Participating institutions

The following CALGB institutions, principal investigators, and cytogeneticists participated in this study:

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Treatment protocols

All patients included in the outcome analyses were treated on Cancer and Leukemia Group B (CALGB) first-line protocols for older (≥60 years) patients with acute myeloid leukemia (AML), and received cytarabine/daunorubicin-based induction therapy followed by cytarabine-based consolidation therapy. None of the protocols included autologous or allogeneic stem cell transplantation in first complete remission (CR). Patients on CALGB 8525 (n=24) were treated with induction chemotherapy consisting of cytarabine in combination with daunorubicin and were randomly assigned to consolidation with different doses of cytarabine followed by maintenance treatment.[1] For older patients, there were no significant differences in disease-free (DFS) or overall survival (OS) among the different consolidation regimens. Patients on CALGB 8923 (n=22) received induction chemotherapy consisting of cytarabine and daunorubicin and were randomly assigned to receive postremission therapy with cytarabine alone or in combination with mitoxantrone, resulting in no significant difference in DFS or OS between both arms.[2] Patients on CALGB 9420 (n=5) and 9720 (n=110) received induction chemotherapy consisting of cytarabine in combination with daunorubicin and etoposide, with (CALGB 9420) or with/without (CALGB 9720) the multidrug resistance protein modulator PSC-833.[3,4] The PSC-833 arm of CALGB 9720 was closed after random assignment of 120 patients because of excessive early deaths, and enrollment continued on the chemotherapy-only control arm. Patients on CALGB 9420 received postremission therapy with cytarabine (2 g/m^2/d) alone, and patients on CALGB 9720 received a single cytarabine/daunorubicin consolidation course and were then randomly assigned to low-dose recombinant interleukin-2 maintenance therapy or none.[5] Interleukin-2 maintenance was not associated with differences in DFS or OS. Patients on

CALGB 10201 (n=73) received induction chemotherapy consisting of cytarabine and daunorubicin, with or without the *BCL2* antisense oblimersen sodium. The consolidation regimen included two cycles of cytarabine (2 g/m^2/d) with or without oblimersen. Preliminary results showed no impact of the antisense therapy on outcome.[6] Patients who enrolled on the treatment protocols also provided written informed consent to participate in the companion protocols CALGB 8461 (prospective cytogenetic companion), CALGB 9665 (leukemia tissue bank) and CALGB 20202 (molecular studies in acute myeloid leukemia), which involved collection of pretreatment bone marrow (BM) aspirates, blood samples, and buccal swabs.

Mutational analyses

To our knowledge, *ASXL1* mutations in AML have exclusively been described in the last coding exon of the gene.[7,8] Previous publications on *ASXL1* mutations have uniformly referred to this exon as exon 12. However, according to the National Center for Biotechnology Information (NCBI) reference sequence database (identifier NM_015338.5), it is numbered exon 14. To avoid potentially confusing inconsistency with the published literature, we use the designation exon 12 throughout the manuscript. Annotation of sequence variants follows the recommendations of the Human Genome Variation Society (www.hgvs.org) and is based on NCBI reference sequence NM_015338.5.

Mononuclear cells from BM and blood samples from patients with cytogenetically normal (CN-) AML were enriched through Ficoll-Hypaque gradient centrifugation, and cryopreserved for later use. Genomic DNA and total RNA were extracted as described previously.[9] The coding portion of *ASXL1* exon 12 was amplified from genomic DNA by polymerase chain reaction (PCR) in three overlapping amplicons, using 30 ng of input DNA for each reaction. PCR conditions and

primers are described in supplemental Table 6. PCR products were treated with exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT, Affymetrix/USB, Cleveland, OH) to degrade residual PCR primers and deoxynucleotides, and 3-4 µl of the cleaned-up PCR product were then used for direct sequencing using amplicon-specific primers (Applied Biosystems 3730 DNA Analyzer and BigDye Terminator Cycle Sequencing Kit version 3.1, Applied Biosystems, Foster City, CA). Screening for mutations in *CEBPA* was performed as reported previously.[9] For patients with double *CEBPA* mutations, the entire coding region was amplified from genomic DNA in a single PCR, and subsequently cloned and sequenced to allow allelic discrimination.

Gene- and microRNA-expression profiling

Gene expression profiles were obtained using Affymetrix HG-U133 plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA). Details regarding sample preparation and array hybridization have been published previously.[10,11] Briefly, summary measures of gene expression were computed for each probe-set using the robust multichip average method, which incorporates quantile normalization of arrays. Expression values were logged (base 2) before analysis. A filtering step was performed to remove probe-sets that did not display significant variation in expression across arrays. In this procedure, a χ^2 test was used to test whether the observed variance in expression of a probe-set was significantly larger than the median observed variance in expression for all probe-sets, using α=.01 as the significance level. A total of 24,649 probe-sets passed the filtering criterion. Normalized expression values were compared between *ASXL1*-mutated (-mut) and *ASXL1*-wild-type (-wt) patients, and a univariable significance level of .001 was used to identify differentially expressed probe-sets. To avoid confounding due to the strong association of mutated *ASXL1* with *NPM1*-wt and absence of *FLT3*-internal tandem duplications (*FLT3*-ITD), this comparison was limited to *NPM1*-wt*/FLT3*-ITD-negative patients. A global test of significance based on a permutation procedure

was performed to determine whether or not the number of differentially expressed probe sets was more than expected by chance; if not, no signature is reported for the comparison. The Gene Set Analysis (GSA) algorithm was used to test whether genes belonging to certain pre-defined gene sets were deregulated in association with *ASXL1* mutation status. Gene sets were obtained from the Broad Institute's Molecular Signatures Database (MSigDB version 3.0, available online at http://www.broadinstitute.org/gsea/msigdb/).[12] We used the curated 'canonical pathways' collection that contains 880 curated gene sets representing canonical biological processes and pathways. Only one probe set per gene was included in the analysis by choosing the probe set with highest average expression across samples. 11,224 probe sets representing as many genes were analyzed. Gene sets that contained fewer than 5 or more than 100 analyzed genes were omitted from analysis, resulting in 820 studied gene sets. A univariable significance level of .005 was used for the comparisons.

