Age-related increase of EED expression in early hematopoietic progenitor cells is associated with global increase of the histone modification H3K27me3

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Abbreviations: EED = Embryonic Ectoderm Development; EZH2 = Enhancer of Zeste 2; SUZ12 = Suppressor of Zeste 12; HSPC = Hematopoietic stem and progenitor cell; HSC = Hematopoietic stem cell; MPP = Multipotent progenitor; LMPP = lymphoid-primed multipotent progenitor; EMP = Erythromyeloid progenitor; MLP = Multilymphoid progenitor; GMP = Granulocyte-macrophage progenitor; EoBP = Eosinophil-basophil progenitor; MEP = Megakaryocyte-erythrocyte progenitor; LM = Lympho-myeloid; EM = Erythro-myeloid; HPC = Hematopoietic progenitor cell; PRC = Polycomb Repressive Complex; ChIP = Chromatin Immunoprecipitation; CFC = Colony-forming cell; PB = peripheral blood; BM = bone marrow; CB = umbilical cord blood

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Abstract

Human hematopoietic stem and progenitor cells (HSPC) from umbilical cord blood exhibit higher differentiation potential and repopulation capacity compared to adult HSPCs. The molecular basis for these functional differences is currently unknown. Upon screening for epigenetic effector genes being differentially expressed in neonatal and adult HSPC subpopulations, the Polycomb Repressive Complex 2 (PRC2) member EED was identified. Even though EED is expressed at comparable amounts in neonatal and adult multipotent HSPCs, early adult lineage committed progenitors of the lympho-myeloid and erythro-myeloid lineages expressed higher EED amounts than neonatal HPCs. We demonstrate that EED overexpression directly leads to higher H3K27me3 levels, a repressive histone modification that is mediated by the PRC2 complex. Quantitative analysis of H3K27me3 levels by FPLC-based ELISA revealed elevated levels in primary blood cells from adults. Besides quantitative changes, gene ontology analysis of the genome-wide H3K27me3 distribution revealed qualitative changes in adult HSPCs with elevated levels in genes associated with non-hematopoietic development pathways. In contrast, H3K4me3 which labels active chromatin was enriched on hematopoietic genes. In vitro differentiation of EED-transfected neonatal HSPCs revealed aberrant expression of the myelopoietic marker CD14, suggesting that EED affects the lymphoid versus myeloid decision processes within the lymphomyeloid lineage. This is in line with lympho-myeloid progenitors having the most pronounced differences in EED expression. Highlighting the dynamic roles of epigenetic modifications in human hematopoiesis, the present data demonstrate shifts in the PRC2-associated histone modification H3K27me3 from birth to adulthood.
Introduction

Transplantation of hematopoietic stem cells (HSCs) is an established part of the therapy for a multitude of hematopoietic malignancies [1]. Commonly used HSC-sources are peripheral blood (PB) of G-CSF mobilized donors, and bone marrow (BM), and umbilical cord blood (CB). Even though CB contains significantly more multipotent hematopoietic stem and progenitor cells (HSPCs) than adult sources [2], far more adult transplants are used for allogeneic HSC transplantation than CB units. In 2012 only 5% of all allogeneic transplantsations in Europe were performed with CB [1]. However, it has been shown that donor age has a major impact on the success of transplantation. In general, patients who received HSCs from younger donors had an improved survival rate after BM transplantation [3].

In mice, aged HSCs have a clearly reduced repopulation potential which has been intensively investigated. Some early studies indicate that aged HSCs intrinsically lose their stem cell potential during aging [4]. This seems to be, at least partly, compensated by an age-associated increase in stem cell number [4]. More recent studies, however, demonstrated that the functional capacity of individual HSCs is not impaired with aging, but that HSC which are intrinsically biased for myelopoiesis tend to dominate the stem cell pool in older mice [5,6]. This is reflected in (I) a pronounced myeloid skewing of hematopoiesis in elderly mice and (II) a preferential repopulation of the myeloid department after HSC transplantation from old donor mice [4,6-10]. It has been confirmed also for humans that the frequency of HSCs in the bone marrow increases during aging and that these HSCs are myeloid biased [11].

During the last years it has become evident that changes in DNA methylation (DNAm) are indicative for the aging of the organism [12-18]. Some of the observed DNAm changes are
certainly due to altered tissue compositions, while there is increasing evidence, that the majority is tissue-independently connected to aging [19]. Especially in the case of the hematopoietic system, it has been shown that age associated changes between CD4⁺ T cells and CD14⁺ monocytes are comparable [15]. Interestingly, it has been observed that age-associated DNA methylation tends to increase on promoters which are known Polycomb Repressive Complex 2 (PRC2) targets and bivalently modified in embryonic stem cells [13-15].

PRC2 is responsible for gene silencing through trimethylation of H3K27 (H3K27me3). It regulates HSC proliferation and differentiation and has also been associated with HSC aging [20-22]. PRC2 consists of several subunits, with the core units Ezh2, Eed, and Suz12 being essential for the enzymatic activity [23-25]. Ezh2 contains a catalytically active SET domain and is responsible for the trimethylation of H3K27, which initiates gene silencing [23-27], while EED is important for recognition of the histone target [28,29].

PRC2 also plays an important role during tumorigenesis. Both, overexpression and loss of PRC2 members have been associated with various forms of blood cancers [30-33]. In the case of myelodysplastic syndrome (MDS), for example, loss of EZH2 or another member of PRC2 is associated with a poor prognosis [34,35]. On the other hand, overactive mutants of EZH2 are responsible for abnormally high levels of H3K27me3 in lymphomas [31-33]. For treatment of the latter, pharmacological inhibitors of EZH2 are currently tested [36,37].

