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Systematic genomic discovery efforts in patients with bone marrow failure due to myelodysplastic syndrome (MDS) has led to the rapid discovery of recurrent somatic genetic alterations underlying these disorders. Remarkably, a large number of these mutations occur in genes whose function is known, or suspected, to be involved in epigenetic regulation of gene transcription. This includes mutations in ASXL1, TET2, and EZH2. The goals of our proposal were to (1) perform functional genetic characterization of these alterations, (2) determine if these alterations are therapeutically targetable, and (3) perform detailed genomic analysis of specific subsets of MDS patients with no known genetic alterations and with severe bone marrow failure to discover additional genetic alterations contributing to MDS pathogenesis. Since funding of this award we have made major progress in (1) understanding the impact of ASXL1 mutations and loss on chromatin (Abdel-Wahab, et al. Cancer Cell 2012), (2) identifying the in vivo biological effects of deletion of Asxl1 and Tet2 alone and in combination with one another (Abdel-Wahab, et al. J Exp Med 2013 (in press)), and (3) identified the genome-wide effects of Asxl1 on transcription (Abdel-Wahab, et al. J Exp Med 2013 (in press) and Abdel-Wahab, O, et al. Leukemia 2013).
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Introduction

Increasing use of genomic discovery efforts in patients with bone marrow failure due to myelodysplastic syndrome (MDS) has led to the rapid discovery of a series of recurrent genetic abnormalities underlying these disorders. Remarkably, a large number of these alterations appear to be in genes whose function is known, or suspected, to be involved in epigenetic regulation of gene transcription. In the last 3 years alone, mutations in the genes TET2, ASXL1, DNMT3a, and EZH2 have all been found to be frequent mutations amongst patients with MDS. Mutations in several of these genes have proven to be important markers of disease outcome with ASXL1 and EZH2 mutations recurrently being identified as adverse prognosticators in MDS patients. Identification of frequent mutations in epigenetic modifiers has also highlighted the fact that a number of these genes encode enzymes and/or result in alterations in enzymatic alterations which may represent novel, tractable therapeutic targets for MDS patients. In this proposal, we originally aimed to identify (a) if mice with genetically engineered deletion of epigenetic modifiers mutated in MDS would serve as valuable murine models of MDS, (b) if mutations in epigenetic modifiers may specifically impact DNA methylation and/or histone post-translational modifications in a manner that is therapeutically targetable, and (c) if additional mutations must exist in patients with specific subsets of MDS with the worst clinical outcome. Since awarding of the proposal, we have made major insights into the epigenomic function of ASXL1 as well as the biological impact of conditional deletion of Asxl1 alone and in combination with other genetic alterations including Tet2 deletions and NRasG12D overexpression. This work has resulted in several publications, multiple oral presentations at national meetings, and has been used as the basis for a proposal recently awarded to me as a Damon Runyon Clinical Investigator Award.

Body

Task 1. “Obtain DoD ACURO approval for the use of animals in the experiments outlined below in Tasks 2 to 4.”

We received approval on 6/27/2013 for initial experiments to generate Asxl1, Tet2, and Ezh2 single and compound knockout mice.

Task 2. “Complete characterization of mice with conditional deletion of Asxl1 alone and Asxl1 combined with Tet2 (Months 1-24) at the work performance site of Memorial Sloan-Kettering Cancer Center.”

We have recently completed generation of mice with deletion of Asxl1, Tet2, or both using multiple differe Cre recombinases. This work has just been accepted for publication in the Journal of Experimental Medicine (Abdel-Wahab, O, Gao, J, Adli, MM, Dey, A, Trimarchi, T, Chung, YR, Kuscu, C, Hricik, T, Ndiaye-Lobry, D, La Fave, LM, Koche, R, Shih, AH, Guryanova, OA, Kim, E, Pandey, S, Shin, JY, Liu, J, Bhatt, PK, Monette, S, Zhao, X, Park, CY, Bernstein, BE, Aifantis, I, Levine, RL. Deletion of Asxl1 Results in Myelodysplasia and Severe Developmental Defects in Vivo. J Exp Med 2013 (in press)) (please see Appendix 2). In brief, we identified the following (please see Appendix 2 for all experimental details and comprehensive data):

- Constitutive loss of Asxl1 results in developmental abnormalities including anopthalmia, microcephaly, cleft palates, and mandibular malformations.
Hematopoietic-specific deletion of Asxl1 results in progressive, multilineage cytopenias and dysplasia in the context of increased numbers of hematopoietic stem/progenitor cells (HSPCs), characteristic features of human MDS. Serial transplantation of Asxl1-null hematopoietic cells results in a lethal myeloid disorder at a shorter latency than primary Asxl1 knockout mice. Asxl1 deletion reduces hematopoietic stem-cell self-renewal, which is restored by concomitant deletion of Tet2, a gene commonly co-mutated with ASXL1 in MDS patients. Compound Asxl1/Tet2 deletion results in an MDS phenotype with hastened death compared to single-gene knockout mice.

Task 3. Continue development of mice with Ezh2 deletion alone and characterize mice with compound deletion of Ezh2/Tet2 and Ezh2/Asxl1 (Months 1-24) at the work performance site of Memorial Sloan-Kettering Cancer Center. We have recently generated mice with Ezh2 deletion in the postnatal compartment (Mx1-cre Ezh2fl/fl) mice and mice with compound deletion of Ezh2 and Asxl1. We are just now beginning to characterize these mice.

Task 4. Determine the epigenetic contribution of Asxl1 and Ezh2 loss to bone marrow failure through Chromatin immunoprecipitation (ChIP) of histone H3 lysine 27 trimethyl (H3K27me3) followed by next-generation sequencing in primary murine hematopoietic cells (Months 1-24) at the work performance site of Memorial Sloan-Kettering Cancer Center.

We have recently completed detailed characterization of the effects of ASXL1 mutations and loss using cell lines (see Appendix 1) and primary cells from knockout mice (see Appendix 2). These results which have been published now in 2 papers (again shown in Appendix 1 and 2) identified the following:

-ASXL1 mutations result in loss of PRC2-mediated histone H3 lysine 27 (H3K27) trimethylation.
-Through integration of microarray data with genome-wide histone modification ChIP-Seq data we identified targets of ASXL1 repression including the posterior HOXA cluster that is known to contribute to myeloid transformation.
-We demonstrated that ASXL1 associates with the Polycomb repressive complex 2 (PRC2), and that loss of ASXL1 in vivo collaborates with NRASG12D to promote myeloid leukemogenesis.
-Asxl1 loss in vivo results in a global reduction of H3K27 trimethylation and dysregulated expression of known regulators of hematopoiesis.
-Combining RNA-seq/ChIP-seq analyses of Asxl1 in hematopoietic cells identified a subset of differentially expressed genes as direct targets of Asxl1.

These findings underscore the importance of Asxl1 in Polycomb-group function, development, and hematopoiesis.

Task 5: Determine the effect of Tet2, Asxl1, and Ezh2 loss to a panel of currently clinically utilized compounds in patients with MDS. Drug panel will include decitabine, 5-azacytidine, lenalidomide, cytarabine, daunorubicin, HDACi (vorinostat, romidepsin, panobinostat, AR-42, trichostatin A), HSP-90 inhibitors (AUY-922, PUH-71), and
parthenolide (Months 1-24) at the work performance site of Memorial Sloan-Kettering Cancer Center.

This work is just now underway.

**Task 5:** Perform candidate gene and exome sequencing on DNA samples from 20 MDS patients with ASXL1 mutations alone (Months 1-6) at the work performance site of Memorial Sloan-Kettering Cancer Center.

This work is just now underway.

**Task 6:** Perform candidate gene and exome sequencing on DNA samples from 40 patients with MDS accompanied by moderate to severe bone marrow fibrosis (Months 1-6) at the work performance site of Memorial Sloan-Kettering Cancer Center.

This work is just now underway.

**Task 7:** Present findings at national meetings and publish in peer-reviewed journals (Month 6-36).

I have given 5 presentations at national meetings on the work performed with funding from this award in the last year (see list of presentations in **Reportable Outcomes** below).

**Key Research Accomplishments**

- Developed and published the first conditional knockout mouse for Asxl1 as well as the first murine model with combined Asxl1 and Tet2 deletion. We believe these models are valuable genetically-accurate murine models of acquired bone marrow failure.
- Confirmed important development functions for Asxl1 with observations that germline deletion of Asxl1 including anophthalmia, microcephaly, cleft palates, and mandibular malformations. This matches human data identifying critical functions for Asxl1 germline mutations in developmental disorder Bohring-Opitz syndrome.
- Identified that hematopoietic-specific deletion of Asxl1 results in progressive, multilineage cytopenias and dysplasia in the context of increased numbers of hematopoietic stem/progenitor cells (HSPCs), characteristic features of human MDS.
- Identified that serial transplantation of Asxl1-null hematopoietic cells results in a lethal myeloid disorder at a shorter latency than primary Asxl1 knockout mice.
- Identified that Asxl1 deletion reduces hematopoietic stem-cell self-renewal, which is restored by concomitant deletion of Tet2, a gene commonly co-mutated with ASXL1 in MDS patients.
- Identified that compound Asxl1/Tet2 deletion results in an MDS phenotype with hastened death compared to single-gene knockout mice.
- Identified ASXL1 mutations result in loss of PRC2-mediated histone H3 lysine 27 (H3K27) tri-methylation.
• Through integration of microarray data with genome-wide histone modification ChIP-Seq data we identified targets of ASXL1 repression including the posterior HOXA cluster that is known to contribute to myeloid transformation.
• Demonstrated that ASXL1 associates with the Polycomb repressive complex 2 (PRC2), and that loss of ASXL1 in vivo collaborates with NRASG12D to promote myeloid leukemogenesis.
• Combined RNA-seq/ChIP-seq analyses of Asxl1 in hematopoietic cells to identify a subset of differentially expressed genes as direct targets of Asxl1.

Reportable Outcomes
Manuscripts:


Abstracts:

Presentations:
2012 American Society of Hematology, Biology of MDS Oral Session, Atlanta, GA.
2013 Clinical Translation of Epigenetics in Cancer Therapy, Asheville NC
Development of novel genetically engineered murine models:
Developed the following murine models of bone marrow failure, all of which are currently published:

Informatics:
- Published gene expression microarray data of knockdown of ASXL1 using shRNA in a variety of ASXL1 wildtype human leukemia cell lines (Abdel-Wahab, O, et al. Cancer Cell 2012). This data has been deposited in a public gene expression repository (*)
- Published genome-wide localization of Asxl1 by chromatin immunoprecipitation followed by next-generation sequencing (ChIP-Seq) technology (Abdel-Wahab, O, et al. J Exp Med 2013 (in press)). This sequencing data will soon be deposited in a public gene expression repository.
- Published RNA-Sequencing data of the effects of Asxl1 deletion in the hematopoietic stem cells (lineage-negative Sca1+ c-KIT+ cells) and myeloid progenitor cells (lineage-negative Sca1- c-KIT+) in one year-old Mx1-cre Asxl1fl/fl and littermate controls (Abdel-Wahab, O, et al. J Exp Med 2013 (in press)). This sequencing data will soon be deposited in a public gene expression repository.
- Published RNA-Sequencing data of the effects of Asxl1 deletion, Tet2 deletion, and combined Asxl1/Tet2 double deletion in the hematopoietic stem cells (lineage-negative Sca1+ c-KIT+ cells) of 6-week-old Mx1-cre Asxl1fl/fl and littermate controls (Abdel-Wahab, O, et al. J Exp Med 2013 (in press)). This sequencing data will soon be deposited in a public gene expression repository.

Funding applied for based on this work:
- Applied for and successfully received a Damon Runyon Clinical Investigator Award to study altered histone modifiers in myeloid malignancies based on all of the above work.

Conclusion
Advancement in sequencing technologies has led to the rapid discovery of recurrent genetic mutations in patients with MDS. Despite this, the functional importance of these mutations in the pathogenesis of MDS as well as the potential importance to the therapy of patients with MDS was previously not well characterized. Since award of this grant we have identified a novel role for ASXL1 in PRC2 function, generated multiple novel genetically-engineered mouse models including some of the first genetically accurate models of MDS. In the ongoing work in this award, we hope to understand the therapeutic implications of mutations in epigenetic modifiers. We will utilize hematopoietic cells from our genetically-engineered mouse models to screen for compounds which specifically target cells bearing genetic defects
common in MDS patients. We have already demonstrated the proof-of-concept of this approach with the observation that Tet2-deficient murine hematopoietic stem cells are preferentially sensitive to HDAC inhibition compared with their wildtype counterparts. We also hope to perform genetic analyses of MDS patients with bone marrow fibrosis (as outlined in the initial award).

References
None

Appendices (please see next page)
ASXL1 Mutations Promote Myeloid Transformation through Loss of PRC2-Mediated Gene Repression

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SUMMARY

Recurrent somatic ASXL1 mutations occur in patients with myelodysplastic syndrome, myeloproliferative neoplasms, and acute myeloid leukemia, and are associated with adverse outcome. Despite the genetic and clinical data implicating ASXL1 mutations in myeloid malignancies, the mechanisms of transformation by ASXL1 mutations are not understood. Here, we identify that ASXL1 mutations result in loss of polycomb repressive complex 2 (PRC2)-mediated histone H3 lysine 27 (H3K27) tri-methylation. Through integration of microarray data with genome-wide histone modification ChIP-Seq data, we identify targets of ASXL1 repression, including the posterior HOXA cluster that is known to contribute to myeloid transformation. We demonstrate that ASXL1 associates with the PRC2, and that loss of ASXL1 in vivo collaborates with NRASG12D to promote myeloid leukemogenesis.

INTRODUCTION

Recent genome-wide and candidate-gene discovery efforts have identified a series of novel somatic genetic alterations in patients with myeloid malignancies with relevance to pathogenesis, prognostication, and/or therapy. Notably, these include mutations in genes with known or putative roles in the epigenetic regulation of gene transcription. One such example is the mutations in the gene Addition of sex combs-like 1 (ASXL1), which is mutated in ≈15%–25% of patients with myelodysplastic

Significance

Mutations in genes involved in modification of chromatin have recently been identified in patients with leukemias and other malignancies. Here, we demonstrate a specific role for ASXL1, a putative epigenetic modifier frequently mutated in myeloid malignancies, in polycomb repressive complex 2 (PRC2)-mediated histone H3 lysine 27 (H3K27) tri-methylation. Through integration of microarray data with genome-wide histone modification ChIP-Seq data, we identify targets of ASXL1 repression, including the posterior HOXA cluster that is known to contribute to myeloid transformation. We demonstrate that ASXL1 associates with the PRC2, and that loss of ASXL1 in vivo collaborates with NRASG12D to promote myeloid leukemogenesis.
syndrome and ≈10%–15% of patients with myeloproliferative neoplasms and acute myeloid leukemia (Abdel-Wahab et al., 2011; Bejar et al., 2011; Gelsi-Boyer et al., 2009). Clinical studies have consistently indicated that mutations in ASXL1 are associated with adverse survival in myelodysplastic syndrome and acute myeloid leukemia (Bejar et al., 2011; Metzeler et al., 2011; Pratcorona et al., 2012; Thol et al., 2011).

