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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), and (3) identified the genome-wide effects of Asxl1 on transcription (Abdel-Wahab, et al. J Exp Med 2013 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 \textit{TET2}, \textit{ASXL1}, \textit{DNMT3a}, and \textit{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 \textit{ASXL1} and \textit{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 \textit{ASXL1} as well as the biological impact of conditional deletion of \textit{Asxl1} alone and in combination with other genetic alterations including \textit{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 \textit{Asxl1}, \textit{Tet2}, and \textit{Ezh2} single and compound knockout mice.

Task 2. “Complete characterization of mice with conditional deletion of \textit{Asxl1} alone and \textit{Asxl1} combined with \textit{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 \textit{Asxl1}, \textit{Tet2}, or both using multiple differe Cre recombinases. This work was recently published in the \textit{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 \textit{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 \textit{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 recently generated mice with Ezh2 deletion in the postnatal compartment (Mx1-cre Ezh2fl/fl) mice and mice with compound deletion of Ezh2 and Asxl1. From these murine models we have identified that:

(i) Hematopoietic stem cells (HSCs) from mice with compound Asxl1/Ezh2 loss have impaired self-renewal compared with HSCs from littermate control mice as well as mice with deletion of either gene alone.

(ii) A high proportion of wildtype mice reconstituted with bone marrow from mice with compound Asxl1/Ezh2 (Mx1-cre Asxl1fl/fl Ezh2fl/fl) deletion die of bone marrow failure within weeks of deletion of these genes. Surviving mice are characterized by anemia and leukopenia as well as morphologic dysplasia.

The above phenotypes of mice with compound deletion of both Asxl1 and Ezh2 are dramatic and we are now working to functionally understand the mechanism by which deletion of these 2 genes impairs HSC function.

**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.

We are now performing these experiments *ex vivo* through use of methylcellulose colony assays. In brief, hematopoietic stem/progenitor cells (HSPCs; lineage-negative Sca1+ c-KIT+ cells) from Tet2 knockout, Asxl1 knockout, Ezh2 knockout, and Tet2/Asxl1 double knockout mice are being plated in methylcellulose with a variety of the above compounds for 7 days. We are evaluating the effects of these compounds on restoring colony formation (for Asxl1 and Ezh2 knockout HSPCs) or reducing colony formation (for Tet2 and Tet2/Asxl1 knockout HSPCs). This work is 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.

In order to complete this task and to inform task #5, we recently performed targeted DNA sequencing on pretreatment DNA samples from a cohort of MDS patients uniformly treated with decitabine. This work, performed in collaboration with MDS clinical expert Dr. Valeria Santini, revealed that ASXL1 mutations frequently co-occur with mutations in the spliceosome-associated protein SRSF2 in patients with MDS/MPN overlap syndromes. This interesting finding suggests an interaction by mutations in the epigenome with mutations in the spliceosome.

**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.

We have now collected samples from 40 such patients with MDS with bone marrow fibrosis and hope to begin performing DNA sequencing soon. We recently helped to generate a DNA next-generation sequencing panel of 300 genes implicated in cancer pathogenesis at our institution. We will apply this sequencing platform to these MDS samples with the hopes of characterizing any novel mutations associated with this unique subtype of MDS.

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

I have given 5 presentations at national/international meetings on the work performed with funding from this award in the last year (see list of presentations in Reportable Outcomes below).
I have also been invited to write several reviews related to the work described in this proposal in well-respected journals including *Blood* and *Haematologica* (cited 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:

2013 National Cancer Research Center, Tokyo Japan
2013 10th International Nikko Symposium, Utsonomiya Japan
2013 Dept. of Cell and Molecular Biology, Chiba University, Chiba Japan
2013 ASH Educational Session on Myeloproliferative Neoplasms
2014 Aplastic Anemia and MDS International Foundation Symposium

Development of novel genetically engineered murine models:
Developed the following murine models of bone marrow failure, all of which are currently published:
- **In vivo** shRNA-mediated depletion of Asxl1 combined with retroviral overexpression of NRasG12D (Abdel-Wahab, O, et al. Cancer Cell 2012)

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). 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)
Recently, major advances in our understanding of the pathogenesis of sporadic myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) have been made through unbiased gene discovery approaches. Similar advances have been made in understanding the genetic basis of rare cases of familial MDS and AML. Currently, germline mutations in genes encoding transcription factors, including RUNX1, CEBPA, and more recently GATA2, have been identified in patients with familial MDS/AML. In patients with familial MDS/AML, however, there is great heterogeneity in the age of disease onset as well as the clinical characteristics of the myeloid malignancy which develops in affected members of such families. For instance, in the largest single survey of disease phenotypes in individuals with germline GATA2 mutations, 50% of patients were without symptoms at the age of 20 and 16% continued to remain without symptoms by the age of 40. In this issue of Haematologica, West et al. begin to unravel the genetic alterations that frequently occur together and collaborate with germline GATA2 mutations to promote the development of MDS and AML.

In 2011, four papers were published identifying heterozygous germline GATA2 mutations as the cause of four previously described clinical syndromes: primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome), RUNX1/CEBPA wild-type familial AML/MDS, monocytopenia and mycobacterial infections (MonoMAC syndrome), and the dendritic cell, monocyte, B and NK, granulocyte colony-stimulating factor receptor, SUZ12, and EP300 deficiency syndrome (DCML deficiency). Since then, approximately 200 patients with germline GATA2 mutations have been described (Table 1), each presenting with a variety of clinical presentations but all with a high risk of developing MDS/AML. In a summary of these studies, approximately 70% of GATA2-deficient individuals appear to develop MDS or AML in their lifetime.

