Reduced BAP1 activity prevents ASXL1 truncation-driven myeloid malignancy *in vivo*

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Materials and methods

Generation of *Bap1* conditional knockout (*Bap1*$^{f/f}$) mice and genetic intercrossing of *Bap1*$^{f/f}$ mice with *Asxl1*^{Y588X}Tg mice. The *Bap1*$^{f/f}$ mice were generated using 129/SvEv mouse ES cells. The *Bap1* alleles were deleted by targeting exons 4-5 (supplemental Figure 1A). Two *LoxP* sites flanking exons 4-5 and a *Frt*-flanked neomycin cassette were inserted (*Loxp-FRT-Neo-FRT-exons 4-5-Loxp*). ES cell clones were screened by PCR and confirmed by sequencing. A positive clone was selected for the blastocysts injection. Male chimeric mice were crossed to C57BL/6 females to screen for germ-line transmission of *Bap1 flox* allele. *Bap1*$^{f/f}$ mice were crossed to a *Flippase* deleter strain (the Jackson Laboratory) to remove the *Frt*-flanked cassette. Subsequently, these *Bap1*$^{f/f}$ mice were crossed with the interferon (IFN)-α-inducible *Mx1cre* transgenic mice (the Jackson Laboratory) to assess the effects of inducible *Bap1* deletion in the hematopoietic cells. *Bap1*$^{f/f}$, *Bap1*$^{f/+}$ and *Bap1*$^{+/+}$ mice were genotyping by PCR with the primers *Bap1*-F (AGCGTGCTTCTGAACTGCAGCAATGTGGAT) and *Bap1*-R (TTCTGAAAGGGCAGTGGTGGCAAATGAGAC) using the following parameters: 94°C for 3 min, followed by 40 cycles of 94°C for 40s, 55°C for 30s, 72°C for 90s, and then 72°C for 10 min. The WT allele was detected at 423 bp, and the flox allele was detected at 520 bp by PCR. *Bap1*$^{f/f}$ mice of mix genetic backgroundwere backcrossed with C57BL/6 mice for more than 6 generations.

Mx1Cre;Bap1$^{f/+}$ mice were crossed with *Asxl1*^{Y588X}Tg mice.[1] *Mx1Cre;Bap1*$^{f/+}$, *Mx1Cre;Bap1*$^{f/+}$;*Asxl1*^{Y588X}Tg, *Asxl1*^{Y588X}Tg and WT mice received four times of intraperitoneal injections of poly(I):poly(C) (pI:pC) (GE Healthcare, 27473201) every other day at a dose of 10 mg/kg of body weight starting at three weeks after birth. Two weeks after the last pI:pC injection, bone marrow (BM) cells were collected. *Cre* recombination was confirmed by PCR with primers to detect a floxed portion of the construct: *Bap1*-RecF (ATTTGTCCTGACCATTAGTGGCGCTCTCCT) and *Bap1*-RecR (TTCTGAAAGGGCAGTGGTGGCAAATGAGAC). The *Bap1* floxed allele recombination was detected at 423 bp. The mRNA level of *Bap1* was examined by quantitative real-time PCR (qRT-PCR) using primers: *Bap1*-F1 (CTCCTGGTGGAAGATTTCGGT) and *Bap1*-R1 (GAGTGGCACAAGAGTTGGGAA). The transgene of *Asxl1*^{Y588X} was identified by

PCR of genomic DNA using primers *Asxl1*^{Y588X}-Tg-F (ACCCGTCAACGGGACGGAC) and *Asxl1*^{Y588X}-Tg-R (CGATCCGGGGGCATATCTGTC). All studies were conducted in accordance with the regulatory guidelines by the Institutional Animal Care and Use Committee at University of Miami Miller School of Medicine.

Phenotypic analyses of the hematopoiesis in mice Peripheral blood (PB) was collected by retro-orbital bleeding and was subjected to an automated blood count (Hemavet System 950FS). For morphological and lineage differential analysis, PB smears were stained with May-Grünwald-Giemsa staining. Morphological analyses of BM cells were performed on cytospins followed by May-Grünwald-Giemsa staining. For histopathology analyses, spleens were dehydrated using ethanol and cleared in xylenes. The specimens were then embedded in melted paraffin and allowed to harden. Thin sections (5 μm) were cut and floated onto microscope slides. For routine assessment, slides were stained with hematoxylin and eosin (H&E). Slides were visualized under a Nikon TE2000-S microscope. Images were taken by a QImaging camera and QCapture-Pro software (Fryer Company Inc.). Chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