Here, we investigated the expression of the PRC2 member EED in different hematopoietic progenitor cell fractions, which were flow-cytometrically purified as multipotent stem and progenitor cells (HSCs/MPPs), lympho-myeloid (LM) progenitors and erythro-myeloid (EM) progenitors according to the revised model of hematopoiesis we proposed recently [2,38-40]. Elevated EED expression was detected in adult but not neonate LM progenitor fractions. A minor increase was also observed in adult EM progenitors, but neither in adult HSC/MPP
fractions or any of the neonate fractions. This was associated with a global increase of the repressive histone mark H3K27me3, which was accompanied by a clear qualitative shift in gene categories occupied by H3K27me3. Additionally, we demonstrate that elevated EED levels reduce formation of NK cells *in vitro* and promote the appearance of myeloid CD14+ cells instead.

**Material and Methods**

**Isolation of hematopoietic stem and progenitor cells (HSPCs)**

Mononucleated cells (MNCs) were obtained by density centrifugation on Ficoll-Hypaque (Biochrom) from umbilical cord blood (CB) of male newborns, as well as from bone marrow (BM) of healthy male adult donors. MNCs from peripheral blood (PB) were collected during leukapheresis after mobilization with granulocyte colony-stimulating factor (G-CSF).

CD34+ hematopoietic stem and progenitor cells (HSPCs) were enriched with immunomagnetic beads specific for CD34 (Miltenyi Biotech) to a purity of > 90%. HSCs/MPPs (CD34+CD133+CD45RA-CD90+), lympho-myeloid (LM) progenitors (CD34+CD133+CD45RA+) and erythro-myeloid (EM) progenitors (CD34+CD133lowCD45RA-) were highly enriched (> 99.5%) via flow-cytometric cell sorting (FACSAriaIIIu, BD Biosciences) from CD34-enriched HSPCs (MACS, Miltenyi Biotech) as described previously [2,38-40]. Experimental details are summarized in Supplementary Figure S2 and Supplementary Table S2. RNA was extracted from all fractions. For ChIP-on-chip analyses CD34+/Lin- cells, i.e. cells that do not express selected markers characteristic for differentiated hematopoietic cells (for detailed information see Supplementary Table S2), were flow cytometrically enriched to a purity of > 99% (MoFlo XPD; Beckman Coulter) and for differentiation assays, a lineage depletion step, excluding cells expressing either CD2,
CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123 or CD235a (Miltenyi Biotech) preceded the CD34+ enrichment. CD34+ cells were either obtained from the negative fraction after CD34+ isolation or, considering the low frequency of CD34+ in peripheral blood (< 0.1%), the complete MNC fraction was used. The study was approved by the University of Düsseldorf and University Hospital Essen Ethics Committees, and all samples were collected after obtaining informed consent of the donors or the donors' mothers, respectively.

**Gene expression analysis**

RNA was isolated with the mirVana (Ambion) or the RNeasy Kit (Qiagen) according to the manufacturers’ instructions. cDNA was either synthesized with the iScript cDNA synthesis Kit (BioRad) using a combination of oligo(dT) and random primers, or generated with MMLV Reverse Transkriptase and oligo(dT) primers (Promega) according to the manufacturers’ instructions, or synthesized with the High-Capacity cDNA RT-Kit (Life Technologies). Expression levels of 37 epigenetic modifiers were measured with a MicroFluidic card (Applied Biosystems) on an ABI 7900HT instrument (Applied Biosystems).

TaqMan Gene Expression Assays (Applied Biosystems) were performed on an ABI 7700 instrument (Applied Biosystems). Further gene expression was determined by qPCR with SYBR Green (Fermentas) on an ABI 7700 instrument (Applied Biosystems). Primer sequences are available upon request. All data were normalized to GAPDH as a housekeeping gene. Mean values were calculated after normalization and significance was determined with two-tailed Student’s t-tests.
H3K27me3 ELISA

Histones were isolated as described previously [41] and H3 was purified via FPLC (for details see supporting methods S1). Purified H3 was resuspended in ELISA Lysis buffer (Cell Signaling Technology) with 1mM PMSF (Roth). 5-10 ng purified histone H3 were analyzed with the PathScan H3K27me3 ELISA Kit (Cell Signaling Technology) according to the manufacturer’s recommendations.

Antibodies

For antibodies used in Western Blot, ChIP-Assay, and flow cytometry, please refer to Supplementary Tables S1 and S2.

Western Blotting

Nuclear extracts were prepared and analyzed by Western Blot. Secondary antibodies against mouse or rabbit IgG were either coupled to a fluorophor (LI-COR Biosciences) or to Horeseradish Peroxidase (Santa Cruz). Fluorescence was analyzed on an Odyssey infrared imager (LI-COR Biosciences instrument) using Odyssey 2.0 software. Ratios between H3K27me3 and H3 were calculated for quantification. Chemiluminescence was detected on an LAS 3000 instrument (Fuji Film).

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) of $1 \times 10^6$ cells was performed essentially as described before [42] with the ChIP-Assay kit from Millipore (Millipore). Immunoprecipitation was performed overnight. Promoter regions of different genes as well as the LINE element were analyzed by real-time PCR with SYBR Green on an ABI 7700 instrument. Primer sequences are available upon request. Data were normalized to the input control and subsequently to total H3.
ChIP-on-chip analysis

For ChIP-on-chip analyses precipitated DNA was amplified with the GenomePlex whole genome amplification kit (Sigma) [43]. 1 µg of DNA was labeled according to the NimbleGen protocol and hybridized to 385k RefSeq NimbleGen Promoter arrays (Roche, NimbleGen) enabling investigation of 24,659 human promoters, each spanning 2,200 bp upstream and 500 bp downstream of the respective transcription start site. For more details see Supplementary Methods S2.