ASXL1 is the human homolog of Drosophila Additional sex combs (Asx). Asx deletion results in a homeotic phenotype characteristic of both Polycomb (PcG) and Trithorax group (TxG) gene deletions (Gaebler et al., 1999), which led to the hypothesis that Asx has dual functions in silencing and activation of homeotic gene expression. In addition, functional studies in Drosophila suggested that Asx encodes a chromatin-associated protein with similarities to PcG proteins (Sinclair et al., 1998). More recently, it was demonstrated that Drosophila Asx forms a complex with the chromatin deubiquitinase Calypso to form the Polycomb-repressive deubiquitinase (PR-DUB) complex, which removes monoubiquitin from histone H2A at lysine 119. The mammalian homolog of Calypso, BAP1, directly associates with ASXL1, and the mammalian BAP1-ASXL1 complex was shown to possess deubiquitinase activity in vitro (Scheuermann et al., 2010).

The mechanisms by which ASXL1 mutations contribute to myeloid transformation have not been delineated. A series of in vitro studies in non-hematopoietic cells have suggested a variety of activities for ASXL1, including physical cooperativity with HP1α and LSD1 to repress retinoic acid-receptor activity and interaction with peroxisome proliferator-activated receptor gamma (PPARγ) to suppress lipogenesis (Cho et al., 2006; Lee et al., 2010; Park et al., 2011). In addition, a recent study using a gene-trap model reported that constitutive disruption of Asxl1 results in significant perinatal lethality; however, the authors did not note alterations in stem/progenitor numbers in surviving mice (Fisher et al., 2010a, 2010b). Importantly, the majority of mutations in ASXL1 occur as nonsense mutations and insertions/deletions proximal or within the last exon prior to the highly conserved plant homeo domain. It is currently unknown whether mutations in ASXL1 might confer a gain-of-function due to expression of a truncated protein, or whether somatic loss of ASXL1 in hematopoietic cells leads to specific changes in epigenetic state, gene expression, or hematopoietic functional output. The goals of this study were to determine the effects of ASXL1 mutations on ASXL1 expression as well as the transcriptional and biological effects of perturbations in ASXL1 which might contribute toward myeloid transformation.

RESULTS

ASXL1 Mutations Result in Loss of ASXL1 Expression

ASXL1 mutations in patients with myeloproliferative neoplasms, myelodysplastic syndrome, and acute myeloid leukemia most commonly occur as somatic nonsense mutations and insertion/deletion mutations in a clustered region adjacent to the highly conserved PHD domain (Abdel-Wahab et al., 2011; Gelsi-Boyer et al., 2009). To assess whether these mutations result in loss of ASXL1 protein expression or in expression of a truncated isoform, we performed western blots using N- and C-terminal anti-ASXL1 antibodies in a panel of human myeloid leukemia cell lines and primary acute myeloid leukemia samples, which are wild-type or mutant for ASXL1. We found that myeloid leukemia cells with homozygous frameshift/nonsense mutations in ASXL1 (NOMO1 and KBM5) have no detectable ASXL1 protein expression (Figure 1A). Similarly, leukemia cells with heterozygous ASXL1 mutations have reduced or absent ASXL1 protein expression. Western blot analysis of ASXL1 using an N-terminal anti-ASXL1 antibody in primary acute myeloid leukemia samples revealed reduced/absent full-length ASXL1 expression in samples with ASXL1 mutations compared to ASXL1 wild-type samples (Figure S1A available online). Importantly, we did not identify truncated ASXL1 protein products in mutant samples using N- or C-terminal directed antibodies in primary acute myeloid leukemia samples or leukemia cell lines. Moreover, expression of wild-type ASXL1 cDNA or cDNA constructs bearing leukemia-associated mutant forms of ASXL1 revealed reduced stability of mutant forms of ASXL1 relative to wild-type ASXL1, with more rapid degradation of mutant ASXL1 isoforms following cycloheximide exposure (Figure S1B). These data are consistent with ASXL1 functioning as a tumor suppressor with loss of ASXL1 protein expression in leukemia cells with mutant ASXL1 alleles.

ASXL1 Knockdown in Hematopoietic Cells Results in Upregulated HOXA Gene Expression

Given that ASXL1 mutations result in loss of ASXL1 expression, we investigated the effects of ASXL1 knockdown in primary hematopoietic cells. We used a pool of small interfering RNAs (siRNA) to perform knockdown of ASXL1 in primary human CD34+ cells isolated from umbilical cord blood. ASXL1 knockdown was performed in triplicate and confirmed by qRT-PCR analysis (Figure 1B), followed by gene-expression microarray analysis. Gene-set enrichment analysis (GSEA) of this microarray data revealed a significant enrichment of genes found in a previously described gene expression signature of leukemic cells from bone marrow of MLL-AF9 knock-in mice (Kumar et al., 2009), as well as highly significant enrichment of a gene signature found in primary human cord blood CD34+ cells expressing NUP98-HOXA9 (Figure S1C and Table S1) (Takeda et al., 2006). Specifically, we found that ASXL1 knockdown in human primary CD34+ cells resulted in increased expression of 145 genes out of the 279 genes, which are overexpressed in the MLL-AF9 gene expression signature (p < 0.05, FDR < 0.05). These gene expression signatures are characterized by increased expression of posterior HOXA cluster genes, including HOXAS-9.

In order to ascertain whether loss of ASXL1 was associated with similar transcriptional effects in leukemia cells, we performed short hairpin RNA (shRNA)-mediated stable knockdown of ASXL1 in the ASXL1-wild-type human leukemia cell lines UKE1 (Figures 1C and 1D) and SET2 (Figure 1D) followed by microarray and qRT-PCR analysis. Gene expression analysis in UKE-1 cells expressing ASXL1 shRNA compared to control cells revealed significant enrichment of the same HOXA gene expression signatures as were seen with ASXL1 knockdown in CD34+ cells (Figure 1C and Table S2). Upregulation of 5′ HOXA genes was confirmed by qRT-PCR in UKE1 (Figure 1D) cells and by western blot analysis (Figure 1D) in SET2 cells expressing ASXL1 shRNA compared to control. Quantitative mRNA profiling (Nanostring nCounter) of the entire HOXA cluster revealed
upregulation of multiple HOXA members, including HOXA5, 7, 9, and 10, in SET2 cells with ASXL1 knockdown compared to control cells (Figure S1D). These results indicate consistent upregulation of HOXA gene expression following ASXL1 loss in multiple hematopoietic contexts.

**ASXL1 Forms a Complex with BAP1 in Leukemia Cells, but BAP1 Loss Does Not Upregulate HoxA Gene Expression in Hematopoietic Cells**

Mammalian ASXL1 forms a protein complex in vitro with the chromatin deubiquitinase BAP1, which removes monoubiquitin from histone H2A at lysine 119 (H2AK119) (Scheuermann et al., 2010). In *Drosophila* loss of either Asx or Calypso resulted in similar effects on genome-wide H2AK119 ubiquitin levels and on target gene expression. Recent studies have revealed recurrent germline and somatic loss-of-function BAP1 mutations in mesothelioma and uveal melanoma (Bott et al., 2011; Harbour et al., 2010; Testa et al., 2011). However, we have not identified BAP1 mutations in patients with myeloproliferative neoplasms or acute myeloid leukemia (O.A.-W., J.P.P., and R.L.L., unpublished data). Co-immunoprecipitation studies revealed an association between ASXL1 and BAP1 in human myeloid leukemia cells wild-type for ASXL1 but not in those cells mutant for ASXL1 due to reduced/absent ASXL1 expression (Figure 2A). Immunoprecipitation of FLAG-tagged wild-type ASXL1 and FLAG-tagged leukemia-associated mutant forms of ASXL1 revealed reduced interaction between mutant forms of ASXL1 and endogenous BAP1 (Figure S2A). Despite these findings, BAP1 knockdown did not result in upregulation of HOXA5 and HOXA9 in UKE1 cells, although a similar extent of ASXL1 knockdown in the same cells reproducibly increased HOXA5 and HOXA9 expression (Figure 2B). We obtained similar results with knockdown of Asxl1 or Bap1 in the Ba/F3 murine hematopoietic cell line (Figure 2C). In Ba/F3 cells, knockdown of Asxl1 resulted in upregulated Hoxa9 gene expression commensurate with the level of Asxl1 downregulation, whereas knockdown of Bap1 does not impact Hoxa expression (Figure 2C). ASXL1 knockdown in SET-2 cells failed to reveal an effect of ASXL1...
loss on H2AK119Ub levels as assessed by western blot of purified histones from shRNA control and ASXL1 knockdown cells (Figure S2B). By contrast, SET2 cells treated with MG132 (25 μM) had a marked decrease in H2AK119Ub, as has been previously described (Dantuma et al., 2006). These data suggest that ASXL1 loss contributes to myeloid transformation through a BAP1-independent mechanism.

Loss of ASXL1 Is Associated with Global Loss of H3K27me3

The results described above led us to hypothesize that ASXL1 loss leads to BAP1-independent effects on chromatin state and on target gene expression. To assess the genome-wide effects of ASXL1 loss on chromatin state, we performed chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) for histone modifications known to be associated with PcG [histone H3 lysine 27 trimethylation (H3K27me3)] or TxG activity [histone H3 lysine 4 trimethylation (H3K4me3)] in UKE1 cells expressing empty vector or two independent validated shRNAs for ASXL1. ChIP-Seq data analysis revealed a significant reduction in genome-wide H3K27me3 transcriptional start site occupancy with ASXL1 knockdown compared to empty vector (p = 2.2 × 10⁻¹⁶; Figure 3A). Approximately 20% of genes (n = 4,686) were initially marked by H3K27me3 in their promoter regions (defined as 1.5 kb downstream and 0.5 kb upstream of the transcriptional start site). Among these genes, ~27% had a 2-fold reduction in H3K27me3 (n = 1,309) and ~66% had a 1.5-fold reduction in H3K27me3 (n = 3,092), respectively, upon ASXL1 knockdown. No significant effect was seen on H3K4me3 transcriptional start site occupancy with ASXL1 depletion (Figure 3A). We next evaluated whether loss of ASXL1 might be associated with loss of H3K27me3 globally by performing western blot analysis on purified histones from UKE1 cells transduced with empty vector or shRNAs for ASXL1 knockdown. This analysis revealed a significant decrease in global H3K27me3 with ASXL1 loss (Figure 3B), despite preserved expression of the core polycomb repressive complex 2 (PRC2) members EZH2, SUZ12, and EED. Similar effects on total H3K27me3 levels were seen following Asxl1 knockdown in Ba/F3 cells (Figure S3A). These results demonstrate that ASXL1 depletion leads to a marked reduction in genome-wide H3K27me3 in hematopoietic cells.
Figure 3. ASXL1 Loss Is Associated with Loss of H3K27me3 and with Increased Expression of Genes Poised for Transcription
(A) ASXL1 loss is associated with a significant genome-wide decrease in H3K27me3 as illustrated by box plot showing the 25th, 50th, and 75th percentiles for H3K27me3 and H3K4me3 enrichment at transcription start sites in UKE1 cells treated with an empty vector or shRNAs directed against ASXL1. The whiskers indicate the most extreme data point less than 1.5 interquartile range from box and the red bar represents the median.
(B) Loss of ASXL1 is associated with a global loss of H3K27me3 without affecting PRC2 component expression as shown by western blot of purified histones from cells with UKE1 knockdown and western blot for core PRC2 component in whole cell lysates from ASXL1 knockdown UKE1 cells.

Cancer Cell
ASXL1 Loss Inhibits PRC2 in Myeloid Leukemia
Detailed analysis of ChIP-seq data revealed that genomic regions marked by large H3K27me3 domains in control cells displayed more profound loss of H3K27me3 upon loss of ASXL1. Genome-wide analysis of the ChIP-Seq data from control and ASXL1 shRNA treated cells revealed that the sites that lose H3K27me3 in the ASXL1 knockdown cells were on average ~6.6 kb in length, while the sites that maintained H3K27me3 were on average ~3.1 kb in length (p < 10−15) (Figure S3B). This is visually illustrated by the reduction in H3K27me3 at the posterior HOXA cluster (Figure 3C) and at the HOXB and HOXC loci (Figure S3C). The association of ASXL1 loss with loss of H3K27me3 abundance at the HOXA locus was confirmed by ChIP for H3K27me3 in control and ASXL1 knockdown cells followed by qPCR (ChIP-qPCR) across the HOXA locus (Figure 3D). ChIP-qPCR in control and knockdown cells revealed a modest increase in H2AK119Ub with ASXL1 loss at the HOXA locus (Figure 3D), in contrast to the more significant reduction in H3K27me3. In contrast to the large decrease in H3K27me3 levels at the HOXA locus with ASXL1 knockdown, a subset of loci had much less significant reduction in H3K27me3, in particular at loci whose promoters were marked by sharp peaks of H3K27me3 (Figure S3D). Intersection of gene expression and ChIP-Seq data revealed that genes overexpressed in ASXL1 knockdown cells were simultaneously marked with both activating (H3K4me3) and repressive (H3K27me3) domains in control cells (Figures 3E and 3F). This finding suggests that the transcriptional repression mediated by ASXL1 in myeloid cells is most apparent at loci poised for transcription with bivalent chromatin domains. Indeed, the effects of ASXL1 loss on H3K27me3 occupancy were most apparent at genes whose promoters were marked by the dual presence of H3K27me3 and H3K4me3 (Figure 3F). We cannot exclude the possibility that H3K4me3 and H3K27me3 exist in different populations within the homogeneous cell lines being studied, but the chromatin and gene expression data are consistent with an effect of ASXL1 loss on loci with bivalent chromatin domains (Bernstein et al., 2006; Mikkelsen et al., 2007).

**Enforced Expression of ASXL1 in Leukemic Cells Results in Suppression of HOXA Gene Expression, a Global Increase in H3K27me3, and Growth Suppression**

We next investigated whether reintroduction of wild-type ASXL1 protein could restore H3K27me3 levels in ASXL1 mutant leukemia cells. We stably expressed wild-type ASXL1 in NOMO1 and KBM5 cells, homozygous ASXL1 mutant human leukemia cell lines, which do not express ASXL1 protein (Figure 4A and Figure S4A). ASXL1 expression resulted in a global increase in H3K27me3 as assessed by histone western blot analysis (Figure 4A). Liquid chromatography/mass spectrometry of purified histones in NOMO1 cells expressing ASXL1 confirmed a ~2.5-fold increase in trimethylated H3K27 peptide and significant increases in dimethylated H3K27 in NOMO1 cells expressing ASXL1 compared to empty vector control (Figure 4B). ASXL1 add-back resulted in growth suppression (Figure 4C) and in decreased HOXA gene expression in NOMO1 cells (Figure 4D). ASXL1 add-back similarly resulted in decreased expression of HOXA target genes in KBM5 cells (Figures S4A and S4B). ChIP-qPCR revealed a strong enrichment in ASXL1 binding at the HOXA locus in NOMO1 cells expressing ASXL1, demonstrating that the HOXA locus is a direct target of ASXL1 in hematopoietic cells (Figure 4E).