Flow cytometry analysis and cell sorting. Total white blood cells were obtained after lysis of PB with red cell lysis buffer (Thermo). Single-cell suspensions from BM, spleen, and PB were stained with panels of fluorochrome-conjugated antibodies (Supplementary Table 2). Flow cytometric analysis of different hematopoietic cell lineages was performed as previously described.[2] The analyses were performed using a BD FACS Canto II or LSR Fortessa flow cytometer. All data were analyzed by FlowJo-V10 software. For cKit$^+$ cell selection, magnetic-activated cell sorting was applied. BM cells were sorted with cKit (CD117) beads. The purity of selected cKit$^+$ cells was routinely over 95%.

Western blotting and immunoprecipitation (IP) assays. 293T cells were transfected with FLAG-Asxl1 full-length (FL) and/or FLAG-Asxl1$^{aa1-587}$ using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015). 48h after transfection, cells were lysed and nuclear fraction were extracted using ProteoExtract® Subcellular Proteome Extraction Kit (EMD MILLIPORE, 539790). IP was performed using a nuclear fraction and anti-BAP1 (Active

Motif, 61501) antibody.[3] After washing with IP buffer (NP40 Cell Lysis Buffer (Thermo Fisher Scientific, FNN0021) for four times, the associated proteins were collected for western blotting analysis.[4] The antibodies used for western blotting are as following: anti-BAP1 (1: 1000, Active Motif, 61501), anti-FLAG (1:2000, Sigma, F3165), anti-H3K27me3 (1:1000, EMD Millipore, 07-449), anti-H2AK119Ub (1:2000, Cell signaling Technology, 8240s), anti-H3 (1:2000, Abcam, ab1791), anti-β-ACTIN (1:2000, Cell signaling Technology, 9542s).

qRT-PCR. Total RNA was isolated from BM cKit$^+$ cells of each mouse genotype and treated with RNase-free DNase to remove contaminating genomic DNA. First-strand cDNA was synthesized. qRT-PCR was performed using Fast SYBR Green master mix (Applied Biosystems). PCR amplifications were performed in triplicate for each gene of interest along with parallel measurements of GAPDH cDNA (internal control). To confirm the specific amplification of the desired PCR product, melting curves were analyzed, and PCR products were separated on a 2% agarose gel. The primers used for the amplification of each gene are shown in supplemental Table 1.

Chromatin immunoprecipitation (ChIP). BM cKit$^+$ cells (2 x 10^6) from each mouse genotype and 32D cells (ATCC) (2 x 10^6 for each condition) of each transduction were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by the addition of lysis buffer, followed by shearing with Bioruptor Pico with water cooler (Diagenode, Seraing, Belgium). The DNA was sheared to an average length of 300-500 bp. Genomic DNA regions of interest were isolated using an antibody against H2AK119Ub (Cell signaling Technology, 8240s). Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65°C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation. After the measurement of DNA concentration with Qubit3.0, the DNA was used for qPCR. The primers used in ChIP-qPCR are shown in supplemental Table 1.

Statistical analysis

Statistical significance was determined by one-way ANOVA for comparisons of all four genotypes of mice, or two-tailed unpaired *t*-test for comparisons between every two genotypes of mice using the Excel program. *P* values of less than 0.05 are considered significantly different. For the *in vivo* experiment, sample size chosen was based on the generalized linear model with Bonferroni multiple comparison adjustment; with the proposed sample size of at least 3 mice per group/genotype. Animals were randomly assigned to each studies. For all *in vitro* experiments, at least two independent experiments with more than three biological replicates for each condition/genotype were performed to ensure adequate power.

References:

1. Yang H, Kurtenbach S, Guo Y, et al. Gain-of-function of ASXL1 truncating protein in the pathogenesis of myeloid malignancies. *Blood*. 2017.
2. Wang J, Li Z, He Y, et al. Loss of Asxl1 leads to myelodysplastic syndrome-like disease in mice. *Blood*. 2014;123(4):541-553.
3. Li J, He F, Zhang P, Wang Q-F, Xu M, Yang F-C. Loss of Asxl2 leads to myeloid malignancies in mice. *Nature Communications*. 2017;8:15456.
4. Zhang P, Xing C, Rhodes SD, et al. Loss of Asxl1 Alters Self-Renewal and Cell Fate of Bone Marrow Stromal Cell, Leading to Bohring-Opitz-like Syndrome in Mice. *Stem Cell Reports*. 2016;6(6):914-925.