For microRNA (miR) microarrays [Ohio State University Comprehensive Cancer Center (OSU_CCC) custom miR array, version 4.0], signal intensities were calculated for each spot, with an adjustment made for local background. Spots that were flagged due to low signal-to-noise ratio on more than 75% of arrays were excluded from analysis. Signal intensities were log-transformed and quantile normalization was performed on arrays using spots for all human and mouse microRNA probes represented on the array. Log-signal intensities from replicate spots (ie, spots representing the same probe) were averaged. For each miR probe, an adjustment was made for batch effects (ie, differences in expression related to the batch in which arrays were hybridized). Further analysis was limited to 460 unique human probes that passed the filtering criterion. A comparison of miR expression was made between *ASXL1*-mut and *ASXL1*-wt patients, using a univariable significance level of .005 to identify differentially expressed miR probes. To avoid confounding due to the strong association of mutated *ASXL1*

with *NPM1*-wt and absence of *FLT3*-internal tandem duplications (*FLT3*-ITD), this comparison was limited to *NPM1*-wt/*FLT3*-ITD-negative patients. A global test of significance based on a permutation procedure was performed to determine whether or not the number of differentially expressed probes was more than expected by chance; if not, no signature is reported for the comparison. All microarray analyses were performed using BRB-ArrayTools Version 3.8.1, developed by Richard Simon, DSc, and Amy Peng Lam.

Definition of clinical endpoints

Clinical endpoints were defined, in accordance with generally accepted criteria, as follows: CR required a BM aspirate with cellularity greater than 20% and maturation of all cell lines, less than 5% blasts and no Auer rods; in the peripheral blood, an absolute neutrophil count of ≥1,500/μL, platelet count of >100,000/μL, and no leukemic blasts; and no evidence of extramedullary leukemia, all of which had to persist for at least 1 month.[13] Patients who died within 30 days of starting therapy were not studied so that resistance as opposed to early death primarily accounted for failure to achieve CR. Relapse was defined by the presence of ≥5% BM blasts, or circulating leukemic blasts, or the development of extramedullary leukemia. DFS was measured from the date of CR until the date of relapse or death; patients alive and in complete remission were censored at last follow-up. OS was measured from the date of study entry until the date of death, and patients alive at last follow-up were censored. Event-free survival (EFS) was measured from the date of study entry until the date of failure to achieve CR, relapse or death, and patients alive and in complete remission at last follow-up were censored.

Multivariable analyses

Multivariable logistic regression models were generated for attainment of CR, and multivariable proportional hazards models were constructed for DFS and OS, using a limited backwards elimination procedure. Variables considered for model inclusion and evaluated in univariable models were: *ASXL1* mutation status, European LeukemiaNet (ELN) genetic category (based on the presence/absence of *FLT3*-ITD, and *NPM1* and *CEBPA* mutations), *ASXL1* mutation-by-ELN group interaction, mutation status of the *TET2, WT1, IDH1*, and *IDH2* genes, *FLT3*-tyrosine kinase domain mutation status, *MLL* partial tandem duplication status, age (as a continuous variable), sex, race (white vs nonwhite), white blood count (WBC), hemoglobin, and platelet count. Variables significant at α=.20 from the univariable analyses were considered for multivariable analyses. For the time-to-event endpoints, the proportional hazards assumption was checked for each variable individually.

Clinical and molecular characteristics and outcomes of* ASXL1*-mut primary CN-AML patients younger than 60 years

Nonsense or frame shift mutations in *ASXL1* exon 12 were found in only six younger primary CN-AML patients. Due to this small number, a detailed analysis of *ASXL1* mutations in younger patients (aged 18–59 years) was not possible. However, it appeared that the clinical and molecular characteristics of *ASXL1*-mut younger patients were largely consistent with our findings in older patients. The six younger *ASXL1*-mut patients were aged 41, 45, 48, 56, 58, and 58 years, and five of the six were male. *ASXL1*-mut younger patients had lower BM blast percentages (median, 53%) than *ASXL1*-wt patients (n=170; median, 68%; *P*=.06). None of the six *ASXL1*-mut patients was *NPM1*-mut, compared with 68% of *ASXL1*-wt patients (*P*=.002). One of the six *ASXL1*-mut patients had a *FLT3*-ITD, and two carried *CEBPA* mutations. Of note, three of the six *ASXL1*-mut patients (50%) had mutated *IDH2*, compared to 10% of *ASXL1*-wt patients (*P*=.02). This finding should be interpreted cautiously in the light of the small numbers, since no similar association was seen in older patients. No other pretreatment patient characteristics showed significant associations with *ASXL1* mutations in younger patients. With regard to treatment outcomes, four of the six younger *ASXL1*-mut patients achieved CR, but three of them relapsed after 14, 21, and 30 months, respectively. The two patients who did not achieve CR and two of the three relapsing patients subsequently died. One patient who achieved CR after induction chemotherapy and subsequently received an autologous stem cell transplant per protocol is alive in CR 8 years after diagnosis. One patient who relapsed and received an allogeneic stem cell transplant is alive 5 years post-transplant.

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Table S1. *ASXL1* exon 12 mutations in 423 patients with primary CN-AML

Patients aged 60 years or older (n=234)			
cDNA sequence change	**Deduced change in protein sequence**	**Number of patients**	**Mutation type**
c.1749G>A	p.(Trp583*)	1	nonsense
c.1772dupA	p.(Tyr591*)	1	frame shift
c.1779dupA	p.(Cys594Metfs*25)	1	frame shift
c.1786dupC	p.(Arg596Profs*23)	1	frame shift
c.1793_1794delTCinsA	p.(Ile598Asnfs*105)	1[†]	frame shift
c.1867C>T	p.(Gln623*)	1	nonsense
c.1900_1922del23	p.(Glu635Argfs*15)	5	frame shift
c.1903G>T	p.(Glu635*)	1	nonsense
c.1911dupC	p.(Thr638Hisfs*20)	1	frame shift
c.1934dupG	p.(Gly646Trpfs*12)	20	frame shift
c.2077C>T	p.(Arg693*)	2[†]	nonsense
c.2100T>A	p.(Tyr700*)	1	nonsense
c.2254_2255delGCinsT	p.(Ala752Phefs*20)	1	frame shift
c.2332C>T	p.(Gln778*)	1	nonsense
c.2348_2349insT	p.(Asp784Glyfs*3)	1	frame shift

