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Supplemental information

**RSPO2 inhibits BMP signaling to promote
self-renewal in acute myeloid leukemia**

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Supplementary Figure S1. RSP02 antagonizes BMP signalling in AML cells.

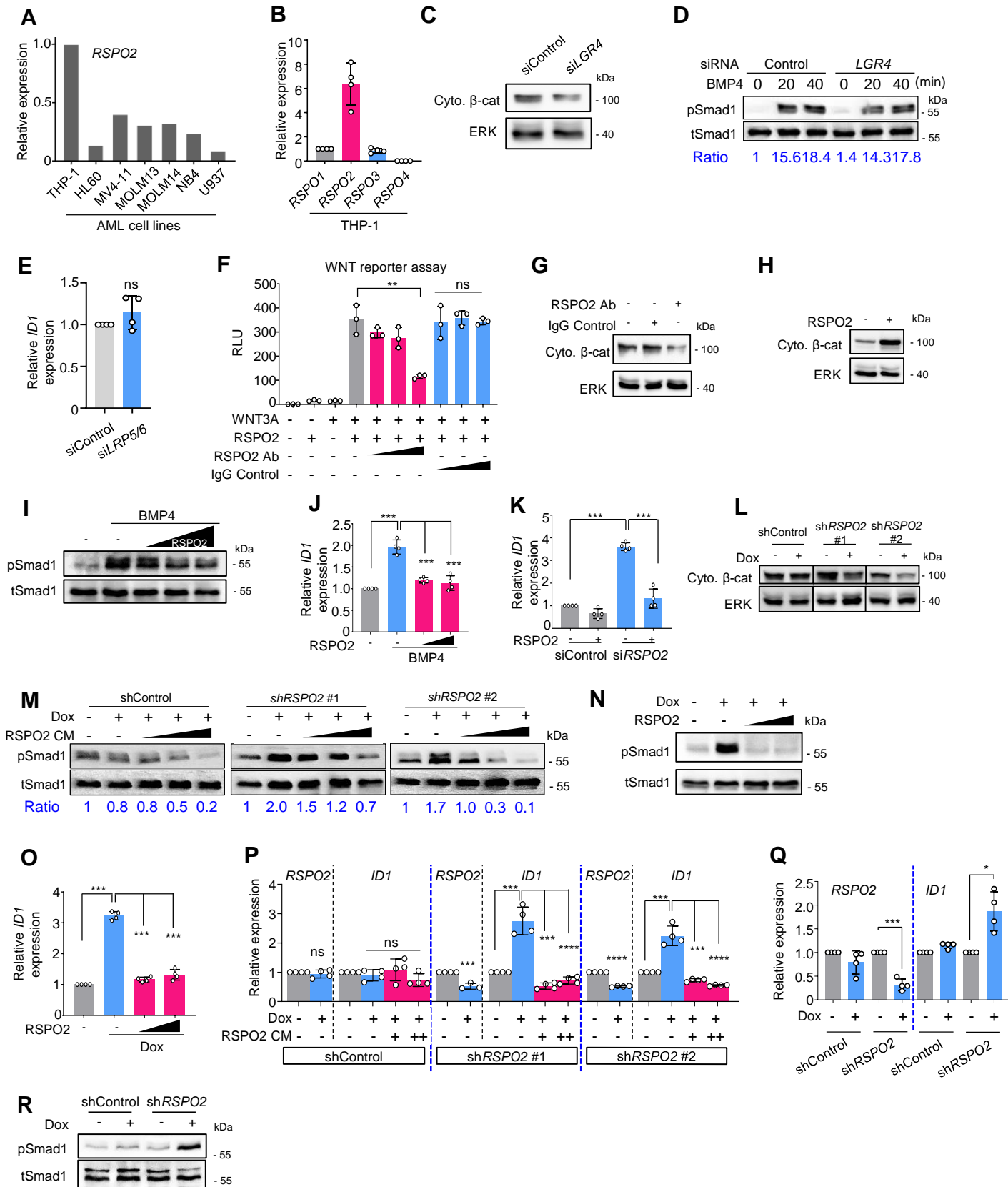


Figure S1: RSPO2 antagonizes BMP signalling in AML cells. Related to Figure 1.

(A) qRT-PCR analysis of *RSPO2* in AML cell lines. n = 1 per group.

(B) qRT-PCR analysis of *RSPO1-4* in THP-1 cells. n = 4 per group.