Lentiviral overexpression of EED

EED cDNA starting with the first ATG [44] was amplified using the following primers: 5’-GATTGACTCGAGAATATGTCCGAGAGGGAAGTGTC-3’ and 5’-CGGCTAGCCGTTATCGAAGTCGATCCCAGCG-3’ and cloned into the pCL6 vector (kind gift of Prof. H. Hanenberg, University Clinic Düsseldorf) directly in front of eGFP, separated by an IRES site. Lentivirus was generated from the pCL6 control vector and the EED containing vector using HEK293T as a producer cell line. Cells were incubated with the virus on Retronectin (Takara) coated plates for 16-72h, washed once and transferred to the appropriate medium. If necessary, transduced cells were flow cytometrically enriched for eGFP expression (MoFlo, Beckman Coulter) in order to obtain pure EED-overexpressing cells for further analyses.

In vitro differentiation assays

CD34+/Lin- cells were isolated from CB using magnetic beads. Cells were transferred on Retronectin coated plates and transduced with the lentivirus. After 16-24h they were replated onto murine EL08 feeder cells and differentiated into NK cells as described previously [45,46]. eGFP expressing cells were flow cytometrically (MoFlo, Beckman Coulter) enriched after 2 weeks of culture and plated on fresh feeder cells for the remaining culture period. At
the end of the cultivation, eGFP expressing cells were again flow cytometrically sorted to gain pure EED overexpressing cells for transcription analyses. Additionally, different surface markers were flow cytometrically analyzed in the eGFP positive population (MoFlo or FC500, Beckman Coulter).

For the CFC assays of the purified HSPC subsets, 200 sorted cells (HSCs/MPPs, LM and EM progenitors; see Supplemental Figure S2 for details) were seeded into 1 ml MethoCult H4434 (StemCell Technologies) in duplicates. Hematopoietic colonies were scored after 14 days as described previously [38,39]. EED-overexpressing cells were cultivated for one week on murine EL08 feeder cells with the starting medium for NK cell generation. Afterwards, eGFP expressing cells were flow cytometrically purified and either 500 or 1,000 eGFP+/CD34+/CD14- or eGFP+/CD34-/CD14- cells were sorted directly into MethoCult GF H4434 medium in triplicates. Colonies were counted in a light microscope (Hund) after 17 days. Afterwards, colonies were manually picked under the light microscope and flow cytometrically analyzed for eGFP expression (FC500, Beckman Coulter).

Results

Differences in EED expression in hematopoietic stem and progenitor cells from newborns and adults

In order to characterize human HSPCs at an epigenetic level, we compared the expression changes of genes encoding epigenetic enzymes which participate in histone modification, remodeling of the chromatin and DNA methylation, in neonatal (CB) and adult (BM/PB) CD34+ cells. This cell population, which is relevant for human stem cell transplantation, comprises a mixture of long-, and short-term hematopoietic stem cells as well as different lineage-specific hematopoietic progenitor cells. [39,47-51]. First, we analyzed the expression of 37 candidate genes in CD34+ HSPCs. For most of the analyzed genes we observed only
minor expression differences (Supplementary Figure S1). However, EED, a core member of Polycomb Repressive Complex 2 (PRC2) and SIRT4, an NAD dependent ADP-ribosylase, were strongly and consistently increased in G-CSF-mobilized peripheral blood (PB) and bone marrow (BM) of adults as compared to newborns (Supplementary Figure S1). Since it is known, that PRC2 plays an important role in hematopoiesis and expression of PRC2 members is often disturbed in hematopoietic malignancies we decided to investigate the expression of EED and other members of PRC2 in more detail.

To verify the initial observations, EED expression levels were analyzed in CD34+ HSPCs isolated from CB (n = 9) and G-CSF-mobilized PB (n = 17, average age: 40 years). mRNA levels of EED were strongly increased in HSPC from adults compared to newborns thus confirming the initial screening experiment (Figure 1A). A similar increase of EED was also observed in CD34+ cells from BM (n = 5, average age: 37 years), thereby demonstrating that the increase was not restricted to G-CSF treated HSPCs (Figure 1A). In fact, analysis of MNC samples from PB (average age: 39.8 years) before and after treatment with G-CSF revealed an inhibitory effect of G-CSF on EED expression (Supplementary Figure S3C).

Analysis of the two other PRC2 core components showed that in case of EZH2, transcript levels were moderately up-regulated, while those of SUZ12 were decreased in mobilized CD34+ HSPCs (Figure 1A). The significant decrease of SUZ12 seems to be at least partly due to the mobilization of HSPCs, since SUZ12 levels decreased considerably following the G-CSF treatment (Supplementary Figure S3C). Importantly, a similar decrease of SUZ12 expression was not seen in untreated HSPC from BM.

Since CD34+ cells provide a heterogenic pool of different progenitor cells that can be separated by combinations of cell surface markers (Figure 1B) [38,39], we decided to analyze
EED expression in defined HSPC subfractions, next. To this end, we purified three different fractions of HSPCs from CD34+ samples from neonatal (CB, n = 4) and adult (BM, n = 3) sources, i.e. HSCs/MPPs (CD34+CD133+CD45RA-CD90+), lympho-myeloid (LM) progenitors (CD34+CD133+CD45RA+) and erythro-myeloid (EM) progenitors (CD34+CD133lowCD45RA-) (Figure 1C) [39,52,53]. EED was found to be expressed at similar levels in adult and neonatal HSCs/MPPs. In contrast, significant higher EED expression rates were detected in adult LM and EM progenitors, thus suggesting that EED becomes specifically up-regulated during lineage commitment in adult but not in neonate HPCs (Figure 1C, Supplemental Figure S2).

Next, we compared the EED expression in CB- (n = 13), PB- (n = 16, average age: 39.9 years) and BM-derived MNCs (n = 3, average age: 36.7 years). This revealed a less pronounced but still highly significant increase of EED in MNCs from adults compared to newborns (Figure 1D). However, it has to be noted that MNCs are a rather heterogeneous cell population and it cannot be excluded that shifts in frequencies of subpopulations contribute to these differences. However, the observed elevated EED expression in HPCs and MNCs in adults underlines the general relevance of this factor for adult hematopoiesis.