**ASXL1 Loss Leads to Exclusion of H3K27me3 and EZH2 from the HoxA Cluster Consistent with a Direct Effect of ASXL1 on PRC2 Recruitment**

We next investigated whether the effects of ASXL1 loss on H3K27me3 was due to inhibition of PRC2 recruitment to specific target loci. ChIP-qPCR for H3K27me3 in ASXL1 knockdown or control revealed a loss of H3K27me3 enrichment at the posterior HOXA locus in ASXL1 knockdown (Figures 5A and 5B). We observed a modest, variable increase in H3K4me3 enrichment at the HOXA locus with ASXL1 depletion in SET2 cells (Figure 5C). We similarly assessed H3K27me3 enrichment in primary bone marrow leukemic cells from acute myeloid leukemia patients, wild-type and mutant for ASXL1, which likewise revealed decreased H3K27me3 enrichment across the HOXA cluster in primary acute myeloid leukemia samples with ASXL1 mutations compared to ASXL1-wild-type acute myeloid leukemia samples (Figure 5D).

Given the consistent effects of ASXL1 depletion on H3K27me3 abundance at the HOXA locus, we then evaluated the occupancy of EZH2, a core PRC2 member, at the HOXa locus. ChIP-Seq for H3K27me3 in native SET2 and UKE1 cells identified that H3K27me3 is present with a dome-like enrichment pattern at the 5’ end of the posterior HOXA cluster (Figure 5A); ChIP-qPCR revealed that EZH2 is prominently enriched in this same region in parental SET2 cells (Figure 5E). Importantly, ASXL1 depletion resulted in loss of EZH2 enrichment at the HOXA locus (Figure 5E), suggesting that ASXL1 is required for EZH2 occupancy and for PRC2-mediated repression of the posterior HOXA locus.

See also Figure S3.

(C) Loss of H3K27me3 is evident at the HOXA locus as shown by ChIP-Seq promoter track signals across the HOXA locus in UKE1 cells treated with an EV or shRNA knockdown of ASXL1.

(D) H3K27me3 ChIP-Seq promoter track signals from HOXAS to HOX13 in UKE1 cells treated with shRNA control or one of 2 anti-ASXL1 shRNAs with location of primers used in ChIP-quantitative PCR (ChIP-qPCR) validation. ChIP for H3K27me3 and H2AK119Ub followed by ChIP-qPCR in cells treated with control or ASXL1 knockdown confirms a significant decrease in H3K27me3 at the HOXA locus with ASXL1 knockdown but minimal effects of ASXL1 knockdown on H2AK119Ub levels at the same primer locations.

(E) Integrating gene-expression data with H3K27me3/H3K4me3 ChIP-Seq identifies a significant correlation between alterations in chromatin state and increases in gene expression following ASXL1 loss at loci normally marked by the simultaneous presence of H3K27me3 and H3K4me3 in control cells.

(F) Loss of H3K27me3 is seen at promoters normally marked by the presence of H3K27me3 alone or at promoters co-occupied by H3K27me3 and H3K4me3 in the control state.
ASXL1 Physically Interacts with Members of the PRC2 in Human Myeloid Leukemic Cells

Given that ASXL1 localizes to PRC2 target loci and ASXL1 depletion leads to loss of PRC2 occupancy and H3K27me2, we investigated whether ASXL1 might physically interact with the PRC2 complex in hematopoietic cells. Co-immunoprecipitation studies using an anti-FLAG antibody in HEK293T cells expressing empty vector, hASXL1-FLAG alone, or hASXL1-FLAG plus hEZH2 cDNA revealed a clear co-immunoprecipitation of FLAG-ASXL1 with endogenous EZH2 and with ectopically expressed EZH2 (Figure 6A). Similarly, co-immunoprecipitation of FLAG-ASXL1 revealed physical association between ASXL1 and endogenous SUZ12 in 293T cells (Figure 6A). Immunoprecipitations were performed in the presence of benzozon to ensure that the protein-protein interactions observed were DNA-independent (Figure 6A) (Muntean et al., 2010). We then assessed whether endogenous ASXL1 formed a complex with PRC2 members in hematopoietic cells. We performed IP for EZH2 or ASXL1 followed by western blotting for partner proteins in SET2 and UKE1 cells, which are wild-type for ASXL1, SUZ12, EZH2, and EED. These co-immunoprecipitation assays all revealed a physical association between ASXL1 and EZH2 in SET2 (Figure 6B) and UKE1 cells (Figure S5). By contrast, immunoprecipitation of endogenous ASXL1 did not reveal evidence of protein-protein interactions between ASXL1 and BMI1 (Figure S5). Likewise, immunoprecipitation of BMI1 enriched for PRC1 member RING1A, but failed to enrich for ASXL1, suggesting a lack of interaction between ASXL1 and the PRC1 repressive complex (Figure 6C).

ASXL1 Loss Collaborates with NRasG12D In Vivo

We and others previously reported that ASXL1 mutations are most common in chronic myelomonocytic leukemia and frequently co-occur with N/K-Ras mutations in chronic
myelomonocytic leukemia (Abdel-Wahab et al., 2011; Patel et al., 2010). We therefore investigated the effects of combined NRasG12D expression and Asxl1 loss in vivo. To do this, we expressed NRasG12D in combination with an empty vector expressing GFP alone or one of two different Asxl1 shRNA constructs in whole bone marrow cells and transplanted these cells into lethally irradiated recipient mice. We validated our ability to effectively knock down ASXL1 in vivo by performing qRT-PCR in hematopoietic cells from recipient mice (Figure 7A and Figure S6A). Consistent with our in vitro data implicating the HoxA cluster as an ASXL1 target locus, we noted a marked increase in HoxA9 and HoxA10 expression in bone marrow nucleated cells from mice expressing NRasG12D in combination with validated mASXL1 knockdown vectors compared to mice expressing NRasG12D alone (Figure 7B).

Expression of oncogenic NRasG12D and an empty shRNA vector control led to a progressive myeloproliferative disorder as previously described (Mackenzie et al., 1999). In contrast, expression of NRasG12D in combination with validated mASXL1 knockdown vectors resulted in accelerated myeloproliferation and impaired survival compared with mice transplanted with NRasG12D/EV (median survival 0.8 month for ASXL1 shRNA versus 3 months for control shRNA vector; p < 0.005; Figure 7C). We also noted impaired survival with an independent mASXL1 shRNA construct (p < 0.01; Figure S6B). Mice transplanted with NRasG12D/Asxl1...
shRNA had increased splenomegaly and hepatomegaly compared with *NRasG12D/EV* transplanted mice (Figures 7D and 7E; Figure S6C). Histological analysis revealed a significant increase in myeloid infiltration of the spleen and livers of mice transplanted with *NRasG12D/Asxl1* shRNA (Figure S6D).

Mice transplanted with *NRasG12D/Asxl1* shRNA, but not *NRasG12D/EV*, experienced progressive, severe anemia (Figure 7F). It has previously been identified that expression of oncogenic *K/N-Ras* in multiple models of human/murine hematopoietic systems results in alterations in the erythroid compartment (Braun et al., 2006; Darley et al., 1997; Zhang et al., 2003). We noted an expansion of CD71high/Ter119high erythroblasts in the bone marrow of mice transplanted with *NRasG12D/Asxl1* shRNA (Figure S6D). We also noted increased granulocytic expansion in mice engrafted with *NRasG12D/Asxl1* shRNA positive cells, as shown by the presence of increased neutrophils in the peripheral blood (Figure S6D) and the expansion of Gr1/Mac1 double-positive cells in the bone marrow by flow cytometry (Figure S6F).

Previous studies have shown that hematopoietic cells from mice expressing oncogenic Ras alleles or other mutations that activate kinase signaling pathways do not exhibit increased self-renewal in colony replating assays (Braun et al., 2004; MacKenzie et al., 1999). This is in contrast to the immortalization of hematopoietic cells in vitro seen with expression of *MLL-AF9* (Somervaille and Cleary, 2006) or deletion of *Tet2* (Moran-Crusio et al., 2011). Bone marrow cells from mice with combined over-expression of *NRasG12D* plus *Asxl1* knockdown had increased serial replating (to five passages) compared to bone marrow cells from mice engrafted with *NRasG12D/EV* cells (Figure 7G). These studies demonstrate that Asxl1 loss cooperates with oncogenic *NRasG12D* in vivo.

**DISCUSSION**

The data presented here identify that ASXL1 loss in hematopoietic cells results in reduced H3K27me3 occupancy through inhibition of PRC2 recruitment to specific oncogenic target loci. Recent studies have demonstrated that genetic alterations in the PRC2 complex occur in a spectrum of human malignancies (Bracken and Helin, 2009; Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010). Activating mutations and overexpression of *EZH2* occur most commonly in epithelial malignancies and in lymphoid malignancies (Morin et al., 2010; Varambally et al., 2002). However, there are increasing genetic data implicating mutations that impair PRC2 function in the pathogenesis of myeloid malignancies. These include the loss-of-function mutations in *EZH2* (Abdel-Wahab et al., 2011; Ernst et al., 2010; Nikoloski et al., 2010) and less common somatic loss-of-function mutations in *SUZ12*, *EED*, and *JARID2* (Score
et al., 2012) in patients with myeloproliferative neoplasms, myelodysplastic syndrome, and chronic myelomonocytic leukemia. The data from genetically-engineered mice also support this concept with Ezh2 overexpression models, revealing evidence of promotion of malignant transformation (Herrera-Merchan et al., 2012), and recent studies demonstrate a role for Ezh2 loss in leukemogenesis (Simon et al., 2012). Thus, it appears that alterations in normal PRC2 activity and/or H3K27me3 abundance in either direction may promote malignant transformation.

Our data implicate ASXL1 mutations as an additional genetic alteration that leads to impaired PRC2 function in patients with myeloid malignancies. In many cases, patients present with concomitant heterozygous mutations in multiple PRC2 members or in EZH2 and ASXL1; these data suggest that haploinsufficiency for multiple genes that regulate PRC2 function can cooperate in hematopoietic transformation through additive alterations in PRC2 function.

Many studies have investigated how mammalian PcG proteins are recruited to chromatin in order to repress gene transcription and specify cell fate in different tissue contexts. Recent in silico analysis suggested that ASXL proteins found in animals contain a number of domains that likely serve in the recruitment of chromatin modulators and transcriptional effectors to DNA (Aravind and Iyer, 2012). Data from ChIP and co-immunoprecipitation experiments presented here suggest a specific role for ASXL1 in epigenetic regulation of gene expression by facilitating PRC2-mediated transcriptional repression of known leukemic oncogenes. Thus, ASXL1 may serve as a scaffold for recruitment of the PRC2 complex to specific loci in hematopoietic cells, as has been demonstrated for JARID2 in embryonic stem cells (Landeira et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009).

Recent data suggested that ASXL1 might interact with BAP1 to form a H2A-K119 deubiquitanase (Scheuermann et al., 2010).
However, our data suggest that ASXL1 loss leads to BAP1-independent alterations in chromatin state and gene expression in hematopoietic cells. These data are consonant with recent genetic studies, which have shown that germline loss of BAP1 increases susceptibility to uveal melanoma and mesothelioma (Testa et al., 2011; Wiesner et al., 2011). In contrast, germline loss of ASXL1 is seen in the developmental disorder Bohring-Opitz Syndrome (Hoischen et al., 2011), but has not, to date, been observed as a germline solid tumor susceptibility locus. Whether alterations in H2AK119 deubiquitanse function due to alterations in BAP1 and/or ASXL1 can contribute to leukemogenesis or to the pathogenesis of other malignancies remains to be determined.

Integration of gene expression and chromatin state data following ASXL1 loss identified specific loci with a known role in leukemogenesis that are altered in the setting of ASXL1 mutations. These include the posterior HOXA cluster, including HOXA9, which has a known role in hematopoietic transformation. We demonstrate that ASXL1 normally serves to tightly regulate HOXA gene expression in hematopoietic cells, and that loss of ASXL1 leads to disordered HOXA gene expression in vitro and in vivo. Overexpression of 5’ HOXA genes is a well-described oncogenic event in hematopoietic malignancies (Lawrence et al., 1996), and previous studies have shown that HOXA9 overexpression leads to transformation in vitro and in vivo when co-expressed with MEIS1 (Kroon et al., 1998). Interestingly, ASXL1 loss was not associated with an increase in MEIS1 expression, suggesting that transformation by ASXL1 mutations requires the co-occurrence of oncogenic disease alleles which dysregulate additional target loci. These data and our in vivo studies suggest that ASXL1 loss, in combination with co-occurring oncogenes, can lead to hematopoietic transformation and increased self-renewal. Further studies in mice expressing ASXL1 shRNA or with conditional deletion of Asxl1 alone and in concert with leukemogenic disease alleles will provide additional insight into the role of ASXL1 loss in hematopoietic stem/progenitor function and in leukemogenesis.

Given that somatic mutations in chromatin modifying enzymes (Dalgliesh et al., 2010), DNA methyltransferases (Ley et al., 2010), and other genes involved in epigenetic regulation occur commonly in human cancers, it will be important to use epigenomic platforms to elucidate how these disease alleles contribute to oncogenesis in different contexts. The data here demonstrate how integrated epigenetic and functional studies can be used to elucidate the function of somatic mutations in epigenetic modifiers. In addition, it is likely that many known oncogenes and tumor suppressors contribute, at least in part, to transformation through direct or indirect alterations in the epigenetic state (Dawson et al., 2009). Subsequent epigenomic studies of human malignancies will likely uncover novel routes to malignant transformation in different malignancies, and therapeutic strategies that reverse epigenetic alterations may be of specific benefit in patients with mutations in epigenetic modifiers.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Human leukemia cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS+1 mM hydrocortisone+10% horse serum (UKE1 cells), RPMI-1640 supplemented with 10% FBS (K562, MOLM13, KCL22, KU812 cells), RPMI-1640 supplemented with 2% FBS (SET2, NOMO1, Monomac-6 cells), or IMDM + 20% FBS (KBm5 cells). For proliferation studies, 1 x 10^3 cells were seeded in 1 ml volume of media in triplicate and cell number was counted manually daily for 7 days by Trypan blue exclusion.

**Plasmid Constructs, Mutagenesis Protocol, Short Hairpin RNA, and Small Interfering RNA**

See Supplemental Information.

**Primary Acute Myeloid Leukemia Patient Samples and ASXL1, BAP1, EZH2, SUZ12, and EED Genomic DNA Sequencing Analysis**

Approval was obtained from the institutional review boards at Memorial Sloan-Kettering Cancer Center and at the Hospital of the University of Pennsylvania for these studies, and informed consent was provided according to the Declaration of Helsinki. Please see Supplemental Information for details on DNA sequence analysis.

