Supplementary information to the article "SOCS2 is part of a highly prognostic 4-gene signature in AML and promotes disease aggressiveness"

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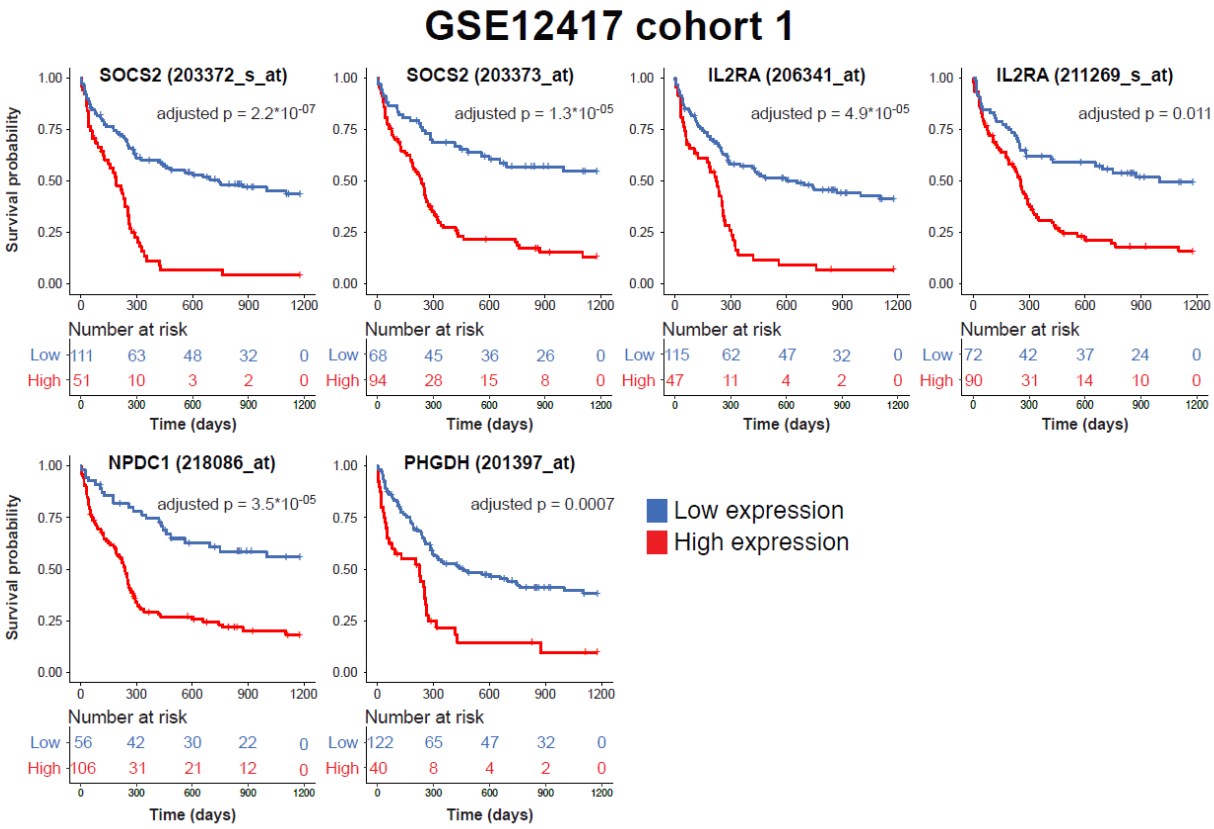
Supplementary Methods

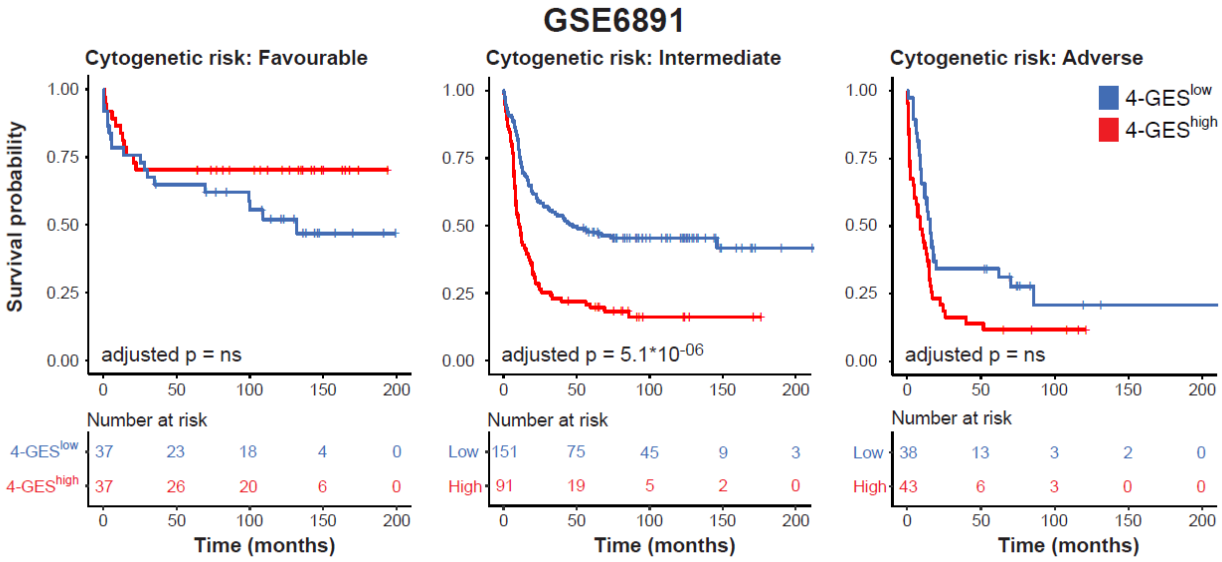
Cell culture, transduction of human myeloid cell lines, and proliferation assay

