transduction. One representative experiment of two replicates is shown, t-test. D. Relative expression of *Igf2bp3*, *Ryk*, *Plag1* and *Hif3a* measured by RT-qPCR in adult cells from B. Ct values were normalized to *B2m* and to the *Runx1-Luc* control, n=2 (two technical replicates each), +/- SEM, Anova.

We focused on the simple genetic configuration of combined losses of *Ezh2* and *Runx1* that was demonstrated to be sufficient to induce transformation in adult cells *in vivo* (see Fig. 8 and section 3.2.2.). Therefore, we transduced Ter119 depleted fetal liver cells with gRNAs, targeting *Ezh2* and *Runx1* or *Runx1* alone (combined with a non-targeting *Luc* control), and measured their growth behavior in liquid culture by cell counting. Strikingly, both populations presented with a comparable proliferation, growing in a moderate manner first and noticeably increasing growth rates after transformation around day 33 (Fig. 9C). In contrast to that, adult LSK cells, transduced in the same manner, showed a similar growth behavior, only when harboring both mutations: the *Ezh2* and the *Runx1* loss. Adult cells, exclusively harboring the *Runx1* loss, did not transform *in vitro* and even stopped growing after 45 days thereby demonstrating a significantly decreased proliferation rate (Fig. 9C). The unaltered proliferation of fetal cells after removing the *Ezh2* gRNA proofs its dispensability for transformation in a fetal setup.

Overall, comparing fetal and adult transformation *in vitro* underlined the importance of the *Ezh2* loss to activate a fetal gene signature in adult but not in fetal *Runx1* loss cells.

3.3.2. Onco-fetal *Ryk*, *Plag1*, *Igf2bp3* and *Hif3a* are upregulated after *Ezh2* deletion in leukemia

The reactivation of fetal gene signatures in adult cells, leading to cancer development, is assumed to be a general oncogenic effect (Kim and Orkin, 2011). Many fetal genes have been demonstrated to be upregulated in tumors thereby driving cancerogenesis. So called onco-fetal genes are defined to be active in fetal development, mainly repressed in adult cells and reactivated during cancer formation (Ahrlund-Richter and Hendrix, 2014). Subsequently, we set out to explore the list of upregulated genes after loss of *Ezh2* in our transplantation setup for the occurrence of these onco-fetal genes. Four known onco-fetal genes were detected in the overlap of highly regulated genes in the Pool 3-*Ezh2* samples compared to Pool 3-*Luc* samples and to *Luc* only samples but were not found regulated in Pool3-*Luc* samples compared to *Luc* only samples: *Igf2bp3*, *Hif3a*, *Ryk* and *Plag1* (Fig. 10A). All four genes are described as important factors in fetal development as well as oncogenes (Halford *et al.*, 2000; Forristal *et al.*, 2010; Bell *et al.*, 2013; Hensen *et al.*, 2004). *Ryk* presented to be 6.5 times upregulated in *Ezh2*^{-/-} samples compared to *Luc* samples (adjP< 0.01) being the highest regulated gene. The upregulation of all indicated onco-fetal genes was validated in RT-qPCR using the same samples as for the microarray (Fig. 10B). Expression changes detected via qRT-PCR surpassed the cDNA microarray results with for instance *Ryk* being upregulated by 20 fold after *Ezh2* loss. To further validate the role of the onco-fetal candidate genes, we next tested gene expression in samples of our *in vitro* proliferation assay (see 3.3.1.), where we also found their upregulation after *Ezh2* deletion in the adult cells (Fig. 9D). Of note, *Igf2bp3*, *Hif3a* and *Ptk7* (like *Ryk*, a Wnt receptor) contributed to the leading edge subset of the GSEA for the gene sets comparing

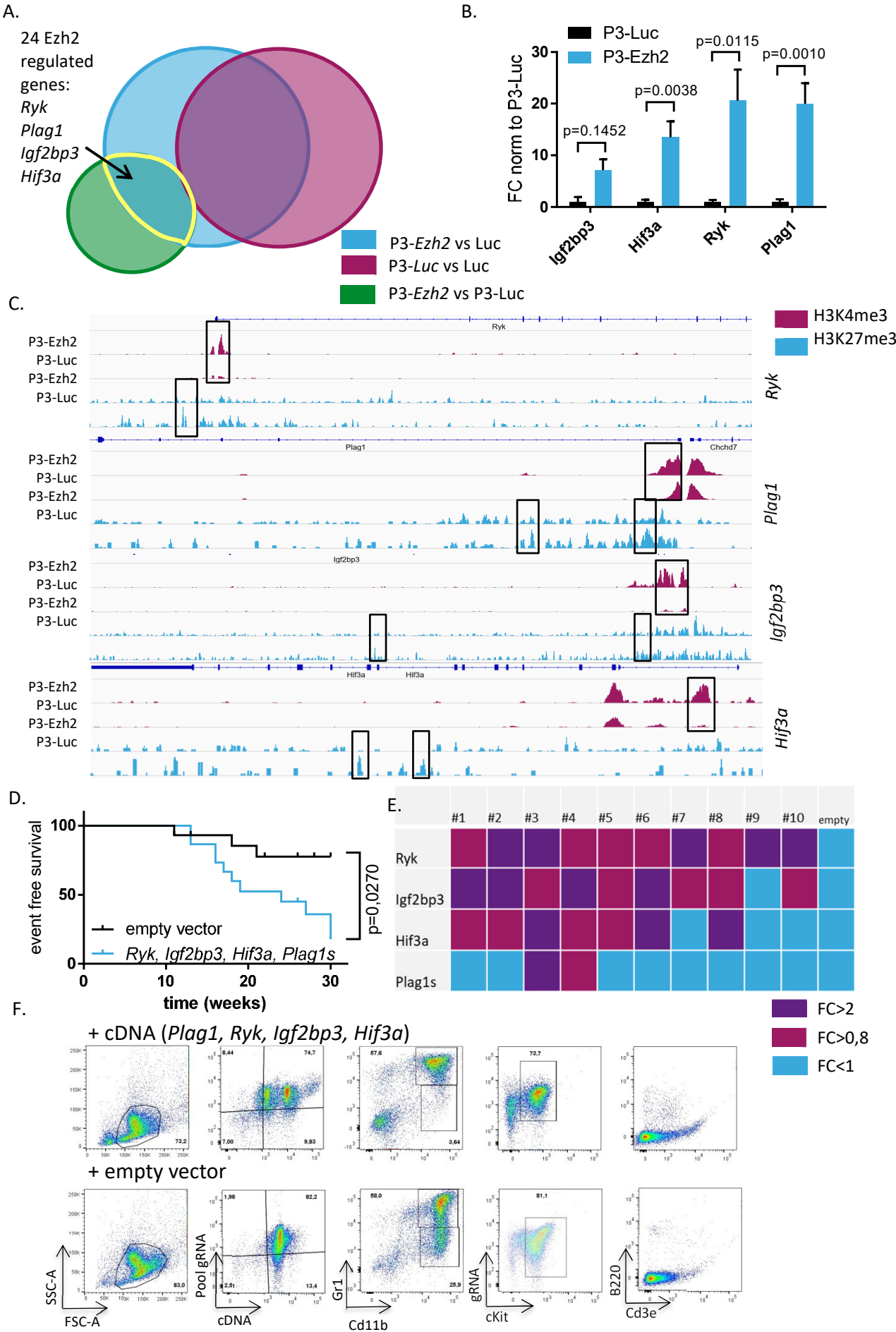


Figure 10. Activation of onco-fetal *Ryk*, *Plag1*, *Igf2bp3* and *Hif3a* after *Ezh2* loss enhances leukemogenesis.

A. Venn diagram illustrating global gene expression measured by cDNA microarray comparing samples transduced with Pool3-*Ezh2*, Pool3-*Luc* and only *Luc* using whole bone marrow from primary transplanted mice, n=4. All genes being regulated with a logFC>1 and adjP<0.05 are considered, onco-fetal genes are labeled. B. RT-qPCR on indicated genes utilizing murine leukemia cells transplanted with P3-*Ezh2* gRNA and P3-*Luc* gRNA, Ct values were normalized to *B2m* and Pool3-*Luc*, n≥4, +SEM, t-test. C. IGV-Genome browser screenshots of indicated genes illustrate ChIP-Seq data using indicated antibodies and whole bone marrow of secondary transplanted mice of pool 3-*Ezh2* and pool 3-*Luc* groups. Squares highlight differing peaks. D. Kaplan-Meier survival curves of primary recipient mice showing leukemia free survival over time. Lines in black indicate Pool 3 + empty vector, lines in blue indicate Pool 3 + cDNAs of *Plag1s*, *Igf2bp3*, *Hif3a* and *Ryk*, n≥10, Mantel Cox. E. Heat map resembling integration of overexpression vectors in gDNA from BM of transplanted mice from D measured by taqman qPCR assays. Colors code for the relative detection of a cDNA compared to HEL single clones (initial transduction <10%), blue indicates <1 fold integration, purple >1 fold, pink > 2 fold integration. Shown are all mice transplanted with the cDNA vectors and one example of the empty vector group. F. Representative dot plots from flow cytometry analysis of leukemic cells from the bone marrow of transplanted mice at the onset of disease. The axis represents SSC or FSC, reporter gene expression or immunophenotypic staining.

fetal and adult HSCs (Fig. 9A). The leading edge subset for the set of genes, expressed in fetal liver and *Ezh2* ko LSKs, also included *Ryk*, *Igf2bp3* and *Hif3a*.

