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The ASXL1-G643W variant accelerates the development of CEBPA mutant acute myeloid leukemia

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Running head: Asx1 lesions collaborate with CEBPA-p30 in AML

Author contributions
TD, MBS, AMH and ASW carried out the experiments. TD, AW, AK, SP and BTP analyzed data. TDA and BP drafted the manuscript which was proofread by all authors. BTP directed the research.

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Abstract

ASXL1 is one of the most commonly mutated genes in myeloid malignancies, including Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML). In order to further our understanding of the role of ASXL1 lesions in malignant hematopoiesis, we generated a novel knock-in mouse model carrying the most frequent ASXL1 mutation identified in MDS patients, p.G643WfsX12. Mutant mice did not display any major hematopoietic defects nor developed any apparent hematological disease. In AML patients, ASXL1 mutations co-occur with mutations in CEBPA and we therefore generated compound CeBpa and Asxl1 mutated mice. Using a transplantation model, we found that the mutated Asxl1 allele significantly accelerated disease development in a CEBPA mutant context. Importantly, we demonstrated that, similar to the human setting, Asxl1 mutated mice responded poorly to chemotherapy. This model therefore constitutes an excellent experimental system for further studies into the clinically important question of chemotherapy resistance mediated by mutant ASXL1.
Introduction

Additional Sex Comb-like 1 (ASXL1) is a frequently mutated gene in myeloid malignancies including Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML)\(^1,\,2\). Moreover, ASXL1 mutations are also highly prevalent in premalignant states such as Clonal Hematopoiesis of Indeterminate Potential (CHIP), demonstrating that ASXL1 lesions are early driver events with the potential to predispose for further malignant transformation\(^3,\,4\). The vast majority of ASXL1 mutations are located in the last exon and are deletions, insertions, or substitutions resulting in stop codon mutations and truncation of the ASXL1 protein\(^1,\,2\). Mutations are always monoallelic and mRNA expression levels are variable. Due to difficulties in detecting ASXL1 in human samples, ASXL1 mutations were originally believed to be loss of function lesions and consequently haploinsufficient\(^5\). However, in more recent work, the truncated protein can indeed be detected, raising the possibility that ASXL1 mutations may act as dominant negative or gain of function variants\(^6\).

Mechanistically, ASXL1 is a dual function epigenetic regulator. Specifically, it interacts directly with the BRCA1-Associated Protein 1 (BAP1) to form a complex which de-ubiquitinates H2AK119Ub, a repressive histone mark deposited by the Polycomb Repressive Complex 1 (PRC1)\(^5,\,7,\,8\). Moreover, ASXL1 interacts with the components of the Polycomb Repressive Complex 2 (PRC2) which deposits the H3K27me3 repressive mark\(^5,\,9\).

The interest in the role of ASXL1 in malignant hematopoiesis has spurred the development of several Asx1 knock-out mouse models\(^10-\,12\) (for a recent review, see\(^13\)) Although these models do not result in identical phenotypes, they all show signs consistent with human MDS. Interestingly, overexpression of a truncated form of Asx1 yielded similar results suggesting that the mutations found in patients result in a dominant-negative version of the protein\(^6\). In line with these findings, several reports have indicated that truncated ASXL1 enhances BAP1 complex activity, thereby promoting depletion of the H2AK119Ub mark and aberrant myeloid differentiation\(^14,\,15\). More refined modeling of ASXL1 function in hematological malignancies has been performed by knock-in of patient-specific ASXL1 lesions into the murine Asx1 locus\(^16,\,17\). These knock-in mice
generally exhibit more subtle phenotypes compared to the complete deletion of
the gene. Although heterozygous knock-in mice do no develop MDS or AML, the
knock-in alleles collaborate with other leukemic drivers such as MN1 and RUNX1
or accelerate AML development in an insertional mutagenesis setting.\textsuperscript{16, 17}

CEBPA is a key myeloid transcription factor which is mutated in approximately
10% of AML patients and biallelic CEBPA mutant AML constitutes a specific AML
subtype.\textsuperscript{18-20} These patients either harbor biallelic N-terminal lesions or, more
frequently, combine these lesions with a C-terminal mutation. Whereas the N-
terminal lesions promote the expression of the N-terminally truncated p30
isoform, C-terminal mutations result in variants that are unable to dimerize and
are consequently inactive. Hence, the genetic lesions in biallelic CEBPA mutant
AML converge at the expression of the N-terminally truncated p30 isoform in the
form of CEBPA-p30 homodimers.\textsuperscript{20} In stark contrast to full-length CEBPA,
CEBPA-p30 is not able to repress E2F-mediated cell cycle progression\textsuperscript{21} and
recent work has also identified specific downstream targets of this oncogenic
CEBPA variant.\textsuperscript{22} Importantly, mice in which CEBPA-p30 expression is driven
from the endogenous Cebpa locus develop AML within the first year of their
lives.\textsuperscript{21} Interestingly, mutations in ASXL1 are frequent in biallelic CEBPA mutant
AML, but how these two sets of lesions interact functionally is currently
unknown.\textsuperscript{23, 24}

In the present work, we generated a novel Asxl1 knock-in line by introducing the
most common disease-associated mutation (p.G643WfsX12) into the murine
Asxl1 allele.\textsuperscript{2, 25} To assess the importance of Asxl1 lesions in the context of
biallelic CEBPA mutated AML, we combined lesions in these two proteins and
found that the ASXL1 mutation accelerated the development of CEBPA-p30
driven AML. Gene expression analysis yielded potential drivers of the
accelerated phenotype. Interestingly, ASXL1 mutated AMLs were largely
refractory to chemotherapy, thereby paralleling the findings from the human
setting.