The packaging cell line Phoenix-GP was maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% Penicillin/Streptomycin (Sigma-Aldrich). U937 and HL60 cells and their derivatives were grown in RPMI 1640 (Invitrogen) containing 10% FBS and 1% Penicillin/Streptomycin/Glutamine (Life Technologies). A codon optimized version of the human *SOCS2* cDNA was cloned into the retroviral vector pMSCV_IRES_GFP using the *XhoI* and *EcoR*I restriction sites. The resulting vector, pMSCV_SOCS2_IRES_GFP, as well as empty pMSCV_IRES_GFP as a control, were transfected into Phoenix-GP cells, along with helper plasmids pMD2.G and pGag-Pol, and retroviral particles were harvested and used to infect U937 and HL60 cells through standard procedures. Three days later, the resulting cell lines U937_Vec, U937_SOCS2, HL60_Vec, and HL60_SOCS2 were sorted for GFP positivity on an Astrios (Beckman Coulter). For the proliferation assays, cells were seeded into 12-well plates at a density of $1.5x10^5$ cells/ml, split at equal ratios when necessary, and total viable cells were counted every 24 h using a CASY counter (Roche Innovates).

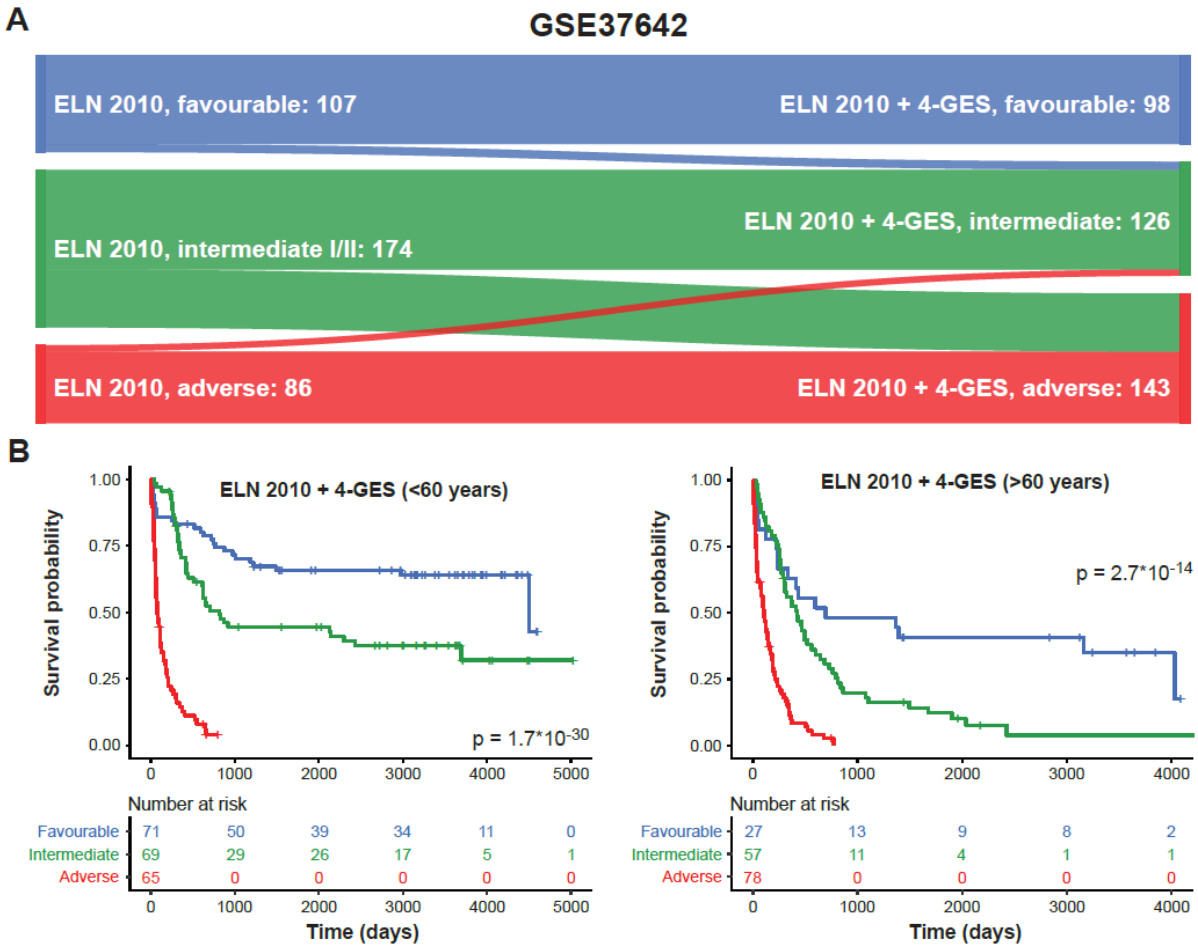
Supplementary Figures



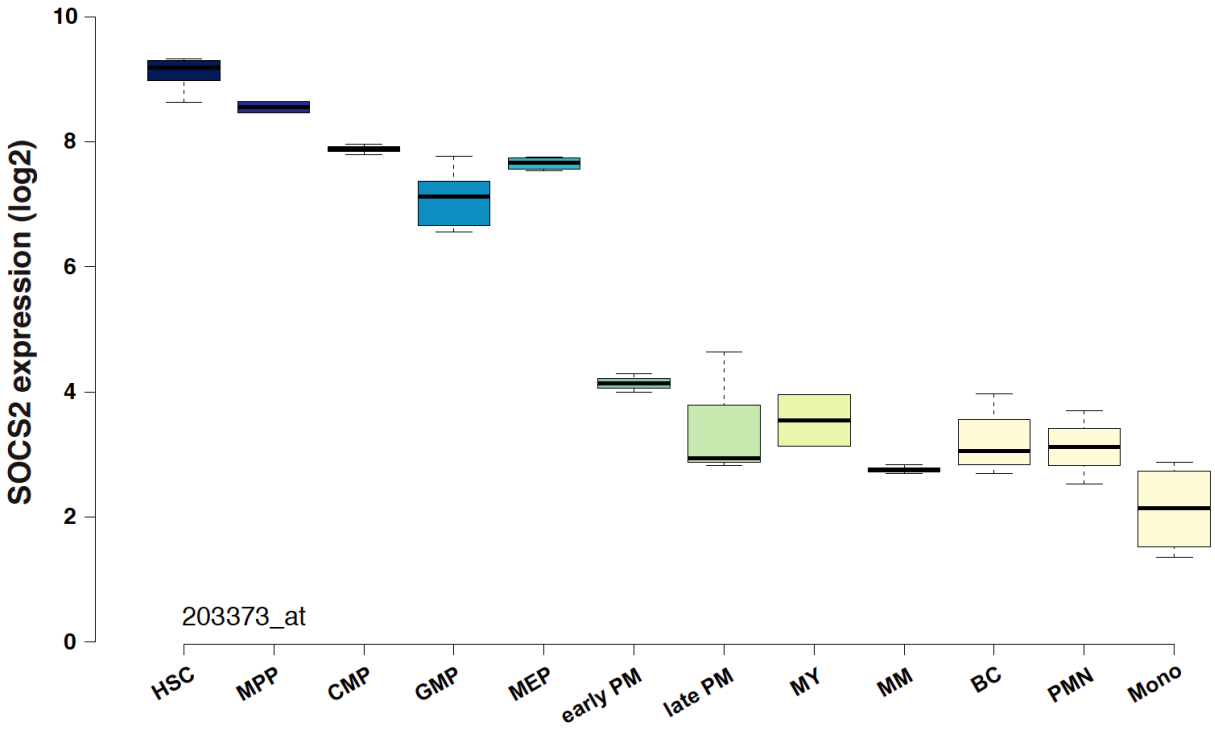
Supplementary Figure S1. Kaplan Meier curves for overall survival of 162 cytogenetically normal AML patients (GSE12417, cohort 1) according to expression values for alternative probe sets for *SOCS2*, *IL2RA*, *NPDC1* and *PHGDH*. Expression values were dichotomized by maximally selected rank statistics. Statistical significance was calculated using the log rank test and p-values were adjusted for multiple testing as described by Altman *et al.* [1].



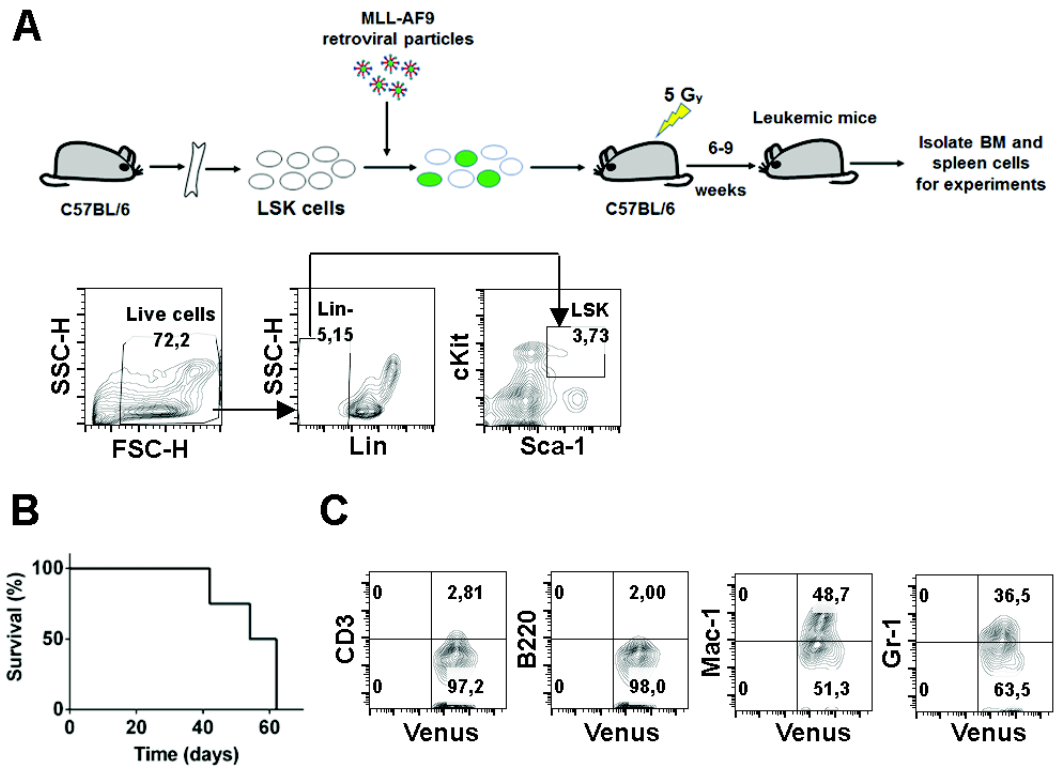
Supplementary Figure S2. Kaplan Meier curves for overall survival of AML patients classified as cytogenetically favourable, intermediate, or adverse (GSE6891, combined cohorts 1 and 2). Patients were stratified into 4-GES^{low} (blue) and 4-GES^{high} (red) subgroups. Statistical significance was calculated using the log rank test and p-values were adjusted for multiple testing as described by Altman *et al.* [1].



