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Supporting Information

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Dendritic cell development requires histone deacetylase activity

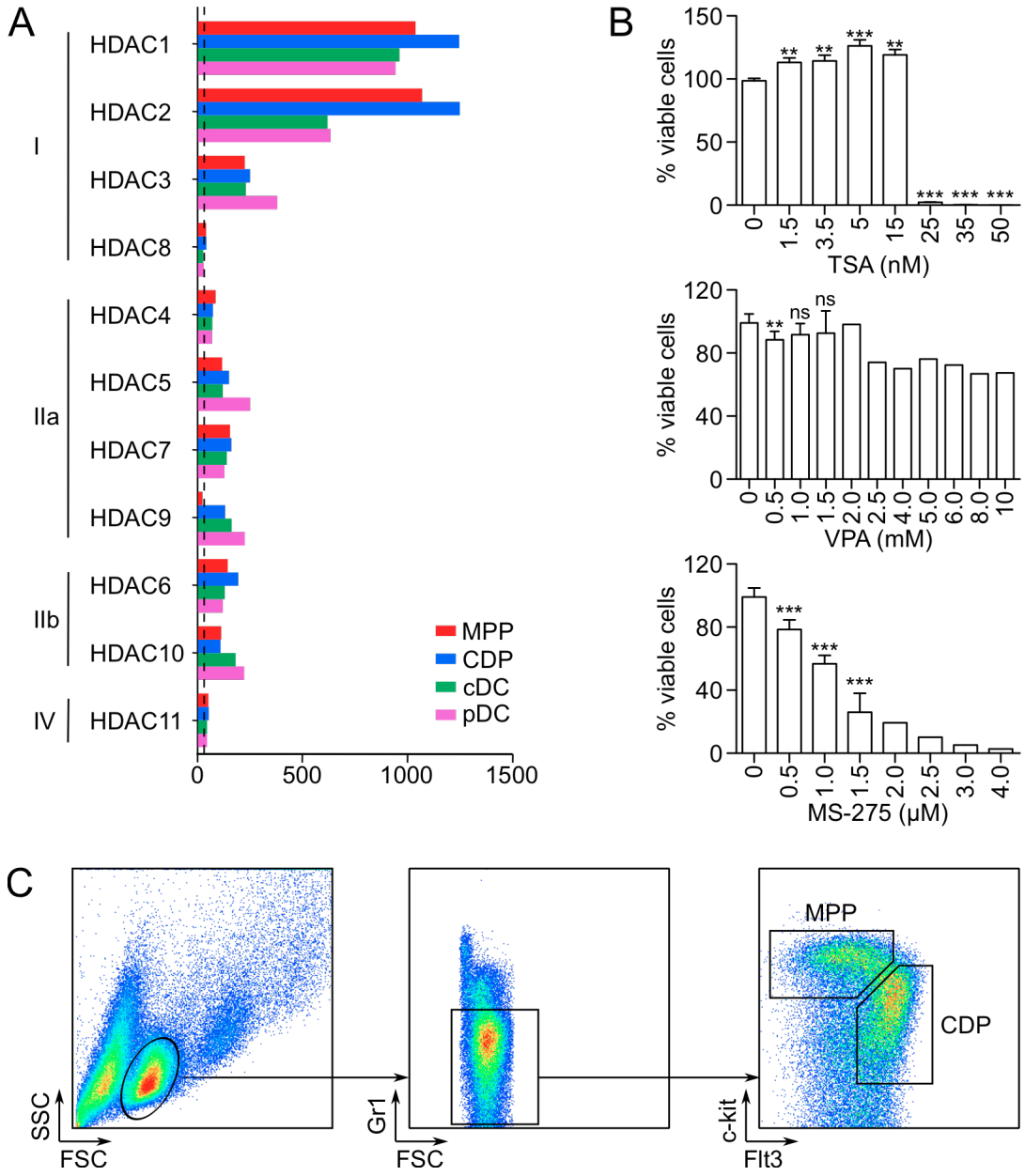

Figure 1. HDAC expression during DC lineage commitment and differentiation and influence of HDAC inhibitors on cell survival and proliferation.

