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Inactivation of *Eed* Impedes *MLL-AF9* Mediated Leukemogenesis Through *Cdkn2a*-Dependent and *Cdkn2a*-Independent Mechanisms in a Murine Model

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Abstract

Polycomb Repressive Complex 2 (PRC2) is a chromatin regulator with central roles in development and cancer. The canonical function of PRC2 is the tri-methylation of histone 3 on lysine residue 27. This epigenetic modification is associated with gene silencing. Both tumor suppressor and oncogenic functions have been reported for PRC2, depending on cellular context. In leukemia mediated by the leukemogenic fusion *MLL-AF9*, complete ablation of canonical PRC2 function by genetic inactivation of the core component Embryonic Ectoderm Development (*Eed*) or by combined pharmacological inhibition of the PRC2 methyltransferases *EZH2* and *EZH1* has a strong antileukemic effect, and this effect has been linked to derepression of the PRC2 target locus *Cdkn2a*. We asked whether inactivation of *Cdkn2a* is sufficient to restore leukemic activity of *Eed*-inactivated *MLL-AF9* leukemia cells, using combined genetic inactivation of *Cdkn2a* and *Eed*. We found that *Cdkn2a* inactivation partially rescues in vitro and in vivo growth of *Eed*-inactivated *MLL-AF9* cells. However, the growth of *Eed*-null *Cdkn2a*-null *MLL-AF9* cells in the absence of *Cdkn2a* remained severely compromised in vitro and in vivo, compared to *Eed*-

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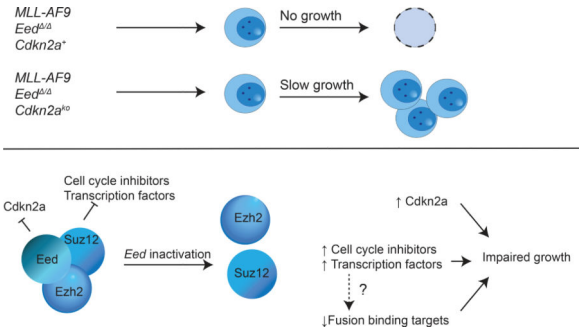
floxed *Cdkn2a*-null counterparts. RNAseq analysis revealed that several genes previously implicated in inefficient growth of *MLL-AF9* transformed cells, including *Gata2*, *Egr1* and *Cdkn2b* were derepressed as a consequence of *Eed*-inactivation. Furthermore, we found that direct binding targets of MLL-fusion proteins are negatively enriched in *Eed*-inactivated *Cdkn2a*-null *MLL-AF9*-transformed cells. Our data show that interference with PRC2 function affects *MLL-AF9* mediated leukemogenesis by both *Cdkn2a*-dependent and *Cdkn2a*-independent mechanisms.

Graphical Abstract



Keywords

Stem Cells; Leukemia; Epigenetics; Polycomb Repressive Complex; MLL

Introduction

Hyperactivity and impairment of the Polycomb Repressive Complex 2 (PRC2) have been described in patients with hematological malignancies, but the underlying mechanisms are incompletely understood. In mice, genetic inactivation of *Ezh2* leads to T-ALL [1] and MDS/MPN [2], which is in keeping with PRC2 alterations in human T-lineage ALL [3, 4] and in myeloid neoplasms [5-7]. Inactivation of the PRC2 methyltransferase *Ezh2* is partially compensated by the less well characterized methyltransferase Ezh1, whereas inactivation of *Eed* leads to complete loss of the canonical PRC2 function, i.e. the di- and tri-methylation of Lysine 27 on Histone 3 [8]. In human *MLL*-rearranged leukemia, to our knowledge PRC2 mutations have not been described. In genetic mouse models of *MLL*-rearranged leukemia, inactivation of *Ezh2* impedes leukemia growth [9, 10] and knockout [9] or knockdown [11] of *Eed* leads to severe compromise of leukemic growth. Pharmacological interference with PRC2 function via stapled peptides targeting *Eed* [12] and via combined pharmacological inhibition of *Ezh2* and *Ezh1* [13] has also recently been demonstrated to interfere with growth of *MLL-AF9* transformed cells. Derepression of the PRC2 silencing target locus *Cdkn2a*, which encodes the tumor suppressors $p16^{ink4a}$ and $p19^{Arf}$, has been implicated as the major mechanism mediating the growth inhibitory effect of impaired PRC2 function in *MLL*-rearranged leukemia [9, 11, 13]. We here test the interplay between PRC2 and *Cdkn2a* stringently in a combined genetic model, asking if genetic inactivation of *Cdkn2a* is indeed able to restore leukemogenic activity in *Eed*-null *MLL-AF9* leukemia.