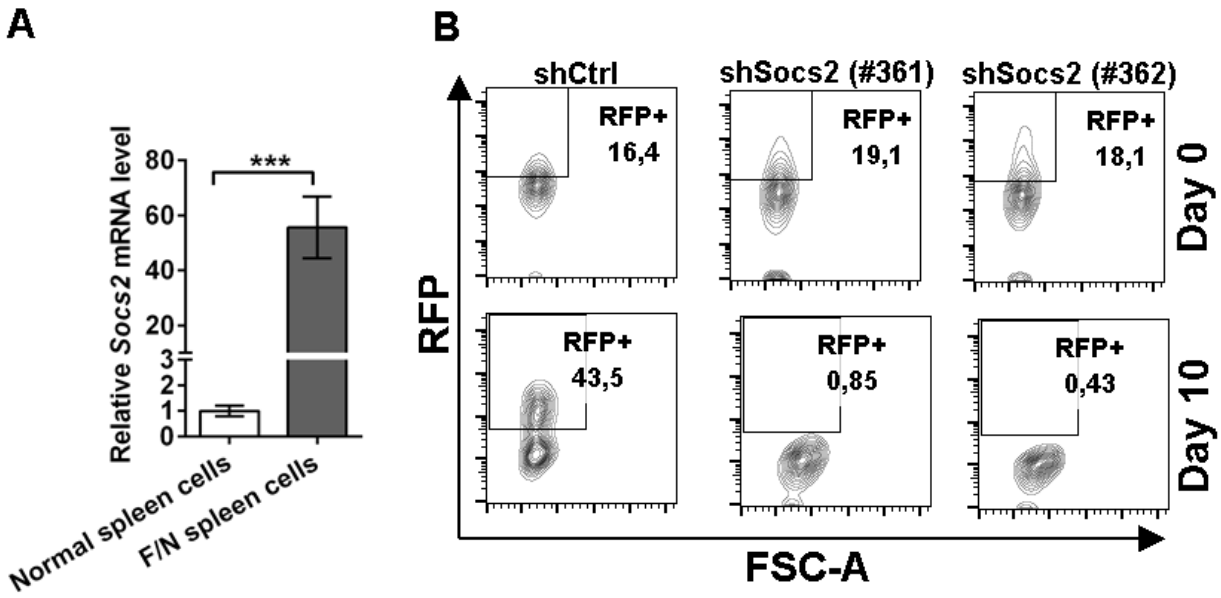
Supplementary Figure S3. The 4-GES is able to refine the ELN classification of AML patients included in the GSE37642 data set. **(A)** A shift of patients from ELN 2010 favourable, intermediate I/II and adverse (left side) to ELN 2010 + 4-GES favourable, intermediate and adverse (right side) is shown. ELN favourable/4-GES^{high} patients with low median OS were re-assigned to ELN intermediate risk group, ELN intermediate/4-GES^{high} patients with low median OS were re-assigned to ELN adverse risk group, and ELN adverse/4-GES^{low} patients with high median OS were re-assigned to ELN intermediate risk group. **(B)** Kaplan Meier curves for overall survival of AML patients <60 years of age (left) and >60years of age (right) stratified into favourable, intermediate, and adverse risk based on the ELN 2010 + 4-GES classification. ELN, European Leukemia Net.



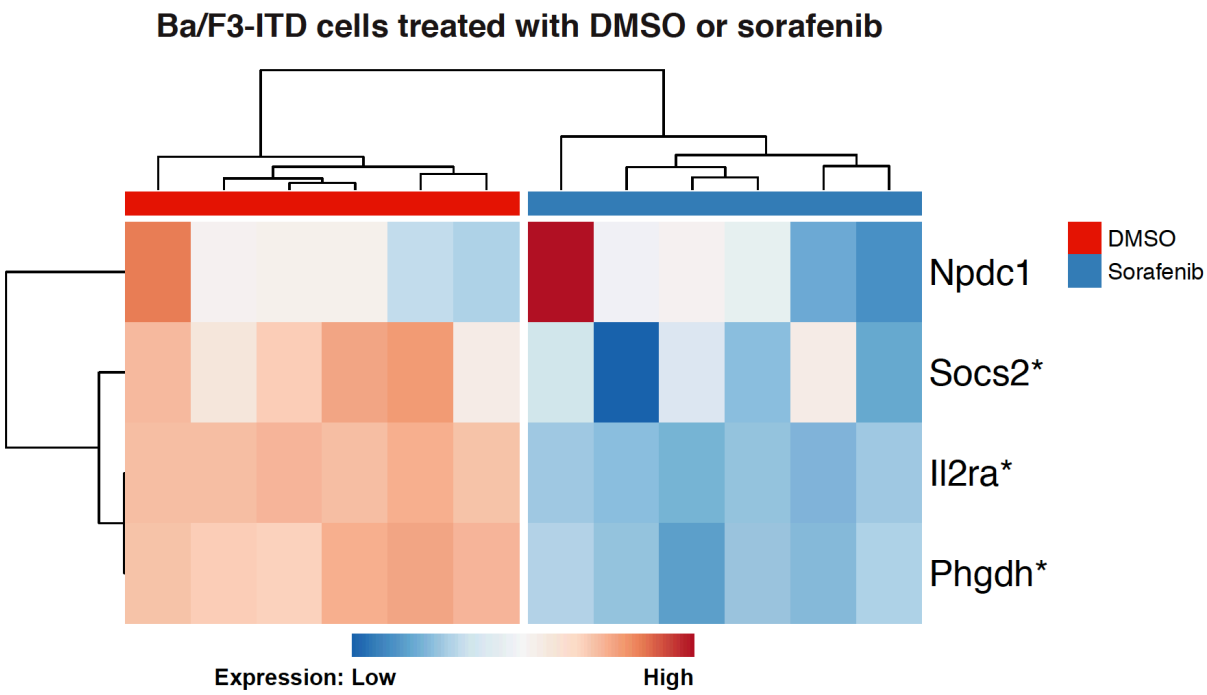
Supplementary Figure S4. SOCS2 (Affymetrix probe set: 203373_at) expression in various hematopoietic cells determined by gene expression microarray analyses. Log2 transformed gene expression values are shown. HSC, Hematopoietic stem cell; MPP, Multipotential progenitors; CMP, Common myeloid progenitor cell; GMP, Granulocyte monocyte progenitors; MEP, Megakaryocyte-erythroid progenitor cell; early_PM, Early Promyelocyte; late_PM, Late Promyelocyte; MY, Myelocyte; MM, Metamyelocytes; BC, Band cell; PMN, Polymorphonuclear cells; Mono, Monocytes