(C) Western blot analysis of cytosolic β -catenin in THP-1 cells upon si*LGR4* transfection.

(D) Western blot analysis of phosphorylated (p) Smad1 and total (t) Smad1 in THP-1 cells treated with siRNA and BMP4 as indicated. After overnight starvation, cells were stimulated with 5 ng/ml BMP4 for 20 and 40 min. Ratio shows relative levels of pSmad1 normalized to tSmad1. min, minute.

(E) qRT-PCR analysis of *ID1* in THP-1 cells treated with siRNA as indicated. n = 4 per group.

(F) TOPflash reporter assay in HEK293T cells treated with WNT3A, RSPO2 and antibody as indicated.

After overnight incubation with WNT3A, RSPO2 and indicated antibodies, luciferase activity was measured. 0.3, 1.0 and 3.0 μ g/ml antibodies were used for the experiment. n = 3 per group. RLU, relative light units.

(G) Western blot analysis of cytosolic β -catenin in THP-1 cells upon antibody treatment as indicated. 3.0 μ g/ml antibodies were used for the experiment.

(H) Western blot analysis of cytosolic β -catenin in THP-1 cells upon treatment with RSPO2 recombinant protein (0.1 μ g/ml) for 24 hrs.

(I) Western blot analysis of phosphorylated (p) Smad1 and total (t) Smad1 in THP-1 cells treated with BMP4 and RSPO2 as indicated. After overnight starvation, cells were co-stimulated with 2.5 ng/mL BMP4 and increasing amount of RSPO2 (0.1, 0.2, 0.4 μ g/ml) for 30 min.

(J) qRT-PCR analysis of *ID1* in THP-1 cells treated with BMP4 (25 ng/ml) and RSPO2 (0.2, 0.4 μ g/ml). n = 4 per group.

(K) qRT-PCR analysis of *ID1* in THP-1 cells treated with siRNA and RSPO2 (0.2 μ g/ml) as indicated. n = 4 per group.

(L) Western blot analysis of cytosolic β -catenin in THP-1 shRNA clones after Dox treatment for 3 days.

(M) Western blot analysis of pSmad1 and tSmad1 in THP-1 clones treated as indicated. Cells were stimulated by Dox with or without increasing amount of RSPO2 conditional medium for 3 days.

(N) Western blot analysis of pSmad1 and tSmad1 in THP-1 sh*RSPO2* #1 cells treated as indicated. Cells were stimulated by Dox with or without increasing amount of RSPO2 recombinant protein (0.2, 0.5 μ g/ml) for 3 days.

(O) qRT-PCR analysis of *ID1* in THP-1 sh*RSPO2* #1 cells treated with as indicated. Cells were stimulated by Dox with or without increasing amount of RSPO2 recombinant protein (0.2, 0.5 μ g/ml) for 3 days. n = 4 per group.

(P) qRT-PCR analysis of *RSPO2* and *ID1* in THP-1 clones with Dox and RSPO2 treatment. Cells were stimulated by Dox with or without increasing amount of RSPO2 for 3 days. n = 4 per group.

(Q) qRT-PCR analysis of *RSPO2* and *ID1* in MOLM14 clones upon Dox treatment for 3 days. n = 4 per group.

(R) Western blot analysis of pSmad1 and tSmad1 in MOLM14 clones upon Dox treatment for 3 days.

Results are presented as the mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 from unpaired t-test for experiments with two groups or one-way ANOVA for experiments with more than two groups.

Supplementary Figure S2. RSPO2 inhibits differentiation of AML cells.