Results and Discussion

We used lineage negative, Sca-1 positive, Kit-positive (LSK-) cells harvested from mice with a homozygously floxed *Eed* locus crossed onto a background of wild type, or hetero- or homozygously inactivated *Cdkn2a*. The mice also carry a ^{lox}Stop^{lox}*ROSA26YFP* Cre-reporter allele [14] permanently activated upon *Cre*-expression (Fig. 1a). We transduced LSK-cells with MSCV-*MLL-AF9*-ires-GFP alone or in combination with a self-excising *Cre*-expressing retroviral vector, thus limiting expression of the Cre-recombinase to a short time window [15]. In 2 independent experiments using 2 separate sets of donor mice (*Cdkn2a*^{ko} vs *Cdkn2a*$^{+/+}$ in experiment 1 and *Cdkn2a*^{ko} vs *Cdkn2a*$^{+/-}$ in experiment 2) we isolated >85 % fully *Eed*-inactivated clones in the C*dkn2a*^{ko} group (Fig. 1b). We were unable to identify any fully *Eed*-inactivated clones in the presence of either one or two intact *Cdkn2a* loci. These data confirm previous findings [9, 11, 13] that *Eed*-inactivated *MLL-AF9* cells with an intact *Cdkn2a*-locus are incapable of in vitro growth and that *Cdkn2a* is a critically important downstream target of PRC2 in leukemia. We went on to further characterize the effects of *Eed* inactivation in *MLL-AF9/Cdkn2a*^{ko} clones. We expanded *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko} control clones and *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} clones with confirmed full excision of floxed *Eed* sequences (Figure 1a-b). *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko} cells formed typical tightly packed colonies, whereas *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells showed a more dispersed growth pattern (Fig. 1c). *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} clones were also noted to have a lower proportion of Kit positive cells by flow cytometry (Fig. 1d). Western blotting confirmed global loss of H3K27me3 as a consequence of *Eed* inactivation (Fig. 1e).. The growth rate of *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells in methylcellulose was reduced to approximately 10% of *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko} control cells (Fig. 1f). While clearly compromised in their growth, *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells were able to serially replate with a replating efficiency of approximately 50% of control (Fig. 1g and Fig. S1c) and grow at a stable rate for several weeks.

We therefore asked whether *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells cause in vivo leukemia. In order to avoid outgrowth of non-deleted escapees, we established clonal lines, and confirmed complete loss of H3K27me3 in clones prior to injection. Complete excision of *Eed* sequences was also evident in the RNAseq gene expression data (Fig. S1a), and was confirmed by RT-qPCR (Fig. S1b). Cells from two *MLL-AF9*/*Eed*^{ff}*/Cdkn2a*^{ko} control clones and four confirmed *MLL-AF9*/*Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} clones where transplanted into syngeneic sublethally irradiated recipients (two recipients per clone). While all the recipients of control cells developed leukemia within an expected time frame, only 2/8 recipients of *MLL-AF9/Eed*$^{\Delta/\Delta}$*Cdkn2a*^{ko} cells (derived from two separate clones) developed leukemia (one additional mouse died from unrelated reasons with low (<1%) but detectable engraftment of *YFP* positive cells in spleen and bone marrow). The onset of leukemia in the *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn*^{ko} group was significantly delayed (Fig. 2a). We confirmed complete inactivation of *Eed* in cells recovered from moribund animals by Western blot for H3K27me3 (Fig. 2b). Immunophenotypic analysis of both *Eed*^{ff}*/Cdkn*^{ko} and *Eed*$^{\Delta/\Delta}$*/Cdkn*^{ko} leukemias was consistent with AML (Fig. 2c and d), without evidence of lineage infidelity with respect to lymphoid surface markers (Fig S2a and b). Interestingly, *MLL-AF9*/*Cdkn2a*^{ko}*/Eed*$^{\Delta/\Delta}$ leukemias showed an increased proportion of Gr-1 and Kit positive cells in