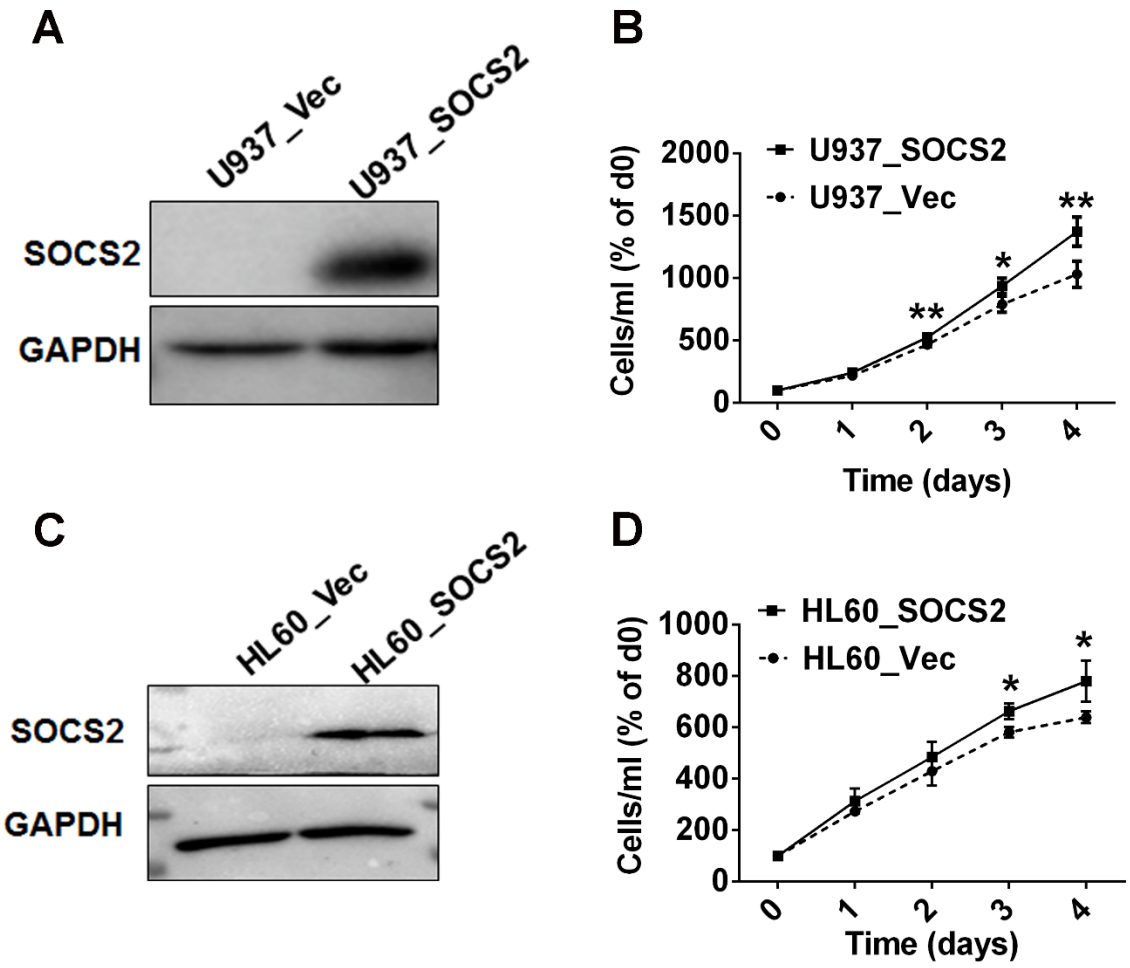
Supplementary Figure S5. *MLL-AF9*-transduced LSK cells cause an aggressive AML-like disease in mice. **(A)** Schematic overview of the experiment. **(B)** Kaplan Meier plot of mice transplanted with *MLL-AF9*-transduced LSK cells (160,000 unsorted cells per mouse, n = 3). (**C**) Flow cytometric analysis of spleen cells from terminally ill mice confirmed the expression of myeloid (Mac-1, Gr-1) and the absence of lymphoid (CD3, B220) markers in LCs ($Venus^+$ cells; representative experiment).



Supplementary Figure S6. *Socs2* is required for proliferation and/or survival of *Flt3*-ITD/*NPM1c* LCs *in vitro*. **(A)** *Socs2* mRNA levels in spleen cells from *Flt3*-ITD/*NPM1c* (F/N) leukemic mice and from healthy mice were determined by qRT-PCR and normalised to those of the housekeeping gene *ß-2-microglobulin* using the $\Delta\Delta C_T$ method. Mean ± SEM of three independent experiments; ***, $p < 0.001$ (Student's two-tailed t-test). **(B)** Representative flow cytometric analysis of RFP^+ cells from shCtrl or shSocs2 transduced *Flt3*-ITD/*NPM1c* LCs. Upper panel, cells on the day of sorting (day 0); lower panel, sorted cells after 10 days in suspension culture (day 10).



Supplementary Figure S7. Expression of *Npdc1*, *Socs2*, *Il2ra* and *Phgdh* in DMSO (n = 6) or sorafenib (n = 6) treated Ba/F3-ITD cells determined using MoGene-1_0-st-v1 microarrays (ArrayExpress ID: E-MTAB-4487). Ba/F3-ITD cells were treated with DMSO or 10nM sorafenib for 24 hours. Log2 transformed gene expression values are shown (blue, low expression; red, high expression). Asterisks indicate statistically significant differences in gene expression between DMSO and sorafenib treated cells (FDR < 0.005).



Supplementary Figure S8. Experimental expression of *SOCS2* promotes proliferation of the malignant human myeloid cell lines U937 **(A, B)** and HL60 **(C, D)**. **(A, C)** Immunoblot analyses demonstrating expression of SOCS2 in U937_SOCS2 and HL60_SOCS2, but not U937_Vec and HL60_Vec cells. **(B, D)** Relative counts of U937_SOCS2, U937_Vec, HL60_SOCS2, and HL60_Vec cells over a period of 4 days. Mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (2-way ANOVA followed by Bonferroni's post-hoc test).