Patients younger than 60 years (n=189)			
cDNA Sequence Change	**Deduced change in protein sequence**	**Number of patients**	**Mutation type**
c.1900_1922del23	p.(Glu635Argfs*15)	1	frame shift
c.1934dupG	p.(Gly646Trpfs*12)	3	frame shift
c.1937delG	p.(Gly646Alafs*57)	1	frame shift
c.2094_2095dupGC	p.(Pro699Argfs*5)	1	frame shift

NOTE: Mutation nomenclature is based on *ASXL1* mRNA reference sequence NM_015338.5 and follows the guidelines of the Human Genome Variation Society, version 2.0 (www.hgvs.org).

[†] One older patient had two *ASXL1* mutations (c.2077C>T and c.1793_1794delTCinsA)

Table S2. List of *ASXL1* exon 12 missense changes not annotated as known SNPs in the dbSNP database (version 132)

Patients aged 60 years or older (n=234)			
cDNA sequence change	**Deduced change in protein sequence**	**Number of patients**	**Comment**
c.2222A>T	p.(Leu741Val)	1	
c.2862C>A	p.(Asp954Glu)	1	
c.2957A>G	p.(Asn986Ser)	2	Described as germline SNP by Bejar et al[15]
c.3306G>T	p.(Glu1102Asp)	5	Described as germline SNP by Abdel-Wahab et al[14]
c.3416C>A	p.(Thr1139Lys)	1	
c.3503G>C	p.(Ser1168Thr)	1	
c.3745A>G	p.(Met1249Val)	1	Described as germline SNP by Abdel-Wahab et al[14] and Bejar et al[15]
c.3793G>T	p.(Asp1265Tyr)	1[†]	
c.3935C>T	p.(Ala1312Val)	2	Described as germline SNP by Bejar et al[15]
c.4183C>G	p.(Leu1395Val)	1	
c.4189G>A	p.(Gly1397Ser)	1[†]	Described as germline SNP by Bejar et al[15]

Patients younger than 60 years (n=189)			
cDNA sequence change	**Deduced change in protein sequence**	**Number of patients**	**Comment**
c.1850T>C	p.(Ile617Thr)	1	
c.2053G>A	p.(Gly685Arg)	1	
c.2395G>T	p.(Asp799Tyr)	1	
c.2597T>C	p.(Leu866Ser)	1	
c.2957A>G	p.(Asn986Ser)	1	Described as germline SNP by Bejar et al[15]
c.3306G>T	p.(Glu1102Asp)	4	Described as germline SNP by Abdel-Wahab et al[14]
c.3745A>G	p.(Met1249Val)	2	Described as germline SNP by Abdel-Wahab et al[14]
c.3973C>T	p.(Leu1325Phe)	1	Subsequently described as germline SNP in dbSNP database (rs6057581)
c.4189G>A	p.(Gly1397Ser)	1	Described as germline SNP by Bejar et al[15]

NOTE: Mutation nomenclature is based on *ASXL1* mRNA reference sequence NM_015338.5 and follows the guidelines of the Human Genome Variation Society, version 2.0 (www.hgvs.org).

[†] One patient had two missense changes (c.3793G>T and c.4189G>A)

Table S3. Relationship of *ASXL1* mutation status with outcomes in primary CN-AML patients aged 60 years or older with mutated or wild-type *CEBPA*

		***ASXL1*-mut**	***ASXL1*-wt**	***P***	**OR/HR (95% CI)**
***CEBPA*-mut patients (n=27)**	No. of patients	10	17		
	Complete remission, no. (%)	5 (50)	13 (76)	.21	0.31 (0.06, 1.64)
	Disease-free survival			ND*	ND
	Median, years	0.4	1.0		
	% Disease-free at 3 years (95% CI)	0	31 (9-55)		
	Overall survival			<.001	5.43 (1.92, 15.36)
	Median (years)	0.8	1.6		
	% Alive at 3 years (95% CI)	0	35 (14-57)		
	Event-free survival			.02	2.66 (1.11, 6.41)
	Median, years	0.2	0.7		
	%Event-free at 3 years (95% CI)	0	24 (7-45)		
***CEBPA*-wt patients (n=193)**	No. of patients	28	165		
	Complete remission, no. (%)	15 (54)	116 (70)	.08	0.49 (0.22, 1.10)
	Disease-free survival			.17	1.46 (0.85, 2.52)
	Median, years	0.7	1.0		
	% Disease-free at 3 years (95% CI)	13 (2-35)	18 (12-26)		
	Overall survival			.11	1.39 (0.93, 2.09)
	Median, years	1.1	1.2		
	% Alive at 3 years (95% CI)	7 (1-20)	22 (16-28)		
	Event-free survival			.03	1.54 (1.03, 2.31)
	Median, years	0.5	0.7		
	% Event-free at 3 years (95% CI)	7 (1-20)	13 (8-18)		

NOTE: Due to small patient numbers, CEBPA single- and double-mutated patients could not be analyzed separately. ND indicates not determined.

* A *P* value for DFS was not calculated in this group, since only five *CEBPA*-mut/*ASXL1*-mut patients achieved complete remission.