the transition from pre-leukemic cell to in vivo leukemia (Fig. 2c-d and Fig. S2c-f). These data demonstrate that in the context of *Cdkn2a* inactivation, *Eed* is not strictly required for *MLL-AF9* leukemia in vitro and in vivo. However, growth of *MLL-AF9/Cdkn2a*^{ko}*/Eed*$^{\Delta/\Delta}$ cells remains severely compromised, suggesting that *Eed*-inactivation results in functionally important gene expression changes other than *Cdkn2a* derepression that adversely affect *MLL-AF9* leukemia growth.

We therefore assessed additional transcriptional consequences of *Eed*-inactivation in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells. RNA-seq was performed on confirmed fully excised *Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} and control *Eed*^{ff}*/Cdkn2a*^{ko} cells prior to injection. Gene set enrichment analysis [16] (GSEA) revealed enrichment of canonical PRC2 targets [17] (Fig. 3a). The cell cycle inhibitor *Cdkn2b*, a PRC2 target (Fig. S3a) was found to be upregulated in *Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells (Fig. 3b). The stem cell associated genes *Gata2 [18]* (Fig. 3c) and *Egr1* (Fig 3d) which are PRC2 targets in murine *MLL-AF9* L-GMP and murine bulk *MLL-AF9* blasts (Fig. S3a), were also upregulated. We also observed negative enrichment of primary *MLL-AF9* binding targets as previously defined by Bernt et al [19] (Fig. 3e) and *MLL-ENL* binding targets as described by Wang et al [20] (Fig. 3f). Downregulation of HoxA9 (Fig. 3g) as a key downstream target of MLL-fusions, as well as the *MLL-AF9* -fusion target *Runx2* (Fig. 3h) were also confirmed by RT-qPCR.

Recent data have suggested combined inhibition of EZH2 and EZH1 as a therapeutic strategy for MLL-leukemia [13]. We compared the gene expression changes observed in our *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} model to the data described by Xu et al. [13]. We found that the genesets behaved analogously in cells treated with a combined EZH2/EZH1 inhibitor, UNC1999 (Fig. S3). Specifically, UNC1999-treated cells showed significant enrichment in PRC2 targets and negative enrichment for MLL-fusion binding targets. Genetic and pharmacologic impairment of PRC2 function is detrimental to MLL-rearranged leukemia cells. This effect has been ascribed to de-repression of the tumor suppressor locus *Cdkn2a*, a documented PRC2 target [9, 11, 13]. We here evaluated the effects of genetic PRC2 inactivation on *MLL-AF9* leukemogenesis in a *Cdkn2a*-null background. Our data demonstrate that the growth disadvantage of *Eed*$^{\Delta/\Delta}$ *MLL-AF9* cells is not entirely dependent on *Cdkn2a* but is rather multifactorial. As expected, in response to genetic inactivation of the PRC2 core component *Eed* canonical PRC2 targets are derepressed, including the known negative regulator of cell cycle Cdkn2b encoding p15. We also found inappropriate expression of developmental transcription factors *Gata2* and *Egr1* which are normally highly expressed in stem-/early progenitor cells. The derepression of stem cell-associated genes may appear to be compatible with, or possibly supportive of a leukemic phenotype. However, the functional relevance of *Gata2* and *Egr1* has been validated by others, and forced expression of *Gata2* [21] and *Egr1* [10] has been linked to impaired growth of *MLL-AF9* leukemia. In keeping with prior reports [21-23] we found that high-level forced expression of *GATA2* alters colony formation and impedes growth of MLL-AF9 transformed *Eed*^{ff}*/Cdkn2a*^{ko} LSK-cells (Fig. S4), thus partially phenocopying inactivation of *Eed*. *MLL-AF9* leukemic stem cells have been suggested to display transcriptional programs partially characteristic of adult hematopoietic stem-cells and partially resembling myeloid progenitors [24]. Our data along with published data suggest that inappropriately high expression of at