Supplementary Table S1. Univariable Cox regression analysis for overall survival of AML patients

Data set	Variable	HR	95% CI	p-value
GSE12417 cohort 1	4-GES^{high} vs. 4-GES^{low}	4.09	2.52-6.64	**$1.3*10^{-08}$**
	Age (years)	1.03	0.76-0.97	**0.0005**
	FAB, M1 vs. others	1.44	0.51-4.07	0.491
	FAB, M2 vs. others	1.1	0.39-3.12	0.858
	FAB, M4 vs. others	0.71	0.24-2.07	0.533
	FAB, M5 vs. others	0.82	0.26-2.58	0.735
	FAB, M6 vs. others	0.61	0.14-2.72	0.516
GSE12417 cohort 2[a]	4-GES^{high} vs. 4-GES^{low}	4.93	1.19-20.35	**0.027**
	Age (years)	1.04	1.01-1.06	**0.006**
GSE6891 cohort 1	4-GES^{high} vs. 4-GES^{low}	2.33	1.68-3.25	**$4.5*10^{-07}$**
	Age (years)	1.01	0.99-1.03	0.066
	FAB, M1 vs. others	0.5	0.2-1.28	0.149
	FAB, M2 vs. others	0.46	0.18-1.17	0.103
	FAB, M4 vs. others	0.48	0.18-1.24	0.127
	FAB, M5 vs. others	0.68	0.27-1.72	0.419
	FAB, M6 vs. others	0.16	0.02-1.41	0.1
	Cytogenetic risk[b]	1.96	1.55-2.46	**$1.2*10^{-08}$**
	Sex	1.22	0.89-1.68	0.226
	FLT3-ITD	1.82	1.29-2.56	**0.0007**
	FLT3-TKD	0.72	0.42-1.25	0.248
	IDH1	0.66	0.32-1.35	0.252
	IDH2	1.15	0.65-2.04	0.627
	KRAS	0.92	0.23-3.7	0.903
	NRAS	0.63	0.36-1.12	0.116
	NPM1c	0.81	0.57-1.15	0.241
	CEBPA (wt/mono/bi)	0.67	0.46-0.98	**0.04**
	CEBPA (yes/no)	0.46	0.2-0.97	**0.042**
	EVI1 expression	1.81	1.09-3.01	**0.022**
GSE6891 cohort 2	4-GES^{high} vs. 4-GES^{low}	2.48	1.7-3.62	**$2.5*10^{-06}$**
	Age (years)	1.02	1-1.03	**0.017**

	FAB, M1 vs. others	0.67	0.3-1.48	0.319
	FAB, M2 vs. others	0.7	0.32-1.53	0.368
	FAB, M4 vs. others	0.64	0.29-1.44	0.285
	FAB, M5 vs. others	0.67	0.3-1.49	0.325
	FAB, M6 vs. others	0.78	0.17-3.69	0.755
	Cytogenetic risk[b]	1.57	1.14-2.16	**0.005**
	Sex	0.91	0.63-1.33	0.64
	FLT3-ITD	1.44	0.97-2.15	0.072
	FLT3-TKD	0.67	0.33-1.38	0.275
	IDH1	1.15	0.6-2.2	0.672
	IDH2	0.59	0.29-1.22	0.158
	NRAS	0.98	0.54-1.78	0.942
	NPM1c	0.96	0.64-1.44	0.86
	CEBPA (wt/mono/bi)	0.76	0.47-1.22	0.255
	CEBPA (yes/no)	0.68	0.3-1.55	0.361
	EVI1 expression	3.52	1.85-6.69	**0.0001**
GSE37642	4-GES^{high} vs. 4-GES^{low}	2.65	2.04-3.45	**$3.1*10^{-13}$**
	Age (years)	1.04	1.03-1.05	**$2.9*10^{-14}$**
	FAB, M2 vs. others	0.75	0.54-1.03	0.077
	FAB, M4 vs. others	0.51	0.36-0.72	**0.0001**
	FAB, M5 vs. others	0.92	0.62-1.36	0.669
	FAB, M6 vs. others	0.78	0.41-1.48	0.449
	FAB, M7 vs. others	1.92	0.47-7.86	0.366
	ELN score[c]	1.43	0.7-1.28	**$2.3*10^{-11}$**
TCGA_LAML	4-GES^{high} vs. 4-GES^{low}	1.91	1.28-2.86	**0.0016**
	Age (years)	1.04	0.97-1.02	**$1.1*10^{-05}$**
	FAB, M1 vs. others	1.14	0.54-2.39	0.737
	FAB, M2 vs. others	1.12	0.53-2.37	0.766
	FAB, M4 vs. others	1.14	0.55-2.28	0.72
	FAB, M5 vs. others	1.56	0.67-3.62	0.303
	FAB, M6 vs. others	2.71	0.59-12.55	0.202
	FAB, M7 vs. others	2.61	0.71-9.59	0.149
	Cytogenetic risk[a]	1.55	1.08-2.23	**0.017**

	Sex	1	0.67-1.5	0.97
	FLT3-ITD	0.97	0.49-1.93	0.934
	FLT3-TKD	1.47	0.71-3.04	0.3
	IDH1	0.69	0.4-1.18	0.178
	RAS	0.78	0.31-1.92	0.584
	NPM1c	1.01	0.65-1.57	0.955

4-GES, 4-gene expression score; HR, hazard ratio; CI, confidence interval; FAB, French American British classification; [a], FAB classification of GSE12417 cohort 2 was excluded from univariable analyses because of low subgroup sample numbers; *FLT3*-ITD, *FLT3* internal tandem duplication; *FLT3*-TKD, *FLT3* tyrosine kinase domain mutation; gene names in italics refer to mutations of the respective genes; mono, monoallelic; bi, biallelic. [b], Assignment to cytogenetic risk groups were included in the respective GEO entries. [c], Assignment to ELN risk groups was provided by T. Herold, University of Munich, Department of Internal Medicine III, Munich, Germany. No relevant patient data were provided in GSE71014; therefore, regression analyses were not performed.