Interestingly, attempts have been made to correlate the risk and outcomes of myeloid malignancy in patients with germline GATA2 mutations with the genotype of the GATA2 mutation present, but this has been limited by the number of patients. Although monosomy 7 clearly appears to be enriched in GATA2-deficient individuals who develop MDS and AML (50% of individuals; Table 1), the first clue to a specific molecular abnormality which might be an important collaborating genetic event for the development of overt myeloid malignancy in GATA2 mutant families came from recent work by Bodor et al. In this prior study of a germline GATA2-mutant kindred, somatic ASXL1 mutations were present exclusively in the two members of the family who developed MDS/AML. This finding strongly suggested that ASXL1 mutations might be an important trigger for the development of overt disease in GATA2-mutated patients.

West et al. performed targeted sequencing of ASXL1 in 48 patients with germline GATA2 mutations and identified heterozygous ASXL1 mutations in 14 of them (29%). Given the rarity of ASXL1 mutations in individuals with myeloid malignancies less than 60 years old, the high frequency of ASXL1 mutations in GATA2-deficient individuals developing MDS/AML is remarkable. Eight different ASXL1 mutations were seen in ten different GATA2-mutant backgrounds. Similar to the pedigree studied by Bodor et al., in this study one pair of sisters had the same GATA2 mutation but only the sister who had an ASXL1 mutation actually developed clinically evident MDS. This finding further underscores the likely collaboration between GATA2 and ASXL1 mutations in promoting the development of MDS. In another informative pedigree here, two sisters with the same GATA2 mutation had discordant ASXL1 genotypes but both developed chronic myelomonocytic leukemia (CMML). This finding further validates the already strong link between the presence of ASXL1 mutations and the clinical phenotype of CMML.

Although this study had a relatively limited number of patients due to the rarity of germline GATA2 mutant patients, the authors were able to determine that overall survival in ASXL1-mutant/GATA2-mutant patients was worse than that in patients with an ASXL1 wild-type/GATA2 mutant genotype. This finding is quite consistent with larger studies in MDS patients revealing an unwavering association between the presence of ASXL1 mutations and adverse outcome. In contrast to de novo MDS, MDS occurring in patients with GATA2 germline mutations are usually hypocellular for age with increased reticulin fibrosis. Interestingly, results from the study by West et al. suggest that the development of ASXL1 mutations coincides with progression from the hypoplastic MDS characteristic of GATA2 deficiency to a more proliferative disease. More sensitive quantitative sequencing, comparing samples during the hypoplastic MDS phase of disease and during acute transformation will be needed to understand how early the ASXL1 mutations occur in the pathogenesis of myeloid disease in these individuals. This is especially relevant since most individuals with co-occurring GATA2 and ASXL1 mutations in this study had additional cytogenetic abnormalities such as monosomy 7.

Further unbiased genome-wide sequencing studies, currently being undertaken by this group and others, are needed to understand the full spectrum of somatic mutations in hematopoietic cells in individuals with disease evolution. For instance, recent whole exome sequencing of serial samples over a 17-year period from a single patient with severe congenital neutropenia progressing to AML showed a number of early and late genetic defects associated with leukemic progression. A nonsense mutation in the gene encoding for granulocyte colony-stimulating factor receptor, CSF3R, appeared to be a clear, early event in the severe congenital neutropenia phase of the disease. In contrast, mutations arising later in the development of AML included mutations in ASXL1, SUZ12, EP300, RUNX1, and an additional mutation in CSF3R. Based on the work by West et al., it is quite plausible that the development of MDS and AML in GATA2 deficient individuals might be similarly driven by a stepwise accumulation of genetic mutations with clonal expansion and selection, with ASXL1 seeming to play a central role.
The strong genetic link between GATA2 and ASXL1 mutations in patients with this rare germline disorder raises the question of the frequency of ASXL1 mutations in diseases marked by somatic GATA2 mutations. For instance, somatic GATA2 mutations have been described in Philadelphia chromosome-negative chronic myeloid leukemia patients at transformation to myeloid blast crisis. Most of these cases were associated with a gain-of-function mutation in GATA2 (Leu359Val) in contrast to GATA2 mutations seen in patients with GATA2-mutant germline syndromes. Somatic GATA2 mutations have also been identified in Philadelphia chromosome-negative chronic myeloid leukemia patients with biallelic CEBPA mutations. From the limited data published on such patients, GATA2 mutant de novo AML does not appear to be significantly associated with ASXL1 mutations. Moreover, GATA2 mutations in de novo AML appear to be associated with a relatively favorable prognosis.

The significant co-occurrence of GATA2 deficiency with ASXL1 mutations at development of MDS/AML strongly suggests a cooperative interaction of these genetic events in promoting hematopoietic transformation. ASXL1 is a Polycomb associated protein which has been shown to affect transcription through effects on the ability of the Polycomb repressive complex 2 to perform histone H3 lysine 27 methylation and also potentially by interacting with the histone H2A lysine 119 deubiquitinase enzyme BAP1. More recently, genome-wide localization studies of ASXL1 by chromatin immunoprecipitation followed by next-generation sequencing showed that ASXL1 localizes strongly to promoter regions of the genome. Moreover, ASXL1 binding strongly overlaps with that of ETS transcription factors. It is now well understood that deletion of key ETS transcription factors, such as PU.1, promotes aggressive myeloid malignancies in vivo. Interestingly even lowering levels of PU.1 to 20% of normal levels promotes leukemogenesis. This observation highlights the importance of transcriptional regulation of ETS target genes in the pathogenesis of myeloid malignancy. Theses facts, taken together with the knowledge that GATA2 interacts with and represses PU.1, possibly suggest that the explanation for the genetic interaction between ASXL1 and GATA2 may lie in the intersection of these factors in transcriptional regulation of key PU.1 target genes. Given the importance of ASXL1 mutations in myeloid malignancies and the development of molecular knowledge regarding GATA2 function in hematopoiesis, future functional work dissecting the interaction of ASXL1 mutations and GATA2 haploinsufficiency may address this hypothesis. It is hoped that further in vitro and in vivo work will elucidate this fascinating genetic interaction identified by West et al.