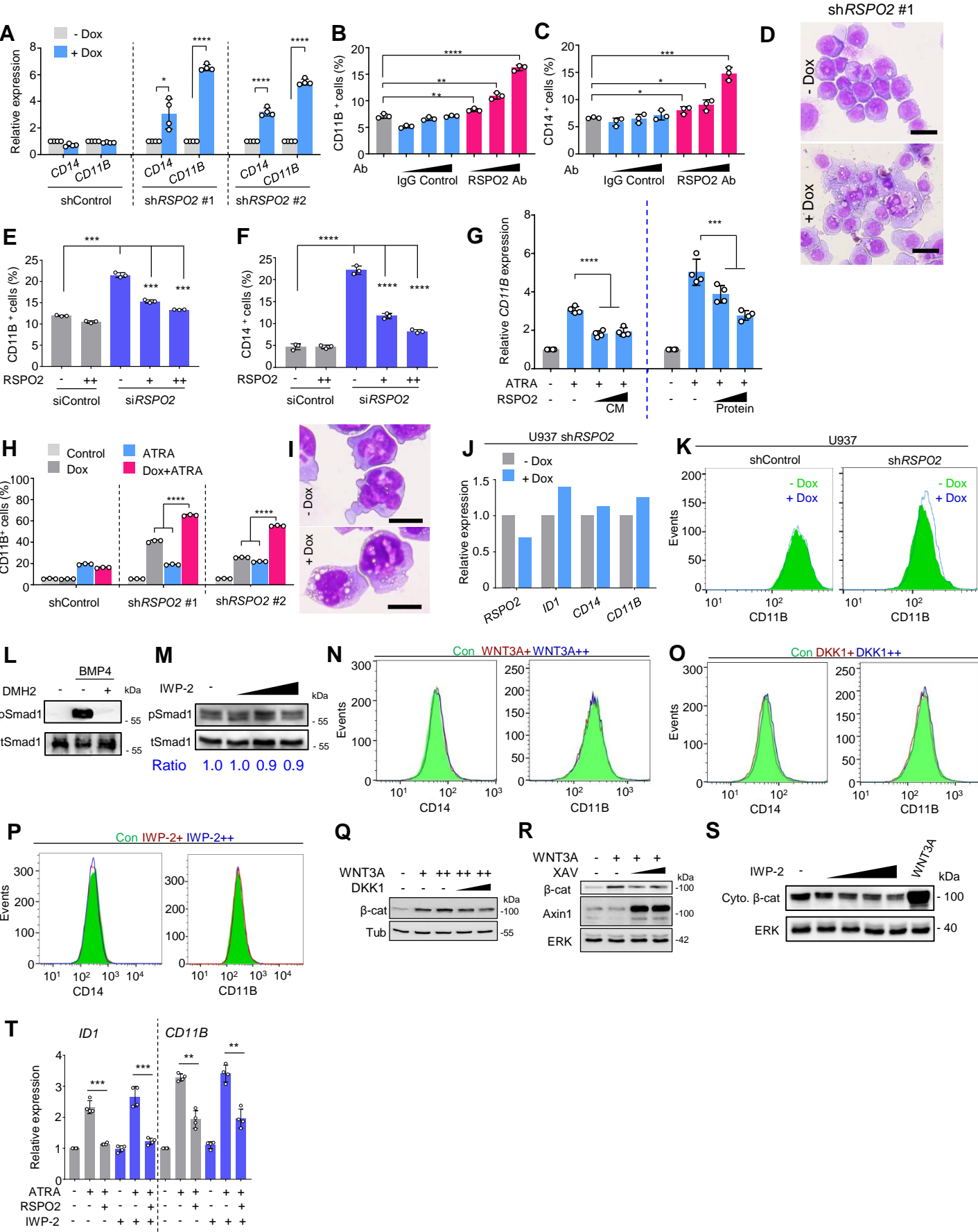


Figure S2. RSPO2 inhibits differentiation of AML cells. Related to **Figure 2**.