Table S4. List of probe sets differentially expressed between 26 *ASXL1*-mut and 39 *ASXL1*-wt CN-AML patients aged 60 years or older with wild-type *NPM1* and no *FLT3*-ITD

Probe set	Gene symbol	Gene name	Fold change: *ASXL1*-mut *v* *ASXL1*-wt	*P*
		Genes up-regulated in patients with mutated *ASXL1*		
225745_at	*LRP6*	low density lipoprotein receptor-related protein 6	4.17	1.00E-07
224435_at	*C10orf58*	chromosome 10 open reading frame 58	3.35	2.30E-06
201667_at	*GJA1*	gap junction protein, alpha 1, 43kDa	3.24	1.08E-04
202437_s_at	*CYP1B1*	cytochrome P450, family 1, subfamily B, polypeptide 1	3.08	1.72E-04
202436_s_at	*CYP1B1*	cytochrome P450, family 1, subfamily B, polypeptide 1	2.70	1.78E-04
204793_at	*GPRASP1*	G protein-coupled receptor associated sorting protein 1	2.66	4.70E-06
201215_at	*PLS3*	plastin 3	2.63	9.55E-05
205174_s_at	*QPCT*	glutaminyl-peptide cyclotransferase	2.61	2.09E-05
228155_at	*C10orf58*	chromosome 10 open reading frame 58	2.58	1.47E-05
219271_at	*GALNT14*	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 14 (GalNAc-T14)	2.57	2.52E-05
202435_s_at	*CYP1B1*	cytochrome P450, family 1, subfamily B, polypeptide 1	2.55	8.48E-05
217901_at	*DSG2*	desmoglein 2	2.50	1.27E-05
202888_s_at	*ANPEP*	alanyl (membrane) aminopeptidase	2.26	2.80E-04
228557_at	*L3MBTL4*	l(3)mbt-like 4 (Drosophila)	2.22	9.50E-06
1557961_s_at	*LOC100127983*	hypothetical protein LOC100127983	2.21	3.52E-04
220617_s_at	*ZNF532*	zinc finger protein 532	2.11	1.15E-04
203543_s_at	*KLF9*	Kruppel-like factor 9	2.09	9.24E-04
228376_at	*GGTA1*	glycoprotein, alpha-galactosyltransferase 1	2.01	9.17E-04

Probe set	Gene symbol	Gene name	Fold change: *ASXL1*-mut *v* *ASXL1*-wt	*P*
225021_at	*ZNF532*	zinc finger protein 532	2.00	4.08E-05
227077_at	*ZNF286A/* *ZNF286B*	zinc finger protein 286A/ zinc finger protein 286B	1.98	4.10E-06
239265_at	*TMEM20*	transmembrane protein 20	1.98	6.00E-07
218236_s_at	*PRKD3*	protein kinase D3	1.98	9.22E-05
222446_s_at	*BACE2*	beta-site APP-cleaving enzyme 2	1.96	1.06E-04
200771_at	*LAMC1*	laminin, gamma 1 (formerly LAMB2)	1.95	2.05E-04
201348_at	*GPX3*	glutathione peroxidase 3 (plasma)	1.95	7.46E-04
1553105_s_at	*DSG2*	desmoglein 2	1.94	3.03E-04
226575_at	*ZNF462*	zinc finger protein 462	1.90	7.00E-07
203146_s_at	*GABBR1*	gamma-aminobutyric acid (GABA) B receptor, 1	1.88	9.71E-05
223170_at	*TMEM98*	transmembrane protein 98	1.86	4.85E-04
229464_at	*MYEF2*	myelin expression factor 2	1.83	4.89E-04
224817_at	*SH3PXD2A*	SH3 and PX domains 2A	1.82	1.51E-04
229116_at	*CNKSR2*	connector enhancer of kinase suppressor of Ras 2	1.80	7.37E-04
239742_at	*TULP4*	tubby like protein 4	1.74	1.47E-04
224327_s_at	*DGAT2*	diacylglycerol O-acyltransferase homolog 2 (mouse)	1.73	2.16E-05
226003_at	*KIF21A*	kinesin family member 21A	1.72	1.90E-04
226064_s_at	*DGAT2*	diacylglycerol O-acyltransferase homolog 2 (mouse)	1.72	4.14E-04
1556682_s_at		Homo sapiens full length insert cDNA clone ZD73H04	1.72	4.36E-04
234199_at		Homo sapiens cDNA FLJ21633 fis	1.72	9.17E-04
204454_at	*LDOC1*	leucine zipper, down-regulated in cancer 1	1.71	3.95E-04
210852_s_at	*AASS*	aminoadipate-semialdehyde synthase	1.71	6.61E-04
240903_at		Transcribed locus Hs.666593	1.71	1.68E-04

Probe set	Gene symbol	Gene name	Fold change: *ASXL1*-mut *v* *ASXL1*-wt	*P*
236565_s_at	*LARP6*	La ribonucleoprotein domain family, member 6	1.70	9.41E-04
243591_at		Transcribed locus Hs.656732	1.69	1.68E-04
218184_at	*TULP4*	tubby like protein 4	1.67	1.27E-04
226925_at	*ACPL2*	acid phosphatase-like 2	1.65	2.98E-04
205606_at	*LRP6*	low density lipoprotein receptor-related protein 6	1.64	1.00E-07
1556650_at		Homo sapiens cDNA FLJ13011 fis	1.64	2.90E-04
1557684_at	*ZNF286A*	zinc finger protein 286A	1.62	1.30E-06
229974_at	*EVC2*	Ellis van Creveld syndrome 2	1.62	4.23E-04
217867_x_at	*BACE2*	beta-site APP-cleaving enzyme 2	1.60	5.95E-04
229194_at	*PCGF5*	polycomb group ring finger 5	1.57	1.64E-04
232664_at	*FLJ12334*	hypothetical gene supported by AK022396; AK097927	1.55	5.00E-07
211564_s_at	*PDLIM4*	PDZ and LIM domain 4	1.54	5.83E-05
201147_s_at	*TIMP3*	TIMP metallopeptidase inhibitor 3	1.53	7.52E-04
213709_at	*BHLHB9*	basic helix-loop-helix domain containing, class B, 9	1.52	5.43E-05
205802_at	*TRPC1*	transient receptor potential cation channel, subfamily C, member 1	1.51	7.68E-04
226029_at	*VANGL2*	vang-like 2 (van gogh, Drosophila)	1.50	6.34E-04
1563453_at		Transcribed locus Hs.683977	1.49	5.41E-04
229623_at	*TMEM150C*	transmembrane protein 150C	1.48	5.88E-04
223311_s_at	*MTA3*	metastasis associated 1 family, member 3	1.47	2.03E-04
226770_at	*MAGI3*	membrane associated guanylate kinase, WW and PDZ domain containing 3	1.47	1.36E-05
209234_at	*KIF1B*	kinesin family member 1B	1.47	9.92E-04
203906_at	*IQSEC1*	IQ motif and Sec7 domain 1	1.47	5.62E-04
1558522_at	*MPP6*	membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	1.42	6.37E-04