In order to strengthen the data, gained by micro array, and to assemble a valid *Ezh2* loss signature in AML development, we complemented expression results with ChIP-Seq data for histone modifications by comparing samples from secondary BM Tx with and without *Ezh2* loss in combination of pool 3. Strikingly, the same onco-fetal genes that were regulated in the expression data also showed differential histone modification patterns between pool 3-*Ezh2* and pool 3-*Luc* samples (Fig. 10C). Genome browser tracks for the genes of *Ryk*, *Plag1*, *Igf2bp3* and *Hif3a* illustrate the expectable decrease of the *Ezh2* corresponding histone mark H3K27me3 after loss of *Ezh2* likely resulting in the activation of the genomic loci (Fig. 10C). For example the *Igf2bp3* gene presented with 16 H3K27me3 peaks in the peak calling of the *Luc* sample but with only nine peaks in the *Ezh2*^{-/-} samples. The activating histone mark H3K4me3 was upregulated in the pool3-*Ezh2* sample, indicated by a higher number and size of peaks, at the loci of *Ryk*, *Igf2bp3*, *Hif3a* and *Plag1* in the peak calling (Fig. 10C) also contributing to an activation of gene expression. The induction of activating histone marks after the removal of inactivating marks for example by the *Ezh2* loss is common at bivalent genes that are normally targeted by *Ezh2*. Overall, these ChIP-Seq results go in line with the expression data from the microarray emphasizing the relevance of the activation of fetal genes, especially of *Ryk*, *Hif3a*, *Plag1* and *Igf2bp3* for the oncogenic function of *Ezh2* deletions. Consequently, we hypothesized that the reactivation of fetal genes, especially of the four examined

candidates, executes the leukemogenic changes of the *Ezh2* loss in the interplay with further mutations. To prove this hypothesis, we set out to mimic the loss of *Ezh2* by replacing it with the overexpression of the four onco-fetal genes *Ryk*, *Igf2bp3*, *Hif3a* and *Plag1*. To this end, we performed BM-TX with the gRNA combinations of *Runx1*, *Bcor*, *Tet2*, *Sh2b3* and *Stag2* in addition to a pool of the four onco-fetal cDNAs (Fig. 10D). Integration of the cDNA vectors in the gDNA of transplanted cells was tested by taqman-assays, which were performed after disease onset using whole BM (see Fig. 10E). As a control, we used an empty expression vector construct combined with the same gRNA combination. The induction of leukemia was only observed in two out of ten mice transduced with pool3 (cotransduced with an E2Chrimson reporter gene on the same vector) and an empty vector control (cotransduced with a GFP reporter gene) (Fig. 10D). Most of the mice presented with healthy hematopoiesis as observed in flow cytometric analysis (Fig. 10F). Interestingly, leukemia was induced in a much more aggressive manner if pool 3 was combined to the onco-fetal cDNA pool (cotransduced with a GFP reporter gene) demonstrated by a higher penetrance (8 out of 10 mice) and a median survival of 23 weeks (Fig 9E). In most cases, leukemias presented with a strong myeloid phenotype in flow cytometry analysis and a high expression of gRNAs and cDNAs, indicated by a high reporter gene signal (E2Chrimson and GFP) (Fig. 10F). The much higher penetrance of leukemia in the mice harboring the cDNAs, hints towards a cooperational effect of the overexpression of onco-fetal genes and mutations induced by gRNAs in pool 3. Since initial transduction rates were low (~10%), we questioned if all cDNAs used in the pooled setup integrated in the gDNA of diseased mice. Possibly, only one or few used candidates (*Ryk*, *Igf2bp3*, *Hif3a* or *Plag1*) exert the leukemic function outcompeting the others during clonal evolution. The amount of vector integrations was measured by taqman qPCR assays normalizing the rate of detection to an initially low transduced (up to 5% transduction rate) HEL single clone (see Fig. 10E). Strikingly, the *Ryk* and the *Igf2bp3* cDNA was detected in all mice, transduced with the cDNA pool (only one exception for *Igf2bp3*), while the *Hif3a* cDNA was lacking in some (3 out of 10) and the *Plag1* cDNA in almost all mice (8 out of 10) (Fig. 10E). This result hints towards a proliferation advantage mediated by the combined overexpression of *Ryk* and *Igf2bp3*. As the *Hif3a* and the *Plag1s* cDNA was not identified in all of the samples, their overexpression might be dispensable for leukemic development. Overall, these transplantation experiments revealed that the overexpression of onco-fetal genes, especially of *Igf2bp3* and *Ryk*, cooperates with pool 3 gRNAs to induce leukemogenesis, and mimics the *Ezh2* loss effect.

3.3.3. *Ezh2*-*Runx1* loss induced leukemia distinguishes from *Bcor*-*Runx1* loss leukemia by the upregulation of onco-fetal *Ryk*, *Igf2bp3*, *Hif3a* and *Plag1*

By microarray analysis, the reactivation of fetal gene signatures, including known onco-fetal genes, was detected as potential driver of the oncogenic effect mediated by the loss of *Ezh2* when combined with gRNAs against *Sh2b3*, *Tet2*, *Stag2*, *Bcor* and *Runx1* (section 3.3.2.). Nevertheless,

reduced complexity screenings in murine transplantations had demonstrated that the loss of *Ezh2* does not require all five cooperating hits to induce leukemogenesis (section 3.2.2): The combination with the loss of *Runx1* was sufficient for the development of a myeloid malignancy. However, the combination of losses in *Runx1* loss and the *Bcor* also cooperated and seem to have a comparable potential to induce a myeloid disease. Consequently, we aimed to analyze molecular differences distinguishing oncogenic effects driven by the two mutational combinations. We hypothesized that the *Ezh2* loss mediated effect of transcriptional upregulation of a fetal gene signature (Fig. 9 and 10) also occurred after combination with the *Runx1* loss only but not in the *Runx1-Bcor* loss combination. Therefore, we performed global gene expression analysis by RNA-Seq utilizing whole bone marrow samples from mice transplanted with double gRNAs (against *Ezh2* and *Runx1*, Fig. 8A), and leukemic cells were FACS sorted for gRNA vector expression (marked by dTomato and E2Chrimson positive population). Analysis for differentially regulated genes clearly revealed a difference in gene expression between leukemic genotypes of *Ezh2*^{-/-} *Runx1*^{-/-} and *Bcor*^{-/-} *Runx1*^{-/-}, respectively (Fig. 11A). 1436 genes were detected to be significantly upregulated while 169 genes were detected to be significantly downregulated in *Ezh2* loss samples compared to *Bcor* loss samples. Strikingly, we found the same onco-fetal genes upregulated after *Ezh2* inactivation as seen in the whole pool samples

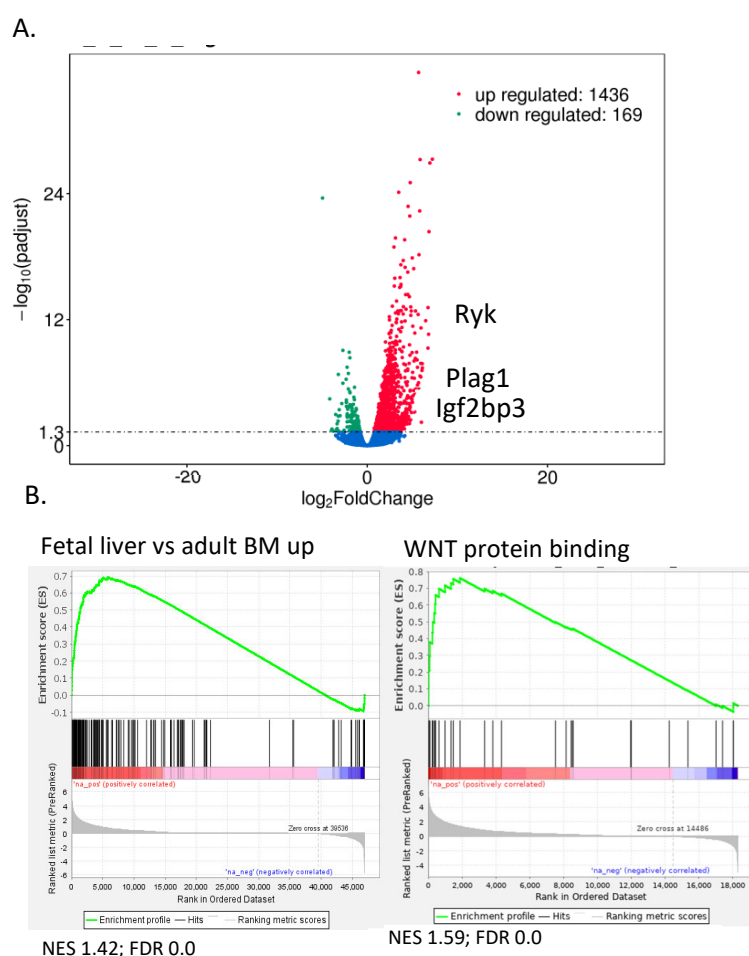


Figure 11. Upregulation of onco-fetal genes distinguishes *Ezh2-Runx1* ko leukemia from *Bcor-Runx1* ko leukemia.

A. Volcano blot illustrating global gene expression measured by RNA-Seq using whole bone marrow from primary transplanted mice comparing leukemias with losses in *Ezh2-Runx1* and losses in *Bcor-Runx1*, $n \geq 3$. Genes upregulated in *Ezh2-Runx1* leukemias are indicated by red dots, genes downregulated are indicated by green dots, $\text{adjP} < 0.05$: *Ryk*: $\text{adjP} = 1.41\text{E-}17$, $\log\text{FC} = 5.16$; *Igf2bp3*: $\text{adjP} = 0.02$, $\log\text{FC} = 2.03$, *Plag1*: $\text{adjP} = 0.009$, $\log\text{FC} = 3.59$. B. Gene set enrichment analysis using RNA-Seq data from A comparing samples of *Ezh2-Runx1* loss with *Bcor-Runx1* loss samples. Gene sets, NES and FDR are indicated.