To further explore if the increase in EED expression is sustained in an aged population, we next compared EED levels in MNCs from elderly donors (n = 12, average age: 74 years) to those of middle-aged donors (n = 19, average age: 39.4 years) and CB (n = 13). Importantly, EED expression levels were not only higher in a substantial proportion of elderly donors, but were also more scattered in older individuals, which is in line with a described stronger epigenetic heterogeneity during aging (Figure 1E) [12].
EED-associated changes in H3K27me3 levels

As PRC2 is responsible for trimethylation of histone H3K27, we were interested to see if the observed increase in *EED* expression in adult cells was associated with higher levels of H3K27me3. To address this question globally, we decided to first measure H3K27me3 levels via ELISA. Notably, recent studies indicate that overall histone levels are reduced not only in aged yeast, but also in senescent human fibroblasts [54,55]. To exclude that a putative loss of core histones interferes with our analysis, we first implemented an FPLC-based purification step for histone H3. Subsequently, equal amounts of FPLC-purified histone H3 were subjected to an H3K27me3-specific ELISA. As shown in Figure 2B, the overall level of H3K27me3 indeed increased significantly in MNCs from newborns to adults (Figure 2B). Due to limited sample material, we could not perform the ELISA on purified histone H3 from HSPCs. Therefore we switched to more efficient ChIP-Assays in order to analyze H3K27me3 in HSPCs. This had the advantage that not only the global H3K27me3 levels but also their distribution over the genomic regions could be analyzed.

It is well known that the H3K27me3 modification is frequently found on repetitive elements such as LINE elements [56]. We thus asked, if the increase in H3K27me3 was mainly due to chromatin modifications occurring at repetitive elements or if known PRC2 target genes were also affected. Therefore, we performed a ChIP-Assay using purified CD34+ HSPCs from newborns and adults (46 years). Similar to the ELISA, analysis of the LINE promoter revealed a substantial increase in H3K27me3 levels at repetitive elements (Figure 2C). Additionally, a strong increase of H3K27me3 levels at the promoter of the estrogen receptor (ESR1), a known PRC2 target gene [57], was observed (Figure 2D). These results gave a first indication that not only repetitive elements, but also gene regions are affected by the observed increase of H3K27me3 levels in HSPCs from adults.
Quantitative and qualitative changes of H3K27me3 occupancy in HSPC from newborns and adults

To investigate H3K27me3 changes on a genome-wide scale, we performed ChIP-on-chip analyses with NimbleGen 385k RefSeq promoter tiling arrays, covering 24,659 human promoters. In order to get highly enriched and consistent populations of HSPCs from newborns and adults, magnetically purified CD34+ cells were further enriched by flow cytometric cell sorting for expression of CD34 and lack of typical cell surface markers for the different hematopoietic lineages (Lin−) (Supplementary Table S2), resulting in a purity of > 99% CD34+Lin− HSPC. ChIP-on-chip experiments were performed with pooled HSPCs from 9 newborns and 5 adults (34-54 years, average age: 43.6 years), respectively. In adults as well as in newborns, H3K27me3 covered only a minor fraction (ca. 13.0%) of all analyzed promoters (Figure 2A, Supplementary table S3 and S4), which is within the range of previous studies analyzing H3K27me3 promoter occupancy in hematopoietic progenitor cells [58]. To confirm that we really precipitated PRC2 targets, we compared our data with a panel of PRC2 target genes defined in 2006 by Lee et al. in human embryonic stem cells [59]. 780 out of the 1,893 genes (41%) described by Lee et al. were associated with H3K27me3 in at least one of our sample groups (Supplementary table S5). This is a high overlap of target genes, considering the fact that different cell types were analyzed with different methods.

As expected from the ELISA and ChIP data, H3K27me3 was enriched in HSPCs from adults compared to those of newborns, with 22.4% of all modified promoters associated with the repressive histone mark only in adults and 17.3% only in newborns (Figure 2A). Additionally, 85% of those promoters, which were associated with H3K27me3 in both samples had a clear predominance of the repressive mark in adults, strengthening the observation of a general increase in H3K27me3 in adult samples (Figure 2A). In embryonic stem cells, the repressive H3K27me3 histone mark is frequently found in combination with the active H3K4me3 modification, thus defining bivalent genes marked for later lineage-specific expression...
Global analyses of H3K4me3 occupancy did not reveal significant changes in the amount of bivalent promoters between HSPCs from newborns and adults (Supplementary table S3 and S6 and data not shown).

We next performed Gene Ontology (GO) analyses to determine functional categories specifically affected by the age-associated increase of H3K27me3. In the adult samples several gene categories that are associated with general functions of the cell such as transcription, cell-cell signaling, or ion transport were stronger associated with the repressive histone mark. More interestingly, H3K27me3 was enriched on genes involved in non-hematopoietic developmental pathways (Figure 2E, F). Notably, in adult HSPCs the activating mark H3K4me3 was predominantly found on promoters involved in hematopoietic development (data not shown).

Altogether, our data indicate a general increase of H3K27me3 between HSPCs from newborns and adults. This also comprises a qualitative shift of functional categories being governed by this histone modification.