(A) Genome-wide gene expression analysis of MPPs, CDPs, cDCs and pDCs. Microarray datasets [28] (GSE22432) were inspected for expression of class I, II and IV HDACs (n = 2, mean). The median expression value of all hybridised genes was calculated and is put as a threshold of gene expression (dashed line). (B) Bone marrow cells were amplified in vitro to obtain MPP/CDP cultures. After 6 days of amplification, TSA, VPA or MS-275 was added at indicated concentrations. 48 hours later, the number of living cells was determined as described in Materials and Methods section. Percentages of viable cells are represented relative to untreated cells and are shown as mean + SD (n = 1 to 9). (C) Gating strategy for MPP and CDP populations. Amplified MPP/CDP cultures are collected, stained for Gr1, c-kit and Flt3 and analysed by flow cytometry. Viable cells are selected based on forward scatter (FSC) and side scatter (SSC). Next, Gr1-positive cells are excluded. Finally, MPPs and CDPs are defined as $Flt3^{-/lo}c\text{-}kit^{hi}$ and $Flt3^{+}c\text{-}kit^{int}$, respectively. ***$p < 0.001$; **$p < 0.01$ versus control.

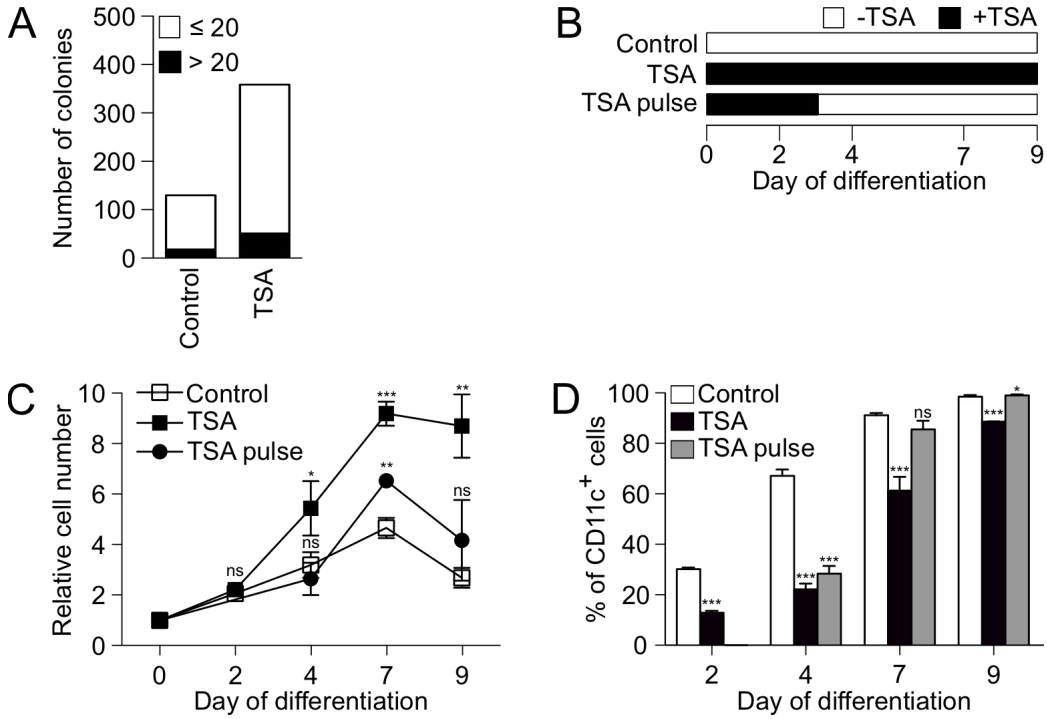

Figure 2. HDAC inhibition restrains DC differentiation.

(A) MPPs/CDPs were induced to differentiate into DCs by addition of Flt3L and treated with 3.5 nM TSA or left untreated. Three days after initiation of differentiation, 5000 cells were seeded into semisolid methylcellulose. The number and size of colonies was determined 3 days later. Colonies were divided into small (≤20, white bars) or large (20<, black bars). (B-D) MPP/CDP cultures were induced to differentiate into DCs with Flt3L. TSA (3.5 nM) was added during differentiation (9 days, TSA) or during the first 3 days only (TSA pulse). Untreated cells were used as control (n = 3). (B) Experimental layout. (C) Total cell numbers were determined with an electronic cell counter device and are depicted (mean ± SD). (D) Cells were stained for CD11c and analysed by flow cytometry. Percentages of $CD11c^+$ cells at each time point of analysis are depicted as mean + SD.