\textbf{Methods}
**Generation of the Asxl1<sup>G643W</sup> knock-in mice**

The Asxl1<sup>G643W</sup> knock-in line was generated using the double nicking CRISPR-Cas9 system in embryonic stem cells (ESCs), followed by blastocyst injection. Two pspCas9n-2A-Puro constructs containing the two Asxl1 target sequences were electroporated into C57BL/6N ESCs together with a 141-mer ssDNA correction template containing the desired mutation. ESCs clones were screened for the presence of the mutation and correctly targeted clones were injected into mouse blastocysts. Please refer to the Supplemental Methods for additional details.

F1 offspring were backcrossed into C57BL/6 and maintained on that background. Animals were housed in SPF facility and all procedures have been approved by the Danish Animal Ethical Committee.

**In vivo AML development**

Bone marrow (BM) cells were retrieved and frozen in FCS with 10% DMSO. For leukemic experiments, stored BM was thawed and 2 million viable cells were transplanted into lethally irradiated (900Gy) recipients by tail vein injection. Three weeks later, recipients were subjected to three intraperitoneal injections with poly-I:C (0.3 mg in 200 µl PBS, GE Healthcare) separated by 48 hours. Recipient mice were monitored for leukemic development and euthanized when moribund. Please refer to the Supplemental Methods for additional details.

For the chemotherapy experiments, we transplanted cohorts of sublethally irradiated recipients with frozen secondary AML. Three weeks after transplant half the mice in each cohort were treated for three days with Cytarabine 50mg/kg and Doxorubicine 1 mg/kg and for two days with Cytarabine 50mg/kg. The remaining mice received PBS as vehicle treatment. 25-30 µl of blood were harvested for analysis three days after the last injection. Leukemic cell numbers were determined by combining cell counting with CD45.1 (recipient)/CD45.2 (donor) flow cytometry. For the survival study, the mice were observed for signs of disease and euthanized when moribund.
Flow cytometry analysis and cell sorting

For blood analysis, 50 µl blood was collected from the facial vein. Erythrocytes were depleted with BD PharmLyse. For bone marrow analysis, cells were collected by crushing tibia, femur and ilium and filtered. Blood or BM nucleated cells were washed in PBS with 3% FCS and stained for 15 min at 4°C. Please refer to the Supplemental Methods for additional details (antibodies and marker combinations).

Statistics

Unpaired t-test was used to compare values in the different groups. Log-rank (Mantel-Cox) test has been used to compare survival distributions. For the chemotherapy data in Figure 5B a one-tailed Mann-Whitney U test was used.

RNA sequencing

Donor derived AML blasts (CD45.2, Ter119-, B220-, CD3-, Mac1low, Gr1low, c-Kit+) were sorted from frozen BM samples into RLT lysis buffer (Qiagen) and RNA was extracted using the RNA Microkit (Qiagen). 100 ng RNA was used for the library generation, using TruSeq-V2 kit (Illumina). The libraries were analyzed by Qbit (ThermoFisher) and Bioanalyzer (Agilent) and pooled in equimolar amounts. Multiplexed samples were sequenced on a NextSeq 500 (Illumina) yielding approximately 35-45 million reads per sample. Please refer to the Supplemental Methods for additional details.
Results

Generation of the $\text{Asxl1}^{G643W}$ knock-in mouse line

In order to model the role of ASXL1 lesions in hematopoietic malignancies in the best possible manner, we decided to generate a mouse line expressing the most common ASXL1 mutation ($G643W_{fsX12}$, from hereon $G643W$) found in MDS patients$^{2, 25}$. Specifically, we used a double nicking CRISPR-Cas9 system in combination with a 141 bp ssDNA donor strand to introduce the c.1934dupG mutation into the endogenous $\text{Asxl1}$ locus. This approach results in the insertion of a $G$ within a stretch of eight $G$'s located in the last exon of $\text{Asxl1}$ which in turn generates a frameshift and an in-frame stop codon 12 codons downstream, thereby precisely mimicking the human situation (Figure 1A). The mutated $\text{Asxl1}^{G643W}$ allele expresses a truncated form of ASXL1 lacking the C-terminal Plant Homeodomain (Figure 1B). Both $\text{Asxl1}^{G643W/+}$ and $\text{Asxl1}^{G643W/G643W}$ mice express elevated levels of $\text{Asxl1}$, demonstrating that the mutated allele escapes nonsense-mediated mRNA decay (Figure 1C). This is in line with the previously observed expression of truncated ASXL1 in patient cells$^{6}$. The increased levels of the mutated mRNA could potentially be the result of a feedback mechanism.

The $\text{ASXL1}^{G643W}$ variant has minimal impact on normal hematopoiesis

We next assessed the impact of the $G643W$ mutation in the context of normal hematopoiesis. Both $\text{Asxl1}^{G643W/+}$ and $\text{Asxl1}^{G643W/G643W}$ mice were born at the expected Mendelian ratios and showed normal lifespan. This suggests that the $\text{ASXL1}^{G643W}$ variant has no impact on embryonic development or aging.