Probe set	Gene symbol	Gene name	Fold change: *ASXL1*-mut *v* *ASXL1*-wt	*P*
238803_at	*HECTD2*	HECT domain containing 2	1.42	6.14E-04
214829_at	*AASS*	aminoadipate-semialdehyde synthase	1.42	6.04E-04
240568_at		Homo sapiens cDNA clone IMAGE:2722444	1.42	7.96E-04
238983_at	*NSUN7*	NOP2/Sun domain family, member 7	1.38	1.60E-04
223621_at	*PNMA3*	paraneoplastic antigen MA3	1.37	4.55E-04
232549_at	*RBM11*	RNA binding motif protein 11	1.36	6.82E-04
225303_at	*KIRREL*	kin of IRRE like (Drosophila)	1.36	5.44E-04
214175_x_at	*PDLIM4*	PDZ and LIM domain 4	1.34	2.60E-04
219740_at	*VASH2*	vasohibin 2	1.33	1.84E-04
1556656_at		Transcribed locus Hs.655421	1.32	2.11E-04
220214_at	*ZNF215*	zinc finger protein 215	1.27	8.19E-04
		Genes down-regulated in patients with mutated *ASXL1*		
230867_at	*COL6A6*	collagen, type VI, alpha 6	0.89	2.46E-04
1562940_at	*DOCK7*	dedicator of cytokinesis 7	0.80	7.73E-04
1556103_at		Homo sapiens cDNA FLJ44114 fis	0.80	7.99E-04
218480_at	*AGBL5*	ATP/GTP binding protein-like 5	0.79	5.96E-04
242517_at	*KISS1R*	KISS1 receptor	0.78	1.15E-04
213643_s_at	*INPP5B*	inositol polyphosphate-5-phosphatase, 75kDa	0.76	9.64E-04
238107_at		Transcribed locus Hs.147507	0.76	3.56E-04
210441_at	*CDC14A*	CDC14 cell division cycle 14 homolog A (S. cerevisiae)	0.75	6.10E-04
206703_at	*CHRNB1*	cholinergic receptor, nicotinic, beta 1 (muscle)	0.73	9.23E-04
209975_at	*CYP2E1*	cytochrome P450, family 2, subfamily E, polypeptide 1	0.71	7.37E-04
244037_at		Transcribed locus Hs.212051	0.70	2.34E-04

Probe set	Gene symbol	Gene name	Fold change: *ASXL1*-mut *v* *ASXL1*-wt	*P*
210594_x_at	*MPZL1*	myelin protein zero-like 1	0.68	4.73E-04
205408_at	*MLLT10*	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10	0.67	6.88E-04
216860_s_at	*GDF11*	growth differentiation factor 11	0.67	4.80E-04
1559048_at	*BAHCC1*	BAH domain and coiled-coil containing 1	0.63	8.00E-05
1559049_a_at	*BAHCC1*	BAH domain and coiled-coil containing 1	0.46	3.50E-06
236553_at		Transcribed locus Hs.649477	0.46	9.00E-07

Table S5. Gene sets representing functional pathways significantly deregulated between *ASXL1*-mut and *ASXL1*-wt CN-AML patients aged 60 years or older with wild-type *NPM1* and no *FLT3*-ITD, as identified by the gene set analysis algorithm.

Gene set representing biological pathway	Number of genes included in analysis	*P*	Direction of deregulation
KEGG: O-GLYCAN BIOSYNTHESIS	17	<.002	upregulated in *ASXL1*-mut
REACTOME: SLC MEDIATED TRANSMEMBRANE TRANSPORT	84	<.002	upregulated in *ASXL1*-mut
REACTOME: CYTOCHROME P450 ARRANGED BY SUBSTRATE TYPE	14	.002	upregulated in *ASXL1*-mut
REACTOME: APOPTOTIC CLEAVAGE OF CELL ADHESION PROTEINS	6	.002	upregulated in *ASXL1*-mut
REACTOME: INORGANIC CATION ANION SLC TRANSPORTERS	46	.002	upregulated in *ASXL1*-mut
BIOCARTA: NO1 PATHWAY	15	.002	downregulated in *ASXL1*-mut
BIOCARTA: DREAM PATHWAY	9	.002	downregulated in *ASXL1*-mut
KEGG: GLYCEROLIPID METABOLISM	35	.004	upregulated in *ASXL1*-mut
REACTOME: TRANSMISSION ACROSS CHEMICAL SYNAPSES	65	.004	upregulated in *ASXL1*-mut
REACTOME: PYRIMIDINE METABOLISM	19	.004	downregulated in *ASXL1*-mut

NOTE: Gene sets were obtained from the ‘curated pathways’ collection of the molecular signatures database (MSigDB), version 3.0. *P* vales are from Efron-Tibshirani's GSA test.

Table S6. List of PCR primers and conditions

Amplicon	Length	Primer sequences	DNA polymerase	Cycling conditions
ASXL1 exon 12 part 1	1225 bp	14_1F: 5′-**CTGAATGGTGTGTTATGGCG-3′**	Phusion Hot Start High-Fidelity DNA Polymerase	98°C, 30s; followed by 37 cycles of 98°C / 10s, 63°C / 30s, 72°C / 30s.
		14_1R: **5′-GGGGCTCAACAGATGGTATG-3′**		
ASXL1 exon 12 part 2	1214 bp	14_2F: 5′-**ACACCTGAATCCTCACCGAC-3′**	AmpliTaq Gold DNA Polymerase	94°C, 300s; followed by 37 cycles of 94°C / 20s, 60°C / 40s, 72°C / 90s.
		14_2R: 5′-**TCCTGTGACATAGCACGGAC-3′**		
ASXL1 exon 12 part 3	1223 bp	14_3F: 5′-**AAAGAATTGCAAGGCAGTCC-3′**	AmpliTaq Gold DNA Polymerase	94°C, 300s; followed by 37 cycles of 94°C / 20s, 60°C / 40s, 72°C / 90s.
		14_3R: 5′-**TATACCCAGGAAACCCCTCC-3′**		

NOTE: Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes/Thermo Fisher Scientific, Lafayette, CO) and AmpliTaq Gold Polymerases (Applied Biosystems, Foster City, CA) were used in their manufacturer-supplied buffers, and reactions were set up in a final volume of 25µl containing 1.6mM Mg^{2+} and 0.5µM of each primer.