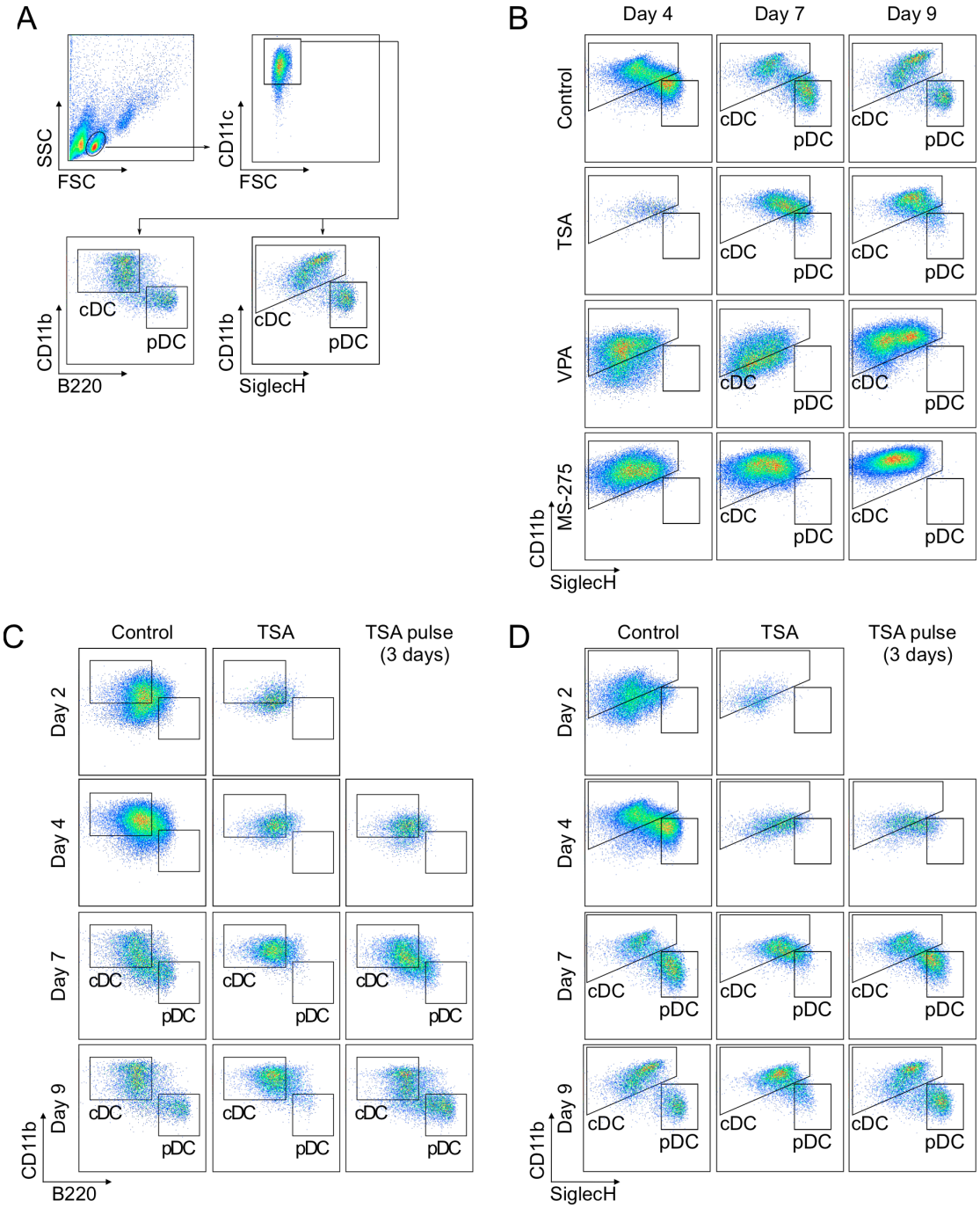

Figure 3. TSA reversibly inhibits DC differentiation.