We next analyzed peripheral blood for the relative frequencies of the major blood lineages. Six months old $\text{Asxl1}^{G643W/+}$ and $\text{Asxl1}^{G643W/G643W}$ mice displayed no major changes within the peripheral blood compared to WT controls (Figure 2A). However, at 18 months, we observed a skewing towards the myeloid lineage in ASXL1 mutated mice (Figure 2B-C, Supplementary Figure S1A). The age-dependent skewing was accompanied by a mild splenomegaly (Figure 2G).
Consistent with the lack of changes in the peripheral blood in young mice, 6 months old \textit{Asxl1}^{G643W/+} and \textit{Asxl1}^{G643W/G643W} neither displayed any changes within the hematopoietic stem cell (HSC) and multipotent progenitor (MPP) compartment, nor within the distribution of mature blood lineages in the bone marrow (Figure 2D-F, Supplementary Figure S1B). Competitive transplantation of ASXL1-mutated bone marrow cells revealed a significant but minor reduction in the ability of \textit{Asxl1}^{G643W/G643W} BM to reconstitute hematopoiesis, suggesting that HSC functionality is only mildly affected (Figure 2H).

Collectively, these findings show that mutation of ASXL1 leads to a mild and age-dependent perturbation of the hematopoietic system in mice.

The ASXL1\textsuperscript{G643W} variant accelerates the development of CEBPA mutant AML

Mutations in \textit{ASXL1} and \textit{CEBPA} are frequently co-occurring in AML patients\textsuperscript{23,24}. To test the potential functional interplay between these factors, we crossed all three \textit{Asxl1} genotypes onto either a \textit{Cebpa}^{fl/p30}; \textit{Mx1Cre} or \textit{Cebpa}^{fl/+}; \textit{Mx1Cre} background resulting in a total of six genotypes. We subsequently transplanted BM from these mice into lethally irradiated recipients and three weeks later induced the deletion of the conditional \textit{Cebpa} allele by injections with polyinosinic:polycytidylic (pIpC) acid (Figure 3A). This strategy facilitates the deletion of the full-length \textit{Cebpa} allele, thereby allowing the \textit{Cebpa}^{p30} allele to exert its oncogenic function. Consistent with previous findings, \textit{Asxl1}^{+/+}; \textit{Cebpa}^{Δ/p30} donor cells sustain the development of AML with a median latency of 43 weeks (Figure 3B)\textsuperscript{26}. Interestingly, both heterozygous and homozygous expression of the ASXL1-G643W variant significantly accelerated CEBPA-mutant driven AML development, with median disease latencies of 37 and 38 weeks, respectively. In contrast, none of the control \textit{Cebpa}^{Δ/+} genotypes lead to AML, irrespective of their \textit{Asxl1} mutation status.

Given that \textit{Asxl1}^{G643W/G643W} animals, compared to their heterozygous counterparts, displayed a somewhat more pronounced phenotype during steady-state hematopoiesis, we decided to focus on this genotype in the context of CEBPA mutant AML. Whereas \textit{Asxl1}^{+/+}; \textit{Cebpa}^{Δ/p30} and \textit{Asxl1}^{G643W/G643W}; \textit{Cebpa}^{Δ/p30} leukemias appeared morphologically identical, the latter displayed a trend towards increased levels of c-Kit (Figure 3C-E, Supplementary Figure S2). This
suggests that the Asxl1 mutation, at least in the context of CEBPA mutant AML, could result in a slightly more immature leukemic phenotype which would be consistent with the more aggressive nature of ASXL1 mutant AML. Collectively, these findings agree with the observed co-occurrence of ASXL1 and CEBPA mutations in human AML and suggest that these lesions cooperate in the development of AML.

The ASXL1$^{G643W}$ variant affects the expression of leukemia relevant pathways

In order to understand the molecular underpinnings of the functional cooperation between ASXL1 and CEBPA mutations, we isolated leukemic blasts from Asxl1$^{+/+}$; Cebpa$^{Δ/p30}$ and Asxl1$^{G643W/G643W}$; Cebpa$^{Δ/p30}$ donor-derived AML and subjected them to gene expression profiling. This analysis revealed that in the context of CEBPA mutant AML, the Asxl1$^{G643W/G643W}$ genotype was associated with the upregulation of 177 genes and the down-regulation of 279 genes (adjusted p-val<0.05; Figure 4A, Supplementary Table S1, Supplementary Figure S3A). Interestingly, 18/30 of the most up-regulated genes encoded pseudogenes, perhaps reflecting a role for ASXL1 in mediating their repression. Gene set enrichment analysis (GSEA) identified gene ontology processes associated with mitosis (chromosome condensation, metaphase/anaphase transition of mitotic cell cycle and others; Fig 4B and Supplementary Table S2) to be up-regulated in the Asxl1$^{G643W/G643W}$ genotype likely reflecting the recent finding of ASXL1 being involved in maintaining sister chromatid separation\textsuperscript{27}. Furthermore, GSEA identified gene ontology terms associated with ribosome biosynthesis (ribosome biogenesis), DNA damage (regulation of DNA damage response, signal transduction by p53 class mediator) and immune activation (antigen processing a presentation of exogenous antigen) to be down-regulated in the Asxl1$^{G643W/G643W}$ genotype (Figure 4C). These findings do not only point to changes in the overall metabolic status of ASXL1 mutant cells, but may also indicate a reduced activation of the immune system as well as a decreased response to genomic insults.

Focusing on individual genes, we noticed a marked up-regulation of Traip in the Asxl1$^{G643W/G643W}$ genotype (log2 fold change = 6.7; Figure 4A, Supplementary
Figure 3A). TRAIP is an E3 ubiquitin ligase which has been shown to be involved in the regulation of the NF-κB pathway, cell proliferation, regulation of the spindle assembly checkpoint, DNA replication fork recovery and more recently as a master regulator of DNA crosslink repair.