- (A) qRT-PCR analysis of *CD14* and *CD11B* in THP-1 clones upon Dox treatment for 3 days. n = 4 per group.
- (B-C) Quantification of FACS analysis for CD11B⁺- or CD14⁺ cells upon antibody treatment of THP-1 cells. 0.3, 1.0 and 3.0 µg/ml antibodies were used for the experiment. n = 3 per group.
- (D) Wright-Giemsa staining of THP-1 sh*RSPO2* #1 cells after Dox treatment for 8 days. Scale bar = 10 µM.
- (E-F) Quantification of FACS analysis for CD14⁺- or CD11B⁺ cells upon siRNA transfection and RSPO2 recombinant protein treatment. n = 3 per group.
- (G) qRT-PCR analysis of *CD11B* in THP-1 cells stimulated by 10 nM ATRA, with or without increasing amount of RSPO2 recombinant protein (0.2, 0.5 µg/ml) or conditioned medium for 3 days. n = 4 per group. ATRA, all-trans retinoic acid. CM, conditioned medium.
- (H) Quantification of FACS analysis for CD11B⁺ cells in THP-1 clones treated as indicated. Cells were stimulated by 1 nM ATRA and Dox for 3 days before analysis. n = 3 per group.
- (I) Wright-Giemsa staining of MOLM14 sh*RSPO2* cells after Dox treatment for 7 days. Scale bar = 10 µM.
- (J) qRT-PCR analysis for *RSPO2*, *ID1*, *CD14* and *CD11B* expression in U937 clone upon Dox treatment for 3 days. n=1 per group.
- (K) FACS analysis for CD11B⁺ cells in U937 clones upon Dox treatment for 3 days.
- (L) Western blot analysis of pSmad1 and tSmad1 in THP-1 cells after BMP4 and BMP receptor inhibitor DMH2 treatment. Cells were stimulated by 10 ng/ml BMP4 for 30 min. 100 nM DMH2 was added for another 30 min incubation before WB analysis.
- (M) Western blot analysis of pSmad1 and tSmad1 in THP-1 cells after overnight IWP-2 (1, 3 and 10 µM) treatment.
- (N-P) FACS plots for CD14⁺- or CD11B⁺ cells in THP-1 cells following overnight treatment with WNT3A (N), DKK1 (O), or IWP-2 (P).
- (Q-S) Western blot analyses in THP-1 cells treated as indicated. XAV, Axin stabilizer XAV 939. 0.01, 0.1, 1, 10 µM IWP-2 were used (S).
- (T) qRT-PCR analysis for *ID1* and *CD11B* expression in THP-1 cells treated as indicated. 10 µM ATRA, 0.01 µM IWP-2 and 0.4 µg/ml RSPO2 were used. n = 4 per group.

Results are presented as the mean ± SD. *p < 0.05, ***p < 0.001, and ****p < 0.0001 from unpaired t-test for experiments with two groups or one-way ANOVA for experiments with more than two groups.

Supplementary Figure S3. RSPO2 deficiency sensitizes THP-1 to chemotherapeutic drug treatment.