(A) Gating strategy for cDCs and pDCs. Cells are stained for CD11c, B220, CD11b and SiglecH and analysed by flow cytometry. Viable cells, determined by forward scatter (FSC) and side scatter (SSC), are further gated for $CD11c^{+}$ cells. cDCs and pDCs are subsequently defined as $CD11b^{hi}B220^{lo}$ and $CD11b^{lo}B220^{hi}$ or as $CD11b^{hi}SiglecH^{lo}$ and $CD11b^{lo}SiglecH^{hi}$, respectively. (B-D) MPP/CDP cultures were induced to differentiate into DCs with Flt3L and exposed to HDAC inhibitors. (B) Cells were treated with 3.5 nM TSA, 0.5 mM VPA or 0.2 µM MS-275 or left untreated. After 4, 7 and 9 days of differentiation cDC and pDC populations were analysed by flow cytometry as described in (A). (C and D) TSA (3.5 nM) was added during 9 days of differentiation (TSA), during the first 3 days only (TSA pulse) or was omitted (as schematically presented in Supporting Information Fig. 2B). DC subset development was followed in time as outlined in (A). Gating for cDCs and pDCs was done using either B220 or SiglecH as pDC marker (C and D, respectively). One representative experiment of at least three independent experiments is depicted.

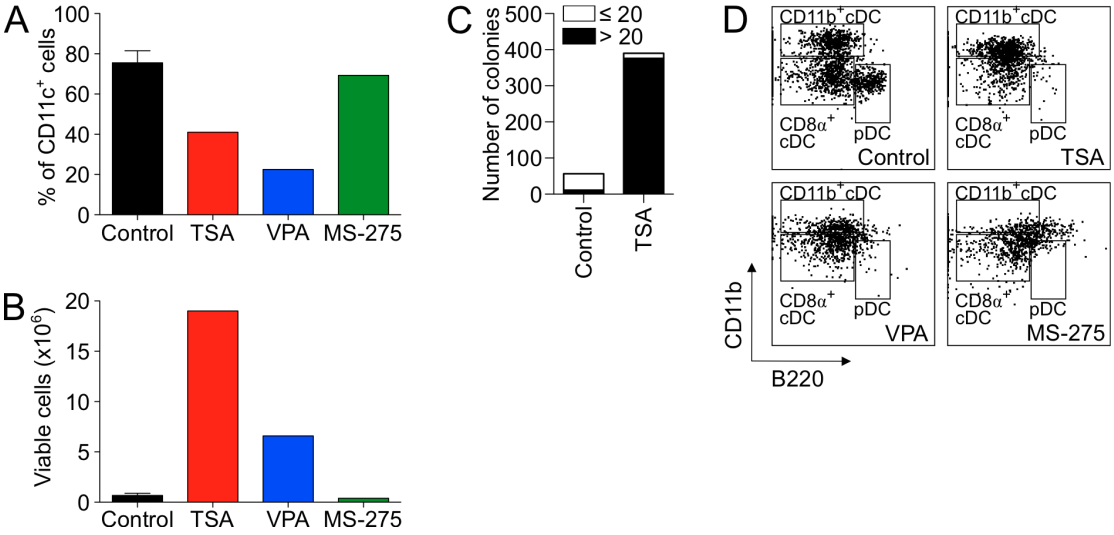

Figure 4. pDC development is blocked upon inhibition of HDAC activity.

Bone marrow cells were cultured with Flt3L without initial amplification in the absence (Control) or presence of 3.5 nM TSA, 0.5 mM VPA or 0.2 μM MS-275. Cell numbers and DC differentiation were monitored in time. (A) Cells were stained for CD11c and analysed by flow cytometry. Numbers of $CD11c^+$ cells are shown as percentage of viable cells. (B) Viable cells were quantified as described in Materials and Methods section and are displayed. (C) After 5 days of differentiation, 5000 cells were seeded into semisolid methylcellulose. Scoring of colonies was done as described in Supporting Information Fig. 2A. (D) Cells were stained for CD11c, CD11b and B220 and analysed by flow cytometry. pDCs, $CD11b^+$ and $CD8\alpha^+$ DCs were identified by the level of B220 and CD11b expression.