Finally, in order to understand the epigenetic characteristics of the genes deregulated in the $Asxl1^{G643W/G643W}$ genotype, we overlaid published ChIP-seq data from $Cebpa^{Δ/p30}$ AML with promoter coordinates from deregulated and constant genes, identified in our gene expression analysis described above. In these ASXL1 proficient cells, the promoters of genes that were up-regulated following mutation of $Asxl1$ (in the context of CEBPA mutant AML), were characterized by low levels of “activating” histone modifications H3K4me3 and H3K27ac as well as by high level of the repressive histone mark H3K27me3 (Figure 4D and Supplementary Figure S3B-C). This combination of epigenetic marks is consistent with the low expression of these genes and previous work has demonstrated that loss of ASXL1 activity is associated with up-regulation of PRC2-repressed genes. Genes that are down-regulated in the $Asxl1^{G643W/G643W}$ genotype are also weakly expressed and display a similar epigenetic signature. The downregulation of these genes could potentially be due to a global decrease in H3K4me3 which has previously been found to be associated with the expression of a C-terminally truncated ASXL1 variant.

Collectively, these data suggest that expression of the ASXL1$^{G643W}$ variant affects the expression of a number of leukemic relevant genes and pathways, consistent with the role of ASXL1 as a broad epigenetic modifier.

The ASXL1$^{G643W}$ variant is associated with increased resistance to chemotherapy in the context of CEBPA mutant AML

Failure to respond to chemotherapy is a major determinant of overall survival in AML and genomic stratification has associated ASXL1 mutations with adverse outcome in human AML. This raises the possibility that AML-associated mutations in ASXL1 specifically impact on the cellular response to chemotherapy. To test this possibility, we used our well-defined experimental set-up to assess the impact of the $Asxl1^{G643W/G643W}$ genotype on the response to induction chemotherapy in the context of CEBPA mutant AML. To this end, we
transplanted $Ax1^+; Cebpa^{3/p30}$ and $Ax1^{G643W/G643W}; Cebpa^{3/p30}$ secondary AML (a total of seven clones) into recipient mice and subjected them to low-dose induction chemotherapy three weeks post transplantation (Figure 5A, Supplementary Figure S4). Compared to the ASXL1$^{WT}$ cohort, ASXL1$^{G643W}$ mice displayed reduced response to chemotherapy as measured by the decrease of leukemic cells in the peripheral blood three days post-treatment cessation (Figure 5B). This translated into an increased latency for the treated ASXL1$^{WT}$ cohort whereas chemotherapy had no impact on survival of the ASXL1$^{G643W}$ mutant cohort (Figure 5C).

Taken together, these findings demonstrate that mutation of ASXL1 renders CEBPA mutated AML largely resistant to chemotherapy.
**Discussion**

Myeloid diseases such as AML are developing from pre-malignant clones which mostly harbor lesions in epigenetic regulators\(^3\,^4\). One of these regulators is ASXL1 which is consequently frequently mutated in both MDS and AML as well as in pre-malignant settings such as CHIP. This mutational profile raises the question whether ASXL1 plays a functional role in full-blown AML or whether it merely provides a fertile ground in which AML can evolve.

There has been considerable confusion concerning the molecular mechanisms by which ASXL1 mutations sustain AML development or hematological deficiencies, specifically whether ASXL1 mutations act as dominant negatives. Some of this confusion is related to the inherent difficulties in detecting ASXL1 by western blotting (issues which we also experienced) thus making it nearly impossible to determine whether the mutated protein is present or not. However, overexpression of ASXL1 variants was associated with hematological malignancies raising the possibility that the mutated protein could act as a dominant negative\(^6\, ^14\, ^15\). Our analysis of heterozygous and homozygous Asxl1\(^{G643W}\) mice in the context of normal hematopoiesis suggests that the Asxl1\(^{G643W}\) variant has a dose-dependent impact in this context. Given that complete loss of Asxl1 leads to more pronounced hematopoietic phenotypes, the most parsimonious explanation from the *in vivo* work is that the ASXL1\(^{G643W}\) variant is hypomorphic or, alternatively, that it exerts a combination of dominant negative and hypomorphic effects\(^10\,-\,^{12}\).

Expression of the Asxl1\(^{G643W}\) variant in the context of CEBPA mutant AML significantly accelerated AML development and was associated with marked resistance to induction chemotherapy. These findings are not only in perfect alignment with the co-occurrence of *CEBPA* and *ASXL1* lesions in human AML, but also with the overall poor prognosis of ASXL1 mutated human AML. Hence the resistance towards chemotherapy is likely underlying the poor prognosis of AXL1 mutated AML\(^33\,-\,^{35}\). It would be interesting to test if other mouse models can recapitulate this behavior beyond CEBPA mutant AML.
Interestingly, gene expression analysis demonstrated that \textit{Asxl1}_{G643W/G643W; Cebpa}^{Δ/p30} exhibited downregulation of signatures associated with immune activation perhaps reflecting that the \textit{Asxl1} lesion renders the developing leukemia less visible to the immune system. We also observed a reduction in activation of the DNA damage pathways in ASXL1 mutated cells which could potentially reflect efficient clearing of ongoing genomic insults. Here our finding of the marked upregulation of \textit{Traip} is of particular interest as the corresponding protein has recently been identified as a master regulator of DNA crosslink repair\textsuperscript{32}. Thus our data strongly suggest that \textit{Asxl1} lesions have functional consequences in the context of CEBPA mutant AML and that they therefore provide more than a fertile ground for AML development.