**Western Blot and Immunoprecipitation Analysis**

Western blots were carried out using the following antibodies: ASXL1 (Clone N-13; Santa Cruz; sc-852833; N-terminus directed), ASXL1 (Clone 2049C2a; Santa Cruz (sc-81053); C-terminus directed), BAP1 (Clone 3C11; Santa Cruz (sc-13576)), BMI1 (Abcam ab14389), EED (Abcam ab4469), EZH2 (Active Motif 39933 or Millipore 07-689), FLA (M2 FLAG; Sigma A2220), Histone H3 lysine 27 trimethyl (Abcam ab6002), Histone H2A Antibody II (Cell Signaling Technologies 2578), Ubiquityl-Histone H2AX (Clone D27C4; Cell Signaling Technologies 8240), RING1A (Abcam ab32880), SUZ12 (Abcam ab12073), and total histone H3 (Abcam ab1791), and tubulin (Sigma, T9026). Antibodies different from the above used for immunoprecipitation include: ASXL1 [clone H105X; Santa Cruz (sc-98302)], FLAG (Novus Biological Products; NBP1-06712), and EZH2 (Active Motif 39901). Immunoprecipitation and pull-down reactions were performed in an immunoprecipitation buffer (150 mM NaCl, 20 mM Tris (pH 7.4–7.5), 5 mM EDTA, 1% Triton, 100 mM sodium orthovanadate, protease arrest (Genotec), 1 mM PMSF, and phenylarsen oxide). To ensure nuclease-free immunoprecipitation conditions, immunoprecipitations were also performed using the following methodology (Muntean et al., 2010): cells were lysed in BC-300 buffer (20 mM Tris-HCl (pH 7.4), 10% glycerol, 300 mM KCl, 0.1% NP-40) and the cleared lysate was separated from the insoluble pellet and treated with MgCl2 to 2.5 mM and benzamidine (Emanuel Merck, Darmstadt) at a concentration of 1,250 U/ml. The lysate was then incubated for 1–1.5 h at 4°C. The reaction was then stopped with addition of 5 mM EDTA. DNA digestion is confirmed on an ethidium bromide agarose gel. We then set up our immunoprecipitation by incubating our lysate overnight at 4°C.

**Histone Extraction and Histone LC/MS Analysis**

See Supplemental Information.

**Gene Expression Analysis**

Total RNA was extracted from cells using QIAGEN’s RNeasy Plus Mini kit (Valencia, CA, USA). cDNA synthesis, labeling, hybridization, and quality control were carried out as previously described (Figueroa et al., 2008). Ten micrograms of RNA was used then used for generation of labeled cRNA according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA, USA). Hybridization of the labeled cRNA fragments and washing, staining, and scanning of the arrays were carried out as per instructions of the manufacturer. Labeled cRNA from CD34+ cells treated with either ASXL1 siRNA or controls were analyzed using the Affymetrix HG-U133-Plus2.0 platform and from UKE1 cells using the Illunina Href8 array. All expression profile experiments were carried out using biological duplicates. “Present” calls in ≥80% of samples were captured and quantile normalized across all samples on a per-chip basis. Raw expression values generated by Genome Studio (Illumina) were filtered to exclude probesets having expression values below negative background in ≥80% of samples. Probesets remaining after background filtering were log2-transformed and quantile normalized on a per-chip basis. qRT-PCR was performed on cDNA using SYBR green quantification in an ABI 7500
Sequence detection system. The sequences of all qRT-PCR primers are listed in the Supplemental Information.

**Chromatin Immunoprecipitation and Antibodies**

ChIP experiments for H3K4me3, H3K27me3, and H3K36me3 were carried out as described previously (Bernstein et al., 2006; Mikkelsen et al., 2007). Cells were cross-linked in 1% formaldehyde, lysed, and sonicated with a Branson 250 Sonifier to obtain chromatin fragments in a size range between 200 and 700 bp. Solubilized chromatin was diluted in ChIP dilution buffer (1:10) and incubated with antibody overnight at 4°C. Protein A sepharose beads (Sigma) were used to capture the antibody-chromatin complex and washed with low salt, LiCl, as well as TE (pH 8.0) wash buffers. Enriched chromatin fragments were eluted at 65°C for 10 min, subjected to cross-link reversal at 65°C for 5 hr, and treated with Proteinase K (1 mg/ml), before being extracted by phenol-chloroform-isoamyl alcohol, and ethanol precipitated. ChIP DNA was then quantified by QuantiT Picogreen dsDNA Assay kit (Invitrogen). ChIP experiments for ASXL1 were carried out on nuclear preps. Cross-linked cells were incubated in swelling buffer (0.1 M Tris pH 7.6, 10 mM KOAc, 15 mM MgOAc, 1% NP40), on ice for 20 minutes, passed through a 16G needle 20 times and centrifuged to collect nuclei. Isolated nuclei were then lysed, sonicated, and immunoprecipitated as described above. Antibodies used for ChIP include anti-H3K4me3 (Abcam ab8580), anti-H3K27me3 (Upstate 07-449), anti-H3K36me3 (Abcam ab9050), and anti-ASXL1 [clone H105X; Santa Cruz (sc-98302)], and Ubiquityl-Histone H2AK119 (Clone D27C4; Cell Signaling Technologies 8240).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.06.032.

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Deletion of Asxl1 Results in Myelodysplasia and Severe Developmental Defects in Vivo

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Condensed title: Conditional deletion of Asxl1 results in MDS

Character count: 45,168
Abstract:
Somatic Addition of Sex Combs Like 1 (ASXL1) mutations occur in 10-30% of patients with myeloid malignancies, most commonly in myelodysplastic syndromes (MDS), and are associated with adverse outcome. Germline ASXL1 mutations occur in patients with Bohring-Opitz Syndrome. Here we show that constitutive loss of Asxl1 results in developmental abnormalities including anopthalmia, microcephaly, cleft palates, and mandibular malformations. By contrast, hematopoietic-specific deletion of Asxl1 resulted in progressive, multilineage cytopenias and dysplasia in the context of increased numbers of hematopoietic stem/progenitor cells (HSPCs), characteristic features of human MDS. Serial transplantation of Asxl1-null hematopoietic cells resulted in a lethal myeloid disorder at a shorter latency than primary Asxl1 knockout mice. Asxl1 deletion reduced hematopoietic stem-cell self-renewal, which was restored by concomitant deletion of Tet2, a gene commonly co-mutated with ASXL1 in MDS patients. Moreover, compound Asxl1/Tet2 deletion resulted in an MDS phenotype with hastened death compared to single-gene knockout mice. Asxl1 loss resulted in a global reduction of H3K27 trimethylation and dysregulated expression of known regulators of hematopoiesis. RNA-seq/ChIP-seq analyses of Asxl1 in hematopoietic cells identified a subset of differentially expressed genes as direct targets of Asxl1. These findings underscore the importance of Asxl1 in Polycomb-group function, development, and hematopoiesis.
Introduction
Candidate gene and genome-wide discovery studies have identified a set of novel disease alleles in patients with myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), and myeloproliferative neoplasms (MPN). These include somatic mutations in genes with a known, or putative role in the epigenetic regulation of gene expression (Shih et al., 2012). Addition of Sex Combs Like 1 (ASXL1) is a Polycomb-associated protein which has been shown to be an essential co-factor for the nuclear deubiquitinase BAP1 (Dey et al., 2012) as well as a critical mediator of the function of the Polycomb repressive complex 2 (PRC2) (Abdel-Wahab et al., 2012). Recurrent somatic loss-of-function mutations and deletions in ASXL1 are observed in MDS, MPN, and AML patients (Gelsi-Boyer et al., 2009). ASXL1 mutations are most common in MDS patients (Bejar et al., 2011; Bejar et al., 2012; Sanada and Ogawa, 2012; Thol et al., 2011) including in 15-20% of MDS patients and in 40-60% in patients with MDS/MPN overlap syndromes (Boultwood et al., 2010; Gelsi-Boyer et al., 2009; Jankowska et al., 2011). ASXL1 mutations are associated with adverse overall survival in MDS, chronic myelomonocytic leukemia (CMML), AML, and MPN (Bejar et al., 2011; Bejar et al., 2012; Itzykson et al., 2013; Metzeler et al., 2011; Patel et al., 2012; Vannucchi et al., 2013), highlighting the relevance of ASXL1 mutations to myeloid transformation and clinical outcome.

More recently, de novo constitutive ASXL1 mutations were identified in children with the developmental disorder Bohring-Optiz Syndrome (Hoischen et al., 2011; Magini et al., 2012). Although these genetic data strongly implicate ASXL1 mutations in myeloid malignancies and in developmental defects, our understanding of the role of Asxl1 in steady-state hematopoiesis, hematopoietic stem/progenitor function, and myeloid malignancies has been limited by a lack of murine model for conditional- and tissue-specific deletion of Asxl1. Fisher et al. investigated the role of Asxl1 in hematopoiesis through the creation and analysis of a model of constitutive Asxl1 deletion with targeted insertion of a neo cassette into the Asxl1 locus (Fisher et al., 2010a; Fisher et al., 2010b). Disruption of Asxl1 expression in this manner resulted in partial perinatal lethality. Analysis of the remaining aged (beyond 15 weeks of age) Asxl1 mutant mice revealed impairment of B and T cell lymphopoiesis and myeloid differentiation. However, constitutive Asxl1 loss did not alter long-term reconstitution in competitive repopulation studies using Asxl1-null fetal liver cells (Fisher et al., 2010a; Fisher et al., 2010b). These results suggested that Asxl1 has an important role in normal hematopoiesis, however the effects of somatic loss of Asxl1in
hematopoietic cells was not evaluated. Here we investigate the effects of \textit{Asxl1} loss in a time- and tissue-dependent manner through the generation of a murine model for conditional deletion of \textit{Asxl1}. We also characterized the effect of \textit{Asxl1} loss on transcriptional output and gene regulation using epigenomic and transcriptomic analysis of hematopoietic stem and progenitor cells from wildtype and \textit{Asxl1} deficient mice.

\textbf{Results}

\textbf{Development of a Conditional \textit{Asxl1} Knockout Allele}

In order to delineate the role of \textit{Asxl1} in development and in hematopoiesis, we generated a conditional allele targeting \textit{Asxl1} \textit{in vivo} (Fig. 1A and Fig. 1B). We utilized ES cell targeting to insert two LoxP sites flanking exons 5-10 of \textit{Asxl1}, as well as an Frt-flanked neomycin selection cassette in the upstream intron (Fig. 1A and Fig. 1B). The generated mice (\textit{Asxl1}^{fl/fl}) were initially crossed to a germline Flp-deleter murine line to eliminate the neomycin cassette, and then subsequently crossed to germline \textit{EIIa-cre} mice, IFN\textgreek{x}-inducible \textit{Mx1-cre}, and hematopoietic-specific \textit{Vav-cre} (all as described below). \textit{Asxl1} protein expression was not detectable in hematopoietic tissue from \textit{Vav-cre} and \textit{Mx1-cre} mice (Fig. 2B) consistent with generation of a knockout allele.

\textbf{Germline \textit{Asx1} loss results in Embryonic Lethality and Craniofacial Abnormalities}

We characterized the effects of constitutive deletion of \textit{Asxl1} by crossing mice bearing floxed \textit{Asxl1} alleles with germline \textit{EIIa-cre} mice. The \textit{Asxl1} floxed allele was completely recombined in \textit{EIIa-cre} \textit{Asxl1}^{fl/fl} mice (data not shown). We observed 100\% embryonic lethality in mice with germline complete deletion of \textit{Asxl1} (\textit{Asxl1}^{+/+}) while mice with heterozygous germline deletion of \textit{Asxl1} (\textit{Asxl1}^{+/\text{Δ}}) were born at expected Mendelian ratios (Fig. 1C). \textit{Asxl1}^{+/\text{Δ}} mice were no longer viable by E19.5 and were characterized by micro-/anopthalmia (seen in 12/12 of homozygous \textit{Asxl1-null} embryos examined) (Fig. 1D and 1E), frequent cleft palates (seen in 5/12 of homozygous \textit{Asxl1-null} embryos examined) (Fig. 1E), and multiple skeletal abnormalities (mandibular hypoplasia, loss of hyoid bone formation, and posterior homeotic transformations) (seen in 4/12 of homozygous \textit{Asxl1-null} embryos examined) (Fig. 1F). \textit{Asxl1}^{+/\text{Δ}} were viable but exhibited craniofacial dysmorphism in 35\% (14/40) of adult \textit{Asxl1}^{+/\text{Δ}} mice examined (Fig. 1G). Immunophenotypic analysis of hematopoietic stem/progenitor and erythroid
precursor cells in fetal liver from control, Asxl1+/+, and Asxl1−/− mice at E14.5 did 
not reveal differences amongst the genotypes (Fig. 1H-I).

**Hematopoietic Specific Deletion of Asxl1 results in MDS**

Asxl1 is expressed throughout the adult hematopoietic compartment (Fig. 2A). In 
order to elucidate the effects of Asxl1 loss on post-natal hematopoiesis, Asxl1fl/fl 
mice were crossed to Vav-cre and IFNα-inducible Mx1-cre transgenic mice for 
conditional deletion of Asxl1 in the hematopoietic compartment (termed as Asxl1 
KO hereafter). In both cases, Asxl1 protein expression was not detectable in 
hematopoietic tissue (Fig. 2B). Mice with hematopoietic-specific deletion of Asxl1 
(Vav-cre Asxl1fl/fl) developed progressive bone marrow (BM) and splenic 
hypocellularity relative to littermate controls (Cre-negative Asxl1fl/fl) beginning at 6 
weeks of age and likewise evident at 24 weeks of age (n=6-10 mice per 
genotype at each timepoint examined) (Fig. 2C). Asxl1 KO mice, but not 
littermate controls, developed progressive leukopenia (Fig. 2D) and anemia (Fig. 
2E) that was most apparent at 6-12 months of age. While the hemoglobin (Hb) in 
Asxl1 KO mice was within normal limits (median of 13.4g/dL, range 12.9- 
14.8g/dL) in mice <6 months of age, between 6-12 months the Hb was a median 
of 8g/dL (range 1.94-13.9g/dL) in KO mice relative to a median of 11.4g/dL in 
age-matched littermate control mice (range, 7.17-14.2g/dL) (n=6-12 mice with 
each genotype at each timepoint examined) (Fig. 2E). Similarly, the white blood 
cell count (WBC) was within normal limits in Asxl1-null mice at less than 6 
months of age (median, 7.64 cells x 10^9/uL; range 4.66-9.6), the WBC count fell 
to a median of 2.51 cells x 10^9/uL (range 0.88-5.18) in Asxl1 KO mice between 
the ages of 6-12 months compared with age-matched littermate Asxl1 wildtype 
mice (median WBC count 4.51 cells x 10^9/uL; range 2.62-13.8) (Fig. 2D). The 
leukopenia was due predominantly to decrements in B220+ mature B-cells, 
CD11b+ Gr1+ neutrophils, and CD11b+ Gr1-negative monocytes as indicated by 
flow cytometric and morphologic analysis of peripheral blood (Fig. 2F-G). The 
age-dependent anemia observed in Asxl1 KO mice was accompanied by an 
increase (median 1.4- to 2-fold) in CD71-positive/Ter119-negative erythroid 
precursor cells in both the bone marrow and spleen consistent with impaired 
erythroid differentiation (Fig. 3A).

Pathologic analysis of Asxl1 KO hematopoietic tissues at 6 months of age 
revealed morphologic dysplasia of circulating myeloid cells (Fig. 3D), frequent 
circulating nucleated red cells (Fig. 3D), hypocellular marrow (Fig. 3B), and 
dysplasia of erythroid precursors (Fig. 3C). Previous characterization of
hematopoetic stem and progenitor cells from patients with MDS (Martínez-Jaramillo and Flores-Figueroa..., 2002; Sawada et al., 1995; Sawada et al., 1993) and murine models of MDS (such as the NUP98-HOXD13 transgenic mouse model (Choi et al., 2008)) have identified an impairment of sorted hematopoietic progenitors to form colonies ex vivo in methylcellulose containing myeloid and erythroid cytokines. Consistent with these prior observations and the impairment in mature myeloid and erythroid differentiation seen in Asxl1-deficient mice, in vitro analysis of sorted myeloid progenitor cells from 6-week-old Asxl1 KO and control mice revealed a clear decrease in colony output of sorted common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), and megakaryocyte/erythroid progenitors (MEP) in KO versus control mice (Fig. 3E-F).