**Histone modification changes are associated with expression changes of key hematopoietic transcription factors**

In order to analyze if the observed changes in histone modifications between HSPCs from newborns and adults correlated with gene expression, we analyzed the transcription levels of several hematopoietic transcription factors (Figure 3A). Importantly, with the exception of RUNX1, expression changes correlated well with histone modification changes (Figure 3B, C and Supplementary Figure S4). Interestingly, the expression of LEF1, which is important for lymphopoiesis [61], was down-regulated in HSPCs from adults, which was accompanied by an increase of the repressive histone mark H3K27me3 at its promoter, while PML, a transcription factor characteristic for myelopoiesis [62] was up regulated, correlating with an
increase in H3K4me3. Additionally, the expression of *HOXA9* and *HOXB4*, which are both important for HSC maintenance and proliferation [63,64], increased in HSPCs from adults, accompanied in both cases by an increase in H3K4me3.

**EED overexpression negatively affects the potential of HSPCs to differentiate along the lymphoid lineage**

In order to establish a direct functional link between the EED increase and the increase of H3K27me3 levels in adult hematopoietic cells, EED was stably overexpressed in the T cell line Jurkat. Overexpression of EED in Jurkat cells did not affect their growth behavior. However upon overexpression in HSPCs, it strongly reduced their proliferation (data not shown). In Jurkat cells, *EED* expression was considerably increased on RNA as well as protein level, while *EZH2* and *SUZ12* levels did not change (Figure 4A, B). Importantly, the ratio of H3K27me3 to total histone H3 increased in cells overexpressing EED (Figure 4B), which indicates that elevated EED levels are sufficient to increase the levels of the repressive histone mark.

In Jurkat cells this effect was only moderate. This made the system suitable for use in the more sensitive primary HSPCs, which were strongly inhibited in their growth-behavior by higher EED-overexpression, but tolerated moderate overexpression-levels (data not shown).

Having established that EED overexpression indeed leads to increased levels of H3K27me3, we next investigated, whether elevated EED levels interfere with hematopoietic differentiation. Therefore, HSPCs isolated from CB were transduced either with an empty control vector or with a vector for EED overexpression. One week after transduction, EED-overexpressing cells were isolated by means of their concomitant expression of the eGFP reporter and submitted to diverging differentiation protocols.

To analyze the influence of EED on myelopoiesis, we performed colony-forming cell (CFC) assays while its influence on lymphopoiesis was monitored by differentiating HSPCs into
natural killer (NK) cells [45,46]. Notably, we did not detect any EED-dependent changes in myelopoiesis in terms of colony number or frequency (Supplementary Figure S5A, B). Here it has to be considered that many of the transduced cells consistently lost eGFP-expression in culture, although they were initially sorted for eGFP expression. This might be due to technical reasons, such as transgene-silencing during differentiation or difficulties of gating very low eGFP-positive cells in flow-cytometry (Supplementary Figure S5C) [65]. As the classical readout for CFC assays is based on light microscopy, which does not distinguish between eGFP positive and negative colonies, it cannot be excluded that this obscures a potentially promoting effect of EED on myelopoiesis.

Lymphoid differentiation was assessed by differentiating EED-transduced cells into NK cells. EGFP-positive and thus EED-overexpressing cells were sorted one week after transfection and differentiated as described previously [45,46]. Flow cytometric analysis at the end of the differentiation was based on the eGFP positive population and showed that lymphoid differentiation was considerably inhibited by EED overexpression. Firstly, the growth rate of cells overexpressing EED was severely reduced under lymphoid, i.e. NK cell promoting conditions. Secondly, the cell surface marker pattern observed on differentiating cells over-expressing EED was strikingly different to that of control cells. After six weeks only 27% of EED-transduced cells expressed the NK cell marker CD56 compared to almost 90% of control cells (Figure 5A, E). Instead, EED-transduced cells contained comparatively large fractions of cells expressing the myeloid marker CD14 (22.6%) (Figure 5A, F). The occurrence of the CD14⁺ subset could be verified on mRNA level by real-time RT PCR (Figure 5D). CD34⁺ HSPCs transduced with the empty vector expressed myeloid markers at the beginning of the NK cell differentiation process (week 2), when only a minor fraction (14%) was positive for CD56. However, these markers vanished during prolonged differentiation (6 weeks), when the stage of mature NK cells was reached (Figure 5G).
Notably, after one additional week of culture, EED overexpressing cells finally developed into mature NK cells expressing characteristic markers such as NKG2A and KIR2DL3 (Figure 5B), suggesting that lymphopoiesis was decelerated but not irreversibly blocked by EED overexpression.

Interestingly, in contrast to Jurkat cells, not only EED expression, but expression of all three core PRC2 members was increased in EED overexpressing in vitro generated NK cells (Figure 5C).

Discussion

In our study, we reveal an increase of the PRC2 member EED in adult human progenitors of both the lympho-myeloid and erythro-myeloid differentiation lineages and MNCs from adults compared to those of newborns. This increase continues, at least in MNCs, until higher ages (up to 87 years) and is accompanied by an increase in heterogeneity of expression levels. Importantly, the increased EED expression could be tightly linked to a genome-wide increase in the corresponding histone modification H3K27me3 by direct analysis of H3K27me3 levels ex vivo as well as experimentally by overexpression of EED in lymphocytes and hematopoietic progenitor cells. Interestingly, expression changes of the other two core PRC2 components, EZH2 and SUZ12, did not follow those of EED, which suggests that the increase of EED alone is sufficient to globally increase H3K27me3 levels. Of note, the expression of JMJD3 and UTX, two factors that are known to demethylate H3K27 [66], followed opposing trends (Supplementary Figure S3A, B).

Our data demonstrate that EED expression is higher in HPCs from adults compared to those of CB and unchanged in HSCs/MPPs. These findings are in contrast to a recent study by Beermann et al. which showed that the expression of PRC2 members in murine HSCs is down-regulated [17] during proliferative aging. While we compared HSPCs from the different
sources CB and adult peripheral blood or bone marrow, Beermann et al. investigated HSCs from young and old mice. In fact, preliminary data from our laboratory indicate that MNCs from children express higher levels of EED than both MNCs from CB and adults, thus corroborating the findings of Beermann et al.