To summarize, we have generated a novel ASXL1\textsuperscript{G643W} mouse model mimicking the most commonly observed \textit{ASXL1} lesion mutation in human MDS and AML patients. Consistent with the co-occurrence of \textit{CEBPA} and \textit{ASXL1} lesions in AML, the ASXL1\textsuperscript{G643W} variant accelerates AML development in the context of CEBPA mutant AML. Finally, the observed resistance towards chemotherapy conferred by the ASXL1\textsuperscript{G643W} variant provides us with an experimental handle for future experiments aimed at its reversal.
References


Figure Legends

Figure 1: Generation of the $Axl1^{G643W}$ mutant mouse line

A. Schematic representation of the three last exons of the $Axl1$ gene. The red star represents the G643W mutation. The "g" inserted in the mutated allele is indicated in red. The protein sequence is in capitals and the asterisk represents the stop codon generated as a result of the frame shift. The blue arrows represent primers used for genotyping (two forward primers have been used to selectively anneal the WT and mutated sequence, respectively).

B. Schematic representation of WT and G643W mutant Asxl1 proteins. The major Plant Homeodomain (PHD) and Additional Sex Combs Homology domain (ASXH) are indicated. Amino acids are numbered. The light blue arrows represent the primers used for quantitative RT-PCRs.

C. The relative expression of $Axl1$ cDNA in $Axl1^{+/+}$, $Axl1^{G643W/+}$ and $Axl1^{G643W/G643W}$ BM cells, determined by quantitative RT-PCR (N=3 mice in each experimental group).

Figure 2: The $ASXL1^{G643W}$ variant has minimal impact on normal hematopoiesis

A. FACS-based analysis of the peripheral blood of 6-months old $Axl1^{+/+}$, $Axl1^{G643W/+}$ and $Axl1^{G643W/G643W}$ mice (N>11 mice in each experimental group). The relative distribution of B-cells (B220), T-cells (CD3), neutrophilic granulocytes (Mac1-Gr1) and other monocyctic/granulocytic cells (Mac1) was analyzed.

B. FACS-based analysis of the peripheral blood of 18-months old $Axl1^{+/+}$, $Axl1^{G643W/+}$ and $Axl1^{G643W/G643W}$ mice (N>5 mice in each experimental group). The relative distribution of B-cells (B220), T-cells (CD3), neutrophilic granulocytes (Mac1-Gr1) and other monocyctic/granulocytic cells (Mac1) was analyzed.

C. Representative FACS profiles of the data from A-B with the gating strategy indicated. The FACS profile represents an $Axl1^{+/+}$ control mouse. See Supplementary Figure S1 for representative FACS plots of $Axl1^{G643W/+}$ and $Axl1^{G643W/G643W}$ animals.
D. FACS-based analysis of bone marrow HSCs and MPPs subsets in 6-months old 
\( Asx1^{+/+} \), \( Asx1^{G643W/+} \) and \( Asx1^{G643W/G643W} \) mice (N>5 mice in each experimental 
group).

E. Representative FACS profiles of the data from D with the gating strategy 
indicated. The FACS profile represents an \( Asx1^{+/+} \) control mouse. See 
Supplementary Figure S1 for representative FACS plots of \( Asx1^{G643W/+} \) and 
\( Asx1^{G643W/G643W} \) animals.

F. Lineage distribution of mature BM subsets in 6-months old \( Asx1^{+/+} \), 
\( Asx1^{G643W/+} \) and \( Asx1^{G643W/G643W} \) mice (N>5 mice in each experimental 
group). The relative distribution of B-cells (B220), T-cells (CD3), neutrophilic 
granulocytes (Mac1-Gr1) and other monocytic/granulocytic cells (Mac1) was 
analyzed.

G. Spleen weights of 18-months old \( Asx1^{+/+} \), \( Asx1^{G643W/+} \) and \( Asx1^{G643W/G643W} \) 
mice (N>5 mice in each experimental group).

H. Competitive BM transplantation of 1:1 mixtures of CD45.2 donor BM cells 
from 6-months old \( Asx1^{+/+} \), \( Asx1^{G643W/+} \) or \( Asx1^{G643W/G643W} \) mice and CD45.1 
competitor cells into lethally irradiated CD45.1 recipient mice. The ratio of 
CD45.2 to CD45.1 in peripheral blood is depicted (N>7 mice in each experimental 
group).

**Figure 3: The ASXL1\(^{G643W}\) variant accelerate CEBPA mutant AML**

A. Schematic outline of the experiment. Briefly, BM was harvested from mice 
with different genotypes and then transplanted into cohorts of irradiated 
recipients. Three weeks after the transplant, mice were injected with plpC and 
subsequently observed for signs of disease development over a period of 60 
weeks.

B. Kaplan-Meyer survival curve of transplanted mice. The arrow indicates the 
time point for injection with plpC. We used a Log-rank (Mantel-Cox) test to 
determine statistical significance (N>7 mice in each experimental group).

C. Giemsa staining of \( Asx1^{+/+}; Cebpa^{3p30} \) or \( Asx1^{G643W/G643W}; Cebpa^{3p30} \) 
leukemic blasts isolated from the BM of moribund mice. A normal aged-matched mouse 
was included as a control.
D. FACS analysis of Asxl1+/+; CebpaΔ/p30 or Asxl1G643W/G643W; CebpaΔ/p30 leukemic blast isolated from transplanted mice. The plot shows the amount of donor-derived c-Kit positive cells. A normal aged-matched mouse was included as a control.

E. Quantification of the data from (D) (N=3 mice in each experimental group).

Figure 4: The ASXL1G643W variant affects the expression of leukemia relevant pathways

A. Volcano Plot depicting gene expression changes in Asxl1G643W/G643W; CebpaΔ/p30 versus Asxl1+/+; CebpaΔ/p30 leukemic blasts.