Figure S1. Survival of CN-AML patients aged 60 years or older with missense changes in *ASXL1* exon 12, compared to *ASXL1*-wt and *ASXL1*-mut patients. (A) EFS in the entire cohort of older CN-AML patients; **(B)** EFS within the ELN Favorable genetic group.

Figure S2. Survival of *ASXL1*-mut and *ASXL1*-wt CN-AML patients aged 60 years or older, according to *CEBPA* mutation status. (A) OS within *CEBPA*-mut and **(B)** within *CEBPA*-wt patients. **(C)** EFS within *CEBPA*-mut and **(D)** within *CEBPA*-wt patients.

Figure S3. Survival of older ELN favorable CN-AML patients with mutated *ASXL1*, compared to *ASXL1*-wt/*TET2*-wt patients and to *ASXL1*-wt/*TET2*-mut patients. (A) OS; **(B)** EFS.

Figure S4. Evidence supporting that *ASXL1* c.1934dupG represents a mutation rather than technical artifact. The figure shows representative chromatograms obtained by sequencing PCR products from a patient with an *ASXL1* c.1934dupG mutation (Patient #288, left side) and a patient without this mutation (Patient #144, right side). c.1934dupG denotes insertion of a guanine (G) nucleotide at coding sequence position 1934. This insertion occurs within a mononucleotide stretch that normally comprises eight G nucleotides. During PCR amplification of DNA, mononucleotide repeats longer than 8-10 nucleotides can lead to the formation of aberrant products due to a phenomenon called ‘polymerase slippage’. Slippage errors most commonly result in products where the mononucleotide repeat is aberrantly shortened (contracted).[16] These aberrant products then may appear as artifacts in subsequent

sequencing reactions, mimicking a deletion or insertion in the DNA template. Some PCR polymerases, including Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes/Thermo Fisher Scientific, Lafayette, CO), have been shown to be relatively resistant to this kind of error, and can reliably amplify homopolymer stretches of 13 or more nucleotides without slippage.[17]

In DNA from the BM of patient #288 (left column), the c.1934dupG mutation was detected in a heterozygous-appearing pattern, both when using a *Taq*-derived DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA; top) and when using the slippage-resistant Phusion DNA polymerase (middle). When DNA from a buccal swab from the same patient was studied using Phusion polymerase (bottom), a wild-type sequence without evidence of an insertion was obtained. In total, buccal swab DNA was available from six patients who had c.1934dupG detected in the BM, and in all cases we obtained a clean wild-type sequence from the germline DNA.

In patient #144 (right side), when PCR was performed on BM DNA using AmpliTaq Gold polymerase (top), the sequencing chromatogram showed some minor peaks most likely representing 'slippage' products after the mononucleotide stretch (highlighted by the red arrows). When the slippage-resistant Phusion polymerase was used to amplify BM DNA from the same patient, a clean wild-type sequence was obtained (below).

For all 23 patients with the c.1934dupG mutation, the finding was confirmed in at least two independent PCR and sequencing reactions. PCR reactions were also repeated for 30 patients where c.1934dupG was not detected initially, and absence of c.1934dupG was confirmed in the repeat analysis in all cases.