(Fig. 10), represented by *Ryk* (logFC=5.16), *Plag1* (logFC=3.59) and *Igf2bp3* (logFC=2.03) (Fig. 11A). Only *Hif3a*, activated in the whole pool setup, was not found in the *Ezh2*^{-/-} *Runx1*^{-/-} samples. Also, the same gene sets of fetal gene signatures were enriched in GSEA of samples with inactivated *Ezh2* and *Runx1* compared to samples with inactivated *Bcor* and *Runx1* (see Fig. 11B), emphasizing their reactivation after *Ezh2* deletion during leukemogenesis. Moreover, the accumulation of Wnt pathway related gene sets in GSEA after *Ezh2* loss was striking, foremost the gene set “Wnt-protein binding” (generated from the gene ontology consortium (Ashburner *et al.*, 2000; The Gene Ontology Consortium, 2019)) with a normalized enrichment score of 1.59 (Fig. 11B). The Wnt signaling pathway is highly important in fetal development and hematopoiesis (Lento *et al.*, 2013; Ruiz-Herguido *et al.*, 2012) so that its upregulation after loss of *Ezh2* might be part of the reactivated fetal gene signature. Moreover, the highly regulated gene *Ryk* codes for a Wnt receptor. These expressional changes clearly demonstrate that the deletion of *Ezh2* in combination with the *Runx1* loss executes different oncogenic programs than the *Bcor* loss distinguishing the two types of leukemia.

3.3.4. The cooperation of deletions in *Ezh2* and *Runx1* induces a myeloid and stem cell like phenotype during leukemogenesis

Global gene expression analysis proofed reactivation of fetal gene signatures after loss of *Ezh2* in multiple experimental setups (Fig. 9-11). However, the molecular contribution of the *Runx1* deletion to leukemogenesis in the context of *Ezh2* inactivation remained elusive so far. These effects must be essential for the development of myeloid leukemia as the *Ezh2* loss alone does not induce any disease (see Fig. 8C). That is why, we set out to distinguish expressional changes mediated by the *Ezh2* loss or the *Runx1* loss only, and changes induced by the cooperation of the two mutations. Effects of collaboration would only be detectable in *Ezh2*^{-/-} *Runx1*^{-/-} samples but not in *Ezh2*^{-/-} *Runx1*^{+/-} or *Ezh2*^{+/-} *Runx1*^{-/-} healthy samples. Expression data from RNA-Seq analysis (see 3.3.3.) on these three groups from murine transplants (*Ezh2*^{-/-} *Runx1*^{-/-}, *Ezh2*^{-/-} *Runx1*^{+/-}, *Ezh2*^{+/-} *Runx1*^{-/-}; single mutated groups were always transduced with a Luc control) was therefore utilized to isolate specific effects by overlapping regulated genes from all possible comparisons which are as followed (Fig. 12A and B):

- i) Isolated effects of *Ezh2* inactivation: overlap of *Ezh2*^{-/-} *Runx1*^{-/-} vs *Ezh2*^{+/-} *Runx1*^{-/-}
and *Ezh2*^{-/-} *Runx1*^{+/-} vs. *Ezh2*^{+/-} *Runx1*^{-/-}
- ii) Isolated effects of *Runx1* inactivation: overlap of *Ezh2*^{-/-} *Runx1*^{-/-} vs *Ezh2*^{-/-} *Runx1*^{+/-}
and *Ezh2*^{+/-} *Runx1*^{-/-} vs. *Ezh2*^{-/-} *Runx1*^{+/-}
- iii) Combinatorial effects of *Ezh2*/*Runx1* inactivation: overlap of *Ezh2*^{-/-} *Runx1*^{-/-} vs
Ezh2^{+/-} *Runx1*^{-/-} and *Ezh2*^{-/-} *Runx1*^{-/-} vs *Ezh2*^{-/-} *Runx1*^{+/-}

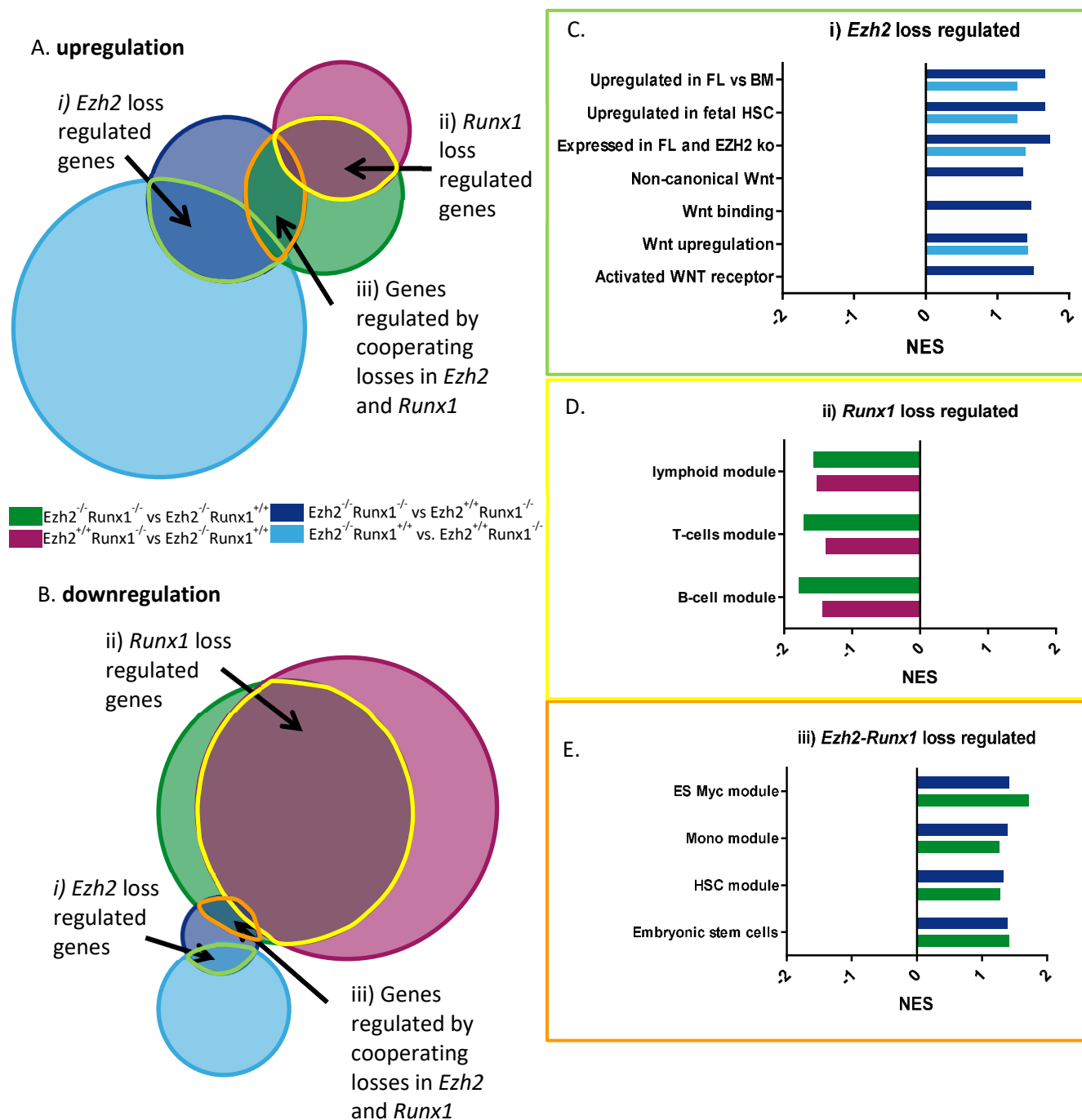


Figure 12. The *Ezh2* and the *Runx1* loss cooperate to induce a myeloid and stem cell like phenotype.

A and B. Venn diagrams illustrating the overlaps of differentially regulated genes measured by RNA-Seq using whole bone marrow from primary transplanted mice comparing *Ezh2*^{-/-}*Runx1*^{-/-}, *Ezh2*^{-/-}*Runx1*^{+/+}, *Ezh2*^{+/+}*Runx1*^{-/-} samples, single mutated groups were always transduced with a Luc non-targeting control, $n \geq 3$, $\log_{2}FC > 1$, $\text{adj}P < 0.05$. Circle sizes are relative to the number of regulated genes, filling colors indicate the comparisons, line colors highlight specific overlaps. A shows upregulated genes, B shows downregulated genes. C, D and E. Summary of gene set enrichment analysis using RNA-Seq data from A and B, gene sets are indicated at the y axis, NES is indicated at the x axis, $\text{FDR} < 0.25$. Bar colors correspond to circle filling color in A and B, colors of squared lines correspond to the highlighted overlaps.

The comparison of the single deletions (*Ezh2*^{-/-} *Runx1*^{+/+} vs. *Ezh2*^{+/+} *Runx1*^{-/-}) delivers transcriptional changes that are mediated by only one of the mutations. Additionally, *Ezh2* loss mediated effects in leukemic samples are identified by isolation of genes that were up- or down regulated after the addition of the *Ezh2* deletion to the single deletion in *Runx1* (*Ezh2*^{-/-} *Runx1*^{-/-} vs *Ezh2*^{+/+} *Runx1*^{-/-}). The overlap of these two comparisons comprises 243 up- and 40 downregulated genes (i) (Fig. 12A and B) highlighting the isolated effects of the *Ezh2* loss. As expected, GSEA revealed an activation of fetal genes and the Wnt signaling pathway in both comparisons (Fig. 12C) confirming our previous results on the *Ezh2* loss driven effects (compare Fig. 9 and 11). Thus, the detected *Ezh2* loss mediated effect complements the already described insights of *Ezh2* loss oncogenicity.