Investigation of the genome-wide distribution of H3K27me3 in HSPCs from newborns and adults by ChIP-on-chip analyses showed that in HSPCs from adults more loci are covered with H3K27me3 than in HSPCs from CB. Additionally, we observed a functional change of gene categories associated with H3K27me3. In the adult samples, H3K27me3 was predominantly enriched at genes connected to development or differentiation pathways of cell types not involved in hematopoiesis. This might reflect an increasing need in adult HSPCs for silencing of non-hematopoietic pathways via H3K27me3. In this context it is noteworthy that whereas the repressive histone mark H3K27me3 increases globally from CB HSPCs to adult HSPCs, several studies have demonstrated an age-associated reduction of DNA methylation levels at the majority of gene loci [67,68]. Notably, although DNA methylation and H3K27me3 are mutually exclusive at numerous promoters under steady-state conditions, experimental inhibition of the DNA methylation machinery results in an efficient silencing of formerly DNA methylated genes via Polycomb-mediated trimethylation of H3K27 [57,69,70]. Based on these observations it could be hypothesized that increasing levels of H3K27me3 fulfill an important compensatory function on genes that lose DNA methylation by keeping them repressed. In this model, aberrant de-repression of genes involved in non-hematopoietic differentiation processes due to loss of DNA methylation would be counteracted by increased H3K27me3 occupancy, which would help to preserve the stem cell characteristics of HSPCs (Figure 6).

PRC2 activity not only ensures repression of aberrantly DNA-demethylated genes but also has the potential to directly recruit the DNA methylation machinery towards its target genes [71]. It is thus likely that an increase of H3K27me3 in HSPCs from adults ultimately results...
in an increase of DNA methylation at specific PRC2 targets. Indeed, a recent study showed that genes known to be PRC2 targets in ES cells are much more likely to become DNA-hypermethylated with age and during carcinogenesis than non-PRC2 target genes. Interestingly, in our data set 93.5% of the investigated age-related PRC2 targets indeed exhibited an increase of H3K27me3 in HSPCs from adults compared to those of CB (Supplementary Figure S6). Although we have not assessed the DNA methylation status of these genes, these data support a close connection between the H3K27me3 mark and DNA hypermethylation and ultimately suggest that elevated H3K27me3 levels could facilitate aberrant DNA methylation of tumor suppressor genes thereby promoting the development of cancer [72] (Figure 6 right panel).

Our investigation of the expression of key hematopoietic transcription factors in the context of the respective histone modification pattern revealed some parallels to the described age-associated myeloid bias of murine and human HSCs [5,7-9,11]. While LEF1 expression, which is important for lymphopoiesis [61], was reduced in HSPCs from adults, the expression of PML, a myelopoietic transcription factor [62], increased in these cells. These divergent expression changes correlated well with the underlying epigenetic structures and the fact that age-associated EED-overexpression was most pronounced in the LM progenitor fraction.

Additionally, they corroborate observations made in murine HSCs which suggest that lymphoid genes are predominantly down-regulated during aging while myeloid genes are up-regulated [4].

Another hallmark of hematopoietic aging is the gradual increase of the stem cell pool [9,11,73]. Concerning this, we observed in adult HSPCs an up-regulation of two members of the HOX gene family, namely HOXA9 and HOXB4, which are both associated with stem cell proliferation. The changes in their expression levels described here correlated with an increase of the activating histone modification mark H3K4me3. Interestingly, HOXA9 has also been associated with the development of acute myeloid leukemia, a hematopoietic cancer with
increasing incidence in older subjects [74,75]. Taken together, our data suggest a link between the increase in EED expression, H3K27me3 levels, and changes in the expression of several transcription factors, which could promote a myeloid bias in hematopoiesis and an expansion of the stem cell pool.

The direct functional consequences of the relative high levels of EED in HPCs from adults are not fully resolved yet. Previous studies demonstrated that Ezh2 overexpression prevented murine HSPC exhaustion during serial transplantation and senescence of high-passage MEFs [76], suggesting that enhanced expression of PRC2 members is connected to a prolonged healthy and functional lifespan of HSPCs. However, other studies indicate that overexpression of Ezh2 in murine HSC can result in myeloproliferative disorders [77]. Together with the observation that a tight Polycomb equilibrium is essential for normal embryonic stem cell development [78], these results suggest, that moderate upregulation of Polycomb action might protect HPC function in older donors but at the cost of a higher risk for malignant transformation due to aberrant silencing of tumor suppressor genes.

Until now the effects of EED overexpression on the differentiation potential of HSPCs have not been directly investigated. Therefore, we analyzed the *in vitro* differentiation potential of human HSPCs overexpressing EED. HSPCs reacted very sensitive to an artificial elevation of EED expression, for example by slowing down their growth rate. This demonstrates that an elaborated Polycomb equilibrium is essential for proper cell growth. To cope with this problem, we worked throughout our study with a system of only moderate EED overexpression.

EED-overexpressing HSPCs did not show any significant changes in their differentiation potential towards the myeloid lineage. In contrast, EED shifted the *in vitro* development of NK cells towards myeloid stages. Expression of the myeloid surface marker CD14 was prominent in EED-overexpressing progenitors at comparatively late stages of differentiation,
when control cultures had already progressed to the stage of mature NK cells. Importantly, EED-overexpressing progenitors were still able to differentiate into NK cells. The pronounced occurrence of myeloid cells in the lymphoid differentiation set-up reflects the in vivo observations that myelopoiesis increases during aging while lymphopoiesis is impaired. Overall, our data indicate that the epigenetic equilibrium is more tightly kept in HPCs from CB than in those from adults. It seems that in HPCs from CBs the epigenetic landscape is in an ideal shape providing an optimal potential for all differentiation pathways. In adults, however, the epigenetic landscape is profoundly different, probably as a reaction to stresses that occur during lifetime. This might lead to a functional impairment of adult HPCs which is aggravated during aging and explains some of the problems occurring with elderly stem cell donors.