Supplementary Table 1. List of primer sequences.

Mouse genotyping and recombination primers	
Bap1-F (P1)	AGCGTGCTTCTGAACTGCAGCAATGTGGAT
Bap1-R (P2)	TTCTGAAAGGGCAGTGGTGGCAAATGAGAC
Bap1-RecF (P3)	ATTTGTCCTGACCATTAGTGGCGCTCTCCT
Bap1-RecR (P2)	TTCTGAAAGGGCAGTGGTGGCAAATGAGAC
Asxl1^{Y588X}-Tg-F	ACCCGTCAACGGGACGGAC
Asxl1^{Y588X}-Tg-R	CGATCCGGGGGCATATCTGTC
Mouse qRT-PCR primers	
Bap1-F	CTCCTGGTGGAAGATTTCGGT
Bap1-R	GAGTGGCACAAGAGTTGGGAA
Hoxa5-F	CTCAGCCCCAGATCTACCC
Hoxa5-R	CAGGGTCTGGTAGCGAGTGT
Hoxa7-F	AAGCCAGTTTCCGCATCTAC
Hoxa7-R	CTTCTCCAGTTCCAGCGTCT
Hoxa9-F	ACAATGCCGAGAATGAGAGC
Hoxa9-R	GTAAGGGCATCGCTTCTTCC
Dcbld1-F	AACTCCGGCTGAACTCAAACG
Dcbld1-R	GCAGCTTTGCACAATAAAGAGGT
Mouse ChIP-qPCR primers	
Hoxa5-ChIP-F	GAGTGCTGGGGTTGGTTAGT
Hoxa5-ChIP-R	AGGTTCCAAAGCTTGAGTCCA
Hoxa7-ChIP-F	GGCCCATAGGTAGTTTGGGAG
Hoxa7-ChIP-R	CCACTTTAGCTCCCAGGGAAA
Hoxa9-ChIP-F	TGGTCGCCTGGCAAATAGAT
Hoxa9-ChIP-R	AAGCACCCTCGACTCAGATG
Dcbld1-ChIP-F	AACTGCCATCATCCCATCAGA
Dcbld1-ChIP-R	TGAAAATCAAAAATTCACCTGGCAC

Supplemental Table 2. List of antibodies for flow cytometry.

Antibody name	Clone	Color	Catalog	Company
Rat anti-Mouse CD34	RAM34	FITC	553733	BD Biosciences
Rat anti-Mouse Ly-6A/E (Sca1)	D7	PE-Cy7	558162	BD Biosciences
Rat anti-Mouse CD117	2B8	APC	553356	BD Biosciences
Rat anti-Mouse CD117	2B8	PerCP-Cy5.5	560557	BD Biosciences
Rat anti-Mouse CD16/CD32	93	APC-Cy7	101328	BioLegend
Mouse Lineage Antibody Cocktail		APC	51-9003632	BD Biosciences
Rat anti-Mouse Ly-6G and Ly-6C	RB6-8c5	PerCP-Cy5.5	552093	BD Biosciences
Rat anti-Mouse CD11b	M1/70	PE	553311	BD Biosciences