Next, we set out to identify *Runx1* loss mediated effects in leukemia by overlapping genes regulated after the *Runx1* deletion in *Ezh2*^{-/-} cells (*Ezh2*^{-/-} *Runx1*^{-/-} vs *Ezh2*^{-/-} *Runx1*^{+/+}) or in wildtype cells (*Ezh2*^{+/+} *Runx1*^{-/-} vs *Ezh2*^{-/-} *Runx1*^{+/+}) (ii): 1021 genes were downregulated and 158 genes were upregulated (Fig. 12 A and B). The high number of downregulated genes hints towards a mainly transcription activating role of *Runx1*. Strikingly, a strong downregulation of lymphoid genes of B- and T cell lineages was clearly associated with a loss in *Runx1* in GSEA (Fig. 12D). The gene set of human lymphoid genes published by (Novershtern *et al.*, 2011), for example, was enriched with a NES of -1.57 in *Ezh2*-*Runx1* ko samples compared to the *Ezh2* ko samples.

Interestingly, the detected specific *Ezh2* and *Runx1* loss driven effects (i and ii) almost do not overlap with each other. The finding, that only eight genes of the specific overlap for the *Ezh2* deletion (i) are also regulated by an additional *Runx1* loss (*Ezh2*^{-/-} *Runx1*^{-/-} vs *Ezh2*^{-/-} *Runx1*^{+/+}) and that only seven genes of the specific overlap for the *Runx1* deletion (ii) are also regulated by an additional *Ezh2* loss (*Ezh2*^{-/-} *Runx1*^{-/-} vs *Ezh2*^{+/+} *Runx1*^{-/-}), emphasizes the specificity of the two effects.

Most importantly, we aimed to isolate the effect of the cooperation of the combined *Ezh2* and *Runx1* loss: we overlapped the genes regulated only in *Ezh2*^{-/-} *Runx1*^{-/-} samples compared to both single knockouts and not overlapping with the comparison of the single knockouts (iii). This yielded 96 genes up- and 25 genes downregulated being specific for the combination and likely marking the cooperative and synergistic effects (Fig. 12A and B). Corresponding overlaps of GSEA results revealed enrichment of embryonic and adult stem cell gene sets as well as an upregulation of myeloid and monocyte related gene sets in both comparisons (Fig. 12E). Gene sets, defined by Chambers and colleagues, for HSCs and monocytes (Chambers *et al.*, 2007), are enriched with an NES above 1 in *Ezh2*^{-/-} *Runx1*^{-/-} samples compared to single knockout samples. Thus, the effect of *Runx1*-*Ezh2* loss collaborations seems to be the activation of myeloid and stem cell gene programs.

These insights in the activation of myeloid genes through the collaboration of deletions in *Ezh2* and *Runx1* and in the repression of lymphoid genes through the *Runx1* loss go in line with the finding of

an induction of a myeloid phenotype after the addition of the *Runx1* loss in murine transplants (Fig. 7 and 8 and section 3.1.2.).

Taken together, gene expression analysis in leukemic samples, harboring the limited number of two mutations (*Runx1* and *Ezh2*), demonstrated the combined effect of onco-fetal, myeloid and HSC gene activation and lymphoid gene inactivation driving *Ezh2-Runx1* mutated leukemogenesis.

4. Discussion

4.1. The mutational interplay of losses in *Ezh2* and *Runx1* in myeloid leukemia

The nature of *EZH2* mutations and deregulation during cancer development and in particular leukemogenesis is highly diverse (Nikoloski *et al.*, 2010; Grubach *et al.*, 2008), indicating a strong context dependency. Therefore, it remains challenging to unravel the cellular and genetic effects influenced by deregulation of *EZH2*. However, huge therapeutic options in exploiting changes in histone modification landscapes open a highly attractive research area on isolating *EZH2*-deregulation caused core signatures.

In myeloid leukemia, loss of *EZH2* function has been described frequently (Ernst *et al.*, 2010; Papaemmanuil *et al.*, 2016), whereas the genetic cooperation and molecular mechanisms executing the oncogenic effects remain elusive. Since the single *EZH2* inactivation is not sufficient to induce leukemia formation (Majewski *et al.*, 2010), it is obvious that additional mutations are needed. Early studies on genetic lesions cooperating with the *Ezh2* loss in myeloid malignancies focused on the interplay with *Tet2* and *Runx1* aberrations (Muto *et al.*, 2013; Sashida *et al.*, 2014) and rather suggested myelodysplastic phenotypes than acute leukemia induction. However, the ongoing extensive research on a whole mutational network around the loss of *EZH2* is crucial in order to develop novel therapeutic options interfering with genetic interactions. Consequently, we aimed in this study to decipher the genetic context in which the *Ezh2* loss functions in an oncogenic manner by cooperating with other specific loss-of-function mutations. Utilizing pooled CRISPR-Cas9 cooperation screenings *in vitro* and *in vivo*, we isolated a combination of gRNAs (*Bcor*, *Tet2*, *Sh3bp3*, *Runx1* and *Stag2*) that potentially exerted oncogenic functions when combined with the loss of *Ezh2* (Fig. 4 and 5, section 3.1.1 and 3.2.1.). By targeted sequencing in transformed *in vitro* derived clones and in leukemic cells we did not only validate the co-occurrence of *Bcor*, *Tet2*, *Sh2b3* and *Runx1* mutations with *Ezh2* deletions but also detected *Stag2* mutations to be dispensable for *Ezh2* loss guided leukemogenesis in murine models. In order to refine functionally cooperative driver mutations, reduced complexity screenings were performed: By combining only one or two gRNAs with the *Ezh2* gRNA, transformative and leukemogenic interplay of mutations in *Ezh2*, *Bcor* and *Runx1* was demonstrated. While this triple mutation induced leukemia of a myeloid phenotype, *Ezh2* loss combined with inactivating mutations in *Bcor* and *Sh2b3* or *Tet2* induced leukemia of a lymphoid phenotype (Fig. 7 and section 3.2.2.). Finally, we demonstrated *Ezh2* and *Runx1* double mutations to be sufficient to cause myeloid leukemia (Fig. 8 and section 3.2.2.).

RUNX1 is an important hematopoietic transcription factor being crucial for HSC development and maintenance by promoting HSC self-renewal (Ito, Bae and Chuang, 2015). Moreover, RUNX1 is needed for lymphopoiesis of B- and T-cell lineages (Guo *et al.*, 2020; Seo *et al.*, 2012) but is also needed for regulation of myeloid differentiation (Imperato *et al.*, 2015). Also, mutations in the

RUNX1 gene are frequently detected in AML patients (Gaidzik *et al.*, 2011; Gaidzik *et al.*, 2016). There are already some reports on the interplay and co-occurrence of *RUNX1* and *EZH2* mutations in myeloid disorders complementing our findings: In MDS, inactivating mutations of *RUNX1* are established to be associated with the loss of chromosome 7q where *EZH2* is located (Christiansen, Andersen and Pedersen-Bjergaard, 2004; Zhang *et al.*, 2017). A cooperation of *RUNX1* and *EZH2* aberrations is probable here as refined analyses revealed the minimal deleted region of 7q in MDS to be centered around the *EZH2* gene and subsequent LOH for *EZH2* is frequent (Hosono *et al.*, 2014; Jerez *et al.*, 2012). A strong correlation between *EZH2* and *RUNX1* mutations was further demonstrated by sequencing a large MDS cohort (Haferlach *et al.*, 2014) reflecting our results on their cooperation. As well in line with our finding on the mutational interaction between *Runx1* and *Bcor*, the same MDS study detected *RUNX1* aberrations to co-occur significantly with *BCOR* mutations (Fig 7). These discoveries in MDS are corroborated by sequencing studies in AML focusing either on *EZH2* or *RUNX1* mutated cases that repeatedly reported the significant detection of their co-occurrence (Stasik *et al.*, 2019; Gaidzik *et al.*, 2016): described combined mutations in *EZH2* and *RUNX1* in patients complement our detection of the sufficiency of this oncogenic combination to induce AML in our murine model underlining the clinical relevance of our findings and proofing a consequence of the interaction in human disease.

The recent study from Stasik and colleagues on the outcome of *EZH2* mutations in 1604 AML cases revealed besides the *EZH2*-*RUNX1* co-mutations the significant co-occurrence of *EZH2* inactivation with mutations in *NRAS*, *ASXL1* and *STAG2* (Stasik *et al.*, 2019). The recurrent combination of *EZH2* and *NRAS* mutations is in agreement of our previous finding that *Nf1*, a regulator of the RAS pathway was co-mutated in mice (Heckl *et al.*, 2014). PRC2 subunit *SUZ12* neighbors *NF1* on chromosome 17q and has been reported to be frequently commonly deleted with *NF1* facilitating formation of several solid cancers (De Raedt *et al.*, 2014). Moreover Gu *et al.* reported recently a synergy of *Ezh2* deletions and *RASG12D* mutants in a murine leukemia model (Gu *et al.*, 2019). However, in the present study only some *Nf1* mutations were detected in *in vitro* derived clones of pool 2 that were not significantly increased. Moreover, an *Asx1* gRNA was included in our *in vitro* screening but its loss was not detected to induce transformation contrasting the codetection in patients. Similar to *EZH2* mutations, *ASXL1* mutations are reported to reduce PRC2 activity so that their deletions might be redundant anyway (Abdel-Wahab *et al.*, 2012). The detected weak correlation for *EZH2* and *STAG2* mutations is surprising since we found *Stag2* mutations to be dispensable in our experimental setup. The absence of cohesion component mutations in murine models but its detection in human leukemia samples has already been reported in ML-DS (Labuhn *et al.*, 2019) and might be explained with different roles of the cohesion complex in mice and humans.