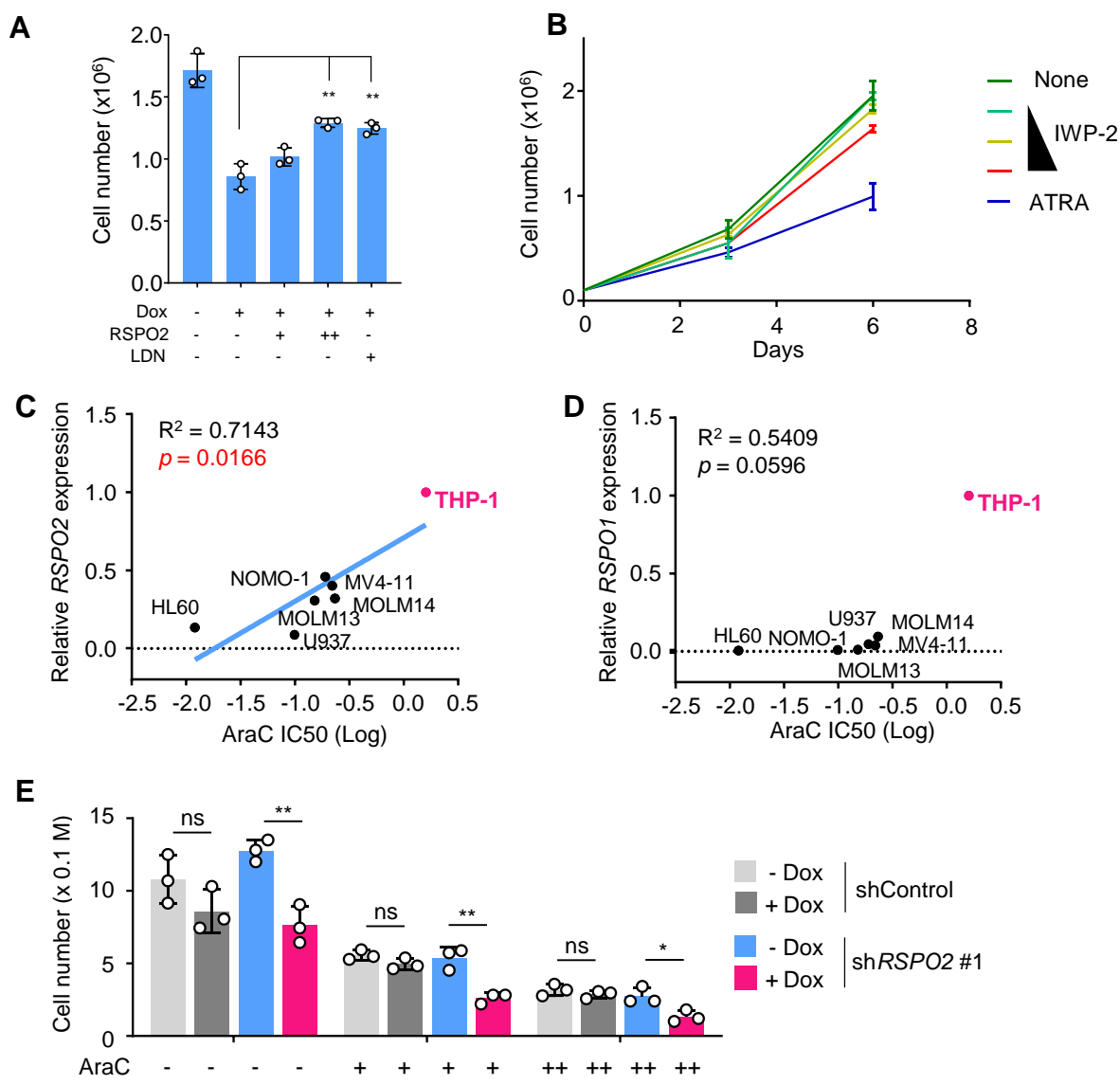


Figure S3. RSPO2 deficiency sensitizes THP-1 to chemotherapeutic drug treatment. Related to Figure 3.

(A) Total cell count of THP-1 shRSPO2 #1 cells treated with Dox, RSPO2 proteins (0.1, 0.2 µg/ml) and 200 nM LDN 193189 for 6 days. n = 3 per group.

(B) Total cell count of THP-1 cells treated with increasing amount of IWP-2 (1, 3, 10 µM) and 10 nM ATRA for 6 days. n = 3 per group.

(C-D) Correlation between the mRNA expression levels of *RSPO2/RSPO1* and AraC logIC₅₀ in different AML cell lines. IC₅₀ was determined after 3 days incubation with increasing amount of AraC.

(E) Total cell count of THP-1 clones treated with Dox and AraC at low (1 µM) or high dose (3 µM) for 3 days. n = 3 per group.

Results are presented as the mean ± SD. *p < 0.05, **p < 0.01 from unpaired t-test.

Supplementary Figure S4. Loss of *RSPO2* induces BMP signalling in primary blood cells.