Supplementary Figure Legends

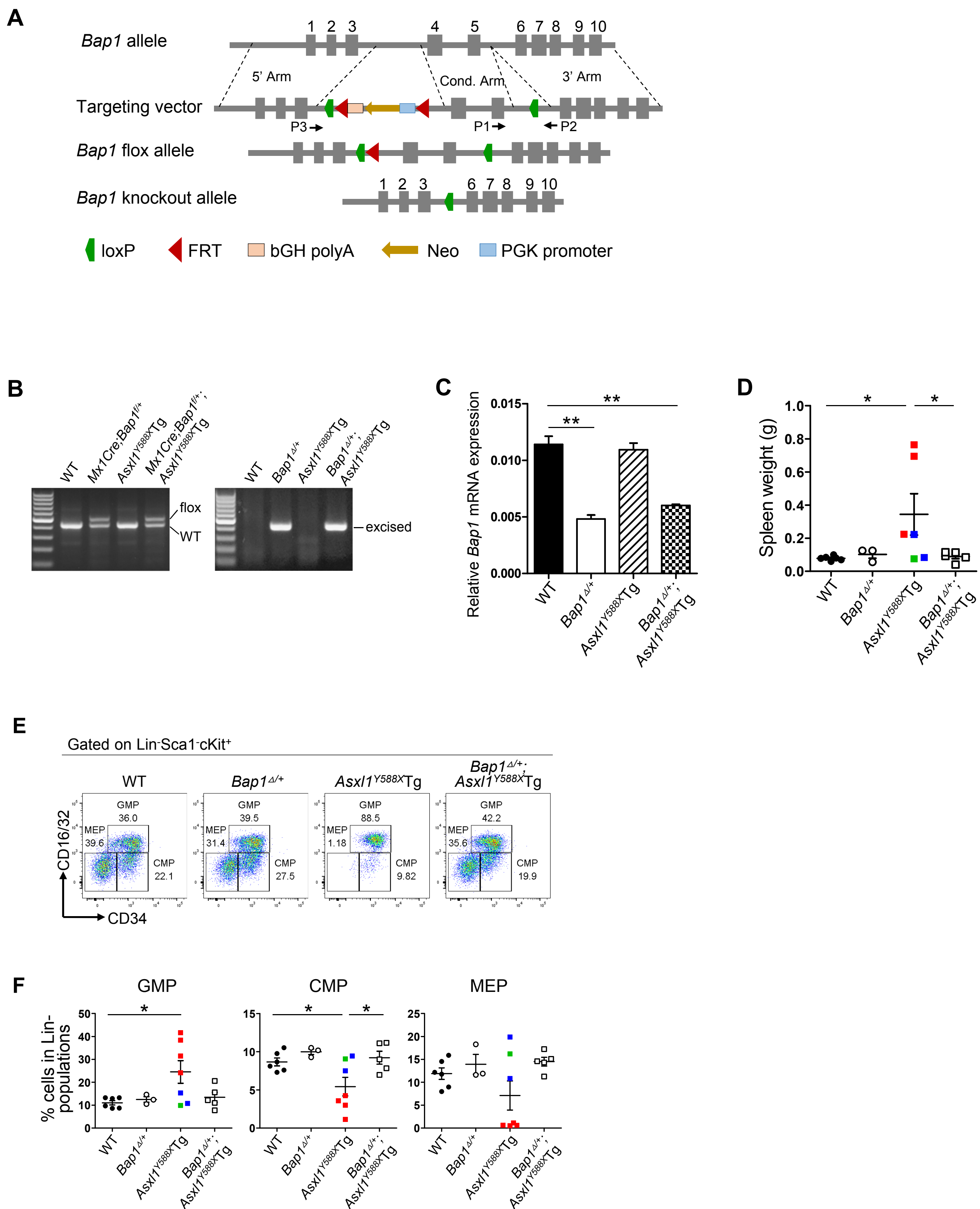
Figure S1. Generation of *$Bap1^{f/f}$* mice and deletion one *Bap1* allele prevents $ASXL1^{aa1\text{-}587}$-driven myeloid malignancies *in vivo.*

(A) Targeting strategy for *Bap1* flox allele mice. *Bap1* exons 4 and 5 were replaced by a *Loxp-FRT-Neo-FRT-exons 4-5-Loxp* cassette after the recombination. Chimeric mice derived from homologous recombinant ES cells were crossed with *Flp* recombinase delete mice to remove the *neo* selection cassette. Black arrows P1 and P2 show the binding sites of the PCR primers used for *Bap1* genotyping. Black arrows P3 and P2 show binding sites of the PCR primers used for *Bap1* floxed allele recombination. (B) Representative PCR analysis show *Bap1* genotyping using genomic DNA (left), and *Bap1* floxed allele after excision 4 weeks post-pI:pC treatment (right). (C) qRT-PCR analysis of the expression levels of *Bap1* in BM $cKit^+$ cells from WT, $Bap1^{\Delta/+}$ and $Bap1^{\Delta/+};Asxl1^{Y588X}$Tg mice. GAPDH was used as a control. Error bars represent mean ± SEM from 3 mice/genotype. mRNA, messenger RNA. (D) Spleen weight of WT, $Bap1^{\Delta/+}$ and $Bap1^{\Delta/+};Asxl1^{Y588X}$Tg mice. Error bars represent mean ± SEM from 3-6 mice/genotype. Mice were 10 months after pI:pC injection. Red square: leukemic mice; blue square: MPN mice; green square: MDS/MPN mice. (E) Flow cytometric analyses of GMP, CMP and megakaryocyte-erythroid progenitor (MEP) in BM $Lin^-Sca\text{-}1^-cKit^+$ populations from representative WT, $Bap1^{\Delta/+}$ and $Bap1^{\Delta/+};Asxl1^{Y588X}$Tg mice. (F) Quantification of the percent GMP, CMP, MEP cells in BM Lin^- cells of mice. Red square: leukemic mice; blue square: MPN mice; green square: MDS/MPN mice. Mice were 10 months after pI:pC injection. One-way ANOVA was used for comparisons of all four genotypes of mice, and the unpaired Students *t* test was used to compare between every two genotypes of mice (*$p < 0.05$, **$p < 0.01$).