least some stem cell related genes has adverse effects in *MLL-AF9* leukemia, suggesting that *MLL-AF9* leukemia requires a precisely regulated balance of stem- and progenitor associated genes. Finally, we observed significant loss of direct binding targets of the leukemogenic *MLL-AF9* and *MLL-ENL* fusion. This decreased expression of binding targets was also observed in published data in response to UNC1999, a combined EZH1/EZH2 inhibitor [13], suggesting that cellular loss of H3K27me3 is responsible for the effect. In summary, impaired PRC2 severely impairs but does not abrogate *MLL-AF9* leukemia growth. The growth inhibitory effect is not only mediated by dysregulation of *Cdkn2a* but also by dysregulation of other genes. Inhibition of PRC2 may be a strategy for the treatment of MLL-rearranged leukemia, especially if synergistic therapeutic interventions can be identified.

Experimental Procedures

For primer sequences, antibodies and detailed experimental procedures please refer to the supplemental materials.

Mice

Animals were maintained at the Animal Research Facility at the University of Colorado Anschutz Medical Campus. Animal experiments were approved by the Internal Animal Care and Use Committee. All mice were maintained on a fully backcrossed C57BL/6 background.

Generation of transformed murine cells and leukemia

See supplement for description of retroviral vectors. ($Lin^{-}Sca\text{-}1^{+}Kit^{+}$ cells were prestimulated in liquid culture in the presence of murine SCF, Flt3L, IL6 and Tpo (Peprotec, Rocky Hill, NJ) and then transduced in the presence on Retronectin (Takara, Madison, WI). Cells were sorted for expression of *GFP* or coexpression of *GFP* and *YFP* and plated in methylcellulose in the presence of IL3, IL6 and SCF (Peprotech). Individual colonies were picked and expanded in 96-well plates. Cells expanded for 18 days and were then injected into syngeneic sublethally (600cGy) irradiated recipients (1 Million cells per recipient). Cell growth and viability were followed by serial cell counts. Antibodies used for flow cytometry and immunoblot detection and qPCR primers are detailed in supplementary methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- We established *MLL-AF9* leukemia with combined genetic inactivation of *Cdkn2a* and *Eed*
- *Cdkn2a* inactivation partially rescues the previously reported growth inhibitory effects of *Eed*-inactivation in *MLL-AF9* cells in vitro and in vivo
- *Cdkn2a*-null *Eed*-null *MLL-AF9* cells remain severely compromised in their growth compared to *Cdkn2a*-null *Eed-ff MLL-AF9* cells
- RNA-seq in *Eed*-null cells demonstrates derepression of Polycomb targets with documented adverse effect on growth of *MLL-AF9* cells
- RNA-seq demonstrate negative enrichment of primary MLL-fusion binding targets in *Eed*-null *MLL-AF9* cells