Jean-Baptiste Micol is an M.D. at the Institut Gustave Roussy (IGR), Villejuif, France and PhD candidate in the laboratories of Professor Eric Solary (IGR) and Dr. Omar Abdel-Wahab at the Memorial Sloan-Kettering Cancer Center, New York, USA. His main field of interest is translational research in myelodysplastic syndromes and acute myeloid leukemia. Omar Abdel-Wahab, MD, is an Assistant Member of the Human Oncology and Pathogenesis Program and the Leukemia Service in the Department of Medicine at Memorial Sloan-Kettering Cancer Center. He is interested in the molecular genetics of leukemia.

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References


Deletion of Asxl1 results in myelodysplasia and severe developmental defects in vivo

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Somatic Addition of Sex Combs Like 1 (ASXL1) mutations occur in 10–30% of patients with myeloid malignancies, most commonly in myelodysplastic syndromes (MDSs), 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 anophthalmia, microcephaly, cleft palates, and mandibular malformations. In contrast, hematopoietic-specific deletion of Asxl1 results in progressive, multilineage cytopenias and dysplasia in the context of increased numbers of hematopoietic stem/progenitor cells, 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 (KO) 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. Moreover, compound Asxl1/Tet2 deletion results in an MDS phenotype with hastened death compared with single-gene KO mice. Asxl1 loss results 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 identify 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.

Candidate gene and genome-wide discovery studies have identified a set of novel disease alleles in patients with myelodysplastic syndromes (MDSs), acute myeloid leukemia (AML), and myeloproliferative neoplasms (MPNs). 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
Asxl1 is a Polycomb–associated protein that has been shown to be an essential cofactor 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, 2012; Thol et al., 2011; Sanada and Ogawa, 2012), including in 15–20% of MDS patients and in 40–60% in patients with MDS/MPN overlap syndromes (Gelsi-Boyer et al., 2009; Boulwood et al., 2010; Jankowska et al., 2011). ASXL1 mutations are associated with adverse overall survival in MDS, chronic myelomonocytic leukemia, AML, and MPN (Bejar et al., 2011, 2012; Metzeler et al., 2011; Patel et al., 2012; Itzykson et al., 2013; 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-Opitz 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 the lack of a mouse model for conditional deletion of Asxl1 (Asxl1f/f) mice (not depicted). We observed 100% embryonic lethality in mice with germline complete deletion of Asxl1 (Asxl1−/−), whereas mice with heterozygous germline deletion of Asxl1 (Asxl1+/-) were born at expected Mendelian ratios (Fig. 1 C). Asxl1+/- mice were no longer viable by embryonic day (E) 19.5 and were characterized by microphthalmia/anophthalmia (seen in 12/12 of homozygous Asxl1−/−embryos examined; Fig. 1, D and E), frequent cleft palates (seen in 5/12 of homozygous Asxl1−/− embryos examined; Fig. 1 E), and multiple skeletal abnormalities (mandibular hypoplasia, loss of hyoid bone formation, and posterior homeotic transformations; seen in 4/12 of homozygous Asxl1−/−embryos examined; Fig. 1 F). Asxl1+/- mice were viable but exhibited craniofacial dysmorphism in 35% (14/40) of adult Asxl1+/- mice examined (Fig. 1 G). Immunophenotypic analysis of HSPCs and erythroid precursor cells in fetal liver from control, Asxl1−/−, and Asxl1+/- mice at E14.5 did not reveal differences among the genotypes (Fig. 1, H and I).

**RESULTS**

**Development of a conditional Asxl1 KO allele**

To delineate the role of Asxl1 in development and in hematopoiesis, we generated a conditional allele targeting Asxl1 in vivo (Fig. 1, A and B). We used embryonic stem (ES) cell targeting to insert two LoxP sites flanking exons 5–10 of Asxl1, as well as an Flp-flanked neomycin selection cassette in the upstream intron (Fig. 1, A and B). The generated mice (Asxl1f/f) were initially crossed to a germline Flp-deleter mouse line to eliminate the neomycin cassette and then subsequently crossed to germline Ela-cre mice, IFN-α–inducible Mx1-cre, and hematopoietic-specific Vav-cre (all as described below). Asxl1 protein expression was not detectable in hematopoietic tissue from Vav-cre and Mx1-cre mice (Fig. 2 B), consistent with generation of a KO allele.

**Germline Asxl1 loss results in embryonic lethality and craniofacial abnormalities**

We characterized the effects of constitutive deletion of Asxl1 by crossing mice bearing floxed Asxl1 alleles with germline Ela-cre mice. The Asxl1 floxed allele was completely recombined in Ela-cre Asxl1f/f mice (not depicted). We observed 100% embryonic lethality in mice with germline complete deletion of Asxl1 (Asxl1−/−), whereas mice with heterozygous germline deletion of Asxl1 (Asxl1+/-) were born at expected Mendelian ratios (Fig. 1 C). Asxl1+/- mice were no longer viable by embryonic day (E) 19.5 and were characterized by microphthalmia/anophthalmia (seen in 12/12 of homozygous Asxl1−/−embryos examined; Fig. 1, D and E), frequent cleft palates (seen in 5/12 of homozygous Asxl1−/− embryos examined; Fig. 1 E), and multiple skeletal abnormalities (mandibular hypoplasia, loss of hyoid bone formation, and posterior homeotic transformations; seen in 4/12 of homozygous Asxl1−/−embryos examined; Fig. 1 F). Asxl1+/- mice were viable but exhibited craniofacial dysmorphism in 35% (14/40) of adult Asxl1+/- mice examined (Fig. 1 G). Immunophenotypic analysis of HSPCs and erythroid precursor cells in fetal liver from control, Asxl1−/−, and Asxl1+/- mice at E14.5 did not reveal differences among the genotypes (Fig. 1, H and I).