Acknowledgments

We are very grateful to the Stem Cell Department of the Red Cross Blood Service West for bone marrow samples, the Department of Gynaecology and Obstetrics of the University Hospital Essen for umbilical cord blood samples, M. Jäger and C. Ziskoven for providing PB of healthy donors older than 55 years. We thank R. Margueron for providing the EED antibody and H. Hanenberg for providing the lentiviral vector. We are also grateful to K. Raba for assistance with flow cytometrically sorting cells. We thank the Research Commission of the University Clinic of Düsseldorf for financial support.

Conflicts of interests

The authors declare no conflicts of interests
References


**Figure Legends**
Figure 1: Expression changes of PRC2 members in HSPCs and MNCs from newborns and adults. Transcript levels were measured by TaqMan gene expression assays and normalized to GAPDH. (A) Human CD34+ HSPCs were isolated from umbilical cord blood (CB, n = 9, white bars), peripheral blood (PB) after G-CSF mobilization (PB, n = 17, average age = 40 years, grey bars) and bone marrow (BM, n = 5, average age = 37 years, striped bars) of healthy male donors and enriched to a purity of > 90%. Fold change was calculated with CB as the control group. Error bars represent the 95% confidence interval. (B) According to the revised human hematopoietic tree multipotent HSCs/MPPs (CD133+ CD34+ CD45RA− CD90+) give rise to lympho-myeloid (CD133+ CD34+ CD45RA−; LMPPs/MLPs/GMPs) and erythro-myeloid (CD133bw CD34 CD45RA−; EMPs/EoBPs/MEPs) progenitors [38,39]. (C) Cells from these three defined HSPC fractions were purified from neonatal (CB; n = 4) and adult donors (BM; n = 3) and analyzed for EED expression. (D) CD34-depleted MNCs were isolated from CB (n = 13, white bars), PB after G-CSF mobilization (n = 16, average age =
39.9 years, grey bars) and BM (n = 3, average age = 43.5 years, striped bars) of healthy male donors. (E) CD34 depleted MNCs were isolated from CB (n = 13), PB with (n = 16, average age 39.9 years) and without (n = 12, average age 74 years) G-CSF mobilization, and BM (n = 3, average age 43.5 years) of healthy donors. Samples were divided into three groups, representing newborns (squares), middle aged, (25-55 years, average age = 39.4 years, triangles) and old (56-90 years, average age = 74 years, circles). Fold change was calculated with CB as the control group. Error bars represent the 95% confidence interval. P-values were calculated with two-tailed Student’s t-tests: (* = p < 0.05; ** = p < 0.01; *** = p < 0.001).

LMPP, lymphoid-primed multipotent progenitor; MLP, multilymphoid progenitor; GMP, granulocyte-macrophage progenitor; EMP, erythro-myeloid progenitor; EoBP, eosinophil-basophil progenitor; MEP, megakaryocyte-erythrocyte progenitor; Neutro, neutrophil; Eos, eosinophil; Bas, basophil; Mac, Macrophage; MK, Megakaryocyte; Ery, Erythrocyte.
Figure 2: Hematopoietic cells of adults have higher H3K27me3 levels than those of newborns. (A) CD34+Lin- HSPC from 9 newborns and 5 adults (average age = 43.6 years)
were flow cytometrically enriched to a purity > 99% and respective samples were pooled. H3K27me3 occupancy of 24,659 human promoters was analyzed via ChIP-on-chip assays using NimbleGen 385k RefSeq promoter tiling arrays. 13.5% of the analyzed promoters were associated with H3K27me3 in at least one sample. Details of the distribution are shown in the right part of (A). The striped sector indicates the proportion of promoters that are only in the adult sample associated with H3K27me3, the grey sector corresponds to promoters that are stronger associated with H3K27me3 in the adult sample, the dotted sector corresponds to promoters that are stronger associated with H3K27me3 in CB, while promoters represented by the white sector are only in CB associated with H3K27me3. (B) Histones were isolated from CD34 depleted MNC from CB (n = 6) and G-CSF-mobilized PB (n = 7, average age = 41.1 years). Equal amounts of FPLC-purified H3 were analyzed in an ELISA specific for H3K27me3. (C, D) ChIP-Assays were performed with purified CD34⁺ HSPCs (> 90%) from CB (pool of 2) and G-CSF-mobilized PB (46 years) with antibodies against H3 and H3K27me3. H3K27me3 occupancy was analyzed for the indicated promoters. All results were first normalized on input and the fold change was calculated with the H3-precipitated DNA as a reference in order to account for changing H3 levels in the different cell populations. Error bars represent the standard deviation of triplicate samples. (E, F) Gene ontology (GO)-analysis for genes associated with H3K27me3 only or stronger in adults. In each panel a selection of 10 gene categories with an FDR < 0.001 are shown.
Figure 3: H3K27me3 influences expression of key hematopoietic transcription factors

(A) Expression analysis of selected transcription factors. CD34+ HSPCs were isolated from CB (n = 4, white bars) and G-CSF mobilized PB (n = 4, average age 46 years, grey bars). Transcript levels were measured by real-time RT PCR and normalized to GAPDH. Fold change was calculated with CB as the control group. Error bars represent the 95% confidence interval. P-values were calculated with two-tailed Student’s t-test: (* = p < 0.05; *** = p < 0.001). (B, C) ChIP-on-chip data for the promoters of two transcription factors were analyzed in detail with SignalMap. Signals for H3K4me3 and H3K27me3 are presented at the tile level in lane 1 and 3, respectively. In lane 2 and 4 peaks have been calculated for the respective histone modifications according to the cumulative coverage (red: FDR < 0.05, yellow FDR < 0.2, grey: FDR > 0.2). Transcription start sites are indicated by arrows.
Age-related increase of EED expression in early hematopoietic progenitor cells is associated with global increase of the histone modification H3K27me3 (doi: 10.1089/scd.2014.0435)