Consistent with the age-dependent development of impaired myeloid and erythroid output in Asxl1 KO mice compared with age-matched littermate controls, Asxl1 KO (Mx1-cre Asxl1fl/fl) mice were found to have infiltration of liver with hematopoietic cells consistent with extramedullary hematopoiesis (EMH) with Asxl1 deletion (Fig. 3G). In order to ascertain if this hematopoietic infiltrate represented inflammatory infiltration of hematopoietic cells versus EMH, we plated 200,000 cells harvested from the liver of Asxl1 KO mice and littermate controls in methylcellulose semi-solid media containing myeloid-erythroid cytokines (rmIL-3, rmSCF, rh-EPO, and rh-IL6). Colonies plated with cells derived from Asxl1 KO mice alone yielded abundant colonies (Fig. 3H-I) demonstrating EMH.

**Cell Autonomous Effects of Asxl1 loss**

Transplantation of whole BM from Asxl1 KO (Vav-cre Asxl1fl/fl) mice into lethally irradiated recipients resulted in a penetrant, lethal hematopoietic disorder (Fig. 4A) indicating that the phenotype induced by Asxl1 loss was cell-autonomous. For example, transplantation of whole BM from 70-week-old primary Asxl1 KO mice into lethally irradiated recipients resulted in death of recipient mice at a median of 50-weeks after transplant (range, 41-74 weeks) whereas no mice transplanted with Asxl1 wildtype BM died during this period of observation. Further, serial transplantation into tertiary recipients resulted in shorter latency disease with mice dying 24-42 weeks after transplant (median of 28 weeks). Transplantation of purified lineage-negative Sca-1+ c-KIT+ (LSK) cells from the BM of secondary recipients led to a lethal myeloid disease in all tertiary transplant recipients with more rapid onset (10.3 median weeks, range 5.1-10.6
weeks) than transplantation of unfractionated BM cells from the same secondary recipients (Fig. 4A). Disease in transplanted mice was characterized by progressive anemia and cachexia (Fig. 4B-C), BM hypocellularity (Fig. 4D), and an increase in the relative frequency of hematopoietic stem and progenitor cells (HSPCs) in both the BM and the spleen (Fig. 4E-F). This was accompanied by splenomegaly due to EMH and effacement of splenic architecture (Fig. 4G-I). Anemia in the KO-transplanted recipient mice was evident even with gross inspection of bones (Fig. 4J). As in primary Asxl1 KO mice this anemia occurred despite an increase in erythroid precursors in both the BM and spleen (Fig. 4K-L), consistent with a block in erythroid differentiation with Asxl1 loss. This block in erythroid differentiation was characterized by a significant increase in CD71+/Ter119 double-positive erythroid precursors in the spleen (Fig. 4L).

Impaired Self-Renewal of Asxl1 Deficient Cells is Rescued by Concomitant Tet2 loss

We next assessed the effects of Asxl1 loss on hematopoietic stem cell (HSC) frequency and function. We observed an increase in the absolute number of immunophenotypically-defined HSPCs in Asxl1 KO (Vav-cre Asxl1fl/fl) mice at 6-weeks of age, including LT-HSC (CD150+CD48-Lin-Sca-1+c-Kit+) cells (Fig. 5A and 5B) (quantified as the total number of live cells per femur). Although the number of immunophenotypic stem/progenitor cells was increased, we observed a decrease in serial plating in vitro in Asxl1 KO cells (Fig. 6A) suggesting a potential defect in self-renewal. To assess the effects of Asxl1 deletion in vivo, 500,000 whole BM nucleated cells from 6-week-old CD45.2 Vav-cre Asxl1fl/fl mice or Asxl1fl/fl littermate controls were transplanted in competition with an equal number of 6-week-old CD45.1 competitor BM cells into lethally irradiated CD45.1 recipient mice (Fig. 5C). Chimerism was assessed based on evaluation of the ratio of CD45.1 to CD45.2 peripheral blood mononuclear cells beginning 2 weeks following transplantation and then monitored on a monthly basis until 16 weeks thereafter. Consistent with the in vitro data, we observed a clear reduction in self-renewal in vivo. Asxl1 KO HSPCs had a significant disadvantage in competitive transplantation that was further accentuated with serial transplantation (Fig. 5C and 5D).

After the final assessment of chimerism in the primary competitive transplantation experiments, primary recipient mice were sacrificed and a serial competitive transplantation experiment was carried out by transplanting 1 million whole bone marrow cells from primary recipient mice into lethally irradiated
CD45.1 secondary recipient mice (Fig. 5D). Serial competitive transplantation revealed an even further competitive disadvantage in Asxl1-deficient HSPCs (Fig. 5D).

Given that MDS is characterized by impaired myeloid differentiation, multilineage cytopenias, and clonal dominance over time, we hypothesized that mutations that occur in concert with ASXL1 deletion/mutation in MDS might compensate for the impaired self-renewal observed with Asxl1 loss. Previous studies have shown that mutations in TET2 are most commonly observed with mutations in ASXL1 in MDS (Bejar et al., 2011; Bejar et al., 2012). We and others previously demonstrated increased hematopoietic self-renewal in Tet2-deficient mice (Ko et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). We analyzed the in vitro and in vivo phenotype of Vav-cre Asxl1fl/fl Tet2fl/fl hematopoietic cells compared with control, Vav-cre Asxl1fl/fl, and Vav-cre Tet2fl/fl mice (Fig. 6A). Colony assays of whole BM cells from the same mice revealed reduced serial replating activity of Asxl1 KO cells but restored serial-replating capacity of cells with compound Asxl1/Tet2 loss (Fig. 6A). More importantly, competitive transplantation studies revealed a competitive advantage for Vav-cre Asxl1fl/fl Tet2fl/fl whole BM compared to matched CD45.1 competitor BM (Figs. 6B-D). These data demonstrate concurrent Tet2 loss restores the self-renewal defect induced by Asxl1 loss.

**Concomitant deletion of Asxl1 and Tet2 in vivo results in MDS**

Given the restoration of self-renewal noted in mice with concomitant deletion of Tet2 and Asxl1 in the context of a competitive transplantation experiment, we investigated the phenotype of mice with compound deletion of Tet2 and Asxl1 compared with mice with deletion of each gene alone. A cohort of primary Cre-negative Asxl1 wildtype Tet2 wildtype, Mx1-cre Asxl1fl/fl (Asxl1 KO), Mx1-cre Tet2fl/fl (Tet2 KO), and Mx1-cre Asxl1fl/fl Tet2fl/fl (double knockout (DKO)) mice were treated with plpC at 4 weeks of life and followed up to 50 weeks following birth (46 weeks following plpC administration). At the end of this observation period, 40% of DKO mice (4/10) and 17.7% of Tet2 KO died (1/6) whereas no Asxl1 KO (0/12) or control mice (0/5) died (Fig. 7A).

For further analyses, in order to obtain a sufficiently large number of mice for each genotype, BM from 6-week-old CD45.2 Mx1-cre Asxl1 wildtype Tet2 wildtype, Mx1-cre Asxl1fl/fl (Asxl1 KO), Mx1-cre Tet2fl/fl (Tet2 KO), and Mx1-cre Asxl1fl/fl Tet2fl/fl (double knockout (DKO)) mice were transplanted into lethally irradiated CD45.1 recipient mice (10 recipient mice per genotype). All recipient
mice (including those transplanted with Mx1-cre Asxl1 wildtype Tet2 wildtype bone marrow) were then treated with plpC 2 weeks following transplantation to delete Tet2 and/or Asxl1. At 72 weeks post-transplant Asxl1-null and/or Asxl1/Tet2 compound-null mice had significantly lower WBC counts and hematocrit compared with wildtype or Tet2 single KO control mice (Fig. 7B-C). As seen in mice with primary deletion of Asxl1, Asxl1 KO mice here had reduced BM cellularity compared with control or Tet2 KO mice (Fig. 7D). However, despite the similar blood counts between Asxl1 KO and DKO mice, the DKO had greater BM cellularity than mice with deletion of just Asxl1 or even Tet2 (Fig. 7D). Examination of the HSPC compartment across mice with the 4 genotypes at 72 weeks indicated a greater total number as well as relative frequency of LSK and myeloid progenitor cells in the BM of DKO mice compared with other groups (Fig. 7E-F). Morphologically, BM of Asxl1 KO mice was characterized by the presence of dysplastic erythroid precursor cells as seen in primary Asxl1 KO mice earlier (Fig. 7G). The BM of DKO mice likewise was characterized by a similar presence of dysplastic erythroid precursors as well as dysplastic myeloid cells (Fig. 7G) but lacked the hypocellularity seen in the BM of mice with Asxl1 deletion alone (Fig. 7D). Histologic analysis of liver tissue revealed increased hematopoietic cell infiltration in DKO mice compared with the other groups (Fig. 7H). The presence of morphologic dysplasia in precursor cells, decrease in peripheral circulating mature cells, and concurrent increased total BM cells and HSPC’s were suggestive of the presence of MDS in the Asxl1/Tet2 compound-deficient mice.

Transcriptional Effects of Asxl1 Loss
In order to understand the basis for the impaired myeloid differentiation and self-renewal observed with Asxl1 loss we performed expression analysis of sorted LSK and MP cells from cytopenic 1 year old Asxl1 KO mice and age-matched littermate controls (Fig. 8A-B; Supplemental Tables 1-2). Analysis of RNA-seq data identified a set of differentially expressed genes in Asxl1 KO LSK cells (797 genes) and MP cells (1095 genes). Integrative analysis identified a set of 75 genes that were differentially expressed in Asxl1 KO LSK and MP cells, including 41 up-regulated and 34 down-regulated genes in Asxl1 KO mice relative to controls (Fig. 8B).

Consistent with previous in vitro data (Abdel-Wahab et al., 2012), we observed increased expression of posterior HoxA genes in Asxl1 KO LSK cells, including HoxA7 and HoxA9 as well as the Hox-associated transcription factors Hes5 and Gdf11 (Fig. 8C). Meis1 was not up-regulated with Asxl1 loss
consistent with prior reports in in vitro systems (Abdel-Wahab et al., 2012) (Fig. 8C). We also noted a progressive, age-dependent increase p16\textsuperscript{INK4a} expression in LT-HSC and multipotent progenitor cells (MPP; lineage-negative Sca-1+ c-KIT+ CD48+ CD150-negative) of Asxl1 KO mice compared with age-matched controls (Fig. 8D). The p16\textsuperscript{INK4a} locus is a known PRC2 target (Bracken et al., 2007; Hidalgo et al., 2012; Jacobs et al., 1999; Tanaka et al., 2012) and in vitro loss of Asxl1 has been linked to defective Polycomb repression and reduced H3K27 methylation (Abdel-Wahab et al., 2012). Given the increase in p16\textsuperscript{INK4a} expression in KO MPP cells from Asxl1 KO mice, we examined the in vivo cell proliferation of MPP cells from 72-week-old Asxl1 KO (Vav-cre Asxl1\textsuperscript{fl/fl}) versus control mice (Cre-negative Asxl1\textsuperscript{fl/fl}) via an in vivo 5-bromo-2-deoxyuridine (BRDU) incorporation assay. The MPP cells of Asxl1 KO mice showed a significant decrease in S-phase compared with littermate control cells (Fig. 8E). Flow-cytometric quantitative assessment of apoptosis in LSK cells of the same mice revealed a significant increase in Annexin-V+/DAPI-negative and Annexin-V+/DAPI-positive LSK cells (Figure 8F; n=5 mice per group) consistent with cell cycle exit and an increase in apoptosis in vivo.

In order to understand what transcriptional differences might exist between mice with compound deletion of Asxl1 and Tet2 relative to mice with deletion of Asxl1, Tet2, or neither gene, we performed RNA-Seq on LSK cells sorted from the BM of 6-week-old Mx1-cre Asxl1\textsuperscript{wildtype} Tet2\textsuperscript{wildtype}, Mx1-cre Asxl1\textsuperscript{fl/fl}, Mx1-cre Tet2\textsuperscript{fl/fl}, and Mx1-cre Asxl1\textsuperscript{fl/fl} Tet2\textsuperscript{fl/fl} mice (Fig. 8G; Supplemental Table 3). Of the 1,744 genes significantly up-regulated in any knockout mice relative to controls, the majority of these genes were shared between Asxl1 KO and Tet2 KO LSKs but not DKO LSKs (32.6% of up-regulated genes (569/1,744 genes)) followed by genes shared between Asxl1 KO, Tet2 KO, and DKO mice (29.2% of up-regulated genes (513/1,363 genes)). Likewise, for the 1,363 significantly down-regulated genes, the majority of these were shared between Asxl1 KO and Tet2 KO LSKs but not DKO LSKs (37.6% of up-regulated genes (513/1,363 genes)) followed by genes shared between Asxl1 KO, Tet2 KO, and DKO mice (28.0% of up-regulated genes (382/1,363 genes)).

We next performed gene set enrichment analysis (GSEA) to identify gene sets enriched in HSPC’s from Asxl1 KO mice or Asxl1/Tet2 DKO mice compared with other groups (Subramanian et al., 2005). We identified gene sets enriched in hematopoietic stem cells (Ramalho-Santos et al., 2002) and apoptosis (http://www.genome.jp/kegg/pathway/hsa/hsa04210.html) in mice with Asxl1 deletion and with concomitant Asxl1/Tet2 deletion (Figure 8H). We also identified
gene sets which were uniquely enriched in Tet2/Asxl1 DKO mice and not seen in the other groups. This prominently included gene sets characteristic of apoptosis signatures, purified HSPCs (Ivanova et al., 2002), cell cycle regulators (http://www.genome.jp/kegg/pathway/hsa/hsa04110.html), and signatures from MLL-rearranged primary leukemias (Mullighan et al., 2007; Ross et al., 2003)(Figure 8I).

**Genome-wide binding of Asxl1 and global effects of Asxl1 loss on the epigenome**

Asxl1 has been shown to interact with epigenetic modifiers known to impact transcription (Dey et al., 2012) (Abdel-Wahab et al., 2012). This includes binding to the core members of the Polycomb Repressive Complex 2 (PRC2) where loss of ASXL1 has previously been found to result in global downregulation of histone H3 lysine 27 (H3K27) methylation (Abdel-Wahab et al., 2012) in vitro and in ex vivo ASXL1 mutant primary patient samples. Consistent with this, H3K27 trimethyl (H3K27me3) levels were significantly reduced after Asxl1 deletion (Fig. 9A) despite sustained expression of the core Polycomb-repressive complex 2 (PRC2) components (Fig. 9B).

Although the effects of Asxl1 loss on transcription due to alterations in histone post-translational modifications has previously been described (Abdel-Wahab et al., 2012), direct transcriptional targets of Asxl1 through characterization of Asxl1 binding throughout the genome has never previously been assessed. We therefore performed chromatin-immunoprecipitation for Asxl1 followed by DNA sequencing (ChIP-Seq) in purified murine myeloid hematopoietic cells. Asxl1 was found to bind to many sites throughout the genome with the majority of significantly enriched Asxl1 peaks (78%) located at CpG-rich transcription start sites (Fig. 9C-E and Supplemental Tables 4-5). Motif enrichment analysis of the Asxl1-binding sites revealed that the top occurring motifs are most similar to known binding sites of Ets family of transcription factors (Fig. 9F; \( p=1e^{-59} \), %target = 40.1%, % background = 21.4%).