Supplementary Table S2. Antibodies used for immunoblot analysis and flow cytometry

Method	Target	Clone/source organism	Fluorophor/ conjugate	Company	Cat. no	Dilution
IB	Human/mouse SOCS2	Rabbit	-	Cell Signaling	2779S	1:1,000
	Human/mouse GAPDH	14C10	-	Cell Signaling	2118S	1:50,000
	Rabbit IgG	Goat	Horseradish peroxidase	Jackson ImmunoResearch	111035008	1:5,000
FC	Mouse Gr-1	RB6-8C5	AF700	Biolegend	108422	1:100
	MouseMac-1	M1/70	AF700	Biolegend	101222	1:100
	Mouse CD3	17A2	AF700	Biolegend	100216	1:100
	Mouse B220	RA3-6B22	AF700	Biolegend	103232	1:100
	Mouse Ter119	TER119	AF700	Biolegend	116220	1:100
	Mouse c-Kit	2B8	PE	Biolegend	105808	1:50
	Mouse c-Kit	2B8	APC-Cy7	Biolegend	105826	1:50
	Mouse Sca-1	D7	BV421	BD Bioscience	562729	1:50
	Mouse Sca-1	D7	PerCP/Cy5.5	Biolegend	122524	1:50
	Mouse CD34	RAM34	FITC	eBioscience	11034182	1:50
	Mouse CD34	MEC14.7	PE/Cy5.5	Biolegend	119312	1:50
	Mouse CD16/CD32	93	PE/Cy7	eBioscience	25016182	1:50
	Mouse Ki-67	16A8	APC	Biolegend	652406	1:50

IB, immunoblot analysis; FC, flow cytometry. Gr-1, Mac-1, CD3, B220, and Ter119 antibodies were combined to define Lin^- cells.

Supplementary Table S3. Genes included in the 4-GES and in 3 published gene expression signatures

4-GES	**L-24**	**M-7**	**W-3**
IL2RA	*ALS2CR8*	*CD34*	*CAP1*
NPDC1	*ANGEL1*	*F2RL1*	*CXCR6*
PHGDH	*ARL6IP5*	*FAM92A1*	*FAM124B*
SOCS2	*BSPRY*	*MIR155HG*	
	BTBD3	*RHOC*	
	C1RL	*SCRN1*	
	CPT1A	*VWA8*	
	DAPK1		
	ETFB		
	FGFR1		
	HEATR6		
	LAPTM4B		
	MAP7		
	NDFIP1		
	PBX3		
	PLA2G4A		
	PLOD3		
	PTP4A3		
	SLC25A12		
	SLC2A5		
	TMEM159		
	TRIM44		
	TRPS1		
	VAV3		

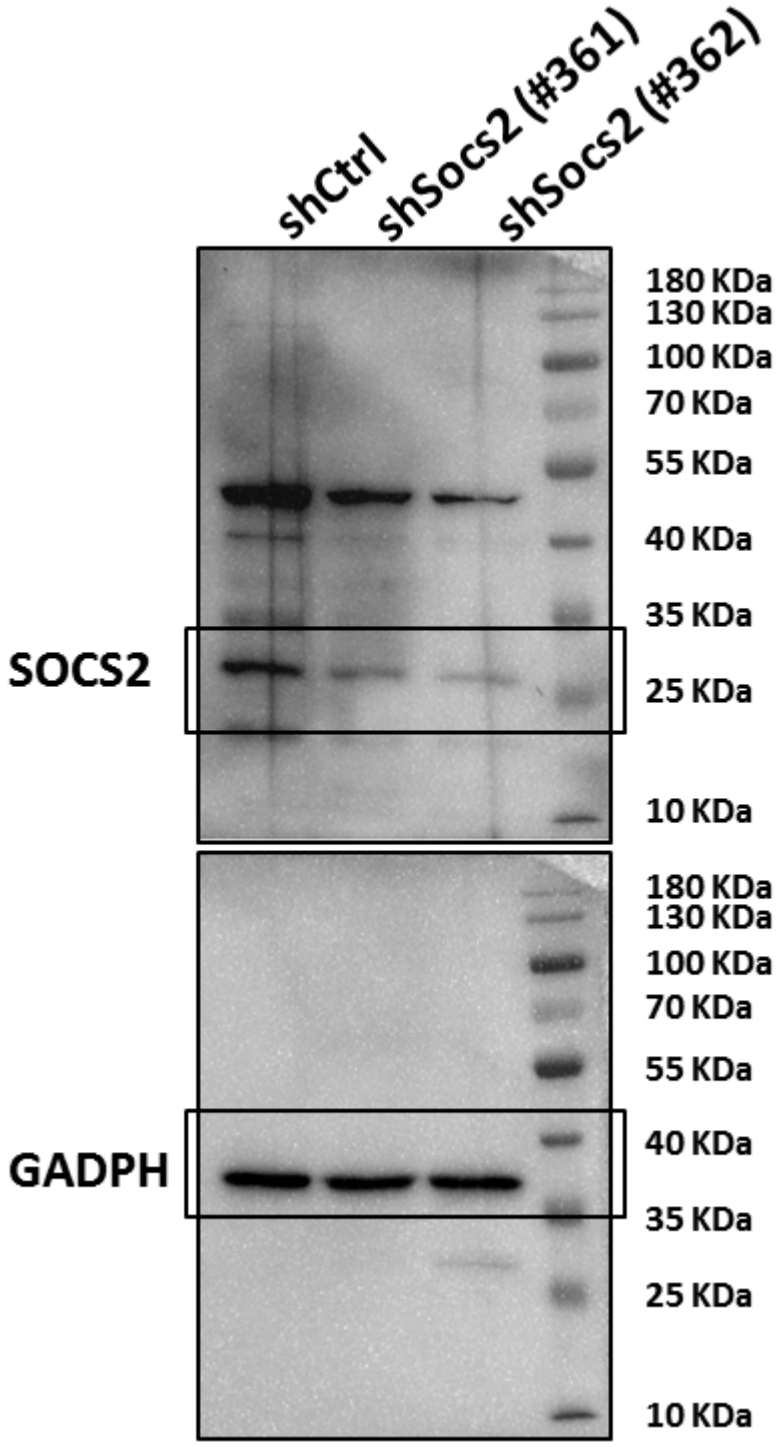
L-24, 24-gene expression signature by Li et al. [2]; M-7, 7-gene expression signature by Marcucci et al. [3]; W-3, 3-gene expression signature by Wilop et al. [4]