The study from Gaidzik and colleagues reported, besides *EZH2* inactivation, *BCOR* aberrations to co-occur with *RUNX1* mutations. In total *BCOR* mutations occurred in 11% of the *RUNX1* mutated patients (Gaidzik *et al.*, 2016) matching our observations on the interplay of losses in *Runx1* and *Bcor*. *BCOR* loss of function mutations were already established to be associated with *RUNX1* mutations in AML (Grossmann *et al.*, 2011), and were recently shown to be especially frequent in myeloid malignancies with *RUNX1* germline mutations following a familial platelet disorder (FPD-MM) (Brown *et al.*, 2020). Initially, we detected a high oncogenic potential of the triple combination of *Ezh2*, *Runx1* and *Bcor* although we could not verify an additive oncogenic effect of the *Bcor* loss (Fig. 5, 7 and 8). To this end, there are no clinical co-occurrences of *EZH2* and *BCOR* mutations described in AML and only one study detected two co-mutated cases in MDS corresponding to better prognosis (Badaat *et al.*, 2020). Our observations on healthy hematopoiesis after mutations in *Ezh2* and *Bcor* match these findings perfectly. If additional *Bcor* mutations in *Ezh2-Runx1* mutated leukemia has even stronger synergistic effects than the exclusive combination of losses in *Runx1* and *Ezh2* remains elusive in clinical leukemia formation.

Additional evidence for the cooperation of *EZH2* and *RUNX1* mutations in human leukemia comes from a study on recently detected *H3K27* mutations in AML, causing a global reduction in *H3K27me2/3* and thereby mimicking the *EZH2* loss. The association with aberrations in the *RUNX1* gene was significant in this context (Lehnertz *et al.*, 2017) again stressing the clinical relevance of our finding. All three described patients presented with co-occurring *RUNX1* mutations, one of them being a *RUNX1-RUNX1T1* translocation and two presenting as point mutations.

While some findings on genetic combination and cooperation of *EZH2* mutations in patients and in our murine model do not present with perfect overlap, our results still cover major findings from human cohorts. Future studies need to focus on humanized functional leukemia models to overcome the discrepancy of murine models and clinical findings.

Mouse models to study cooperating effects of the *Ezh2* loss are to this end very rare revealing the novelty of our approach and results. The *RUNX1* mutant *RUNX1S291fs*, that retains DNA binding but lacks transactivation like the human *RUNX1A* isoform (Watanabe-Okochi *et al.*, 2008), was already studied in combination with the *Ezh2* loss in mice causing MDS development (Sashida *et al.*, 2014). However, in this model the *Ezh2* loss prevented the mice from developing secondary AML, which is in contrast to our findings. As complete loss-of-function mutations were induced in our model (compare 3.1.1. and 3.2.1.), by utilizing a *Runx1* gRNA targeting the DNA binding domain, we might have studied a more drastic effect compared to the *RUNX1S291fs* mutant that enabled another effect of the *Ezh2* loss. Another functional report on the cooperation of the *Runx1* and the *Ezh2* loss comes from Booth and colleagues focusing on lympho-myeloid leukemia. Here, simultaneous introduction of *Ezh2* and *Runx1* homozygous deletions in early thymic progenitors (ETPs), that are

lymphoid progenitors with retained myeloid potential, promoted leukemia initiation (Booth *et al.*, 2018). The leukemia driving effect of the *Ezh2-Runx1* loss interplay seems to be identical to our observations even though differing phenotypes are produced. By targeting ETPs, leukemia with combined lymphoid and myeloid marker expression was produced by Booth *et al.* while we detected an enhanced expression of myeloid markers only by targeting LSKs. An explanation for this difference could be the difference between the target cell populations: ETPs might be already more biased towards the lymphoid side than LSK cells.

A genetic screening *in vitro* for cooperating genetic inhibitions of the *Runx1* loss in hematopoietic transformation identified the loss of *Pcgf1* as a major event (Ross *et al.*, 2012). *Pcgf1* is part of the PRC1.1 which also contains *Bcor* as an essential subunit. In line with these findings we identified a cooperation of *Runx1* and *Bcor* inactivation in murine leukemia formation. Noteworthy, the study by Ross and colleagues did not show any experiments *in vivo* so that our study significantly expanded the understanding on their mutational combination.

Additionally, knockdown of *Tet2* is already described to interact with the *Ezh2* loss in order to induce myelodysplastic disorders in mice (Muto *et al.*, 2013) but no full AML development was observed in this study. The addition of the *Tet2* gRNA to the *Ezh2-Bcor* gRNA combination led to a marker negative or lymphoid leukemic disease in our study (see Fig. 7). A reason for this phenotypic difference of *Tet2* mutated hematologic malignancies might be the additional *Bcor* mutation in our setup that might favor lymphoid programs (see Fig. 8B and E) which can only be reverted by an additional *Runx1* mutation but not by a *Tet2* loss.

However, we could demonstrate for the first time that the interplay between the *Runx1* and the *Ezh2* loss is sufficient to exert an oncogenic function to induce myeloid leukemia formation in mice. Furthermore, our multiplexed modeling enabled us to show that mutations in *Bcor*, *Tet2*, *Sh2b3*, or other genes can contribute to the acceleration leukemia development and may be required for successful clonal evolution in patients but are dispensable for basic transformation in murine models. Clinically, leukemia formation results from the occurrence of the combination of at least three mutations in a single initiating cell. Normally these genetic aberrations cover all mutational classes (proliferation, differentiation and epigenetics) (Ley *et al.*, 2013; Takahashi, 2011) so that our detected combination of *Runx1* and *Ezh2* aberrations lacks the third mutation deregulating growth signaling. However, growth factor affecting mutations are the supposedly least stable class of mutations in studies of *de novo* and relapsed AML, indicating a modulatory role with characteristics of interchangeability. Especially, our analysis of *Ezh2* loss driven effects revealed the activation of fetal gene sets (see 3.3.1. and further discussed in 4.2.) leading to a faster cycling of cells and thereby suggesting a dual role of the *Ezh2* loss in proliferation and epigenetics in AML. Even though we limited the number of initiating cells by using a pooled transduction strategy and mainly monoclonal

leukemia development was detected by flow analysis and NGS sequencing, clonal evolution in patients might still be in the need for a third driving mutations contrasting clonal evolution in our experimental setup.

In the mutational context of our findings, especially in combination with *Runx1* mutations, we provided compelling evidence for the requirement for an inactivated *Ezh2* in order to induce leukemogenesis. Nevertheless, *Ezh2* aberrations might play a different role in other genetic contexts explaining its varying functions in hematopoietic cancer development as a tumor suppressor or an oncogene. In *MLL-Af9* leukemia, for example, *EZH2* expression has essential functions as its inactivation disrupts leukemic proliferation (Tanaka *et al.*, 2012; Neff *et al.*, 2012). The *EZH2* activating mutation Y614F interacts with *BCL6* overexpression and p53 inactivation in lymphoma development (Souroullas *et al.*, 2016). In the context of *Flt3*-IDT mutation and p53 inactivation the *Ezh2* loss did not show a tumor driving but a chemo resistance mediating effect (Göllner *et al.*, 2017) revealing different functions for *EZH2* in different mutational situations.

The complementation of biological findings in mouse models and sequencing data in patients is a reasonable approach but direct translation remains challenging. Elucidating the role of a combined *EZH2-RUNX1* loss in patients seems to be inevitable based on our findings in order to determine optimal therapy for these cases. Identification of the presence of *RUNX1* and *EZH2* mutations in different disease stages, clonal analysis on the order of their occurrence and the identification of additional mutations in clinical samples might be helpful here. Rescue experiments in double mutated (*Ezh2* and *Runx1* loss) AML samples or knockdown experiments in single mutated (only *Runx1* or *Ezh2* loss plus additional mutations) AML samples in PDX models might be crucial to understand the effect of this mutational combination in humans.

4.2. The reactivation of onco-fetal genes guides *Ezh2*-loss leukemogenesis

EZH2 is an epigenetic writer and influences histone modifications throughout the genome so that the effect of its loss will result in multifaceted and varying effects depending on the cellular context and additional genetic aberrations. Therefore, we aimed to understand the molecular mechanisms governing leukemogenesis in the specific cellular and genetic setup of our study, which is clinically relevant and precisely defined (see chapter 4.1). The changes in gene expression, induced by loss of *EZH2*, leading to the transformation of HSCs appear to be especially important. Only the understanding of the complex molecular changes in specific genetic situations will allow the development of individual therapies counteracting these oncogenic alterations. That is why, we studied gene expression and histone modification profiles (H3K27me3 and H3K4me3) after *Ezh2* loss in the combination of the whole pool 3 (*Bcor*, *Runx1*, *Sh2bp3*, *Stag2*, *Tet2*) as well as in the well-defined genetic situation of only *Ezh2* and *Runx1* mutated leukemia. By comparing murine leukemias, harboring only mutations induced by pool 3, to leukemias, additionally harboring an *Ezh2* loss, we

isolated specific expression changes mediated by mutations in *Ezh2*. One major finding was the activation of fetal gene signatures (Fig. 9 and section 3.3.) including the upregulation of four well-known onco-fetal genes *Plag1*, *Igf2bp3*, *Hif3a* and *Ryk* (Halford *et al.*, 2000; Forristal *et al.*, 2010; Bell *et al.*, 2013; Hensen *et al.*, 2004) (Fig. 9A). Activation of *Plag1*, *Igf2bp3* and *Ryk* was also found in the case of combined *Ezh2* and *Runx1* losses only but presented to be exclusively driven by the *Ezh2* loss (Fig. 10 and 11) suggesting them to be direct *Ezh2* target genes. All four genes are *bona fide* onco-fetal proteins as being mainly expressed during fetal development and operating as oncogenes when reactivated in adult cells (Ahrlund-Richter and Hendrix, 2014). The oncogenic function of the upregulation of these four genes was validated in our study by the overexpression in murine transplants leading to an enhanced leukemic development in combination with pool 3 (Fig. 9E).