B-C. GSEA plots for selected gene sets which are either up-regulated (B) or down-regulated (C) in Asxl1G643W/G643W; CebpaΔ/p30 versus Asxl1+/+; CebpaΔ/p30 leukemic blasts.

D. Boxplots showing the ChIP-signal levels of selected marks surrounding the TSS (+/- 500 bp) for genes that are either up- or down-regulated in Asxl1G643W/G643W; CebpaΔ/p30 versus Asxl1+/+; CebpaΔ/p30 leukemic blasts (Supplementary Table S1). The data is derived from a previous CEBPA mutant (CebpaΔ/p30, Asxl1 WT) dataset. Gene expression levels as determined by RNA-seq are also indicated. Up: upregulated genes in Asxl1G643W/G643W vs. Asxl1+/+ leukemic blasts (FDR 0.05, log2FC > 0, baseMean >10, n=105), down: downregulated genes in Asxl1G643W/G643W vs. Asxl1+/+ (FDR 0.05, log2FC < 0, baseMean >10, n=201), neutral: neutral genes (-0.01 < log2FC < 0.01, baseMean >10, n=165) in Asxl1G643W/G643W vs. Asxl1+/+: P-values levels < 0.05 (*), 0.01(**), 0.001(***) are indicated

Figure 5: The ASXL1G643W variant is associated with increased resistance to chemotherapy

A. Schematic outline of the chemotherapy treatment set-up. Briefly, sub-lethally irradiated recipient mice were transplanted with BM cells harvested from leukemic mice. 21 days after transplant, recipient mice were treated with standard induction chemotherapy for 5 days. Three days later, blood was harvested for analysis and leukemic mice were subsequently monitored for disease development.
B. Analysis of peripheral leukemic numbers in mice after chemotherapy/vehicle treatment. Each data point represents the fold change difference between the vehicle-treated and chemotherapy-treated groups for a given clone (n=12 mice per clone). The two different genotypes, Asxl1+/+; CebpaAp30 and Asxl1G643W/G643W CebpaAp30, were represented by 4 and 3 clones, respectively. The responses of individual clones are indicated in Supplementary Figure S4. A one-tailed Mann-Whitney U test was used to assess statistical significance.

C. Kaplan-Meyer survival curves of leukemic mice after chemotherapy/vehicle treatment. The data represent the aggregate of two different leukemic clones (n=6 recipients of each clone, clones 1-2 and 5-6) for each of the two different genotypes (Asxl1+/+; CebpaAp30 and Asxl1G643W/G643W; CebpaAp30).
Figure 1

A

- Diagram showing gene structure with exons labeled 11, 12, and 13.
- DNA sequence: a ggg ggg ggg tgg ccc ggg tgg atc cgg cag tgg ggc cat cga tga.
- G G G W P G W I R Q W G H R *

B

- Comparison of WT (wild type) and ASXL1<sup>G643W</sup> proteins.
- WT: 1514 amino acids.
- ASXL1<sup>G643W</sup>: 654 amino acids.
- Gray box represents ASXH domain.
- Blue box represents PHD domain.

C

- Bar chart showing Asx/1 expression levels.
- Bars for Asx/1, G643W, and G643W/G643W samples.
- Relative expression values on the y-axis range from 0.0000 to 0.006.

Figure 3

A. Bone Marrow harvest and freezing
   Transplant and Cre activation
   Follow disease development

B. Survival of transplanted mice
   - Asxl1^{+/+}; Cebpa^{1/p30}
   - Asxl1^{G643W}; Cebpa^{1/p30}
   - Asxl1^{G643W/G643W}; Cebpa^{1/p30}
   - Asxl1^{+/+}; Cebpa^{1/+}
   - Asxl1^{G643W}; Cebpa^{1/+}
   - Asxl1^{G643W/G643W}; Cebpa^{1/+}

C. Normal BM
   Asxl1^{+/+}; Cebpa^{1/p30}
   Asxl1^{G643W/G643W}; Cebpa^{1/p30}

D. Normal BM
   Singlets; CD45.1^+; CD45.2^-
   c-Kit^+
   Mac-1

E. c-Kit^+ (Percent of donor-derived)
   p = 0.0657

Genotype
Supplementary information:

The ASXL1-G643W variant accelerates the development of CEBPA mutant Acute Myeloid Leukemia

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Content:
Supplemental Methods
Four Supplemental Figures
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Supplemental Methods

Additional information regarding the generation of the Asxl1G643W knock-in mice
The two Asxl1 target sequences (5'-GGCCACCACT GCCATCGGAG-3' and 5'-GTGGTAACCT CTCGCCCTC-3') were designed using the online tool ([https://gt-scan.csiro.au](https://gt-scan.csiro.au)). In order to generate the two guide RNA-expressing constructs, two pairs of complementary oligos with overhangs (5'-ACACCGGCCCA CCACTGCCAT CGGAGG-3' and 5'-AAAACCTCCG ATGGCAGTGG TGGCCG-3', 5'-ACACCGTGTT AACCTTCGTC CCCTCG-3' and 5'-AAAACGAGGG GCGAGAGGTT ACCACG-3') were annealed and cloned into the pSpCas9n-2A-Puro vector (Addgene #48141) using the BsbI restriction site as previously described. The sequence of the 141-mer ssDNA template is the following: (5'-AGGACCCTCG CAGACATTAA AGCCCGTGCT TTGCAGGCTC GGGAGCGAG AGGTTACCAC TGCAATCGAG AGACGGCCAC CACTGCCATT GGGGAGGG GGTGACCCGG GTGGATCCGG CAGTGGGGCC ATCGATGAGG G-3').