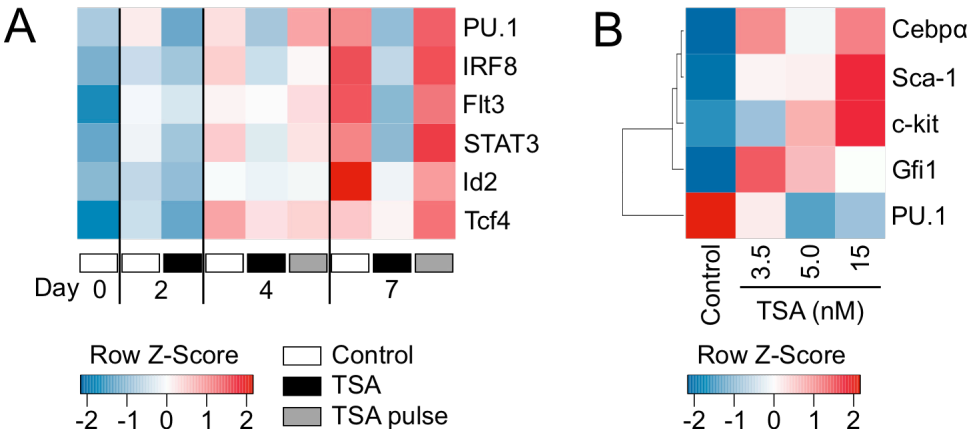

Figure 5. Impact of HDAC inhibition on gene expression.

(A) MPP/CDP cultures were induced to differentiate into DCs with Flt3L and treated with TSA in pulse-chase experiments as shown in Supporting Information Fig. 2B. RNA was isolated at different time points during differentiation. (B) MPP/CDP cultures were treated with varying concentrations of TSA or were left untreated. RNA was isolated after 48h. (A and B) Gene expression was analysed by RT-qPCR. ΔΔCt values were calculated relative to GAPDH and are presented in heatmap format (blue: expression levels below median; white: expression levels equal to median; red: expression levels above median). Data are mean of three independent experiments.

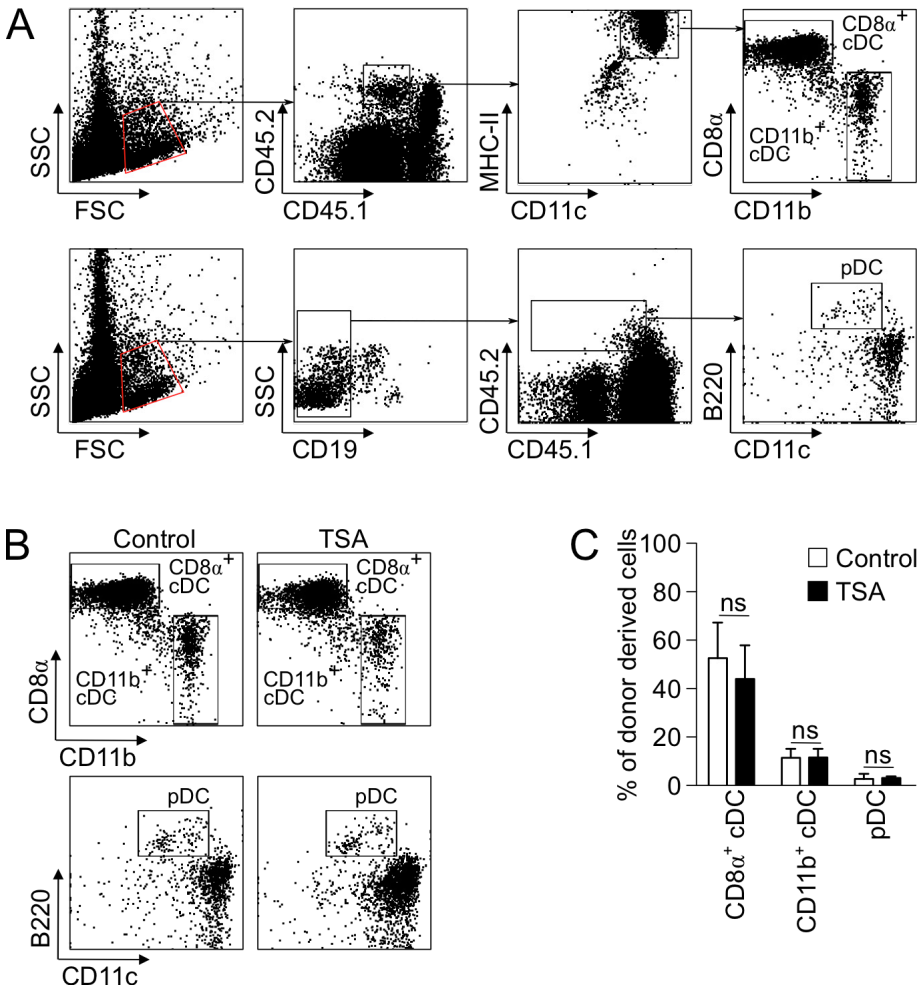

Figure 6. TSA-treated cells give rise to DCs but no other lineages *in vivo*.