A significant subset of genes with dysregulated expression in Asxl1 KO LSK/MP cells were confirmed as direct targets of Asxl1 in our ChIP-Seq analysis (14 up-regulated genes and 9 down-regulated genes) (Table 1).

**Discussion**

Here we identify that conditional deletion of Asxl1, a gene commonly mutated in human MDS, in hematopoietic cells resulted in the development of
progressive anemia and leukopenia with concomitant multilineage myeloid dysplasia in vivo. Asxl1 deletion was associated with an increase in the frequency and total number of HSPCs, increased apoptosis, and altered cell cycle distribution of HSPC’s in vivo. Asxl1 loss also led to a reduction in myeloid colony output. MDS is characterized by variable cytopenias due to ineffective production of mature granulocyte, erythroid, and/or megakaryocyte populations and a risk of transformation to AML. Functional characterization of primary samples from patients with MDS has identified an expansion of the primitive hematopoietic stem cell compartment (comprised of long-term and short-term HSC’s) (Pang et al., 2013; Will et al., 2012), the presence of specific genetic alterations throughout the diseased clone originating in the most immature HSC’s (Nilsson et al., 2000; Nilsson et al., 2007; Pang et al., 2013; Tehranchi et al., 2010; Will et al., 2012), dysplastic clonogenic activity of HSCs with reduced in vitro colony formation from MDS-derived HSC’s (Martinez-Jaramillo and Flores-Figueroa…, 2002; Sawada et al., 1995; Sawada et al., 1993; Will et al., 2012), and an increase in the frequency and absolute number of HSPC’s in the setting of decreased mature circulating cells with a concomitant increase in apoptosis of HSPCs in MDS patients (Pang et al., 2013; Sawada et al., 1995; Sawada et al., 1993). Taken together, the phenotype of Asxl1 loss recapitulates these central features of human MDS.

One aspect of the Asxl1 conditional knockout mouse model which differs from human MDS is the BM hypocellularity observed in Asxl1 KO mice, in contrast to the increased BM cellularity in most MDS patients. Nevertheless, the impaired production of mature myeloid and erythroid cells in the context of an increased relative and total numbers of HSPCs in Asxl1 conditional knockout mice does recapitulate key features of human MDS. Moreover, concomitant loss of Asxl1 and Tet2 which are commonly mutated in concert in human MDS, resulted in increased BM cellularity and disease severity with pathologic evidence of multi-lineage dysplasia.

In contrast to MPN and AML, there are few previously described models of MDS, and to date no models of MDS based on mutations in recurrently mutated MDS disease alleles. The most widely utilized model of MDS is based on transgenic expression of a NUP98-HOXD13 fusion allele (Lin et al., 2005). Although this model has many of the characteristic features of human MDS, the NUP98-HOXD13 fusion was identified in a young patient with therapy-related AML (Raza-Egilmez et al., 1998) and has not been identified in MDS patients to date. By contrast, ASXL1 mutations occur in 15-20% of patients with MDS.
Prior studies of the effects of Asxl1 loss on development and hematopoiesis were performed using a constitutive Asxl1 KO mouse model (Fisher et al., 2010a; Fisher et al., 2010b). Our conditional model allows for evaluation of the effects of post-natal deletion of Asxl1 and obviates the problems associated with a high-frequency of perinatal lethality in mice with constitutive Asxl1 deletion. Notably, Fischer et al. identified a 72% reduction in the expected number of Asxl1 homozygous KO mice by post-natal day 21; when mice were backcrossed >8 generations to a consistent genetic background, KO mice were 100% embryonic lethal, preventing analysis of adult constitutive Asxl1 KO mice. Similar to the germline model, we observed an age-dependent decrease in mature B lymphocytes, splenomegaly due to extramedullary hematopoiesis, and decreased formation of myeloid and erythroid colonies from Asxl1 KO cells (Fisher et al., 2010a; Fisher et al., 2010b). In addition, Fisher et al. observed dysregulated expression of HoxA genes and homeotic transformation of homozygous Asxl1-mutant embryos (Fisher et al., 2010a; Fisher et al., 2010b), consistent with the current Asxl1 germline and conditional KO models described in this report.

Despite these similarities, a number of differences exist, however, between the two models. First, no reproducible differences in peripheral blood counts, bone marrow cellularity or bone marrow cell morphology were seen in Asxl1 constitutive KO mice. In contrast, mice with conditional, homozygous, post-natal deletion of Asxl1 developed leukopenia, anemia, myelo-erythroid dysplasia and bone marrow hypocellularity starting at 6 months of life. This could be due to differences in the strain of the mice analyzed, the timing of Asxl1 loss, or cell non-autonomous effects observed in the constitutive knockout model.

Of note, the more profound hematologic abnormalities seen with serial competitive and non-competitive transplantation of Asxl1 conditional KO hematopoietic cells here have no counterpart in the reports of the prior constitutive KO model as serial transplantation was not performed in the prior studies. The marked impairment in serial transplantability observed with Asxl1 loss is consistent with the progressive defects in hematopoietic stem cell function observed in mice with other alterations in other Polycomb-group function (Ohta et al., 2002).

Although non-competitive transplantation studies demonstrated that the MDS phenotype was cell autonomous, Asxl1-deficiency was associated with a defect in hematopoietic stem cell self renewal in competitive transplantation assays and in in vitro colony formation. These data suggested that concurrent
genetic or epigenetic alterations in ASXL1-mutant MDS cells must promote self-renewal to allow for clonal dominance of MDS cells. Indeed, concurrent Tet2 loss restored the self-renewal defect induced by Asxl1 loss. Moreover, mice with concomitant loss of Asxl1 and Tet2 developed a larger increase in HSPCs, increased BM cellularity, and decreased numbers of circulating mature cells compared with single gene knockout mice. This phenotype is consistent with more severe MDS and suggests a functional interdependency between these two disease alleles in MDS. Subsequent studies may identify additional disease alleles which can rescue the self-renewal defect seen in ASXL1-deficient stem cells, and may lead to the identification of additional mutational interdependencies in MDS and in other malignant contexts.

We previously demonstrated loss of ASXL1 in vitro results in global downregulation in H3K27me3 (Abdel-Wahab et al., 2012), the repressive histone modification placed by the PRC2. Here we demonstrate Asxl1 deletion in the hematopoietic compartment results in reduced H3K27me3 in vivo. We used ChIP-Seq for Asxl1 itself to show that Asxl1 is enriched at gene-promoter regions throughout the genome suggesting a potential role for Asxl1 in direct regulation of gene transcription. In addition, motif enrichment analysis of Asxl1-binding sites revealed enrichment in known binding sites of the Ets family of transcription factors. The significant overlap between genome-wide binding of Asxl1 and Ets family members is a critically supportive of the importance of Asxl1 in hematopoiesis as Ets family of transcription factors are very well understood to play a key role in the growth, survival, differentiation, and activation of hematopoietic cells (Choi et al., 2008; Koschmieder et al., 2005; Mizuki et al., 2003; Steidl et al., 2006; Vangala et al., 2003). Deletion, mutation, and translocation of ETS family members are well-described in myeloid malignancies (Gilliland, 2001), including ETV6 mutations/translocations in MDS and CMML (Haferlach et al., 2012) and loss-of-function mutations of PU.1 in AML (Mueller et al., 2002). Moreover, common oncogenic events seen in patients with myeloid malignancies have been demonstrated to transform myeloid cells through suppression of expression of key Ets members. For example, FLT3-ITD mutations and the AML1-ETO (t(8;21)) fusion oncoprotein have been demonstrated to suppress PU.1 expression and function (Mizuki et al., 2003; Vangala et al., 2003). In addition, down-regulation of PU.1 expression results in impaired myeloid differentiation (Rosenbauer et al., 2004; Steidl et al., 2006). Further work to understand the involvement of individual Ets family members and/or a shared transcriptional program between Asxl1 loss and Ets family...
member loss (Steidl et al., 2006) in the pathogenesis of ASXL1-mutant myeloid malignancy will be critical.

Taken together, our studies reveal that deletion of Asxl1 results in craniofacial and skeletal developmental abnormalities and mice with hematopoietic-specific Asxl1 loss developed hallmark features of MDS, including progressive ineffective hematopoiesis, impaired myeloid differentiation, multilineage dysplasia, and increased apoptosis and altered cell cycle regulation of HSPCs. Given the paucity of murine models of human MDS based on known, recurrent MDS disease alleles, we believe the development of a genetically accurate model of MDS will inform subsequent studies aimed to elucidate the molecular basis for MDS and to develop novel therapies for MDS patients.
Acknowledgments

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Author Contributions


Competing Financial Interests

The authors declare no competing financial interests.
Figure 1: Generation of a conditional Asxl1 allele and characterization of mice with constitutive Asxl1 loss. (A) Schematic depiction of the targeted Asxl1 allele. Exons 5-10 are targeted and flanked by LoxP sites upon Frt-mediated deletion of the Neo cassette. (B) Verification of correct homologous recombination of Asxl1 targeted allele using Southern blots on targeted ES cells. (C) Enumeration of offspring derived from mating Ella-cre Asxl1+/Δ parents reveal that homozygous constitutive deletion of Asxl1 (Asxl1Δ/Δ) is associated with 100% prenatal lethality. Although Asxl1Δ/Δ mice are observed at 14.5 and 18.5 days post-coitus (dpc), fetal mice with germline homozygous loss of Asxl1 are characterized by lack of eyes as shown by gross pathology (D) and tissue sections at E14.5 and E18.5 (E; section 1 and 2). Germline Asxl1 null mice are also characterized frequent cleft palates (E; section 2). (F) Skeletal preparations from germline Asxl1 null mice surviving to day E20.5 reveal frequent skeletal abnormalities including hypoplastic mandibles (asterisk), lack of hyoid bone (arrowhead), and lower lumbar/sacral posterior homeotic transformations (arrow). (G) Gross phenotype of Ella-cre Asxl1+/Δ and littermate control mice revealing bilateral micro-opthalmia in Ella-cre Asxl1+/Δ mice. (H) Immunophenotyping of fetal liver at day 14.5 days post-coitus (dpc) reveals no major alterations in the relative frequency of lineage-negative Sca-1+ Kit+ (LSK), multipotent progenitors (MPP; LSK, CD48+, CD150- cells), and long-term hematopoietic stem cells (LT-HSC; LSK, CD48-, CD150+ cells) between mice with germline loss of 0, 1, or 2 copies of Asxl1. FACS analysis was performed with 3-5 independent fetal liver samples per genotype. (I) Likewise, FACS analysis of fetal liver at 14.5 dpc reveals no major alterations in the relative frequency of CD71+ single-positive, CD71/Ter119 double-positive, or Ter119 single-positive cells with constitutive loss of Asxl1. Antibody stainings are as indicated and cells were gated on live cells in the parent gate. Error bars represent ± SD.

Figure 2: Conditional deletion of Asxl1 results in age-dependent leukopenia, and anemia. (A) qRT-PCR showing relative expression level of Asxl1 in purified progenitor and mature mouse hematopoietic stem and progenitor subsets. (B) Verification of Mx1-cre (left) and Vav-cre (right) mediated deletion of Asxl1 at the level of protein expression in Western blot of splenocytes. (C) Enumeration of nucleated cells in bilateral femurs and tibiae or whole spleens of control (Asxl1fl/fl) and Asxl1 hematopoietic-specific knockout (KO) mice (Vav-cre Asxl1fl/fl) reveals
significant decrease in hematopoietic cells in both compartments at 6 weeks as well as 24 weeks of age (n=6-10 mice per genotype at each timepoint examined). (D, E). Enumeration of peripheral white blood cells (WBC) (D), and hemoglobin (Hb) (E) reveals time-dependent development of leukopenia and anemia with post-natal deletion of Asxl1 (performed using Mx1-cre Asxl1fl/fl mice or Cre-negative Asxl1fl/fl controls). Counts in aged Asxl1 KO mice are compared to age-matched controls as well as younger KO and control mice (n=6-12 mice per genotype at each timepoint examined). (F,G) Flow cytometric enumeration of B220+, CD11b+/Gr1+, CD3+, and CD11b+/Gr1- cells in the peripheral blood of >6 month-old Mx1-cre Asxl1fl/fl (KO) and Asxl1fl/fl (C) mice (n=5 mice per genotype were utilized for FACS analysis of peripheral blood). Right panel reveals peripheral blood FACS analysis. Antibody stainings are as indicated and cells were gated on live cells in the parent gate.

**Figure 3:** Deletion of Asxl1 results in myeloid and erythroid dysplasia and impaired progenitor differentiation consistent with myelodysplasia. (A) Relative frequency of CD71-positive/Ter119-negative erythroid precursors in bone marrow (BM) and spleen of 6.5 month-old Mx1-cre Asxl1fl/fl (KO) and Cre-negative Asxl1fl/fl control mice revealing a modest but statistically significant increase erythroid precursors in both compartments (expressed as percentage of live cells) (n=3-5 mice per genotype in each tissue type examined by FACS analysis). (B) Histologic (H&E) analysis of Mx1-cre Asxl1fl/fl and Cre-negative Asxl1fl/fl control BM from 6-month old littermate mice illustrating marrow hypocellularity (scale bar represents 50 µm). (C) BM cytopspins (Wright-Giemsa) from the same mice illustrating erythroid precursor dysplasia (arrows indicating erythroid precursors with prominent irregular nuclear contours) in KO mice and normal morphology in littermate controls (scale bar represents 10 µm). (D) Representative morphology of peripheral blood myeloid cells (top) and nucleated red blood cells (bottom) in KO mice (Wright-Giemsa stain) (scale bar represents 5 µm). (E) Number of colonies formed 7 days after plating of 1,000 common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), or megakaryocyte/erythroid progenitor (MEP) cells into methylcellulose from 6-week-old Vav-cre Asxl1fl/fl and littermate control (Cre-negative Asxl11fl/fl control) mice. Experiment was performed in biological duplicate. (F) Photograph of methylcellulose colony plate 7 days after plating of MEP cells from 6-week-old KO and control mice. (G) Histologic analysis by H&E stain liver from 72-week-old Mx1-cre Asxl1fl/fl mice and littermates revealing extramedullary myeloid infiltrates
in liver of KO mice. (H) Number of colonies formed 7 days after plating of 200,000 nucleated cells harvested from the liver of 72-week-old Mx1-cre Asxl1<sup>fl/fl</sup> or littermate control mice in methylcellulose containing rmIL-3, rm-SCF, rh-IL6, rh-EPO (liver cells from n=5 mice per genotype plated in methylcellulose). (I) Photomicrograph of colonies grown from cells taken from the liver and plated in methylcellulose is shown on right (scale bar represents 200 µm). Error bars represent ± SD. Asterisk represents p<0.05 (Mann-Whitney U test).