Plag1 is a developmentally regulated zinc-finger transcription factor showing high overall fetal expression that drops after birth (Hensen *et al.*, 2004). *Plag1* ko mice present with a growth retardation and reduced fertility (Hensen *et al.*, 2004). Initially, *PLAG1* was discovered as an oncogene in pleomorphic adenoma being consistently rearranged (Kas *et al.*, 1997). In AML, *Plag1* overexpression is described to cooperate with the *Cbfb-MYH11* fusion gene in mice and an increased *PLAG1* expression was detected in 20% of human AML samples (Landrette *et al.*, 2005). *Plag1* mainly exerts its oncogenic function by activating the transcription of the *Igf2* gene thereby increasing Igf2 growth signaling which is itself implemented in embryogenesis and carcinogenesis (Voz *et al.*, 2000). Only recently, a fetal regulation pathway between HMGA, *PLAG1* and IGF2 has been established (Abi Habib *et al.*, 2018). In a recent study Basheer *et al.* already demonstrated *Plag1* as a target gene of *Ezh2* being upregulated upon *Ezh2* inactivation leading to AML formation (Basheer *et al.*, 2019). These results are identical to our findings in microarrays and ChIP-Seq analysis (Fig. 9). However, our murine Tx experiments revealed that the *Plag1* overexpression is not necessarily needed in the presence of additional onco-fetal gene activation in order to induce leukemia formation (Fig. 9E). Noteworthy, Basheer and colleagues utilized retroviral overexpression models of the fusion oncogenes *RUNX1-RUNX1T1* and *MLL-AF9* on HSPCs with an already deleted *Ezh2* gene. In contrast to our study, the *Ezh2* loss was not tested for the transformation of HSCs but rather for the acceleration of leukemia onset, since both tested oncogenes were able to elicit overt leukemia alone. A second onco-fetal candidate in our study was *Igf2bp3*, further stressing the importance of the Igf2 signaling pathway in *Ezh2* loss leukemogenesis, since *Igf2bp3* regulates *Igf2* activity by binding its mRNA and promoting its synthesis (Dai *et al.*, 2011). *De novo* synthesis or severe upregulation of *IGF2BP3* has been described for various cancer types although its expression is normally restricted to embryogenesis (Lederer *et al.*, 2014; Nielsen *et al.*, 1999). Most of its other target mRNAs, including *MYC* and *CDK6* presented with onco-fetal and tumor driving characteristics (Viswanathan *et al.*, 2009; Palanichamy *et al.*, 2016). In hematopoiesis, *IGF2BP3* has been demonstrated to interact with the

lin28/let7c pathway (Wang *et al.*, 2019a) a network between microRNAs and RNA binding proteins playing a central role in regulating fetal hematopoiesis and promoting transformation (Copley *et al.*, 2013). *Ezh2* has been found to co-repress *Igf2bp3* together with *let7c* to maintain adult cell fate (Oshima *et al.*, 2016). Oshima *et al.* detected an upregulation of expression and a downregulation of H3K27me3 signatures at the *Igf2bp3* gene in fetal cells, as well as after *Ezh2* loss in adult LSK cells matching our finding in murine AML after *Ezh2* deletion. Beside the Wnt receptor *Ryk*, *Igf2bp3* was the only cDNA detected in all murine leukemia when mimicking the *Ezh2* loss effect by their overexpression stressing the importance of *Ryk* and *Igf2bp3* in mediating oncogenic effects of the *Ezh2* inactivation.

The pseudokinase *Ryk* is mainly activated by Wnt5a being mostly associated with non-canonical Wnt signaling (Green, Nusse and van Amerongen, 2014). Keeping HSCs in a quiescent state to enhance repopulation capacity is an important function of *Ryk* in hematopoiesis (Povinelli and Nemeth, 2014) explaining its detected upregulation in leukemia (Alvarez-Zavala *et al.*, 2016). The Wnt pathway in general has been connected to fetal HSC development (Richter, Traver and Willert, 2017) and to leukemogenesis since being upregulated by fusion genes in AML for example (Müller-Tidow *et al.*, 2004). The overexpression of *PLAG1* in pleiomorphic adenomas has also been shown to activate Wnt signaling by upregulating β -catenin expression (Zhao *et al.*, 2006). Consequently, the upregulation of *Ryk* and *Plag1* after *Ezh2* loss in our experimental setup hints towards an activation of the Wnt pathway supporting AML development. The upregulation of the Wnt pathway was validated as an *Ezh2* loss effect in GSEA utilizing expression data from the leukemic samples harboring *Ezh2* and *Runx1* mutations (Fig. 11C). Additionally, overactivation of the Wnt pathway after PRC2 inhibition has been recently shown to deplete cancer stem cells in multiple myeloma (Nakagawa *et al.*, 2019). The correlation of Wnt activation and *EZH2* inactivation in this study goes in line with our finding although we could detect a cancer promoting and not depleting effect, maybe due to exclusive targeting of *Ezh2* (instead of joint *EZH1* and 2 inhibition) in our model.

The fourth upregulated onco-fetal protein after *Ezh2* loss is *Hif3a*. Hypoxia inducible factors (HIFs) are transcription factors activated by hypoxic conditions promoting the expression of target genes that are involved in erythropoiesis, apoptosis and proliferation. Low oxygen conditions are mainly found in embryogenesis so that *Hif* functions are of special importance in the fetus (Dunwoodie, 2009). The overexpression of *HIFs* has been demonstrated to push cancer cells towards a more stem cell like phenotype (Heddleston *et al.*, 2009) and has been detected in AML (Wang *et al.*, 2011; Deeb *et al.*, 2011). *HIF3a* has not been studied much in specific but has been proven to be implemented in erythropoiesis (Novershtern *et al.*, 2011) so that our study is the first one to establish *Hif3a* in leukemogenesis and as a potential *Ezh2* target gene. Only *Hif1a* has been recently described to be

repressed by *Ezh2* in coordination with the lncRNA *HITT* leading to disturbed hypoxic adaptation (Wang *et al.*, 2019b).

Overall, we established a set of four onco-fetal proteins functioning as oncogenes since inducing leukemia upon overexpression in cooperation with additional mutations. Their overexpression is detected after *Ezh2* loss and has the potential to replace it (Fig. 9E). Taking the lower initial transduction rates that go along with the usage of large cDNA vectors, compared to gRNA vectors, into account, the kinetics of leukemia development after overexpression of the four onco-fetal genes resembled the kinetics after *Ezh2* loss (see Fig. 5B and 9D). The slightly more moderate effect in leukemic induction compared to an actual *Ezh2* deletion might also be explained by only overexpressing four candidate genes while *Ezh2* represses a whole signature of fetal genes. If these four genes are direct *Ezh2* target genes, or if they are secondly regulated for example by each other, cannot be sufficiently clarified here but reduced H3K27me3 levels (Fig. 9C) hint towards direct targeting by *Ezh2*. Additional ChIP experiments against *Ezh2* would help to understand its role in regulation of *Plag1*, *Igf2bp3*, *Hif3a* and *Ryk*.

As mentioned before, the four presented onco-fetal genes are only part of the reactivation of a whole fetal signature after *Ezh2* loss. Expression analysis of the *Ezh2* loss only and in the context of the whole pool 3 (*Bcor*, *Tet2*, *Runx1*, *Sh2bp3* and *Stag2*) or *Runx1* inactivation clearly revealed that this effect is exclusively due to the *Ezh2* loss, and not due to the mutational cooperation of *Ezh2* and *Runx1*. Oshima and colleagues already demonstrated not only the regulation of *Igf2bp3* but the regulation of a huge set of onco-fetal genes by *Ezh2* and *let7c* (Oshima *et al.*, 2016) in non-malignant cells. *Let7* was already described to target a number of onco-fetal genes (Boyerinas *et al.*, 2008). Consequently, Oshima *et al.* proposed the upregulation of fetal genes as a general oncogenic mechanism of the *Ezh2* loss (Oshima *et al.*, 2016) by providing a proliferation advantage (Ahrlund-Richter and Hendrix, 2014). However, their study did not show any functional experiments and their

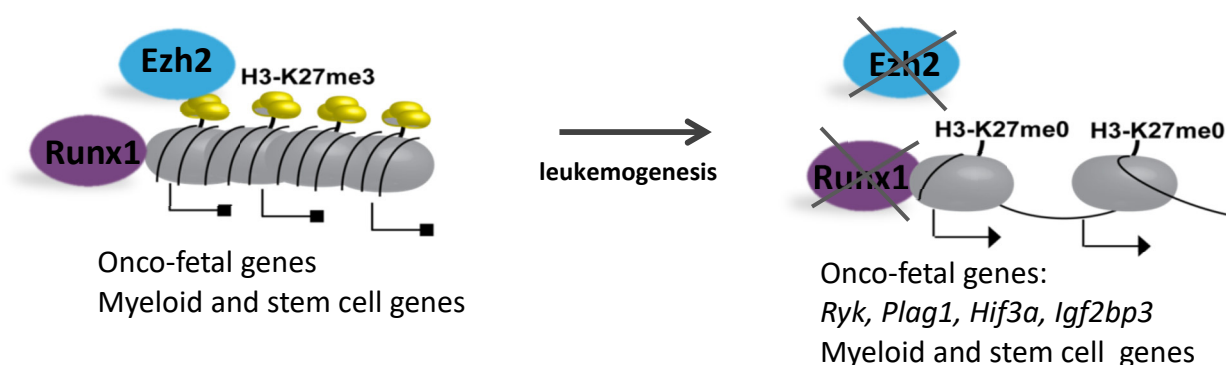


Figure 13. Graphical summary. *Ezh2* and *Runx1* target onco-fetal, myeloid and stem cell genes to repress their transcription. Upon loss of *Ezh2* and *Runx1* onco-fetal, myeloid and stem cell genes are activated and lead to the development of a myeloid malignancy.

conclusions were solely derived from healthy cells thereby disregarding the possibility that these effects may not be transferred to the malignant setting. By comparing the transforming effect of the *Ezh2* loss in fetal and adults cells and by replacing the *Ezh2* loss by onco-fetal overexpression we thus clarified this oncogenic mechanism of the *Ezh2* inactivation in AML. Future experiments may aim for overexpression of larger cDNA libraries concentrating on more potential onco-fetal genes. Focused CRISPR-Cas9 screenings in established *Ezh2*^{-/-} *Runx1*^{-/-} leukemias, targeting all potential onco-fetal candidate genes, may also be a promising approach to isolate the disease driving factors.