Supplementary Table S4. Univariable Cox regression analysis for overall survival of AML patients with respect to the 4-GES and 3 published gene expression signatures

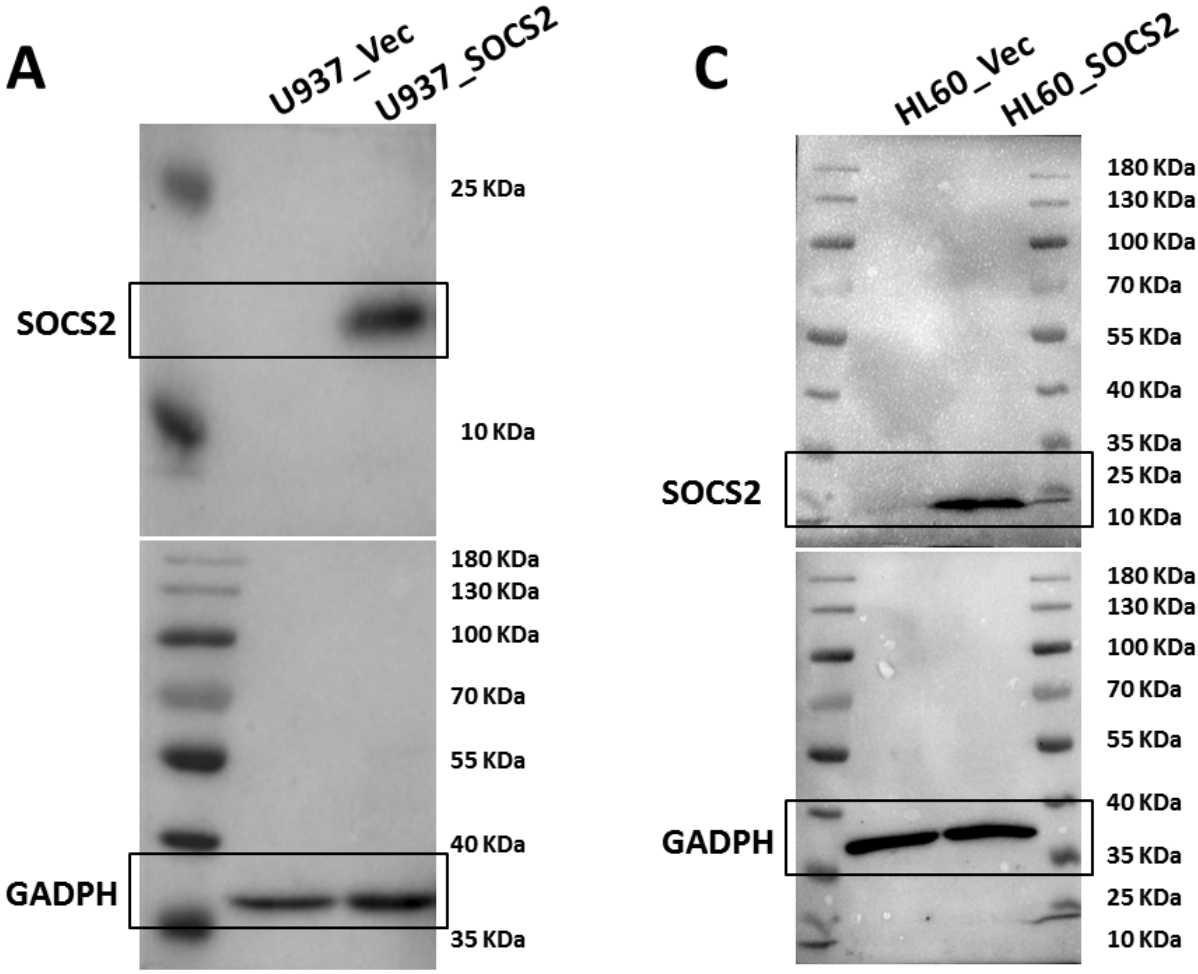
	4-GES		L-24		M-7		W-3	
Data set	**HR**	**p**	**HR**	**p**	**HR**	**p**	**HR**	**p**
GSE12417, cohort 1	**4.09**	**$1.3*10^{-08}$**	**2.19**	**0.00012**	na	na	**1.47**	**0.03**
GSE12417, cohort 2	**4.93**	**0.027**	1.63	0.097	**2.68**	**0.001**	**1.97**	**0.016**
GSE37642	**2.62**	**$1.2*10^{-13}$**	**1.59**	**0.00011**	na	na	1.15	0.171
GSE6891, cohort1	**2.34**	**$4.5*10^{-07}$**	**1.71**	**0.001**	**1.95**	**$6.3*10^{-05}$**	1.34	0.082
GSE6891, cohort2	**2.48**	**$2.4*10^{-06}$**	**1.69**	**0.007**	**1.63**	**0.011**	1.26	0.249
GSE71014	**3.28**	**0.002**	1.74	0.102	**2.7**	**0.005**	1.37	0.418
TCGA_LAML	**1.91**	**0.002**	**1.78**	**0.006**	1.16	0.471	**2.19**	**0.01**

L-24, 24-gene expression signature by Li et al. [2]; M-7, 7-gene expression signature by Marcucci et al. [3]; W-3, 3-gene expression signature by Wilop et al. [4]; HR, hazard ratio; na, not applicable (2 signature genes not represented on U133A arrays). Significant p-values and corresponding HRs are indicated in bold.

Addendum to main Figure 5B: full length blot image



Addendum to supplementary Figures S8A and S8C: full length blot images



Supplementary References

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4 Wilop, S. *et al.* A three-gene expression-based risk score can refine the European LeukemiaNet AML classification. *J Hematol Oncol* **9**, 78, doi:10.1186/s13045-016-0308-8 (2016).