**Hematopoietic-specific deletion of Asxl1 results in MDS**

Asxl1 is expressed throughout the adult hematopoietic compartment (Fig. 2 A). To elucidate the effects of Asxl1 loss on postnatal hematopoiesis, Asxl1f/f mice were crossed to Vav-cre and IFN-α–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. 2 B). Mice with hematopoietic–specific deletion of Asxl1 (Vav-cre Asxl1f/f) developed progressive BM and splenic hypocellularity relative to littermate controls (Cre− Asxl1f/f) beginning at 6 wk of age and likewise evident at 24 wk of age (n = 6–10 mice per genotype at each time point examined; Fig. 2 C), Asxl1 KO mice, but not littermate controls, developed progressive leukopenia (Fig. 2 D) and anemia (Fig. 2 E) that was most apparent at 6–12 mo of age. Although the hemoglobin (Hb) in Asxl1 KO mice was within normal limits (median of 13.4 g/dl, range 12.9–14.8 g/dl) in mice <6 mo of age, between 6 and 12 mo the Hb was a median of 8 g/dl (range 1.9–13.9 g/dl) in KO mice relative to a median of 11.4 g/dl in age-matched littermate control mice (range 7.17–14.2 g/dl). Similary, the WBC count was within normal limits in Asxl1−/−null mice at <6 mo of age (median of 7.64 × 109 cells/µl, range 4.66–9.6 × 109 cells/µl), the WBC count fell to a median of 2.51 × 109 cells/µl (range 0.88–5.18 × 109 cells/µl)
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+/− parents. (D and E) Gross pathology (D) and tissue sections (E) of Asxl1−/− mice at 14.5 and 18.5 d postcoitus (dpc). (F) Analysis of skeletal preparations from germline Asxl1-null mice surviving to E20.5 including hypoplastic mandibles (asterisk), lack of hyoid bone (arrowhead), and lower lumbar/sacral posterior homeotic transformations (arrow). (G) Gross phenotype of Ella-cre Asxl1+/− and littermate control mice on bilateral microphthalmia. Bars: (D) 2 mm; (E and F [top]) 1 mm; (F, bottom) 2.5 mm; (G) 0.5 cm. (H) Immunophenotyping of fetal liver at 14.5 dpc on relative frequency of LSK cells, MPP cells (LSK, CD48−, CD150− cells), and LT-HSCs (LSK, CD48−, CD150+ cells) between mice with germline loss of 0, 1, or 2 copies of Asxl1. FACS analysis was performed with three to five independent fetal liver samples per genotype. (I) FACS analysis of fetal liver at 14.5 dpc reveals 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- and Vav-cre-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 KO mice (Vav-cre Asxl1fl/fl) at 6 as well as 24 wk of age (n = 6–10 mice per genotype at each time point examined). (D and E) Enumeration of peripheral WBCs (D) and Hb (E) with postnatal deletion of Asxl1 (performed using Mx1-cre Asxl1fl/fl mice or CreAsxl1fl/fl controls). Counts in aged Asxl1 KO mice are compared with age-matched controls as well as younger KO and control mice (n = 6–12 mice per genotype at each time point examined). (F and G) Flow cytometric enumeration of B220+CD11b−Gr1− cells in the peripheral blood of >6-mo-old Mx1-cre Asxl1fl/fl (KO) and Asxl1fl/fl (C) mice (n = 5 mice per genotype were used for FACS analysis of peripheral blood). The right panel reveals peripheral blood FACS analysis. Antibody stainings are as indicated, and cells were gated on live cells in the parent gate. (A and C–F) Error bars represent ±SD (A and F); mean ± SEM is shown (C–E); *, P < 0.05; **, P < 0.001 (Mann–Whitney U test).
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 HSPCs from patients with MDS (Sawada et al., 1993, 1995) and mouse models of MDS (such as the NUP98-HOXD13 transgenic mouse model [Choi et al., 2008]) have

Asx11 KO mice was accompanied by an increase (median 1.4–2-fold) in CD71+/Ter119− erythroid precursor cells in both the BM and spleen, consistent with impaired erythroid differentiation (Fig. 3A).