The involvement of EZH2 in the repression of fetal genes during differentiation and embryogenesis and their reactivation during cancer development is discussed intensively at the moment (Zaidi *et al.*, 2017). EZH2 is needed to establish bivalent promoter marks on the one hand to silence lineage specific genes in undifferentiated cells and to repress fetal and stemness genes on the other hand at the onset of differentiation (Mikkelsen *et al.*, 2007). Bivalent promoter marks at stemness and proliferation genes are replaced by DNA methylation in fully differentiated and developed cells in the adult (Mohn *et al.*, 2008; Mikkelsen *et al.*, 2007). These two kind of opposing target genes may explain the dual role of EZH2 in leukemogenesis depending on the cellular and genetic contexts. Onco-fetal genes are inactivated by DNA methylation and repressive chromatin marks in the adult but become demethylated and poised at the onset of cancer priming them for reactivation (Zaidi *et al.*, 2017). For instance, a bivalent promoter landscape similar to the one in embryonic stem cells has been detected in a breast cancer cell line (Messier *et al.*, 2016). Consequently, these genes will undergo a massive activation of expression upon the loss of *EZH2* and the repressive H3K27me3 marks. These findings and considerations are reflected by our data as we also found the activation of embryonic stem cell genes (Fig. 8B) and the establishment of the activating part of bivalent promoter marks (H3K4me3) after *EZH2* loss and leukemia initiation (Fig. 9C). This model would suggest the *EZH2* loss to occur after the establishment of a pre-leukemic state as a secondary mutation, which was not studied here, but would suggest to perform comparative studies in the future where *EZH2* mutations are introduced early or late in the study design (e.g. during secondary Tx).

Overall, these reports and our data clearly reveal the activation of genes, implicated in fetal development, after *Ezh2* loss during cancer formation. Therefore, we hypothesized that the deletion of *Ezh2* is redundant in fetal transformation as embryonic genes are already active and intrinsically cycle faster. This hypothesis was supported by high expression of our onco-fetal candidate genes in murine fetal liver HSPCs compared to adult HSPCs. By comparing fetal and adult transformation *in vitro*, we demonstrated that the *Ezh2* loss indeed only adds transformative potential in adult cells (Fig. 8 and section 3.3.1.). Only fetal hematopoiesis was demonstrated to depend on *Ezh2* with its loss resulting in a disruption of hematopoiesis. Probably, the *Ezh2* inactivation can be partly compensated by *Ezh1* in the adult suggesting that the *Ezh2* loss cannot lead to hyperproliferation and

cancer formation in the fetus (Mochizuki-Kashio *et al.*, 2011). Nevertheless, *EZH2* mutations are described for pediatric myeloid cancers such as JMML and MLDS (Caye *et al.*, 2015; Yoshida *et al.*, 2013; Labuhn *et al.*, 2019) which are believed to already originate in the fetus. Still, the *EZH2* loss might only occur after fetal development in the neonate or in a juvenile stage leading to the maintenance of fetal transcriptional programs that are normally silenced by *EZH2* after birth. In order to clarify the role of *Ezh2* in fetal, juvenile, adult hematopoiesis and leukemia formation, ChIP-Seq and expression analysis in patient samples, and in developmental stage-specific mouse models, would be helpful and irreplaceable. Studies on the formation of bivalent promoter marks in embryogenesis and in tumor development, as well as on the order of the events leading to a derepression of onco-fetal genes, would shed more light into the molecular functions of *Ezh2* in fetal and cancer development. Furthermore, the exploitation of the *Ezh2* loss driven activation of fetal genes for therapy appears to be an exciting avenue. If loss of *EZH2* in AML results in fetal hematopoietic characteristics, pediatric treatment protocols would be worth testing in adults. Additionally many attempts have been made to interfere with the formation of bivalent promoter marks by inhibiting LSD1, EZH1/2, PRMT5 and DOT1L (Dawson, 2017) despite of the fact that genome wide effects on transcriptional control might be unpredictable and difficult to guide. As activating histone marks are established after the *EZH2* loss, their removal, for example by WDR5 inhibition, might reverse the *EZH2* loss driven effects. Candidates for onco-fetal therapy targets have been reported and tested (Ahrlund-Richter and Hendrix, 2014) although the question on the effectiveness of targeting single genes while whole signatures are upregulated remains unanswered. Genetic and pharmaceutical screening for candidates of the onco-fetal signature, actually conferring a growth disadvantage after inactivation, might help to identify useful targetable therapeutic options.

4.3. Activation of myeloid and HSC gene programs by the cooperation of *Ezh2* and *Runx1* mutations

In CRISPR-Cas9 cooperation screenings we demonstrated the oncogenic cooperation between loss of function mutations in *Ezh2* and *Runx1* during leukemogenesis (Fig. 7 and section 3.2.2.). Subsequently, we aimed to differentiate the molecular events triggered by loss of *Ezh2*, loss of *Runx1*, or their combined effect. Identification of cooperating functions would allow the development of strategies that interfere with the *Ezh2-Runx1* mutational collaboration as therapeutic options. Differential expression analysis in bone marrow samples, carrying only single or the double mutations, helped answering this question. In general we detected a massive upregulation of genes after the *Ezh2* loss and a massive downregulation of genes after the *Runx1* loss (Fig. 11) reflecting the repressive functions of *Ezh2* and the role as a transcriptional activator for *Runx1* in our setup (in general *Runx1* can have activating and repressive functions (Mevel *et al.*, 2019)). While the inactivation of *Ezh2* led to an upregulation of onco-fetal genes including the Wnt

pathway (Fig. 11C and section 3.3.3.), the addition of the *Runx1* loss seemed to be mainly responsible to convey myeloid disease fate by inactivating lymphoid genes (Fig. 11C). Based on these expression data, the inactivation of lymphoid genes is an effect solely accounted to the loss of *Runx1*, whereas the activation of myeloid and stem cell expression programs is triggered by the interplay of the *Ezh2* and the *Runx1* loss. These findings were further supported by immune-phenotyping of leukemias of different genetic configurations in our study. Only by adding the *Runx1* gRNA, a myeloid cell fate was triggered while all tested genetic combinations lacking the *Runx1* mutations induced preferentially a lymphoid malignancy (Fig. 6 and 7 and section 3.2.2.). Importantly, adding the *Runx1* gRNA in secondary transplantations to pre-malignant lymphoid-biased bone marrow shifted the disease phenotype into a myeloid direction. Several studies already underlined the need of Runx1 function for the proper development of B- and T-cells (Guo *et al.*, 2020; Seo *et al.*, 2012). Conditional knockouts of *Runx1* in adult hematopoiesis in mice revealed inhibition of CLPs with severely impaired B- and T-cell development (Gowney *et al.*, 2005). Obviously, *Runx1* is needed for the regulation of lymphoid genes and its loss leads to their inactivation, necessarily, leading to a myeloid cell fate. These findings go in line with our GSEA analysis of *Runx1* mutated samples (Fig. 11E) which also highlight a downregulation of lymphoid genes. In the study of Gowney *et al.*, *Runx1* deficient mice developed a mild myeloproliferative phenotype, which might develop into a full-blown AML by the interplay of additional genetic aberrations like the *Ezh2* loss considering our data. Another study revealed a function for the isolated *Runx1* depletion in promoting myeloid cell fate by upregulation of myeloid transcriptional programs (Behrens *et al.*, 2016) whereas our study demonstrated the requirement for additional *Ezh2* mutations for these transcriptional activations. Presumably, only the additive derepressing effects of both deletions together fully activated myeloid and stemness genes in a sufficient manner to lead to malignant growth of myeloid cell fate in our model (Fig. 11D). Not only the removal of H3K27me3, but also the removal of the repressive Runx1, is strictly needed to activate myeloid and stem cell genes in a sufficient amount for leukemia onset.

Booth *et al.* observed similar mechanisms in ETPs since the combined knockout of *Ezh2* and *Runx1* led to a more frequent activation of myeloid and HSC genes than in wild type ETPs although lymphoid genes stayed expressed in their setup (Booth *et al.*, 2018). The remaining expression of lymphoid genes in ETPs, compared to the inactivated state in bone marrow after *Ezh2* and *Runx1* loss in our study, might be due to the differences of the initiating cells. ETPs already harbor a more lymphoid bias than LSK cells that were targeted in our experimental setup.

Besides being an important transcriptional regulator in lymphopoiesis RUNX1 is also crucial to regulate myeloid differentiation together with its downstream transcription factor PU1 (Imperato *et al.*, 2015) so that the consequent activation of myeloid genes after its loss is astonishing. However, the complicated dual role of RUNX1 as transcriptional activator and repressor, for example by

recruiting PRC1 complexes in megakaryocytes (Yu *et al.*, 2012), might explain these effects of its deletion. All mechanisms, induced by the losses of *Ezh2* and the *Runx1*, seem to be especially specific for their individual interplay due to the high amount of differential gene regulation between the *Ezh2*^{-/-} *Runx1*^{-/-} leukemic samples and the *Bcor*^{-/-} *Runx1*^{-/-} leukemias (Fig. 10).

As only the combination of mutations in *Runx1* and *Ezh2* leads to the development of a myeloid malignancy and the single mutations do not, it is evident that all transcriptional effects, mediated by deletions in *Ezh2* and *Runx1* alone or by their combination, respectively, are obligatory and potentiate each other. Nevertheless, the full molecular processes, guiding this interplay, remain to be unraveled. Sequential ChIP-Seq analysis of *Ezh2* and *Runx1* would contribute useful information in order to determine genes that are directly targeted by *Runx1* and *Ezh2* simultaneously and would exclude secondary effects. Moreover, protein interaction studies and reporter assays would help to determine the exact role of the combination of H3K27me3 and *Runx1* removal at their target genes.