MPP/CDP cultures were induced to differentiate with Flt3L in the absence (Control) or presence of 3.5 nM TSA (TSA). After 3 days cells were adoptively transferred into NOD-SCID-IL2rg^{null} mice and 6 days later donor (CD45.2$^+$) cells in spleen were analysed by flow cytometry. (A) Gating strategy for splenic CD11b$^+$ cDCs, CD8α^+ cDCs and pDCs. (B) Representative plots of cDCs (upper panels) and pDCs (lower panels) derived from TSA-treated or untreated cells. (C) Quantification of DC subsets as percentage of donor cells is depicted (n = 3, mean ± SD). $p > 0.05$: ns, not significant versus control (Student's t-test).

Table 1. Primer sequences for RT-qPCR

Gene	Primer Sequence	Reference
C/ebpα	For 5'-GCAGGAGGAAGATACAGGAAGCT-3'	[50]
	Rev 5'-ACACCTAAGTCCCTCCCCTCTAAA-3'	
c-kit	For 5'-AGGCTTTGCTGCTGTCTCC-3'	[51]
	Rev 5'-GGAACTCGCTACCCTGGAAT-3'	
Flt3	For 5'-TGAAGACCTCCTTTGCTTTGCG-3'	
	Rev 5'-TGACCAACACATTCCTGGCTGC-3'	
GAPDH	For 5'-ACCTGCCAAGTATGATGACATCA-3'	[50]
	Rev 5'-GGTCCTCAGTGTAGCCCAAGAT-3'	
Gata2	For 5'-CACAAGATGAATGGACAGAACC-3'	[52]
	Rev 5'-ACAGGTGCCCGCTCTTCT-3'	
Gfi1	For 5'-CAGCTTACCGAGGCTCCCGACAGG-3'	[53]
	Rev 5'-CAAGACCGCTCCATGCATAGGGCTT-3'	
Id1	For 5'-ACCCTGAACGGCGAGATCA-3'	[54]
	Rev 5'-TCGTCGGCTGGAACACATG-3'	
Id2	For 5'-AAAACAGCCTGTCGGACCAC-3'	[55]
	Rev 5'-CTGGGCACCAGTTCCTTGAG-3'	
Id3	For 5'-AGCTTAGCCAGGTGGAAATCCT-3'	[55]
	Rev 5'-TCAGCTGTCTGGATCGGGAG-3'	
IRF8	For 5'-TGACACCAACCAGTTCATCCGAGA-3'	[56]
	Rev 5'-TGCTCTACCTGCACCAGAATGAGT-3'	
PU.1	For 5'-GGAGAAGCTGATGGCTTGG-3'	[57]
	Rev 5'-CAGGCGAATCTTTTTCTTGC-3'	
Sca-1	For 5'-TGGGTACTAAGGTCAACGTGAAGACTTCC-3'	[30]
	Rev 5'-TGGAGGTCATTGGGAGGACCATCAG-3'	
STAT3	For 5'-TGCGGAGAAGCATTGTGAGTG-3'	
	Rev 5'-AGACGGTCCAGGCAGATGTTG-3'	
Tcf4	For 5'-CAATCCTTCAACTCCTGTGGGC-3'	
	Rev 5'-CGTCCAGTCTTTCCAAACGGTC-3'	

Table 2. Primer sequences for ChIP-qPCR with an H3K9ac-specific antibody.