Pathological analysis of Asx11 KO hematopoietic tissues at 6 mo of age revealed morphological 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).
Figure 4. Serial noncompetitive transplantation of Asxl1-null cells results in lethal myelodysplastic disorder. (A) Kaplan-Meier survival curve of recipient mice transplanted with 70-wk-old Vav-cre Asxl1fl/fl or Cre− Asxl1fl/fl littermate control whole BM after secondary and tertiary transplantation. Also shown is the survival of mice transplanted with purified LSK cells in tertiary transplantation (tertiary transplant of Asxl1fl/fl control LSK cells is not shown; no recipient mice from this group died by 40 wk [n = 5]). Cre− Asxl1fl/fl littermate controls were similarly transplanted in parallel in each
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−/− mice, in vitro analysis of sorted myeloid progenitor (MP) cells from 6-wk-old Asxl1 KO and control mice revealed a clear decrease in colony output of sorted common MPs (CMPs), granulocyte/macrophage progenitors (GMPs), and megakaryocyte/erythroid progenitors (MEPs) in KO versus control mice (Fig. 3, E and 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 (Vav-cre Asxl1fl/fl) mice were found to have infiltration of liver with hematopoietic cells consistent with extramedullary hematopoiesis (EMH) with Asxl1 deletion (Fig. 3 G). To ascertain whether 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 semisolid media containing myeloid-erythroid cytokines (rniIL-3, rniSCF, rh-EPO, and rh-IL6). Colonies plated with cells derived from Asxl1 KO mice alone yielded abundant colonies (Fig. 3, H and 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. 4 A), indicating that the phenotype induced by Asxl1 loss was cell autonomous. For example, transplantation of whole BM from 70-wk-old primary Asxl1 KO mice into lethally irradiated recipients resulted in the death of recipient mice at a median of 50 wk after transplant (range 41–74 wk), whereas no mice transplanted with Asxl1 WT BM died during this period of observation. Furthermore, serial transplantation into tertiary recipients resulted in shorter latency of disease, with mice dying 24–42 wk after transplant (median of 28 wk). Transplantation of purified lineage− 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 (median of 10.3 wk, range 5.1–10.6 wk) than transplantation of unfractionated BM cells from the same secondary recipients (Fig. 4 A). Disease in transplanted mice was characterized by progressive anemia and cachexia (Fig. 4, B and C), BM hypocellularity (Fig. 4 D), and an increase in the relative frequency of HSPCs in both the BM and the spleen (Fig. 4, E and F). This was accompanied by splenomegaly caused by EMH and effacement of splenic architecture (Fig. 4, G–I). Anemia in the KO-transplanted recipient mice was evident even with gross inspection of bones (Fig. 4 J). As in primary Asxl1 KO mice, this anemia occurred despite an increase in erythroid precursors in both the BM and spleen (Fig. 4, K and 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. 4 L).

**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 wk of age, including long-term HSCs (LT-HSC; CD150+ CD48− Lin− Sca-1+ c-KIT+) (Fig. 5, A and B; 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. 6 A), suggesting a potential defect in self-renewal. To assess the effects of Asxl1 deletion in vivo, 500,000 whole BM nucleated cells from 6-wk-old CD45.2 Vav-cre Asxl1fl/fl mice or Asxl1fl/fl littermate controls were transplanted in competition with an equal number of 6-wk-old CD45.1 competitor BM cells into lethally irradiated CD45.1 recipient mice (Fig. 5 C).
Chimerism was assessed based on evaluation of the ratio of CD45.1 to CD45.2 peripheral blood mononuclear cells beginning 2 wk after transplantation and then monitored on a monthly basis until 16 wk 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. 5, C and D).

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

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, 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 Asxl1KO/Tet2KO hematopoietic cells compared with control, Vav-cre Asxl1KO, and Vav-cre Tet2KO 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 experiments revealed a competitive advantage for Vav-cre Asxl1KO/Tet2KO whole BM compared with matched CD45.1 competitor BM (Figs. 6, B–D). These data demonstrate that concurrent Tet2 loss restores the self-renewal defect induced by Asxl1 loss.
Figure 7. Concomitant deletion of Asxl1 and Tet2 results in myelodysplasia in mice. (A) Kaplan-Meier survival curve of primary Cre−/− Asxl1 WT (n = 5), Mx1-cre Asxl1 KO (n = 12), Mx1-cre Tet2 KO (n = 6), Mx1-cre Asxl1 KO + Tet2 KO (n = 10 mice per genotype). Mice were treated with polyI:polyC at 4 wk after birth and then followed for 50 wk. (B) Peripheral WBC count and differential of recipient mice transplanted with BM from 6-wk-old Mx1-cre Asxl1 WT Tet2 WT (control; C), Mx1-cre Asxl1 KO (Asxl1 KO), Mx1-cre Tet2 KO (Tet2 KO), and Mx1-cre Asxl1 KO + Tet2 KO (Asxl1/Tet2 DKO) mice 66 wk after transplantation.
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− Asxl1fl/fl Tet2fl/fl, Mx1-cre Asxl1fl/fl (Asxl1 KO), Mx1-cre Tet2fl/fl (Tet2 KO), and Mx1-cre Asxl1fl/fl Tet2fl/fl (double KO [DKO]) mice were treated with polyinosinic-polycytidylic acid (polyI:polyC) at 4 wk of life and followed up to 50 wk after birth (46 wk after polyI:polyC 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. 7 A).

For further analyses, to obtain a sufficiently large number of mice for each genotype, BM from 6-wk-old CD45.2 Mx1-cre Asxl1 WT Tet2 WT, Mx1-cre Asxl1fl/fl (Asxl1 KO), Mx1-cre Tet2fl/fl (Tet2 KO), and Mx1-cre Asxl1fl/fl Tet2fl/fl (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 WT Tet2 WT BM) were then treated with polyI:polyC 2 wk after transplantation to delete Tet2 and/or Asxl1. At 72 wk after transplantation, Asxl1-null and/or Asxl1/Tet2 compound-null mice had significantly lower WBC counts and hematocrit compared with WT or Tet2 single KO control mice (Fig. 7, B and 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. 7 D). 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. 7 D). Examination of the HSPC compartment across mice with the four genotypes at 72 wk indicated a greater total number as well as relative frequency of LSK and MP cells in the BM of DKO mice compared with other groups (Fig. 7 E and 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. 7 G). The BM of DKO mice likewise was characterized by a similar presence of dysplastic erythroid precursors as well as dysplastic myeloid cells (Fig. 7 G) but lacked the hypocellularity seen in the BM of mice with Asxl1 deletion alone (Fig. 7 D). Histological analysis of liver tissue revealed increased hematopoietic cell infiltration in DKO mice compared with the other groups (Fig. 7 H). The presence of morphological dysplasia in precursor cells, decrease in peripheral circulating mature cells, and concurrent increased total BM cells and HSPCs were suggestive of the presence of MDS in the Asxl1/Tet2 compound–deficient mice.