As already mentioned, the therapeutic intervention of this interplay is very attractive in the specific, frequently occurring, case of *Runx1-Ezh2* co-mutated AML. Discovering therapeutic path that counteract the mechanisms, specifically initiated by the *Runx1-Ezh2* mutational interplay, remains promising. According to our expression data, there are many genes commonly regulated by both factors besides myeloid and stem cell specific genes that might be worth targeting pharmaceutically or genetically. Inhibition of factors, antagonizing the *Ezh2* function and thereby interrupting the transcriptional interplay of *Ezh2* and *Runx1* mutations, like trithorax proteins, might also be worth testing and a promising therapeutic path. The removal of activating chromatin marks that are set after *Ezh2* loss, like WDR5 inhibition, could prevent the activation of *Runx1-Ezh2* shared target genes after their loss.

Overall, our study is the first one to prove the coexistence of three mechanisms guiding the molecular functions of a combined deletion of *Ezh2* and *Runx1*. The *Ezh2* loss mediated upregulation of onco-fetal genes, *Runx1* loss mediated downregulation of lymphoid genes, and upregulation of stem cell and myeloid genes by their combination present to be equally and simultaneously crucial for AML development.

4.4. Outlook

Aiming to uncover core transformative mechanisms of recurrently detected PRC2 repression in AML, this thesis isolated the potent leukemogenic properties of combined *Ezh2*- and *Runx1*-loss and leveraged this model to highlight the distinct and synergistic molecular events guiding the way from normal to malignant hematopoiesis. While reactivation of onco-fetal gene signatures and repression of lymphoid fate decisions were found to be distinct actions of *Ezh2*- and *Runx1*-loss, respectively, only their combined inactivation enabled stem cell self-renewal and myeloid determination. Human AML sequencing studies supposed an interaction of these mutations already, but lacked the

necessary biological proof of cooperation and the molecular mode in patients so far. However, in order to translate the findings of this thesis to the actual situation in patients, studies in a human system will be crucial. Genetic studies in human Cd34+ cord blood cells and in *EZH2* or *RUNX1* mutated PDX samples by knockout or overexpression may provide additional insights into the relevance of our findings in human disease. Moreover, humanized models will help to verify species transferability of our molecular findings and will be a crucial prerequisite to for preclinical testing of therapeutic approaches. The already here performed gene expression analyses revealed an activation of myeloid- and stem cell genes upon the collaboration of *Runx1* and *Ezh2* mutations and will serve as a benchmark for future studies and drug development. However, serial ChIP experiments of *RUNX1* and *EZH2* are highly recommended for future studies to discern direct, shared targets of *EZH2* and *RUNX1* from secondary events. Protein interaction studies may also help to elucidate a possible physical interaction and additional factors that are needed for this joint transcriptional repression. If complete transcriptional activation of myeloid and stemness genes after loss of the repressive factors *EZH2* and *RUNX1* required the recruitment of counteracting factors, as suggested by the detection of the activating mark H3K4me3 in our data, would be answered by ChIP-Seq experiments for example for WDR5 (enabling H3K4me3). In this case, pharmaceutical inhibition of the position of activating histone marks might prevent the full establishment of the *RUNX1-EZH2* loss cooperation and might be worth testing. Additionally, we identified the activation of onco-fetal genes as an oncogenic effect of the *Ezh2* loss. In order to further understand this mechanism the discrimination of *Ezh2* target genes in adult and fetal cells of all stages of differentiation by gene expression and ChIP-Seq analysis might be interesting. Moreover, our gene expression and ChIP-Seq data revealed a whole list of activated oncogenic drivers in *Ezh2* loss leukemia, above the presented onco-fetal candidates, on which leukemic cells might depend. Subsequent CRISPR-Cas9 screenings, utilizing these *Ezh2* loss signatures, in murine or human leukemias might identify vulnerabilities that could be targeted pharmaceutically or genetically. Overall, our discoveries and insights on the oncogenic mechanisms of the *Ezh2* deletion offer many starting points for intensified studies on vulnerabilities in PRC2-loss AML paving the way for novel therapeutic approaches in the future.

5. References

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6. Appendix

6.1. List of abbreviations

AGM	Aorta-gonadmesonephrocs
AML	Acute myeloid leukemia
av	average
BM	Bone marrow
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP-Sequencing
CMP	Common myeloid progenitor
CLP	Common lymphoid progenitor
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
dTom	dTomato
ETP	Early thymic progenitor
FACS	Fluorescent activated cell sorting
FC	Fold change
Fig.	Figure
FL	Fetal liver
FPKM	Fragments per kb per million
FSC	Forward scatter
gDNA	genomic DNA
GMP	Granulocyte-monocyte progenitor
gRNA	guide RNA
GSEA	Gene set enrichment analysis
HE	Hematoxylin and eosine
HSC	Hematopoietic stem cell
JMML	Juvenile myelo-monocytic leukemia
ko	knockout
lncRNA	long non-coding RNA
LSK	Lineage negative, Sca1 positive, Kit positive cells
LT-HSC	Long term HSC
LOF	Loss of function

MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythrocyte progenitor
ML-DS	Myeloid leukemia of Down syndrome
MNC	Mononuclear cell
MOI	Multiplicity of infection
MPP	multipotent progenitors
mRNA	messenger RNA
NES	normalized enrichment score
neg	negative
NGS	Next generation sequencing
P	Pool
PAGE	Polyacrylamide gel electrophoresis
Pb	Pacific blue
PcG	Polycomb group protein
PCR	Polymerase chain reaction
PDX	Patient derived xenograft
PRC	Polycomb repressive complex
PRE	Polycomb responsive element
pos	positive
qPCR	quantitative PCR
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RT	Reverse transcription
RT	room temperature
SSC	Sideward scatter
ST-HSC	Short term HSC
T-ALL	Acute T lymphoid leukemia
Tx	Transplantation
WHO	World health organization

6.2. Curriculum vitae

Dorit Schneider, Hebbelstr. 14, 30177 Hannover
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 Born on August 11th, 1990 in Engelskirchen
 German

Academic education

2015/10 – 2020/04	Scientific employee and PhD student at the Department for Pediatric Hematology and Oncology at the Hanover Medical School, laboratory of Prof. Dr. Heckl: <i>“Deciphering the oncogenic network of PRC2-loss guided leukemogenesis”</i> . Part of the PhD Program “Molecular Medicine” of the Hanover Biomedical Research School, grade of the Intermediate Exams: very good.
2013/10 - 2015/09	Master of Science in Human biology with a major in Tumor biology at the Philipps University Marburg (final grade 1.3). Master thesis at the Institute for Molecular biology and Tumor research, laboratory of Prof. Dr. Suske: <i>“Studying the Chromatin recruitments of the polycomb protein L3mbtl2”</i> .
2010/10 – 2013/07	Bachelor of Science in Biomedical Sciences at the Philipps University Marburg (final grade 1.4). Bachelor Thesis at the Institute for Molecular biology and Tumor research, laboratory of Prof. Dr. Suske: <i>“Chromatin recruitment of the transcription factor Sp3”</i> .
2001/08 – 2010/07	Gymnasium Bayreuther Str., Wuppertal, Abitur (final grade 1.1)
1997/08 – 2001/07	Primary school in Schwelm und Wuppertal

Additional academic activities

2019/08 u. 2018/05	Talks on „Interrogating leukemogenesis with CRISPR-Cas9 genome-editing“ as a part of a Mini symposium of IDT (Integrated DNA technologies Inc.) and the LISA summer school.
2017/09 – 2017/12	Research stay at the Children’s Research Institute at UT Southwestern, Dallas, USA, laboratory of Prof. Dr. Jian Xu to learn the method of ChIP-Seq.
2012/09 – 2015/08	Study adviser for biomedical studies at the Philipps University Marburg.
2013/05 – 2015/06	Tutor supervising practical and theoretical training in genetics, biochemistry and molecular biology.
2011/02 – 2014/06	Internships in the following laboratories of the Philipps University Marburg: Prof. Dr. Dr. Jürgen Daut (Institute for Physiology), PD Dr. Cornelia Brendel (Clinics for Hematology, Oncology and Immunology), Prof. Dr. Robert Mandic (Clinics for Otorhinolaryngology).

Conferences and Prizes

Yearly meeting of the International society for Experimental Hematology (ISEH) 2019 in Brisbane, Australia, Talk *“Loss of Ezh2 cooperates with loss of Bcor, Runx1 and Tet2 and reactivates a fetal gene signature”*, honors of the T. Ray Bradley Award (2nd prize New Investigator Award PhD students).

Yearly meeting of the International society for Experimental Hematology (ISEH) 2017 in Frankfurt, Germany, Talk *“Deciphering the oncogenic network of PRC2 loss guided leukemogenesis”*, honors of the 2nd prize of pre-meeting workshop.

International Symposium on Acute Leukemias (ISAL) 2017 in München, Talk, *“Deciphering the oncogenic network of PRC2 loss guided leukemogenesis”*, honors of one of three best free contributions.

Additional courses

2018	Seminar on “Drug development in the pharmaceutical Industry”
	Seminar on presentation and rhetoric techniques: “Personal Presence” by INT Media
2017	Course on “Good manufacturing praxis (GMP)” and “Good Laboratory Praxis (GLP)” in cooperation with the Fraunhofer Institute ITEM
2016	Course on “Scientific Writing”
2015	Basics in Laboratory Animal Science (corresponds to the demands of the Directive 2010/63/EU)

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6.4. Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently, in compliance with “the policy of Hannover Medical School on the safeguarding of good scientific practice and procedural rules for dealing with scientific misconduct” and that I have not submitted it at any other university worldwide.

Herewith, I agree that MHH can check my thesis by plagiarism detection software as well as randomly check the primary data. I am aware that in case of suspicion, ombudsman proceedings according to § 9 of MHH ‘Guidelines of Hannover Medical School to guarantee good scientific practice and dealing with scientific fraud’ will be initiated. During such proceedings, the PhD process is paused.

Hannover, April 2020

Dorit Schneider