Gene	Primer Sequence	Reference	Genomic Localisation of Product	5'-End of Product Relative to Exon1 (bp)	3'-End of Product Relative to Exon1 (bp)
Flt3	For 5'-AGGTTCCTCCCCCTCTGCT-3'		chr5:148,211,941- chr5:148,211,991	+74	+124
	Rev 5'-CACTGACCACAGATTCCCTCG-3'				
Gfi1	For 5'-CCTAAATCCGAAGGGAAATGAGC-3'		chr5:108,155,576 - chr5:108,155,713	-2328	-2232
	Rev 5'-CGTCCGCAGAAAGAAACCTG-3'				
Id1	For 5'-CCTTATCTCGCTCTGGTGTTCACAG-3'		chr2:152,562,683 - chr2:152,562,779	+673	+769
	Rev 5'-GGAGTCCATCTGGTCCCTCAGTG-3'				
Id2	For 5'-TGGGGAGAAAGAGGATAAAAGGCAG-3'		chr12:25,780,241 - chr12:25,780,312	+645	+716
	Rev 5'-TGTGAAAGGAGGAGGGGGGC-3'				
Id3	For 5'-AGTCGGTTTAGGGAAAAAGCCAAG-3'		chr4:135,700,376 - chr4:135,700,501	+639	+764
	Rev 5'-GGGATGAGAAGGGACAGCGTG-3'				
IRF8	For 5'-TCACTGGGGGACACAAGGGAAC-3'		chr8:123,259,673 - chr8:123,259,797	-603	-479
	Rev 5'-CTGGATGGAGGATGACCGCC-3'				
PU.1	For 5'-GGCTCCTGAGGCAAAGGCTATTAG-3'		chr2:90,937,554 - chr2:90,937,618	+600	+664
	Rev 5'-CCACAAACTGAATGTTGGGGACC-3'				
Tcf4	For 5'-CGGATGTGAATGGATTACAATG-3'	[58]	chr18:69,504,192 - chr18:69,504,375	+46	+229
	Rev 5'-ATTGTTCTTCGGTCTTGTTGGT-3'				

Table 3. Primer sequences for ChIP-qPCR with a PU.1-specific antibody.

Gene	Recognition Site (kb)	Primer Sequence	Reference	Genomic Localisation of Product	5'-End of Product Relative to Exon1 (bp)	3'-End of Product Relative to Exon1 (bp)
PU.1	-15.7	For 5'-TTTCTGGGAGGAGGCAGGAAGTAG-3'		chr2:90921148 - chr2:90921281	-15806	-15673
		Rev 5'-AAAGGGGTGGGGGGAATGC-3'				
PU.1	-13.7	For 5'-AGGCAGAGCACACATGCTTC-3'	[59]	chr2:90923217 - chr2:90923355	-13737	-13599
		Rev 5'-CTTCTGGGCAGGGTCAGAGT-3'				
PU.1	-12.6	For 5'-CACACGGAGTCAGAGCGGGCAG-3'	[59]	chr2:90924755 - chr2:90924901	-12199	-12053
		Rev 5'-AGGAAAGAGGAAGCCATGGGGAGA-3'				
PU.1	-10.3	For 5'-GCTCTGAAAAGCACCGTTTCC-3'	[59]	chr2:90926654 - chr2:90926783	-10300	-10171
		Rev 5'-CTGTGTTGGACCTGCAAGGAGT-3'				
PU.1	-9	For 5'-GGAAGCAGCTCTTGTCCTTGG-3'	[59]	chr2:90927766 - chr2:90927891	-9188	-9082
		Rev 5'-AGTGATGTGGCCAGGAGGTGA-3'				
IRF8	-50	For 5'-CGGGGAGGGAAAAGCAATCAG-3'		chr8:123210058 - chr8:123210121	-50218	-50155
		Rev 5'-TGCGGGCGAGCACACAAC-3'				
IRF8	-16	For 5'-CCTCCAAGCGAAAACCAGAATAGAG-3'		chr8:123243993 - chr8:123244099	-16283	-16177
		Rev 5'-TGGCAGGGTCAAAGTGAAATCG-3'				
IRF8	+27	For 5'-CCCAAAGGTCCAGAGATGACACG-3'		chr8:123287211 - chr8:123287297	+26935	+27021
		Rev 5'-TGAGGTGGCAGGAAGGAGAAGC-3'				
Flt3	+0.1	For 5'-AGCACTGCGCCAGTTCAG-3'	[7]	chr5:148211938 - chr5:148212045	+37	+127
		Rev 5'-CGTCACTGACCACAGATTCC-3'				
Flt3	+11	For 5'-GCATCACATCATCAGCCACATAGG-3'		chr5:148200761 - chr5:148200897	+11189	+11304
		Rev 5'-TTGTCCCTGGTTCCCCTCTCAG-3'				
Flt3	+37	For 5'-TCTCCTACCCTCAAGCCCCATTC-3'		chr5:148174363 - chr5:148174502	+37586	+37702
		Rev 5'-GCTCTTTCCTGTGTTCGCAGTCTG-3'				
Flt3	+46	For 5'-AGGATGGGACAGAGTGGGAAGTG-3'		chr5:148165900 - chr5:148166058	+46027	+46165
		Rev 5'-GGGGCAGTCGTGAGCAAAGAC-3'				

Materials and Methods

DNA microarray analysis

Microarray datasets of MPPs, CDPs, cDCs and pDCs were retrieved from [28] (GSE22432). For each HDAC, the highest expressed probeset was determined.