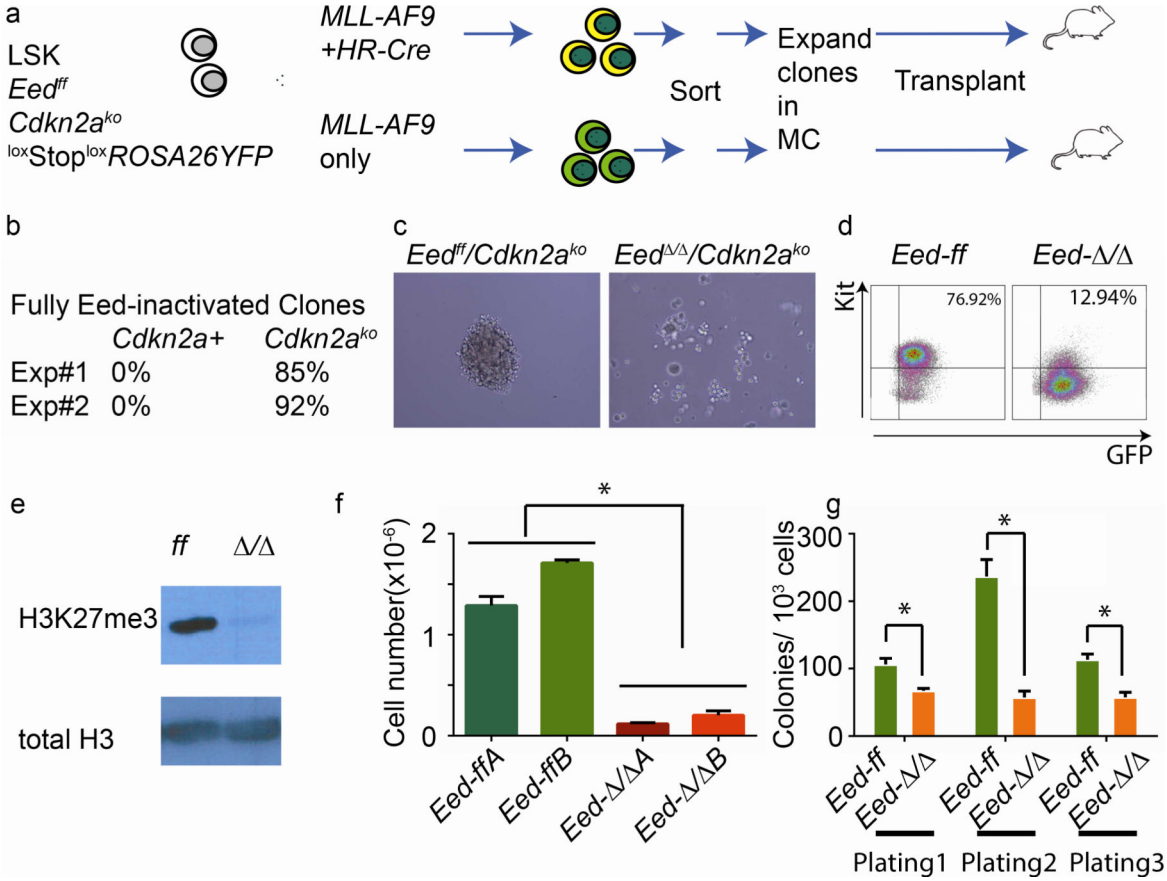

Fig. 1. Genetic inactivation of *Cdkn2a* partially rescues genetic inactivation of *Eed* in *MLL-AF9* cells in vitro

a) Schematic depiction of experimental design. Co-transduction with an *MLL-AF9* encoding vector and retroviral self-excising Hit-and Run *Cre* vector (*HR-Cre*) was used to excise floxed *Eed* sequences. MC; methylcellulose. *Cre* expression leads to activation of the ROSA26-YFP reporter which was used for sorting of *Eed*-inactivated cells. b) Fully deleted $Eed^{\Delta/\Delta}$ *MLL-AF9* clones can be established in the absence, but not presence of an intact homozygous or heterozygous *Cdkn2a*-locus. c) *MLL-AF9/*Eed^{ff}*/*$Cdkn2a^{ko}$ clones have the typical packed colony morphology while *MLL-AF9/*$Eed^{\Delta/\Delta}$*/*$Cdkn2a^{ko}$ cells grow more dispersed. d) Lower proportion of Kit positive cells in *MLL-AF9/*$Eed^{\Delta/\Delta}$*/*$Cdkn2a^{ko}$ compared to *MLL-AF9/*Eed^{ff}*/*$Cdkn2a^{ko}$ cells. A separate pair of clones showed 89.1% Kit-positive cells for Eed^{ff} cells versus 53.6% for $Eed^{\Delta/\Delta}$ cells. e) complete absence of H3K27me3 in *MLL-AF9/*$Eed^{\Delta/\Delta}$*/*$Cdkn2a^{ko}$ cells (Western blot). f) *MLL-AF9/*$Eed^{\Delta/\Delta}$*/* $Cdkn2a^{ko}$ cells grow more slowly in methylcellulose compared to *MLL-AF9/*Eed^{ff}*/*$Cdkn2a^{ko}$ cells. 5000 cells were plated per well in Methylcellulose and growth factors SCF, IL3 and IL6 and total cell number was determined 4 days later. Error bars indicate standard deviation (SD). g) Approximately 50% average reduction in plating efficiency in *MLL-AF9/*$Eed^{\Delta/\Delta}$*/* $Cdkn2a^{ko}$ clones compared to *MLL-AF9/*$Eed^{\Delta/\Delta}$*/*$Cdkn2a^{ko}$ clones. 2 *MLL-AF9/*$Eed^{\Delta/\Delta}$*/* $Cdkn2a^{ko}$ clones and 3 *MLL-AF9/*$Eed^{\Delta/\Delta}$*/*$Cdkn2a^{ko}$ clones were plated in triplicate day 0, and replated in triplicate on day 5 and day 10. Error bars indicate SEM. * $p<0.05$ (t-test).