The following primers were used for genotyping: Asxl1wtF (5'-GGCCCGGGTG GATCC-3'), Asxl1mutF (5'-GGCCCGGGTG GAGGT-3') and Asxl1CommonR (5'-ACTGGAGTTT GGGAGGACAG-3').

Additional information regarding in vivo AML development
To assess the impact of the Asxl1G643W allele on the development of CEBPA mutant AML, we used our Asxl1G643W knock-in, the Cebpa0/+, the Cebpa0/+ and Mx1-Cre lines to generate Asxl1G643W / Cebpa mutant compound lines. The following genotypes were used for experiments: Asxl1+/+; Cebpa0/+, Asxl1G643W/+, Cebpa0/+, Asxl1G643W/+, Cebpa0/+, Asxl1+/+, Cebpa0/p30, Mx1Cre, Asxl1G643W/+, Cebpa0/p30, Mx1Cre and Asxl1G643W/ , Cebpa0/p30, Mx1Cre.

Competitive transplant
BM cells were collected, filtered and counted. 500,000 live and nucleated donor cells (Asxl1+/+, Asxl1G643W/+ and Asxl1G643W/G643W; CD45.2) and 500,000 competitor cells (Asxl1+/+, CD45.1) were mixed and transplanted into cohorts of eight lethally irradiated recipients. Recipient mice were analysed four months later for blood
chimerism. For the analysis, the output CD45.2/CD45.1 ratios were divided by the input ratios of the injection mix to obtain output-to-input ratios.

*Additional information regarding flow cytometry analysis and cell sorting*

For blood analysis, the following antibody cocktail was used: CD3-FITC, B220-PerCP, Mac1-APC and Gr1-PE (BD Bioscience).

For bone marrow analysis, the following antibodies were used: CD45.1-FITC and CD45.2-PE (BD Bioscience), Flt3-PE-CF594 (e-Bioscience), CD150-BV650 (e-Bioscience), Mac1-PE-Cy5 (e-Bioscience), Gr1-PE-Cy5 (BD Bioscience), B220-PE-Cy5 (BD Bioscience), CD3-PE-Cy5 (BD Bioscience), Ter119-PE-Cy5 (BD Bioscience), Sca1-APC (BD Bioscience), CD48-PE-CF7 (e-Bioscience), c-Kit-Alexa780 (e-Bioscience), Mac1-APC (BD Bioscience), Gr1-PE (BD Bioscience), Ter119-FITC (BD Bioscience), FcgRII/III-Alexa700 (Invitrogen). To assess BM chimerism in the transplantation experiments, blood or BM cells were stained with CD45.1-FITC and CD45.2-PE (both BD Bioscience).

The following marker combinations were used to define the analysed populations:
- LT-HSCs (Ter119-, B220-, CD3-, Mac1-, Gr1-, Sca1+, c-Kit+, Flt3-, CD48-, CD150+);
- ST-HSCs (Ter119-, B220-, CD3-, Mac1-, Gr1-, Sca1+, c-Kit+, Flt3-, CD48-, CD150-);
- MPP2 (Ter119-, B220-, CD3-, Mac1-, Gr1-, Sca1+, c-Kit+, Flt3-, CD48+, CD150+);
- MPP3 (Ter119-, B220-, CD3-, Mac1-, Gr1-, Sca1+, c-Kit+, Flt3-, CD48+, CD150-);
- MPP4 (Ter119-, B220-, CD3-, Mac1-, Gr1-, Sca1+, c-Kit+, Flt3+);
- CFUs (Ter119-, B220-, CD3-, Mac1-, Gr1-, c-Kit+, Sca1-, CD105+, CD150+);
- GMPs (Ter119-, B220-, CD3-, Mac1-, Gr1-, c-Kit+, Sca1-, FCgRII/III+);
- MkPs (Ter119-, B220-, CD3-, Mac1-, Gr1-, c-Kit+, Sca1-, CD41+).

For assessment of myeloid progenitor populations in leukemic animals, the following strategy was applied: BM cells isolated as described above were incubated with anti-CD45.2-biotin (BD Pharmingen), washed once and stained with the following antibody cocktail: Mac1-FITC (BD Pharmingen), CD41-PE (eBioscience), Gr1-PECy5 (Invitrogen), B220-PE-Cy5 (eBioscience), CD3e-PE-Cy5 (eBioscience), Sca1-PerCP-Cy5.5 (eBioscience), CD105-PE-Cy7 (BioLegend), CD150-APC (BioLegend), FcgRII/III-Alexa700 (Invitrogen), c-Kit-APC-eFlour780 (Invitrogen), CD45.1-eFlour450 (eBioscience), Streptavidin-Q-dots655 (Life Technologies). Donor-derived cells were gated as CD45.1·CD45.2+. 
Cell sorting was performed using a FACSAriaI whereas analytical stains were analysed using LSRII or FACSCanto instruments (all BD Biosciences). All analyses were carried out using the FlowJo software (BD Bioscience).

**Quantitative reverse transcription PCR**

RNA was extracted using the RNAeasy Mini kit (Qiagen) following the manufacturer’s instructions. cDNA was synthesized using the ProtoScript cDNA synthesis kit (New England Biolabs). Real-time PCR was performed in a LightCycler 480 (Roche) using the SYBR Green I PCR Master Mix (Roche). The following primers were used: mAsxl1F (5’-CAGCCGCTA AAGAGGAGCC-3’), (5’-TCCGGGGGCA TATCTGTAA -3’), mTraipF (5’- CAAAGTGTGACCAGGAGATCA-3), mTraipR (5’-CTCATTGTCGCCGGAGG-3’), mFhl3F (5’-CTCGTCAAAGTGCCAGGAAT-3’), mFhl3R (5’-CCAGTGTGCCTGGCTTTGAT-3’), mClcnkaF (5’-CTGGTCCTGGGCTTCAGC-3’), mClcnkaR (5’-AGAGAGTGCAATGCTCCGC-3’), mAnglt7F (5’-CGTCTTACCACACCAAGACA-3’), mAnglt7R (5’-GCAGCAGTGGATCAGTACCAGCC-3’), mActinF (5’-CTCCAGCGCTTCTCCTCC-3’) and mActinR (5’-TGCTAGGGCT GTGATCTCCT-3’).