Figure S2.

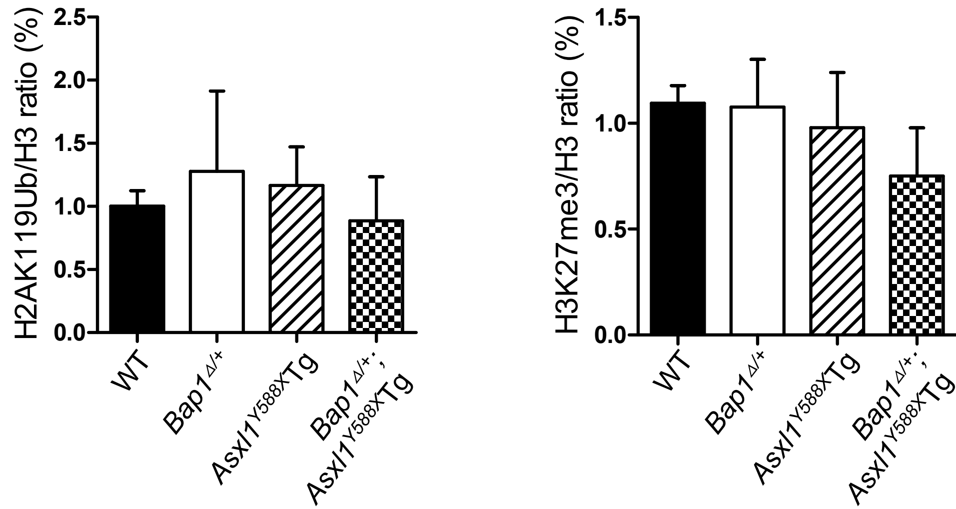
(A) The ratio of protein expression of H2AK119Ub/H3 and H3K27me3/H3 in the BM $cKit^+$ cells of WT, $Bap1^{\Delta/+}$, $Asxl1^{Y588X}$Tg, and $Bap1^{\Delta/+};Asxl1^{Y588X}$Tg mice. Error bars represent mean ± SEM. Similar data were obtained from two to three independent experiments. (B)

ChIP-qRCR analysis show of the relative H2AK119Ub occupancy at the promoter regions of *Hoxa5*, *Hoxa7*, *Hoxa9* and *Dcbld1* genes in 32D cells expressing HA-tagged ASXL1$^{aa1-587}$. Error bars represent mean ± SEM. Similar data were obtained from two independent experiments. One-way ANOVA was used for comparisons of all four genotypes of mice, and the unpaired Students *t* test was used to compare between every two genotypes of mice (**$p < 0.01$).



Guo et al. supplemental Figure S1

A



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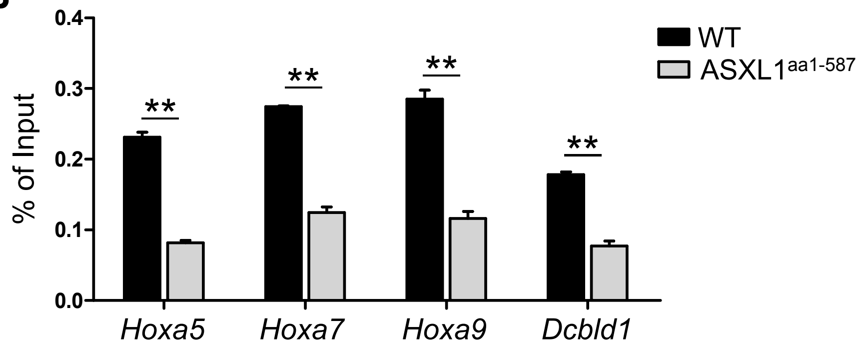

Guo et al. supplemental Figure S2