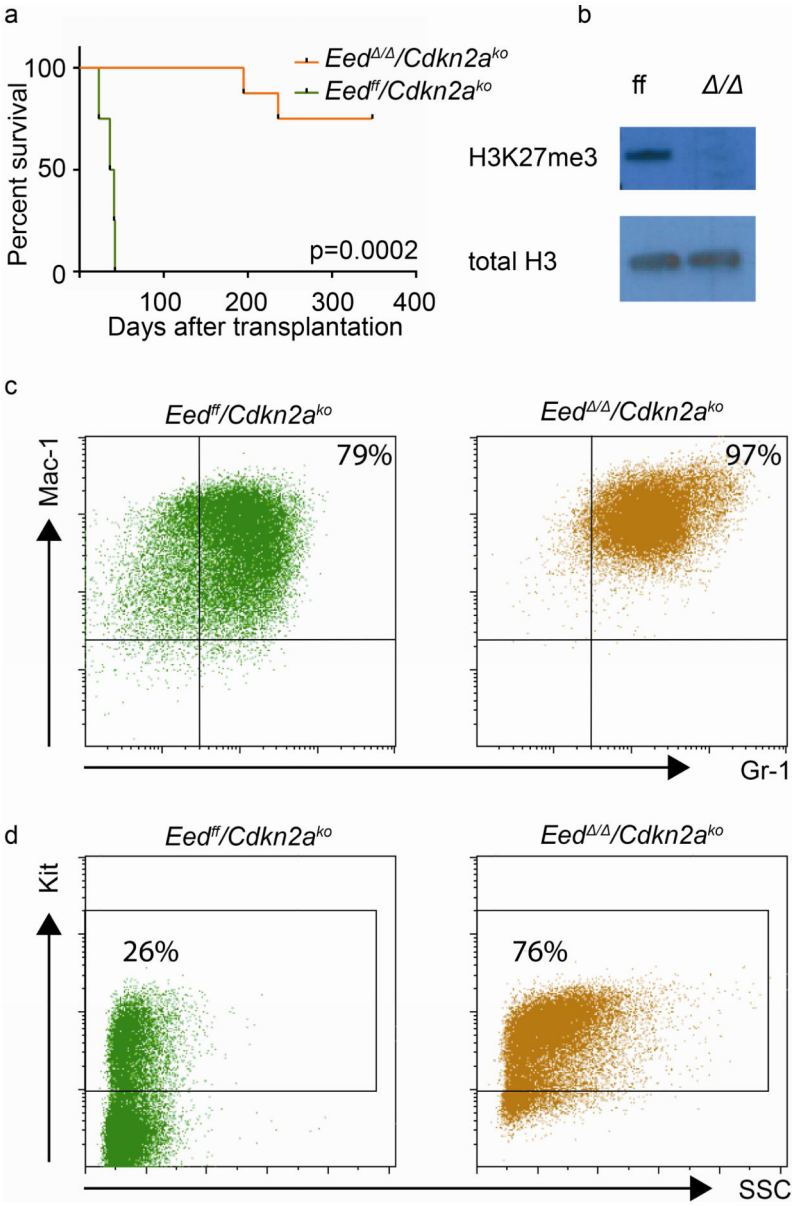

Fig. 2. Genetic inactivation of *Cdkn2a* partially rescues genetic inactivation of *Eed* in *MLL-AF9* cells in vivo

a) *MLL-AF9/Eed*$^{\Delta/\Delta}$ leukemia can be established on a *Cdkn2a*^{ko} genetic background in vivo with incomplete penetrance and significantly prolonged latency compared to *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko} controls. b) complete absence of H3K27me3 in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells in vivo (Western blot). c) Gr-1 and Mac-1 expression in vivo in *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko} (green, gated on live GFP$^+$ cells) and *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} (yellow, gated on live GFP$^+$YFP$^+$ cells) blasts. d) Kit expression in vivo in *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko} blasts (green, gated on live GFP$^+$ cells) and *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} *blasts* (yellow, gated on live GFP$^+$YFP$^+$ cells).