Figure S1

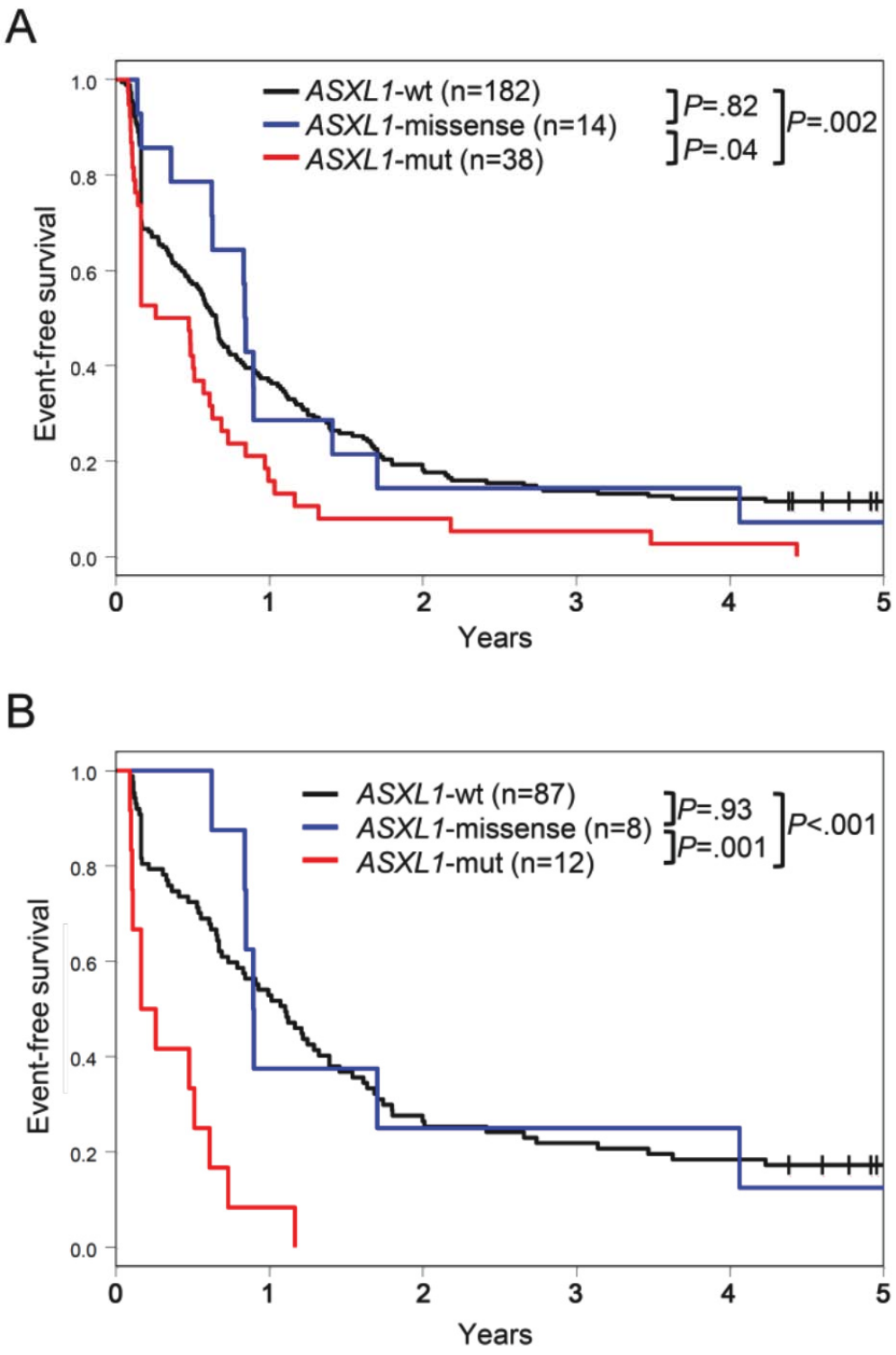

A
ASXL1-wt (n=182)
ASXL1-missense (n=14)
ASXL1-mut (n=38)
P=.82
P=.04
P=.002
Event-free survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Years
B
ASXL1-wt (n=87)
ASXL1-missense (n=8)
ASXL1-mut (n=12)
P=.93
P=.001
P<.001
Event-free survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Years

Figure S2

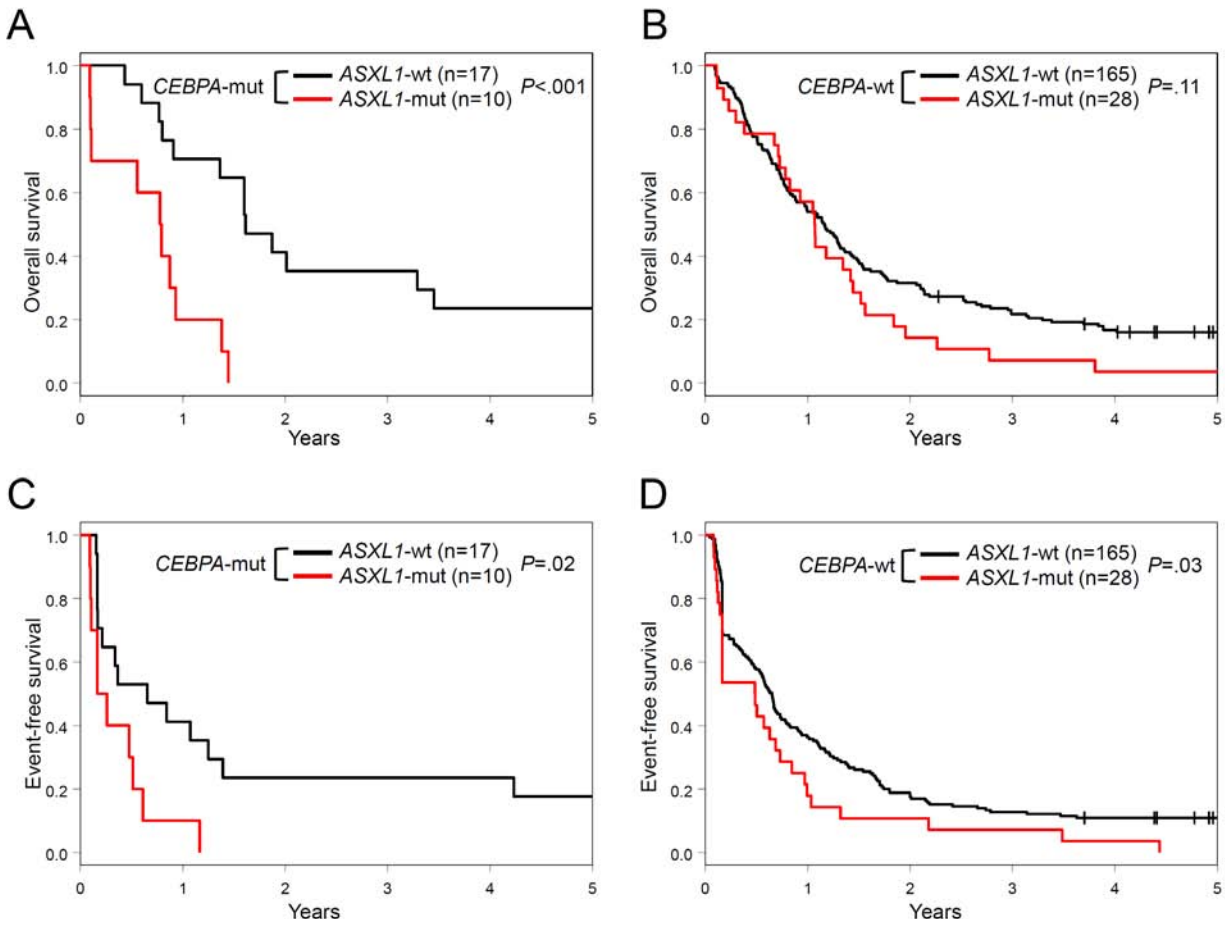

A
CEBPA-mut
ASXL1-wt (n=17)
ASXL1-mut (n=10)
P<.001
Overall survival
Years
B
CEBPA-wt
ASXL1-wt (n=165)
ASXL1-mut (n=28)
P=.11
Overall survival
Years
C
CEBPA-mut
ASXL1-wt (n=17)
ASXL1-mut (n=10)
P=.02
Event-free survival
Years
D
CEBPA-wt
ASXL1-wt (n=165)
ASXL1-mut (n=28)
P=.03
Event-free survival
Years