Figure 4: Serial non-competitive transplantation of Asxl1-null cells results in lethal myelodysplastic disorder. (A) Kaplan-Meier survival curve of recipient mice transplanted with 70-week-old Vav-cre Asxl1<sup>fl/fl</sup> or Cre-negative Asxl1<sup>fl/fl</sup> littermate control whole bone marrow following secondary and tertiary transplantation. Also shown is the survival of mice transplanted with purified lineage-negative, Sca-1+, c-Kit+ (LSK), cells in tertiary transplantation (tertiary transplant of Asxl1<sup>fl/fl</sup> control LSK cells is not shown; no recipient mice from this group died by 40 weeks (n=5)). Cre-negative Asxl1<sup>fl/fl</sup> littermate controls were similarly transplanted in parallel in each experiment. 4-6 recipient mice were transplanted in each experiment. (B) Hematocrit over time of secondary recipient mice transplanted with Asxl1-null or littermate control whole bone marrow in a non-competitive manner. Dashed bar represent lower-limit of normal hematocrit for C57/B6 mice (n=4-6 mice per genotype at each time point). (C) Body weight of secondarily transplanted mice at 50 weeks following transplantation (n=4 mice per genotype). (D) Bone marrow histopathology of secondary recipient mice transplanted with Asxl1-null or littermate control whole bone marrow at 50 weeks (scale-bar represents 50 µm). (E) Relative frequency of lineage-negative, Sca-1+, c-Kit+, (LSK), multipotent progenitor (MPP; LSK+, CD150-, CD48+), and long-term hematopoietic stem cells (LT-HSC; LSK+ CD150+ CD48-) in bone marrow and spleen at 50 weeks following non-competitive secondary transplantation. Frequencies are expressed as frequency of live cells (n=4 mice per genotype examined for FACS experiments). (F) Relative frequency of myeloid progenitors (lineage-negative c-Kit+ Sca-1-), common myeloid progenitor (CMP; lineage-negative, c-Kit+, Sca-1-, FcγR-, CD34+), granulocyte-macrophage progenitor (GMP; lineage-negativec-Kit+Sca-1-, FcγR+CD34+), and megakaryocyte-erythroid progenitor (MEP; lineage-negativec-Kit+Sca-1-FcγR-CD34-) cells at 50 weeks following non-competitive secondary transplantation. Frequencies are expressed as frequency of live cells. (G) Photographs of spleens from secondary
recipient mice transplanted with Vav-cre Asxl1<sup>fl/fl</sup> or Cre-negative Asxl1<sup>fl/fl</sup> littermate control whole bone marrow 50 weeks following lethal irradiation. (H) Weight of the same spleens as shown in (G) (n=4 mice per genotype). (I) Histopathology of spleens from secondary recipient mice transplanted with Asxl1-null or wild-type littermate control whole bone marrow 50 weeks following non-competitive secondary transplantation revealing loss of normal splenic architecture (scale bar represents 50 µm). (J) Photographs of representative femur (top) and tibia (below) from secondary recipient mice transplanted with Vav-cre Asxl1<sup>fl/fl</sup> or Cre-negative Asxl1<sup>fl/fl</sup> littermate control whole bone marrow 50 weeks following non-competitive secondary transplantation. (K) Relative quantification of CD71+/Ter119-negative and CD71/Ter119 double-positive cells from bone marrow and spleen of secondary recipient mice transplanted with Vav-cre Asxl1<sup>fl/fl</sup> or Cre-negative Asxl1<sup>fl/fl</sup> littermate control whole bone marrow 50 weeks following non-competitive secondary transplantation. Frequencies are expressed as percentage of live cells (n=4 mice per genotype examined by FACS analysis). (L) Representative FACS plots of data shown in (K) from splenocytes. Staining is as shown and live cells were gated in parent gate. Error bars represent ± SD. Asterisk represents p<0.05 (Mann-Whitney U test).

Figure 5: Asxl1<sup>-/-</sup> mice have increased stem/progenitor cells but impaired self-renewal. (A) Flow cytometric enumeration of bone marrow lineage-negative Sca-1+ c-Kit+ cells (LSK), long-term hematopoietic stem cells (LT-HSC; LSK CD150+ CD48-), and multipotent progenitor cells (MPP; LSK CD150- CD48+) in wild-type (Asxl1<sup>fl/fl</sup>) and knockout (Vav-cre Asxl1<sup>fl/fl</sup>) mice at 6 weeks of age reveals a significant increase in immunophenotypically-defined LSK and LT-HSC cells (n=4-6 mice per genotype as indicated). Data are expressed as total number of live cells per femur. (B) Representative FACS analysis of bone marrow stem cell populations of Asxl1<sup>-/-</sup> (Vav-cre Asxl1<sup>fl/fl</sup>) and wild-type (Asxl1<sup>fl/fl</sup>) at 6 weeks. Antibody stains are as indicated and parent gate is live, lineage-negative cells. (C) Schematic depiction of the competitive transplantation assay. Asxl1<sup>fl/fl</sup> and Vav-cre Asxl1<sup>fl/fl</sup> are positive for CD45.2 while wild-type competitor cells are positive for CD45.1. Recipient mice are also CD45.1. Representative FACS plots of the percentage of CD45.1 versus CD45.2 total chimerism in the peripheral blood of recipient animals at 16 weeks following competitive transplantation is shown. (D) Percentage of CD45.1 versus CD45.2 total chimerism in the peripheral blood of recipient animals at 4 weeks and 16 weeks in primary competitive transplant and serial secondary competitive transplants.
are shown (n=5 recipient mice for each genotype) (C: control and KO: Asxl1 knockout). Experiment was performed in biological duplicate. Asterisk represents p<0.05 (Mann-Whitney U test).

Figure 6: Combined loss of Asxl1 and Tet2 rescues the impaired self-renewal of Asxl1-deficient hematopoietic stem cells (HSCs). (A) Enumeration of colonies and serial replating capacity of 20,000 whole BM cells from 6-week-old littermate mice with hematopoietic-specific deletion of Asxl1 (Vav-cre Asxl1fl/fl), Tet2 (Vav-cre Tet2fl/fl), or both (Vav-cre Asxl1fl/fl Tet2fl/fl) reveals increased replating capacity of Asxl1-null/Tet2-null cells compared with Asxl1-null cells alone. At the same time, Asxl1/Tet2 double-knockout cells have impaired colony formation compared with Tet2 single-knockout cells at extended replating. The same whole BM cells were also used for in vivo competitive transplantation. (B) Schematic depiction of the competitive transplantation experiment. Control, Vav-cre Asxl1fl/fl, Vav-cre Tet2fl/fl, and Vav-cre Asxl1fl/fl Tet2fl/fl cells are positive for CD45.2 while wild-type competitor cells are positive for CD45.1. On the right, monthly assessment of donor chimerism in the peripheral blood of recipient animals is shown up to 16 weeks post-transplant (n=5 recipient mice were used for each genotype and experiment was performed in biological duplicate). 16-week chimerism was significantly higher in Tet2−/− transplanted mice compared with all other genotypes. p-value determined by Mann-Whitney U test. (C) Representative FACS analysis of peripheral blood of mice transplanted with each genotype at 16 weeks. Staining schemes are as indicated and parental gate was live cells. (D) Proportion of CD45.2+ peripheral blood cells of each lineage at 16 weeks in mice transplanted with each genotype (n=5 mice analyzed for each genotype) as determined by FACS analysis. Each competitive transplantation experiment was performed in biological duplicate with 5 recipient mice per genotype in each experiment. Error bars represent ± SD. Asterisk represents p<0.05 (Mann-Whitney U test).

Figure 7: Concomitant deletion of Asxl1 and Tet2 results in myelodysplasia in mice. (A) Kaplan-Meier survival curve of primary Cre-negative Asxl1fl/fl (n=5), Mx1-cre Asxl1fl/fl (n=12), Mx1-cre Tet2fl/fl (n=6), Mx1-cre Asxl1fl/fl Tet2fl/fl (n=10 mice per genotype). Mice were treated with pIpC at 4 weeks following birth and then followed for 50 weeks. (B) Peripheral white blood cell (WBC) count and differential of recipient mice transplanted with bone marrow from 6-week-old Mx1-cre Asxl1 wildtype Tet2 wildtype (control; C), Mx1-cre Asxl1fl/fl (Asxl1 KO),
Mx1-cre Tet2^{fl/fl} (Tet2 KO), and Mx1-cre Asxl1^{fl/fl} Tet2^{fl/fl} (Asxl1/Tet2 DKO) mice 66 weeks after transplantation (68 weeks after polyinosinic-polycytidylic acid (pIpC) administration to recipient mice) (n=10 mice per genotype). Asxl1 KO and Asxl1/Tet2 DKO mice had significantly lower WBC counts compared with control and Tet2 KO mice. Differential was determined by flow cytometric analysis of peripheral blood. (C) Hematocrit and (D) total number of nucleated bone marrow (BM) cells of same mice as shown in (B). (E) Representative flow cytometric assessment of relative frequencies of myeloid progenitor and lineage-negative Sca-1+ c-KIT+ (LSK) cells in 72-week-old mice. Parent population was live, lineage-negative cells. (F) Total numbers of LSK and myeloid progenitor cells (lineage-negative Sca-1- c-Kit+) in mice from each genotype at 72-weeks of age. This was determined by flow-cytometric quantification of living LSK and MP cells from c-KIT enriched BM cells harvested from spine plus bilateral femurs, tibiae, and humeri of each mouse from each genotype at 72 weeks of age (n=3 mice per group). (G) Wright-giemsa stain of BM representative erythroid precursor from cytospins of 72-week-old control, Asxl1 KO, Tet2 KO, or Asx1/Tet2 DKO mice. Dysplasia of erythroid precursors is seen in Asxl1 KO and Asxl1/Tet2 DKO genotypes as evidenced by erythroid precursors with prominent multinuclearity and nuclear fragmentation (arrows; scale bar represents 5 µm). (H) Representation histologic sections of liver from 72-week-old control, Asxl1 KO, Tet2 KO, or Asx1/Tet2 DKO mice revealing hematopoietic cell infiltrates in the Asxl1 KO, Tet2 KO, or Asxl1/Tet2 DKO mice suggestive of extramedullary hematopoiesis in all genotypes except control (scale bar represents 50 µm). Hematopoietic cell infiltrate is most prominent in the Asx1/Tet2 DKO mice. For (A) and (B) n=10 mice per group, for all (C)-(H) n=3 mice per group. Error bars represent ± SD. Asterisk represents $p<0.05$ (Mann-Whitney U test).

**Figure 8: Identification of genes significantly dysregulated with deletion of Asxl1 alone and in concert with deletion of Tet2 and their functional impact.**

(A) Volcano plot of differentially expressed transcripts from RNA-Seq data of 1-year old control versus littermate Asxl1 knockout (KO; Mx1-cre Asxl1^{fl/fl}) lineage-negative Sca-1+ c-Kit+ (LSK) cells and myeloid progenitor (lineage-negative Sca-1- c-Kit+) cells (experiment included cells from 2 individual mice per genotype).

(B) Venn diagrams of genes significantly up-regulated and down-regulated with Asxl1 loss in lineage-negative Sca-1+ cKit+ (LSK) and myeloid progenitor (MP; lineage-negative, Sca-1-negative, cKit+) cells from 1 year-old Mx1-cre Asxl1^{fl/fl} mice and littermate Cre-negative controls as identified in (A). (C) Quantitative
real-time PCR (qRT-PCR) analysis of \textit{HoxA} and \textit{Hox}-associated transcription factor genes in LSK cells of 1-year old Cre-negative \textit{Asxl1}^{fl/fl} control versus littermate \textit{Vav-cre Asxl1}^{fl/fl}. (D) qRT-PCR analysis of \textit{p16}^{NK4a} in long-term hematopoietic stem cells (LT-HSC; lineage-negative, Sca-1+, c-Kit+, CD150+, CD48-) and multipotent progenitor cells (MPP; lineage-negative, Sca-1+, c-Kit+, CD150-, CD48+) from 6-weeks and 6-months old control (C) versus littermate \textit{Vav-cre Asxl1}^{fl/fl} (KO) mice. (E) Cell cycle analysis of MPPs from 72-week-old \textit{Vav-cre Asxl1}^{fl/fl} or littermate Cre-negative \textit{Asxl1}^{fl/fl} control mice with \textit{in vivo} 5-bromo-2-deoxyuridine (BRDU) administration. Representative FACS plot analysis showing gating on MPP cells followed by BRDU versus DAPI stain is shown on left (parent gate is lineage-negative Sca-1+ c-KIT+ (LSK) cells). Relative quantification of the percentage of MPP cells in S-phase, G2/M-phase, and G0/1-phase is shown on right (n=5 mice per group) and reveals modest but statistically significant decrement in the proportion of MPP cells from KO mice in S-phase relative to controls. (F) Assessment of proportion of hematopoietic stem/progenitor cells undergoing apoptosis was performed by Annexin V/DAPI stain of LSK cells from 72-week-old \textit{Vav-cre Asxl1}^{fl/fl} mice or Cre-negative \textit{Asxl1}^{fl/fl} littermate controls. Representative FACS plot analysis showing gating on LSK cells followed by Annexin-V versus DAPI stain is shown on left (parent gate is lineage-negative cells). Relative quantification of the percentage of annexin V+/DAPI- and annexin V+/DAPI+ LSK cells is shown on right (n=5 mice per group) and reveals increase in annexin V+ cells in LSK cells from KO mice relative to controls. (G) Comparison of significant differentially expressed genes in LSK cells from 6-week-old \textit{Mx1-cre Asxl1}^{fl/fl}, \textit{Mx1-cre Tet2}^{fl/fl}, or \textit{Mx1-cre Asxl1}^{fl/fl} \textit{Tet2}^{fl/fl} relative to controls (or \textit{Mx1-cre Asxl1} wildtype \textit{Tet2} wildtype). 99 genes are uniquely downregulated in \textit{Asxl1/Tet2} double-knockout mice relative to all other genotypes (left) whereas 49 genes are significantly up-regulated (right). (H) Gene set enrichment analysis (GSEA) of overlapping and statistically significant gene sets enriched in the LSK cells of mice with deletion of \textit{Asxl1} alone or with combined \textit{Asxl1} and \textit{Tet2} deletion identifies enrichment of apoptosis and stem cell signatures as shown. (I) Gene sets uniquely enriched in mice with concomitant deletion of \textit{Asxl1} and \textit{Tet2} relative to all other genotypes as determined by GSEA. Asterisks denoted $p<0.05$. $p$-value determined by Mann-Whitney U test. Error bars represent ± SD.

\textbf{Figure 9: Effect of \textit{Asxl1} loss \textit{in vivo} on histone H3 lysine 27 trimethylation (H3K27me3) and identification of \textit{Asxl1} regulated genes by chromatin}
immunoprecipitation followed by sequencing (ChIP-Seq). (A) Loss of Asxl1 in vivo is associated with global loss of H3K27me3 as revealed by H3K27me3 Western blot in splenocytes of 6-week-old Vav-cre Asxl10/0 mice. (B) Levels of core-Polycomb repressive complex 2 members Ezh2, Suz12, and Eed in splenocytes of same mice as shown in (A). (C) Characterization of Asxl1 binding sites identified by anti-Asxl1 ChIP-seq analysis in murine wild-type bone-marrow derived macrophages. (D) Heatmap representation of Asxl1 ChIP-Seq signal centered around transcription start sites (TSS) (+/- 2kb) of CpG (left) and non-CpG (right) promoters. (E) Average Asxl1 ChIP-Seq signal density of CpG-(blue) and non-CpG (red) promoters centered around TSS +/- 10kb. (F) Motif enrichment analysis of Asxl1 binding sites identified significant enrichment of Ets transcription factor binding sites ($p=1e^{-59}$, % target=40.1%, and % background=21.4%).
Table 1: Genes directly regulated by Asxl1 as determined by differential expression in hematopoietic stem and progenitor cells with Asxl1 deletion and directly bound by Asxl1.