Transcriptional effects of Asxl1 loss

To understand what transcriptional differences might exist between mice with compound deletion of Asxl1 and Tet2 (68 wk after polyI:polyC administration to recipient mice; n = 10 mice per genotype). Differential was determined by flow cytometric analysis of peripheral blood. (C and D) Hematocrit (C) and total number (D) of nucleated BM cells of same mice as shown in B. Horizontal lines indicate the mean. (E) Representative flow cytometric assessment of relative frequencies of MP and LSK cells in 72-wk-old mice. Parent population was live, lineage− cells. (F) Total numbers of LSK and MP cells (lineage− Sca-1− c-Kit+/hi) in mice from each genotype at 72 wk 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 wk of age (n = 3 mice per group). (G) Wright-Giemsa stain of BM representative erythroid precursor from cytopsins of 72-wk-old control, Asxl1 KO, Tet2 KO, or Asxl1/Tet2 DKO mice bars: (G) 5 μm; (H) 50 μm. For A and B, n = 10 mice per group; for C–H, n = 3 mice per group. Error bars represent ±SD; *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-yr-old control versus littermate Asxl1 KO (Mx1-cre Asxl1fl/fl) LSK and MP (lineage−, Sca-1−, c-Kit+) cells (experiment included cells from two individual mice per genotype). (B) Venn diagrams of genes significantly up- and down-regulated with Asxl1 loss in LSK and MP (lineage−, Sca-1−, cKit+) cells from 1-yr-old Mx1-cre Asxl1fl/fl mice and littermate.
relative to mice with deletion of Asxl1, Tet2, or neither gene, we performed RNA-Seq on LSK cells sorted from the BM of 6-wk-old Mx1-cre Asxl1 WT/Tet2 WT, Mx1-cre Asxl1 WT/Tet2 fl/fl, and Mx1-cre Asxl1 fl/fl/Tet2 WT mice (Fig. 8 G and Table S3). Of the 1,744 genes significantly up-regulated in any KO 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 [510/1,744 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 HSPCs from Asxl1 KO mice or Asxl1/Tet2 DKO mice compared with other groups (Subramanian et al., 2005). We identified gene sets enriched in HSCs (Ramalho-Santos et al., 2002) and apoptosis (http://www.genome.jp/kegg/pathway/hsa/hsa04110.html), and signatures from cell cycle regulators (http://www.genome.jp/kegg/pathway/hsa/hsa04210.html) in mice with Asxl1 deletion and with concomitant Asxl1/Tet2 deletion (Fig. 8 H). We also identified gene sets that 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 HSCPs (Ivanova et al., 2002), cell cycle regulators (http://www.genome.jp/kegg/pathway/hsa/hsa04110.html), and signatures from MLL-rearranged primary leukemias (Fig. 8 I; Ross et al., 2003; Mullighan et al., 2007).

**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 (Abdel-Wahab et al., 2012; Dey et al., 2012). This includes binding to the core members of the PRC2, where loss of ASXL1 has previously been found to result in global down-regulation 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. 9 A) despite sustained expression of the core PRC2 components (Fig. 9 B).

Although the effects of Asxl1 loss on transcription caused by alterations in histone posttranslational modifications have previously been described (Abdel-Wahab et al., 2012), direct transcriptional targets of Asxl1 through characterization of Asxl1 binding throughout the genome have never previously been assessed. We therefore performed chromatin immunoprecipitation (ChIP) for Asxl1 followed by DNA sequencing (ChIP-Seq) in purified mouse 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 (TSSs; Figs. 9, C–E; and Tables S4 and S5). Motif enrichment analysis of the Asxl1-binding sites revealed that the top occurring motifs are most similar to known binding sites of the Ets family of transcription factors (Fig. 9 F; P = 1 × 10–59, percent target = 40.1%, and percent 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 and 9 down-regulated genes; Table 1).

**DISCUSSION**

Here we show 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 HSPCs in vivo. Asxl1 loss also led to a reduction in myeloid colony output. MDS is characterized by variable cytopenias caused by ineffective production of mature granulocyte, erythrocytoid, 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 HSC compartment (comprised of long-term and short-term HSCs; Will et al., 2012; Pang et al., 2013), the presence of specific genetic alterations throughout the diseased clone originating in the most immature HSCs (Nilsson et al., 2000, 2007; Tehranchi et al., 2007) controls as identified in A. (C) qRT-PCR analysis of HoxA and Hox-associated transcription factor genes in LSK cells of 1-yr-old Cre−/− Asxl1fl/fl control versus littermate Vav-cre Asxl1fl/fl. (D) qRT-PCR analysis of p16Ink4a in LT-HSCs (lineage−, Sca-1−, c-Kit+, CD150+, CD48−) and MPP cells (lineage−, Sca-1−, c-Kit+, CD150+, CD48+) from 6-wk- and 6-mo-old control (C) versus littermate (WT) and with concomitant deletion of T-cell (Tet2−/−) or Asxl1 alone (Asxl1−/−) or with combined Tet2 and Asxl1 deletion. (I) Gene sets uniquely enriched in mice with concomitant deletion of Asxl1 and Tet2 relative to all other genotypes (left), whereas 49 genes are significantly up-regulated (right).
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,b). Our conditional model allows for evaluation of the effects of postnatal deletion of Asxl1 and obviates the problems associated with a high frequency of perinatal lethality in mice with constitutive Asxl1 deletion. Notably, Fisher et al. (2010a,b) identified a 72% reduction in the expected number of Asxl1 homozygous KO mice by postnatal day 21; when mice were backcrossed more than eight 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 caused by EMH, and decreased formation of myeloid and erythroid colonies from Asxl1 KO cells (Fisher et al., 2010a,b). In addition, Fisher et al. (2010a,b) observed dysregulated expression of HoxA genes and homeotic transformation of homozygous Asxl1 mutant embryos, consistent with the current Asxl1 germline and conditional KO models described in this study.