Figure S3

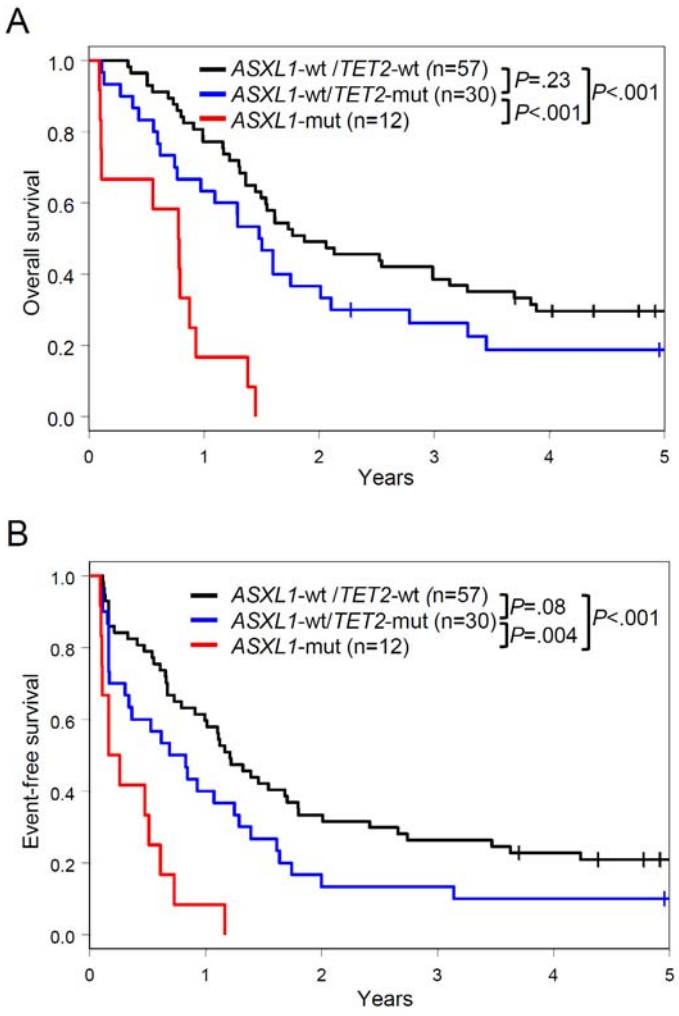
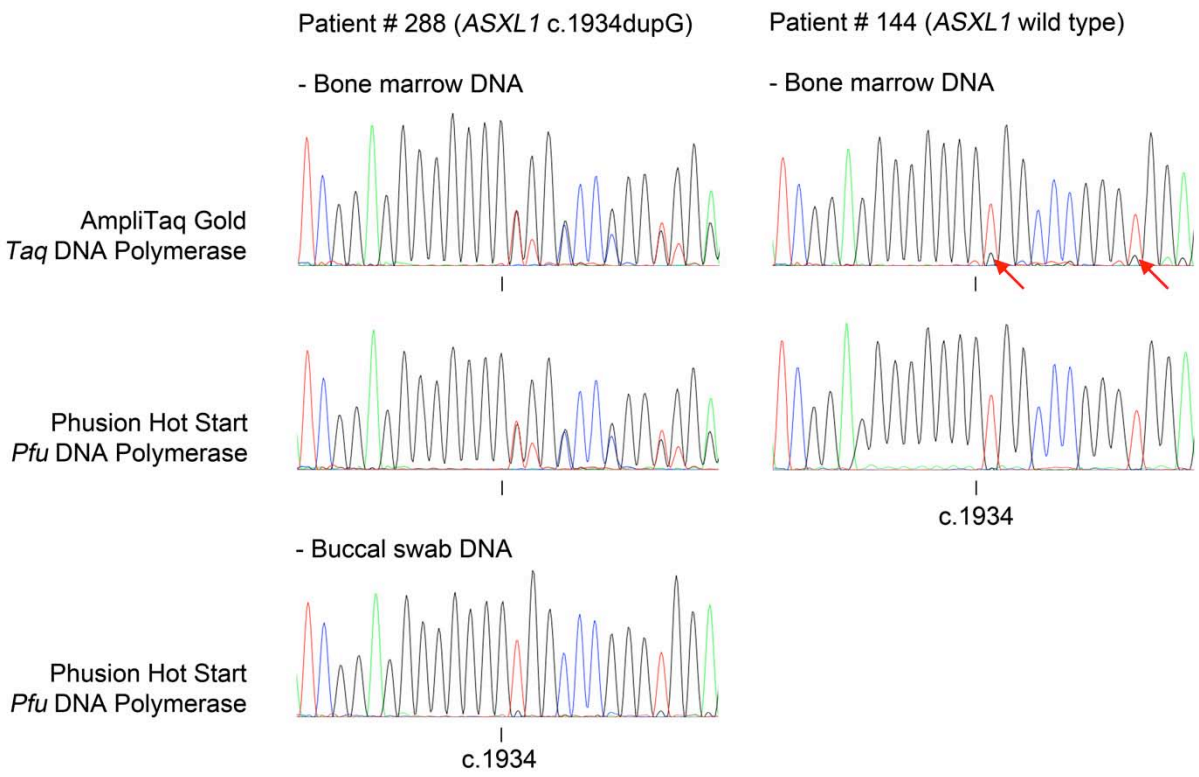

A
ASXL1-wt /TET2-wt (n=57)
ASXL1-wt/TET2-mut (n=30)
ASXL1-mut (n=12)
P=.23
P<.001
P<.001
Overall survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Years
B
ASXL1-wt /TET2-wt (n=57)
ASXL1-wt/TET2-mut (n=30)
ASXL1-mut (n=12)
P=.08
P=.004
P<.001
Event-free survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Years

Figure S4


Patient # 288 (*ASXL1* c.1934dupG)
Patient # 144 (*ASXL1* wild type)
- Bone marrow DNA
- Bone marrow DNA
AmpliTaq Gold
Taq DNA Polymerase
Phusion Hot Start
Pfu DNA Polymerase
c.1934
- Buccal swab DNA
Phusion Hot Start
Pfu DNA Polymerase
c.1934