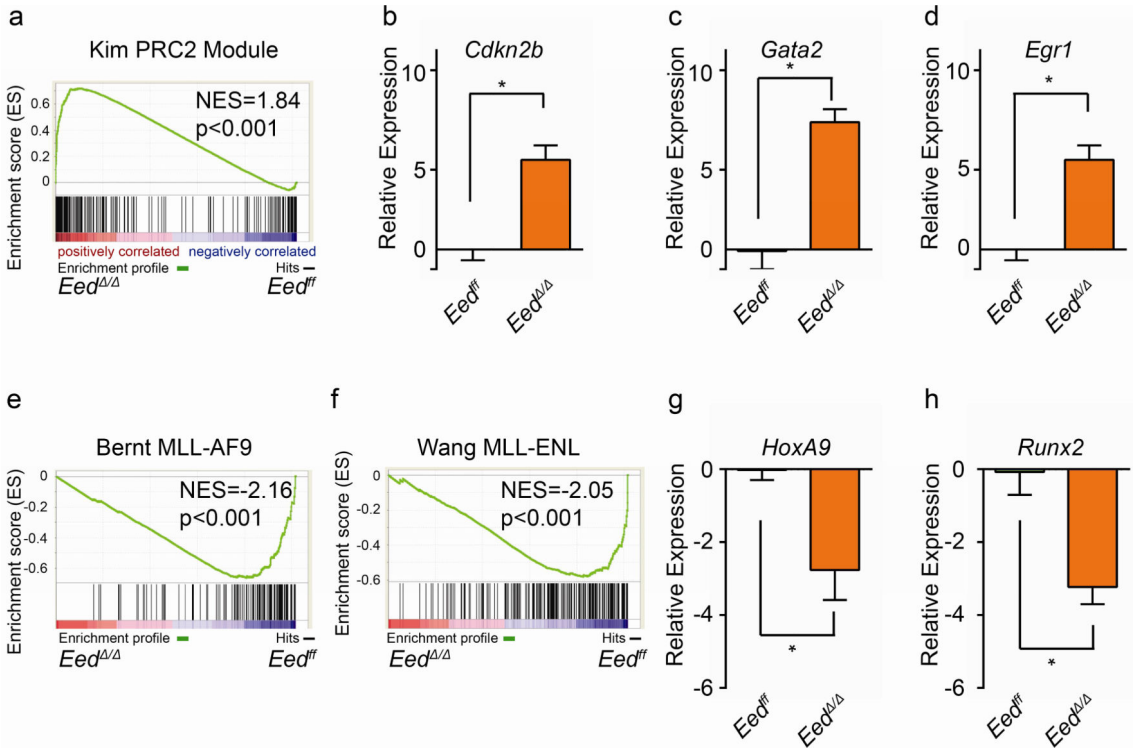

Fig. 3. Transcriptional changes in response to *Eed*-inactivation

a) Polycomb Repressive Complex 2 (PRC2) binding targets are enriched in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells compared to *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko} cells. b)-d) Increased expression as measured by RT-qPCR of PRC2 binding targets *Cdkn2b* (b), *Gata2* (c) and *Egr1* (d) in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} clones. (n=2, *Eed*^{ff} and n=4 for *Eed*^{ko}). e) Negative enrichment (i.e. decreased expression) of *MLL-AF9* fusion binding targets in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells. d) Negative enrichment (i.e. decreased expression) of *MLL-ENL* fusion binding targets in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} cells. g) Decreased expression of *HoxA9* in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} clones (*Eed*^{ff}, n=2, and *Eed*$^{\Delta/\Delta}$ n=4). h) Decreased expression of *MLL-AF9* binding target *Runx2* in *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} clones (*Eed*^{ff}, n=2, and *Eed*^{ko} n=3). Relative expression values are expressed as log2 of *MLL-AF9/Eed*$^{\Delta/\Delta}$*/Cdkn2a*^{ko} compared to *MLL-AF9/Eed*^{ff}*/Cdkn2a*^{ko}. *p<0.05.