One aspect of the Asxl1 conditional KO mouse model that 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 KO 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 pathological evidence of multilineage 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 used 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. In contrast, ASXL1 mutations occur in 15–20% of patients with MDS. Despite these similarities, several differences exist between the two models. First, no reproducible differences in peripheral blood counts, BM cellularity, or BM cell morphology were seen in Asxl1 constitutive KO mice. In contrast, mice with conditional, homozygous, postnatal deletion of Asxl1 developed leukopenia, anemia, myelodysplasia, and BM hypopcellularity starting at 6 mo of life. This could be caused by differences in the strain of the mice analyzed, the timing of Asxl1 loss, or cell–nonautonomous effects observed in the constitutive KO model.

Of note, the more profound hematologic abnormalities seen with serial competitive and noncompetitive transplantation...
...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 KO 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 that 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 that loss of ASXL1 in vitro results in global down-regulation in H3K27me3 (Abdel-Wahab et al., 2012), the repressive histone modification placed by the PRC2. Here we demonstrate that Asxl1 deletion in the hematopoietic compartment results in reduced H3K27me3 in vivo. We used ChIP-Seq for Asxl1 itself to show that Asxl1 gene transcription. In addition, motif enrichment analysis of

**Table 1.** Genes directly regulated by Asxl1 as determined by differential expression in HSPCs with Asxl1 deletion and directly bound by Asxl1

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FDR, false discovery rate. Differentially expressed genes in RNA sequencing of MPs (lineage + Sca-1^- c-KIT+ cells) from 1-yr-old Mx1-cre Asxl1^fl/fl mice relative to Cre^- Asxl1^fl/fl littermate controls. Differentially expressed genes in RNA sequencing of LSK cells (lineage + Sca-1^- c-KIT+ cells) from 1-yr-old Mx1-cre Asxl1^fl/fl mice relative to Cre^- Asxl1^fl/fl littermate controls. Data derived from anti-Asxl1 ChIP-Seq in WT C57BL/6J BMDMs.
Axl1-binding sites revealed enrichment in known binding sites of the Ets family of transcription factors. The significant overlap between genome-wide binding of Axl1 and Ets family members is critically supportive of the importance of Axl1 in hematopoiesis as the Ets family of transcription factors are very well understood to play a key role in the growth, survival, differentiation, and activation of hematopoietic cells (Mizuki et al., 2003; Vangala et al., 2003; Koschmieder et al., 2005; Steidl et al., 2006; Choi et al., 2008). Deletion, mutation, and translocation of ETS family members are well-described in myeloid malignancies (Gilliland, 2001), including ETV6 mutations/translocations in MDS and chronic myelomonocytic leukemia (Hafelerich 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, FLI1-ITD mutations and the AML1-ETO (t(8;21)) fusion oncoprotein have been shown 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 Axl1 loss and ETS family member loss (Steidl et al., 2006) in the pathogenesis of ASXL1 mutant myeloid malignancy will be critical.

Collectively, our experiments reveal that deletion of Axl1 results in craniofacial and skeletal developmental abnormalities and mice with hematopoietic-specific Axl1 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 mouse 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.

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 at New York University School of Medicine and Memorial Sloan–Kettering Cancer Center.

Generation of Axl1-deficient mice. The Axl1 allele was deleted by targeting exons 5–10. Two LoxP sites flanking exon 5–10 and an Flp-flanked neomycin selection cassette were inserted in the upstream intron (Fig. 1 A). 10 μg of the targeting vector was linearized by NotI and then transfected by electroporation of BAC-BA1 (C57BL/6 × 129/SvEv) hybrid ES cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombining 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 × 129/SvEv; Hybrid) mouse strains was used as WT controls. Positive ES clones were expanded and injected into blastocysts.

The generated mice (Axl1fl/fl) were initially crossed to a germline Flip-deletor (The Jackson Laboratory), to eliminate the neomycin cassette, and subsequently to the IFN-α-inducible Msx-1cre (The Jackson Laboratory), the hematopoietic-specific Vav-cre, and the germline Etsa-cre (Kühn et al., 1995; Lakso et al., 1996; Stadtfeld and Graf, 2005). Mice were backcrossed for six generations to C57BL/6 mice.

Axl1fl/fl, Axl1fl/+ , and Axl1+/+ littersmate mice were genotyped by PCR with primers Axl1-F3 (5’-CAAGCGTTTACACGATTTC-3’) and Axl1-R3 (5’-AGGGAAAGGGACAGAATGAC-3’) using the following parameters: 95°C for 4 min, followed by 35 cycles of 95°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and then 72°C for 5 min. The WT allele was detected as a band at 200 bp, whereas the 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 (Axl1–ReFeF, 5’-ACGGCCGGCTTTAGTGTACACG-3’, and Axl1–ReFeR, 5’-AGTAAGTGGCAGGTCGCTT-3’) using the same parameters as above.

In vivo experiments. Msx-1cre Axl1fl/fl conditional and Cre+ Axl1fl/fl control mice received five intraperitoneal injections of polyI:polyC every other day at a dose of 20 mg/kg of body weight starting at 2 wk after birth. For the hematopoietic-specific Vav-cre lane, Axl1fl/fl Vav-cre−/− and Axl1fl/+ Vav-cre−/− mice were analyzed between 3 and 60 wk of age. BM, spleen, and peripheral blood were analyzed by flow cytometry. Formalin-fixed paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E). 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. Park). Deletion of the Axl1 allele and transcript was measured by genomic PCR and Western blot analysis.