Cell culture

Reversibility of the TSA effect during DC differentiation was analysed in pulse-chase experiments, with 3 days TSA treatment (3.5 nM) at the start of differentiation followed by chase without TSA.

Colony formation assay

5000 cells were plated in semisolid methylcellulose (MethoCult GF M3434, StemCell Technologies) containing insulin, transferrin, SCF, IL-3, IL-6, and erythropoietin. Colony size and numbers were analysed after 3 days. Colonies with less than 20 cells were defined as small, colonies with more than 20 cells were defined as large.

Adoptive transfers

MPP/CDP cultures were induced to differentiate with Flt3L in the presence or absence of 3.5 nM TSA. On day 3 of differentiation, cells were collected, washed with PBS and 5×10^6 cells were injected i.v. into sublethally irradiated (2.5 Gy) CD45.1$^+$ NOD-SCID-IL2rg^{null} mice. 6 days after injection, mice were sacrificed and donor cells (CD45.2$^+$) were analysed in spleen. CD8α^+ and CD11b$^+$ cDCs were identified as CD11c$^+$MHC-II$^+$CD11b$^-$CD8α^+ and CD11c$^+$MHC-II$^+$CD11b$^+$CD8α^-, respectively. pDCs were gated as CD11c$^+$CD19$^-$B220$^+$ cells. Three recipient mice per group were analysed. Additional antibodies used were MHC-II (M5/114.15.2), CD8α (53-6.7), CD19 (1D3), CD45.1 (A20), CD45.2 (104). All antibodies were obtained from eBioscience.

References

50 **Tagoh, H., Schebesta, A., Lefevre, P., Wilson, N., Hume, D., Busslinger, M. and Bonifer, C.**, Epigenetic silencing of the c-fms locus during B-lymphopoiesis occurs in discrete steps and is reversible. *EMBO J* 2004. 23: 4275-4285.

51 **Massa, S., Balciunaite, G., Ceredig, R. and Rolink, A. G.**, Critical role for c-kit (CD117) in T cell lineage commitment and early thymocyte development in vitro. Eur J Immunol 2006. 36: 526-532.

52 **Pearson, S., Sroczynska, P., Lacaud, G. and Kouskoff, V.**, The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. *Development* 2008. 135: 1525-1535.

53 **Rathinam, C. and Klein, C.**, Transcriptional repressor Gfi1 integrates cytokine-receptor signals controlling B-cell differentiation. *PLoS One* 2007. 2: e306.

54 **Kautz, L., Meynard, D., Monnier, A., Darnaud, V., Bouvet, R., Wang, R. H., Deng, C. et al.**, Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. *Blood* 2008. 112: 1503-1509.

55 **Saika, S., Ikeda, K., Yamanaka, O., Flanders, K. C., Ohnishi, Y., Nakajima, Y., Muragaki, Y. et al.**, Adenoviral gene transfer of BMP-7, Id2, or Id3 suppresses injury-induced epithelial-to-mesenchymal transition of lens epithelium in mice. *Am J Physiol Cell Physiol* 2006. 290: C282-289.

56 **Nardi, V., Naveiras, O., Azam, M. and Daley, G. Q.**, ICSBP-mediated immune protection against BCR-ABL-induced leukemia requires the CCL6 and CCL9 chemokines. *Blood* 2009. 113: 3813-3820.

57 **Weigelt, K., Ernst, W., Walczak, Y., Ebert, S., Loenhardt, T., Klug, M., Rehli, M. et al.**, Dap12 expression in activated microglia from retinoschisin-deficient retina and its PU.1-dependent promoter regulation. *J Leukoc Biol* 2007. 82: 1564-1574.

58 **Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Canamero, M., Blasco, M. A. et al.**, The Ink4/Arf locus is a barrier for iPS cell reprogramming. *Nature* 2009. 460: 1136-1139.

59 **Zarnegar, M. A. and Rothenberg, E. V.**, Ikaros represses and activates PU.1 cell-type-specifically through the multifunctional Sfpi1 URE and a myeloid specific enhancer. *Oncogene* 2012. 31: 4647-4654.