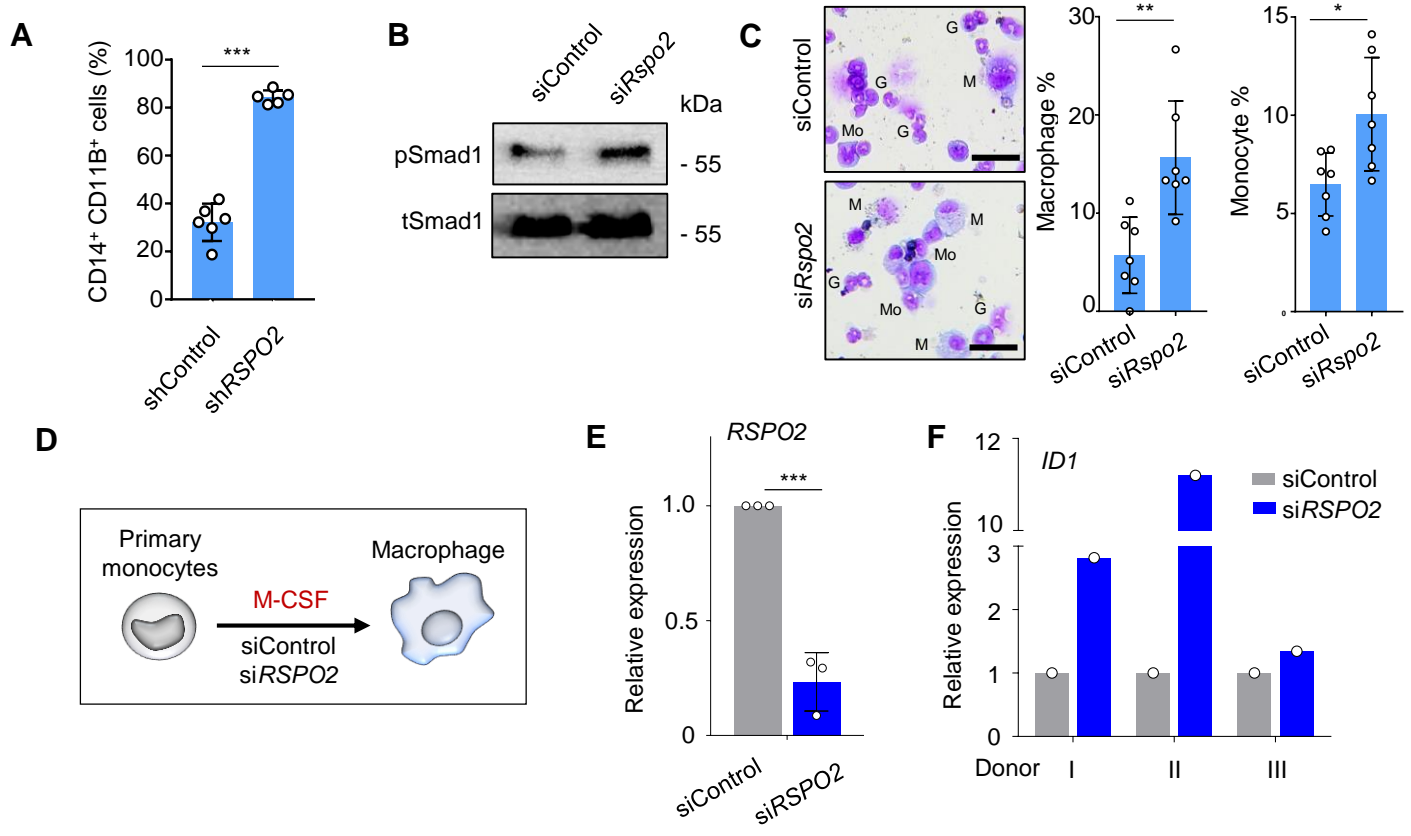


Figure S4. Loss of *RSPO2* induces BMP signalling in primary blood cells. Related to **Figure 4**.

(A) Quantification of FACS analysis on cells collected from colony formation assays (**Figure 4F**). n = 5/6 per group.

(B) Western blot analysis of phosphorylated (p) Smad1 and total (t) Smad1 in mouse bone marrow cells after siRNA transfection.

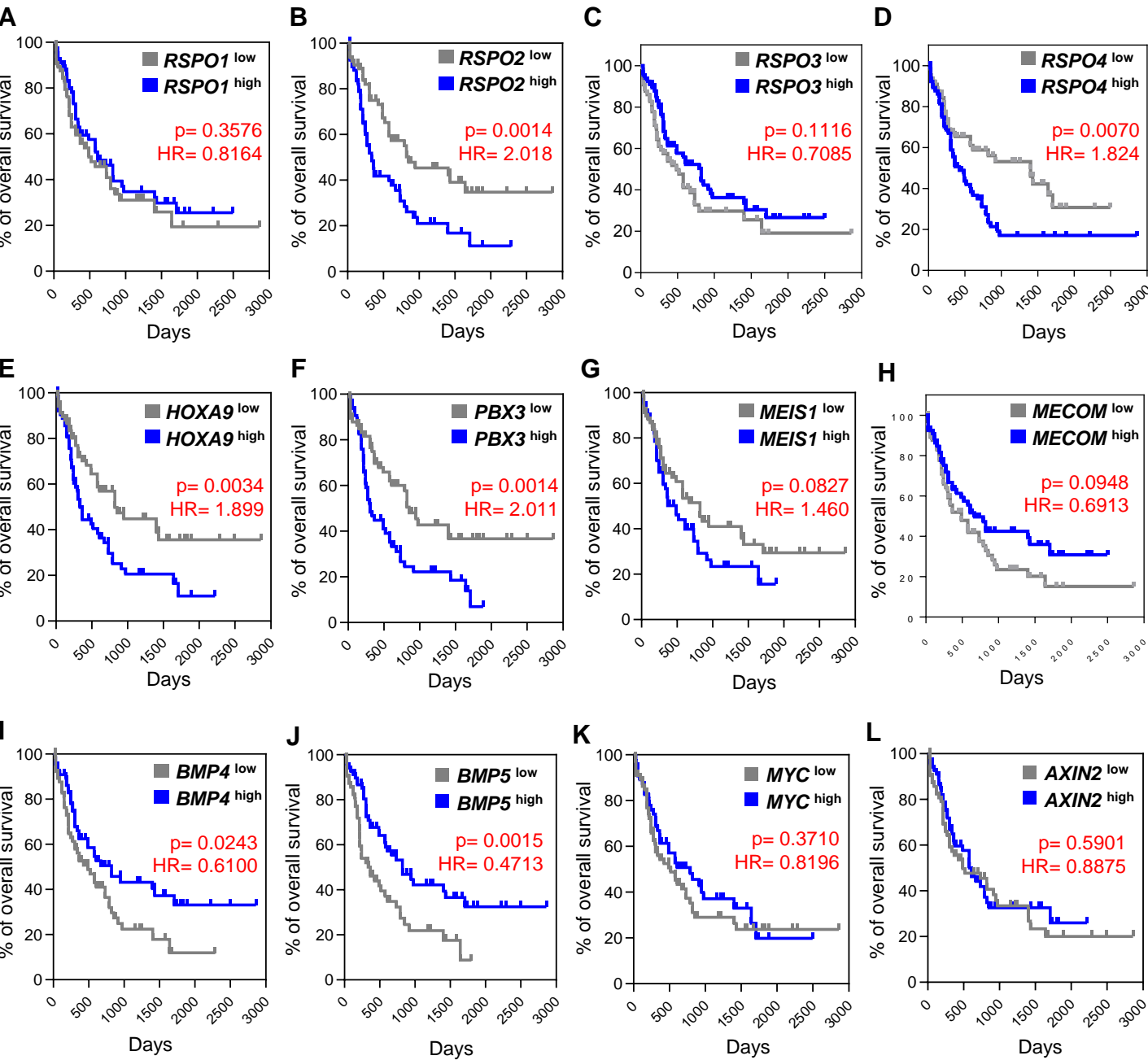
(C) Wright-Giemsa staining of mouse bone marrow cells upon siRNA transfection and M-CSF stimulation. Percentage of macrophages and monocytes was quantified from seven different fields based on the morphological characterization. Scale bar = 10 μ M. M, macrophage. Mo, monocyte. G, granulocyte.