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1) Differentially expressed genes in RNA sequencing of myeloid progenitors (lineage-negative, Sca-1-negative c-KIT-positive cells) from 1 year-old Mx1-cre Asxl1fl/fl mice relative to Cre-negative Asxl1fl/fl littermate controls.

2) Differentially expressed genes in RNA sequencing of LSK cells (lineage-negative, Sca-1-positive c-KIT-positive cells) from 1 year-old Mx1-cre Asxl1fl/fl mice relative to Cre-negative Asxl1fl/fl littermate controls.

3) Data derived from anti-Asxl1 chromatin immunoprecipitation followed by next-generation (ChIP-Seq) in wildtype C57/B6H bone marrow-derived macrophages.
Materials and Methods

Animals
All animals were housed at New York University School of Medicine or at Memorial Sloan-Kettering Cancer Center. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees (IACUCs) at New York University School of Medicine and Memorial Sloan-Kettering Cancer Center.

Generation of Asxl1-Deficient Mice
The Asxl1 allele was deleted by targeting exons 5-10. Two LoxP sites flanking exon 5-10 and a Flrt-flanked neomycin selection cassette were inserted in the upstream intron (Fig. 1A). Ten micrograms of the targeting vector was linearized by NotI and then transfected by electroporation of BAC-BA1 (C57BL/6 x 129/SvEv) hybrid embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones. Secondary confirmation of positive clones identified by PCR was performed by Southern blotting analysis. DNA was digested with BamHI and electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a probe targeted against the 3’ or 5’ external region. DNA from C57BL/6 (B6), 129/SvEv (129), and BA1 (C57BL/6 x 129/SvEv) (Hybrid) mouse strains was used as wild-type controls. Positive ES clones were expanded and injected into blastocysts.

The generated mice (Asxl1fl/fl) were initially crossed to a germline Flp-deletor (Jackson Laboratories), to eliminate the neomycin cassette, and subsequently to the IFNγ-inducible Mx1-cre (Jackson Laboratories), the hematopoietic-specific Vav-cre, and the germline Ella-cre(Kuhn et al., 1995; Lakso et al., 1996; Stadtfeld and Graf, 2005). Mice were backcrossed for six generations to C57BL/6 mice.

Asxl1fl/fl, Asxl1fl/+, and Asxl1+/+ littermate mice were genotyped by PCR with primers Asxl1-F3 (5’–CAGCCGTTTTACCACAGTTT-3’) and Asxl1-R3 (5’-AGGGAAAGGGACAGAATGAC-3’) using the following parameters: 95°C for 4 min, followed by 35 cycles of 95°C for 45 sec, 56°C for 45 sec, and 72°C for 1 min, and then 72°C for 5 min. Wild-type allele was detected as a band at 200 base pair (bp) while floxed allele was detected as a band of 380 bp. Excision
after Cre recombination was confirmed by PCR with primers to detect a floxed portion of the construct (Asxl1-RecF: 5’–ACGCCGGCTTAAGTTGACG-3’ and Asxl1-RecR: 5’–GACTAAGTGGCGTGGTGCT-3’) using the same parameters as the above.

**In Vivo Studies**

*Mx1-cre Asxl1*^fl/fl^ conditional and Cre-negative *Asxl1*^fl/fl^ control mice received five intraperitoneal injections of poly(I:C) every other day at a dose of 20 mg/kg of body weight starting at 2 weeks post-birth. For the hematopoietic-specific *Vav-cre* line, *Asxl1*^fl/fl^*Vav-cre*^+, and *Asxl1*^fl/fl^*Vav-cre-^ mice were analyzed between 3 and 60 weeks of age. Bone marrow, spleen, and peripheral blood were analyzed by flow cytometry. Formalin-fixed paraffin-embedded tissue sections were stained with hematoxylin and eosin. Peripheral blood was smeared on a slide and stained using the Wright-Giemsa staining method. Tissue sections and blood smears were evaluated by a hematopathologist (C.Y.P.) Deletion of the *Asxl1* allele and transcript was measured by genomic PCR and Western blot analysis.

**Bone Marrow Transplantation**

Freshly dissected femurs and tibias were isolated from *Asxl1*^fl/fl^ CD45.2+ or *Vav-cre*+*Asxl1*^fl/fl^ CD45.2+ mice. Bone marrow was flushed with a 3 cc insulin syringe into PBS supplemented with 3% fetal bovine serum. The bone marrow was spun at 0.5 x g by centrifugation at 4°C, and red blood cells were lysed in ammonium chloride-potassium bicarbonate lysis buffer for 5 min. After centrifugation, cells were resuspended in PBS plus 3% FBS, passed through a cell strainer, and counted. Finally, 0.5 x 10^6^ total bone marrow cells of *Asxl1*^fl/fl^ CD45.2+ or *Vav-cre*+ *Asxl1*^fl/fl^ CD45.2+ mice were mixed with 0.5 x 10^6^ wild-type CD45.1+ support bone marrow and transplanted via tail-vein injection into lethally irradiated (two times 450 cGy) CD45.1+ host mice. Chimerism was measured by FACS in peripheral blood at 4 weeks post-transplant (week 0, pre-polyI-polyC). Chimerism was followed via FACS in the peripheral blood every 4 weeks (week 0, 4, 6, 8, 12, and 16 after polyI-polyC injection). Additionally, for each bleeding, whole blood cell counts were measured on a blood analyzer, and peripheral blood smears were scored. Chimerism in the bone marrow, spleen, and thymus was evaluated at 16 weeks via animal sacrifice and subsequent FACS analysis. The above procedure was also repeated with *Asxl1*^fl/fl^ CD45.2+, *Vav-cre*+ *Asxl1*^fl/fl^ CD45.2+ mice, *Vav-cre*+ Tet2^fl/fl^ CD45.2+, and *Vav-cre*+ *Asxl1*^fl/fl^ Tet2^fl/fl^ CD45.2+ mice for competitive transplantation of mice with loss of Asxl1, Tet2, or both. For non-
competitive transplantation studies, 1 x 10^6 total bone marrow cells of Asxl1^fl/fl^ CD45.2+, littermate Vav-cre+ Asxl1^fl/fl^ CD45.2+ mice, or littermate Mx1-cre+ Asxl1^fl/fl^ CD45.2+ mice were injected into lethally irradiated (two times 450 cGy) CD45.1+ host mice. Similarly, for LSK transplants, 1,000 FACS-sorted LSK cells from secondarily transplanted Asxl1 KO or control mice were transplanted into lethally irradiated CD45.1 host mice. Recipient mice were then followed until moribund or 80-weeks following transplantation.

**In Vitro Colony-Forming Assays**

LSK, CMP, GMP, and MEP cells were sorted from the bone marrow of Asxl1^fl/fl^ and littermate Vav-cre+ Asxl1^fl/fl^ mice and seeded at a density of 500 cells/replicate for LSK cells and 1,000 cells/replicate for CMP, GMP, and MEP subsets into cytokine-supplemented methylcellulose medium (Methocult, M3434; STEMCELL Technologies). Colonies propagated in culture were scored at day 7. Representative colonies were isolated from the plate for cytospins. Remaining cells were resuspended, counted, and a portion was taken for replating (20,000 cells/replicate) for a total of 7 platings. Cytospins were performed by resuspending in warm PBS and spun onto the slides at 350 x g for 5 min. Slides were air-dried and stained using the Giemsa-Wright method.

**Antibodies, FACS, and Western Blot Analysis**

Antibody staining and FACS analysis was performed as previously described(Klinakis et al., 2011). Bone marrow or spleen mononuclear cells were stained with a lineage cocktail comprised of antibodies targeting CD4, CD8, B220, NK1.1, Gr-1, CD11b, Ter119, and IL-7Rα. Cells were also stained with antibodies against c-Kit, Sca-1, FcγRII/III, and CD34. Cell populations were analyzed using a FACS-LSRII (Becton Dickinson) and sorted with a FACSAria instrument (Becton Dickinson). All antibodies were purchased from BD-Pharmingen or eBioscience. We used the following antibodies: c-Kit (2B8), Sca-1 (D7), Mac-1/CD11b (M1/70), Gr-1 (RB6-8C5), NK1.1 (PK136), Ter-119, IL7-Rα(A7R34), CD34 (RAM34), FcγRII/III (2.4G2), CD4 (RM4-5), CD4 (H129.19), CD8 (53-6.7), CD45.1 (A20), CD45.2 (104), CD150 (9D1), CD48 (HM48-1).

The following antibodies were used for Western blot analysis: Asxl1 (Clone N-13; Santa Cruz (sc-85283), Ezh2 (Millipore 07-689), Suz12 (Abcam ab12073), EED (Abcam ab4469), H3K27me3 (Abcam ab6002), total H3 (Abcam ab179), and tubulin (Sigma, T9026).
**Cell Cycle and Apoptosis Analyses.** For cell cycle analysis, the BRDU-APC kit was used (BD Pharmingen 557892) according to the manufacturer’s protocol. Mice were treated with 1mg BRDU intraperitoneally followed by harvest of BM cells 24 hours later. For evaluation of apoptosis, the Annexin V-FITC apoptosis detection kit was used (BD Pharmingen 556570) according to the manufacturer’s recommendations. DAPI was used as counterstain in both BRDU and annexin V experiments.

**Histological Analyses.** Mice were sacrificed and autopsied, then dissected tissue samples or were fixed for 24h in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin blocks were sectioned at 4µm and stained with haematoxylin and eosin. Images were acquired using a Zeiss Axio Observer A1 microscope (Zeiss).

**Peripheral Blood Analysis.** Blood was collected by retro-orbital bleeding using heparinized microhematocrit capillary tubes (Fisher). Automated peripheral blood counts were obtained using a HemaVet 950 (Drew Scientific) following standard manufacturer's instruction. Differential blood counts were realized on blood smears stained using Wright-Giemsa staining and visualized using a Zeiss Axio Observer A1 microscope (Zeiss).

**RNA Sequencing (RNA-Seq) and Quantitative Real-Time PCR Analysis**
Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and cDNA was synthesized using the SuperScript First-Strand Kit (Invitrogen). Quantitative PCR was performed using SYBR green iMaster and a LightCycler 480 (Roche). For RNA-Seq analysis, Fastq files were aligned to mm9 using TopHatV1.4 with default parameters. Differential expression tests were done using the Cuffdiff module of Cufflinks with RefSeq genes provided as an annotation (-N, -u and –M options engaged). We considered genes that had a q<0.05 to be significantly different between genotypes.

**Chromatin Immunoprecipitation Sequencing (ChIP-Seq) and Analysis**
Since low chromatin yields from hematopoietic stem/progenitor cell populations precluded ChIP-Seq studies, bone marrow derived macrophages (BMDM) from wildtype C57BL/6 mice were used as a surrogate to identify genome-wide Asxl1 binding sites. The antibody used for Asxl1 ChIP-Seq studies was from Santa Cruz (sc-85283). ChIP was performed as described previously (Dey et al., 2012).
ChIP and input DNAs were prepared for amplification by converting overhangs into phosphorylated blunt ends and adding an adenine to the 3' ends. Illumina adaptors were added and the library was size-selected (175-225 bp) on an agarose gel. The adaptor-ligated libraries were amplified for 18 cycles. The resulting DNA libraries were purified, quantified, and tested by qPCR at the same specific genomic regions as the original ChIP DNA to assess quality of the amplification reactions. DNA libraries then were sequenced on the Illumina Genome Analyzer II.

Sequenced reads were aligned to the reference genomes (mm9) using bowtie with maximum two mismatches, keeping only uniquely mapping reads. Peak calling was performed using MACS1.4 with the following options: -p 1e-7, --nomodel True, --shiftsize 100, --keep-dup 1. Peaks were assigned to genes using bedtools. We considered Asxl1-bound genes to be any mouse RefSeq entry containing a peak overlapping the gene or 2kb upstream of the TSS. ChIP-Seq read profile and heatmap densities were generated using genomic-tools. Mouse RefSeq and CpG island annotations were downloaded from the UCSC table browser (genome.ucsc.edu).

**Skeletal preparations.** Skeletal preparations were performed as described previously (de Pontual et al., 2011).
References


Martínez-Jaramillo, G., and E. Flores-Figueroa.... 2002. Comparative analysis of the in vitro proliferation and expansion of hematopoietic progenitors from patients with aplastic anemia and myelodysplasia. *Leukemia research*


factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. Blood 101:270-277.


Figure 1

Panel A: Schematic of Axl1 gene structure showing exons and introns. The positions of primers are indicated as arrows.

Panel B: Western blot analysis showing bands corresponding to WT and KO samples for both short and long arms.

Panel C: Table summarizing gene expression data for different days and genotypes.

Panel D: Photographs showing developmental stages of embryos labeled as "Asxl1 wildtype" and "Asxl1Δ/Δ" at Day 14.5.

Panel E: Images of Ets1 1/f/f embryos labeled as "Asxl1 Δ/Δ" at different developmental stages.

Panel F: Images of Ets1 1/f/f embryos labeled as "Asxl1 Δ/Δ" showing differences in morphology.

Panel G: Photographs of mice labeled as "Ets1 cre+" and "Asxl1 wt/Δ" showing phenotypic differences.

Panel H: Bar graph comparing the percentage of live cells in different genotypes.

Panel I: Heatmaps illustrating cell distributions for different genotypes at different developmental stages.

Figure 1 legend:
- Asxl1 1/f/f
- Asxl1 Δ/Δ
- Ets1 cre+
Figure 2

A: Relative fold-change

B: Western Blot

C: Number of cells × 10^6/µL

D: WBC count (x10^3/µL)

E: Hb (g/dL)

F: Number of cells × 10^6/µL

G: Flow cytometry analysis

Legend:
- Asxl1^+/+ bone marrow (BM)
- Asxl1^+/+ spleen
- Vav-cre + Asxl1^+/+ bone marrow (BM)
- Vav-cre + Asxl1^+/+ spleen

**Peripheral blood, gated on live cells:**

- B220
- CD11b+ Gr1+
- CD11b+ Gr1-
- CD3
Figure 3
Figure 4
Figure 5

A. 

B. Gated on lineage-negative

C. 

D. 

Figure 5
Figure 7
Figure 8
Figure 9

(A) Western blots showing the expression of Asxl1, Tubulin, H3K27me3, and Total H3 in Asxl1fl/fl and Vav-cre+ Asxl1fl/fl conditions.

(B) Western blots showing the expression of Asxl1, Ezh2, Suz12, and Eed in Asxl1fl/fl and Vav-cre+ Asxl1fl/fl conditions.

(C) Pie chart showing the percentage distribution of gene body, non-CpG promoter, intergenic, and CpG promoter regions.

(D) Heat maps showing the expression levels of CpG promoters and non-CpG promoters.

(E) Graph showing the average read density around the transcription start site (TSS) for CpG promoters and non-CpG promoters.

(F) Graph showing the information content for the sequence CAGAAAGT with a p-value of 1e^-59.

% target = 40.1%
% background = 21.4%