BM transplantation. Freshly dissected femurs and tibias were isolated from Axl1fl/fl CD45.2+ or Vav-cre+ Axl1fl/fl CD45.2+ mice. BM was flushed with a 3-cm insulin syringe into PBS supplemented with 3% fetal bovine serum. The BM was spun at 0.5 g by centrifugation at 4°C, and RBCs were lysed in ammonium chloride-potassium bicarbonate lys buffer for 5 min. After centrifugation, cells were resuspended in PBS plus 3% PBS, passed through a cell strainer, and counted. Finally, 0.5 × 106 total BM cells of Axl1fl/fl CD45.2+ or Vav-cre+ Axl1fl/fl CD45.2+ mice were mixed with 0.5 × 106 WT CD45.1+ support BM 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 wk after transplant (week 0, pre-polypolyC). Chimerism was followed via FACS in the peripheral blood every 4 wk (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 BM, spleen, and thymus was evaluated at 16 wk via animal sacrifice and subsequent FACS analysis. The above procedure was also repeated with Axl1fl/fl CD45.2+, Vav-cre+ Axl1fl/fl CD45.2+, Vav-cre– Tet2fl/fl CD45.2+, and Vav-cre– Axl1fl/fl Tet2fl/fl CD45.2+ mouse for competitive transplantation of mice with loss of Axl1, Tet2, or both. For noncompetitive transplantation experiments, 106 total BM cells of Axl1fl/fl CD45.2+, Vav-cre– Axl1fl/fl CD45.2+, littersmate Vav-cre– Axl1fl/fl CD45.2+, or littersmate Msx-1cre– Axl1fl/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 Axl1 KO or control mice were transplanted into lethally irradiated CD45.1+ host mice. Recipient mice were then followed until moribund or 80 wk after transplantation.

In vitro colony-forming assays. LSK, CMP, GMP, and MEP cells were sorted from the BM of Axl1fl/fl and littersmate Vav-cre– Axl1fl/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 retransplanted and
counted, and a portion was taken for replating (20,000 cells/replicate) for a total of seven platings. Cytospins were performed by resuspending in warm PBS and spun onto the slides at 350 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). BM 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 (BD) and sorted with a FACSaria instrument (BD). All antibodies were purchased from BD 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, IL-7Rα (A7R34), CD34 (RAM34), FcγRII/III (2.4G2), CD4 (RM4-5), CD8 (H129.19), CD8α (3.6-7), CD45.1 (A20), CD45.2 (104), CD150 (9D4), and CD48 (HM48-1). The following antibodies were used for Western blot analysis: Asxl1 (clone N-13; Santa Cruz Biotechnology, Inc.), Ezh2 (EMD Millipore), Suz12 (Abcam), EED (Abcam), H3K27me3 (Abcam), total H3 (Abcam), and tubulin (Sigma-Aldrich).

Cell cycle and apoptosis analyses. For cell cycle analysis, the BrdU–APC kit was used (BD) according to the manufacturer’s protocol. Mice were treated with 1 mg BrdU intraperitoneally, followed by harvest of BM cells 24 h later. For evaluation of apoptosis, the Annexin V–FITC apoptosis detection kit was used (BD) 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, and then dissected tissue samples were fixed for 24 h in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Paraffin blocks were sectioned at 4 μm and stained with H&E. Images were acquired using an Axio Observer A1 microscope (Carl Zeiss).

Peripheral blood analysis. Blood was collected by retroorbital bleeding using heparinized microhematocrit capillary tubes (Thermo Fisher Scientific). Automated peripheral blood counts were obtained using a Hemavet 950 (Drew Scientific) according to standard manufacturer’s instructions. Differential blood counts were realized on blood smears stained using Wright–Giemsa staining and visualized using an Axio Observer A1 microscope.

RNA-Seq and quantitative real-time PCR (qRT-PCR) analysis. Total RNA was isolated using the RNasy 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, Faqt files were aligned to mm9 using TopHatV1.4 with default parameters. Differential expression tests were performed 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.

ChIP-Seq analysis. Because low chromatin yields from HSPC populations precluded ChIP-Seq experiments, BMDMs from WT C57BL/6 mice were used as a surrogate to identify genome-wide Asxl1 binding sites. The antibody used for Asxl1 ChIP-Seq experiments was obtained from Santa Cruz Biotechnology, Inc. 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 adapters were added and the library was size-selected (175–225 bp) on an agarose gel. The adapter–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 2 kb upstream of the TSS. ChIP-Seq read profile and heat map densities were generated using genomic tools. Mouse RefSeq and CpG island annotations were downloaded from the UCSC Genome Bioinformatics Table Browser.

Skeletal preparations. Skeletal preparations were performed as described previously (de Pontual et al., 2011). Online supplemental material. Table S1, included as a separate PDF file, shows differentially expressed transcripts between 1-yr-old Mx1-floxed Asxl1fl/fl and Asxl1fl/fl LSK cells by RNA sequencing. Table S2, included as a separate PDF file, shows differentially expressed transcripts between 1-yr-old Mx1-floxed and Asxl1fl/fl MPs by RNA sequencing. Table S3, included as a separate PDF file, shows differentially expressed genes in LSK cells from 6- to 8-week-old Mx1-floxed Asxl1fl/fl, Mx1-floxed Ter119+, and Mx1-floxed Asxl1fl/fl Ter119−. Table S4, included as a separate PDF file, shows regions with significant Asxl1 binding by anti-Asxl1 ChIP-Seq. Table S5, included as a separate PDF file, shows genes with significant Asxl1 binding ±2 kb from the TSS as determined by anti-Asxl1 ChIP-Seq. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131141/DC1.

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References