(D) Scheme of experimental setup for human primary monocytes differentiation.

(E-F) qRT-PCR analysis for *RSPO2* and *ID1* expression in human primary monocytes upon siRNA transfection. (E), data pooled from three different donors. (F), data showing *ID1* expression in three different donors separately.

Results are presented as the mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 from unpaired t-test.

Supplementary Figure S5. High *RSPO2* expression is a predictor for poor prognosis in AML.



M Summary gene expression association with overall survival

GDC-TCGA AML	<i>RSPO1</i>	<i>RSPO2</i>	<i>RSPO3</i>	<i>RSPO4</i>	<i>HOXA9</i>	<i>PBX3</i>	<i>MEIS1</i>	<i>MECOM</i>	<i>BMP4</i>	<i>BMP5</i>	<i>MYC</i>	<i>AXIN2</i>
Low (n)	65	63	66	66	46	67	69	66	66	64	67	64
High (n)	67	69	66	66	43	65	63	66	66	68	65	68
Hazard ratio (HR)	0.8164	2.018	0.7085	1.824	1.899	2.011	1.460	0.6913	0.6100	0.5037	0.8196	0.8875
Median survival ratio	1.245	0.392	1.691	0.3026	0.408	0.3571	0.5912	1.372	1.681	2.246	1.529	1.243
Log-rank p	0.3576	0.0014	0.1116	0.0070	0.0034	0.0014	0.0827	0.0948	0.0243	0.0015	0.3710	0.5901
Significance	ns	**	ns	**	**	**	ns	ns	**	**	ns	ns

Figure S5. High *RSPO2* expression is a predictor for poor prognosis in AML. Related to **Figure 5**.

(A-L) Kaplan-Meier plot of AML patients stratified by different gene expression levels (low and high according to the median).

(M) Table showing the number of patients analyzed (n), hazard ratio (HR), median survival ratio and significance of survival differences. Log-rank test was used for statistical analysis.

Supplementary Figure S6. Loss of *RSPO2* reduces tumor burden in an AML xenograft model.