**RNA sequencing analysis**

RNA-seq reads were processed with the bcbio RNA-seq pipeline (https://github.com/bcbio/bcbio-nextgen) and the bcbioRNASeq R package (https://github.com/hbc/bcbioRNASeq). Transcript abundance estimates were obtained using Salmon 3, summarized to gene level and imported into R using tximport 4. Differential gene expression analysis between Asxl1+/+; CebpaD/p30 and Asxl1G643W/G643W; CebpaD/p30 AML cells was performed using DESeq2 with standard parameters 5. Shrunken log fold changes were used for visualization in the volcano plot and as ranking metric in gene set enrichment analysis (GSEA). GSEA was carried out using clusterProfiler 6, testing all gene ontology (GO) biological process gene sets containing 15-500 genes. Data has been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE133314.
**ChIP-seq analysis**

ChIP-seq data from CEBPA mutant AML was derived from 7. The mean ChIP enrichment between replicates was calculated using deepTools (v3.2.1) with options (bamCompare --operation mean) 8. The mean bigWig plots were lifted over from mm9 to mm10 using CrossMap (v0.3.4) 9. The heatmaps and line plots were created by deepTools computeMatrix (reference-point --upstream 5000 --downstream 5000 --missingDataAsZero) and plotHeatmap (--boxAroundHeatmaps yes --colorMap Blues --sortUsing mean).

For the promoter analysis, the expressed transcripts, overlapping between RefSeq and Ensembl were kept, and if a gene contained several isoforms, the longest coding sequence per gene was kept. The low-expressed genes were filtered at average of the normalized count values, taken over all samples (baseMean) > 10. Further, the groups of differentially expressed genes were defined at FDR-adjusted p-value cutoff 0.05 and log2 fold change (log2FC) 0. In contrast to differentially expressed genes, the neutral genes were defined by absolute value of log2FC < 0.01. The plots were visualized using R packages ggplot210 and ggpubr (https://CRAN.R-project.org/package=ggpubr).

**Supplemental References**

Supplemental Figures

Supplementary Figure S1

A. Representative FACS analysis of peripheral blood derived from 6-months old AxslITm/+, AxslITmG643W/+ and AxslITmG643W/G643W mice.

B. Representative FACS analysis of bone marrow HSCs and MPPs subsets in 6-months old AxslITm/+, AxslITmG643W/+ and AxslITmG643W/G643W mice.
Supplementary Figure S2 – related to Figure 3
A. FACS analysis of Asxl1<sup>+/+</sup>; Cebpa<sup>D100</sup> or Asxl1<sup>G643W/G643W</sup>; Cebpa<sup>D100</sup> bone marrow isolated from transplanted mice. The plot shows that all c-Kit<sup>+</sup> progenitors are GMPs, irrespectively of the leukemic genotype.
B. Quantification of the data from (A)
Supplementary Figure S3 – related to Figure 4

A. qRT-PCR of selected differentially expressed genes.
B. The ChIPseq signal surrounding differentially expressed genes between Asxl1+/+; Cebpa^{bp10} and Asxl1^{G643W/G643W}; Cebpa^{bp10} leukemic samples represented by line plots. C. The data from (B) represented as a heatmap. Up: upregulated genes in Asxl1^{G643W/G643W} vs. Asxl1^{+/+} leukemic blasts (FDR 0.05, log2FC > 0, baseMean >10, n=105), down: downregulated genes in Asxl1^{G643W/G643W} vs. Asxl1^{+/+} (FDR 0.05, log2FC < 0, baseMean >10, n=201), neutral: neutral genes (-0.01 < log2FC < 0.01, baseMean >10, n=165) in Asxl1^{G643W/G643W} vs. Asxl1^{+/+}.
Supplementary Figure 4 – related to Figure 5

Analysis of leukemic mice after chemotherapy/vehicle treatment indicating the relative numbers of leukemic cells in the blood. The data represent the behavior of seven different leukemic clones (n=12 recipients of each clone, half treated with vehicle and half with chemotherapy). To facilitate comparison of leukemic engraftment between clones, the vehicle samples (PBS) were set to 100 for each clone. The fold reductions in leukemic cell numbers following chemotherapy are shown for each clone. These values constitute the foundation for the data in Figure 5B. Error bars indicate standard errors for the 6 technical replicates for each condition (PBS/chemo) of each clone.
Legends to Supplementary Tables

**Supplementary Table S1 on Excel file**
Differentially expressed genes between \(\text{Asxl1}^{+/+}; \text{Cebp}^{Δp39} \) and \(\text{Asxl1}^{G643W/G643W}; \text{Cebp}^{Δp39} \) AML (adjusted p-value<0.05).

**Supplementary Table S2 on Excel file**
GSEA representing differentially expressed pathways between \(\text{Asxl1}^{+/+}; \text{Cebp}^{Δp39} \) and \(\text{Asxl1}^{G643W/G643W}; \text{Cebp}^{Δp39} \) AML (q-value<0.05).