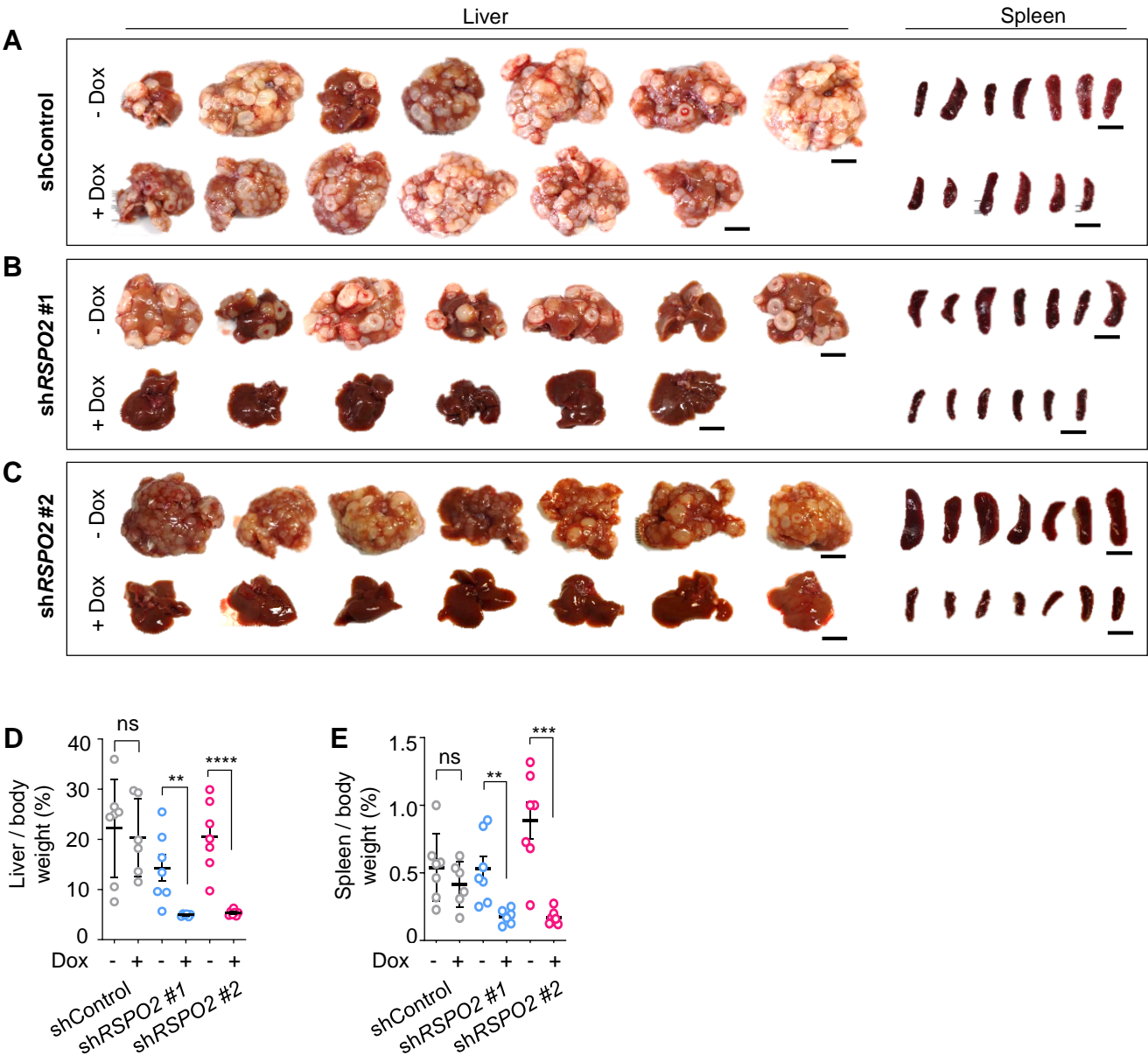


Figure S6. Loss of *RSPO2* reduces tumor burden in an AML xenograft model. Related to **Figure 6**.

(A-C) Images of livers and spleens harvested from mice xenografted with THP-1 cell lines expressing the indicated Dox-inducible shRNAs treated with (+) or without (-) Dox. Scale bar = 1 cm.

(D-E) Liver and spleen weights from mice xenografted with THP-1 cell lines expressing the indicated Dox-inducible shRNAs treated with (+) or without (-) Dox. Organ weights were normalized to total body weight of the corresponding mouse. Samples were analyzed on day 50 post cell-injection for shControl, day 57 post cell-injection for sh*RSPO2*-1 and day 42 post cell-injection for sh*RSPO2*-2 separately. Samples from Dox treated and not treated group were analyzed at the same time.

Results are presented as the mean \pm SD. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ from unpaired t-test.