This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.
Figure 4: EED overexpression correlates with increased H3K27me3 levels in Jurkat cells. Jurkat cells were stably transfected with the EED vector (grey bars) or an empty control vector (white bars). (A) Expression levels were measured by real-time RT PCR and normalized to GAPDH. Fold change was calculated with the empty vector as a control. Error bars represent the standard deviation of triplicate samples. (B) Jurkat cells overexpressing EED were analyzed by Western Blot with the indicated antibodies. H3 and H3K27me3 levels were measured with secondary antibodies coupled to fluorescence dyes in order to calculate the ratio between H3K27me3 and H3.
Figure 5: Differentiation of CD34⁺/Lin⁻ HSPCs into Natural Killer (NK) cells.

CD34⁺/Lin⁻ HSPCs were isolated from CB and transduced with either the empty control vector or the EED-overexpression vector, and differentiated into NK cells. Transduced cells were flow-cytometrically enriched for eGFP expression after 2 weeks and at the end of the differentiation period to ensure that expression analyses were only performed on cells that
indeed overexpress EED. (A, B) Surface marker expression of eGFP positive cells was analyzed flow-cytometrically after 6 (A) or 7 (B) weeks of differentiation. Empty-vector transduced control cells are shown in the upper, EED-overexpressing cells in the lower row. (C, D) Transcript levels of the indicated factors were analyzed in eGFP-positive cells (all cells in case of wildtype (wt)) after 6 weeks of culture by real-time RT PCR and normalized to GAPDH. Fold change was calculated relative to cells infected with the empty control vector. (E, F) Percentage of eGFP positive cells expressing the indicated marker on their surface after 6 (white bars) and 7 (grey bars) weeks of NK cell differentiation culture. (G) Percentage of empty vector transfected eGFP positive control cells expressing the indicated marker on their surface after 2 (white bars) and 6 (grey bars) weeks of NK cell differentiation culture.
Figure 6: Schematic view of possible interactions between Polycomb-mediated gene silencing and DNA methylation.

When DNA methylation decreases globally, as is observed e.g. during aging, formerly repressed genes become activated. Concomitantly, however, Polycomb-mediated H3K27me3 increases and is able to compensate for the loss of DNA methylation, thus sustaining the repressed state (left panel). Although increased Polycomb action may prevent aberrant expression of normally silenced genes, it may also have detrimental effects, when aberrantly silencing genes that are expressed in young individuals such as tumor-suppressors (right panel). Additionally, recruitment of DNA methyltransferases through Polycomb Factors may reinforce the silent state independently of the former DNA methylation status.
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Figure S1: Screening for epigenetic factors with different expression levels in CD34\(^+\) HSPCs from newborns and adults. CD34\(^+\) HSPCs were isolated from cord blood (CB, n = 4), peripheral blood after G-CSF mobilization (PB, n = 5, average age = 44.6 years, green bars) and bone marrow (BM, n = 1, 44 years, purple bars) of healthy male donors and enriched to a purity of > 90%. Expression levels were measured on a MicroFluidic Card and normalized to GAPDH. Fold change was calculated with CB as the control group. Expression of EED and SIRT4 increased strongly and consistently in PBMC as well as in BM during aging (black arrows).
Age-related increase of EED expression in early hematopoietic progenitor cells is associated with global increase of the histone modification H3K27me3 (doi: 10.1089/scd.2014.0435)

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Figure S2: Definition and characterization of analyzed human HSPC subsets. (A) Gating-strategy and (B) subsequent quantification of multipotent HSC/MPP, lympho-myeloid (LM) and erythro-myeloid (EM) HSPC subpopulations in CD34+ cell fractions derived from umbilical cord blood (CB) and from bone marrow (BM). (C) Isolated HSPC subpopulations were tested in CFC assays, and all three sub-fractions showed a comparable lineage-output between both CB and BM samples. All results are in good agreement with previous data [1,2].
Figure S3: Expression changes of Polycomb antagonists JMJD3 and UTX in HSPCs and MNC from newborns and adults. Transcript levels were measured by TaqMan gene expression assays and normalized to GAPDH. (A) CD34+ HSPCs were isolated from CB (n = 9, white bars), PB after G-CSF mobilization (n = 17, average age = 40 years, grey bars) and BM (n = 5, average age = 37 years, striped bars) of healthy male donors and enriched to a purity of > 90 %. (B) CD34 depleted MNC were isolated from CB (n = 13, white bars), PB after G-CSF mobilization (n = 16, average age = 39.9 years, grey bars) and BM (n = 3, average age = 43.5 years, striped bars) of healthy male donors. Fold change was calculated with cordblood as the control group. Error bars represent the 95% confidence interval. (C) Transcript levels of the indicated factors were analyzed in MNC isolated from 4 healthy male donors (average age = 39.8 years) before (white bars) and after mobilization (grey bars) with G-CSF. Transcript levels were measured by real-time RT PCR and normalized to GAPDH. Fold change was calculated in relation to lymphocytes before treatment. P-values were calculated with two-tailed Student’s t-test: (*) = p < 0.05; (**) = p < 0.01; (***) = p < 0.001.)
Age-related increase of EED expression in early hematopoietic progenitor cells is associated with global increase of the histone modification H3K27me3 (doi: 10.1089/scd.2014.0435)

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Figure S4: Changes of histone modifications at selected promoters during aging.
ChIP-on-chip data for the promoters of several transcription factors were analyzed in detail with SignalMap. For each factor signals for H3K4me3 and H3K27me3 are presented at the tile level in lane 1 and 3, respectively. In lane 2 and 4 peaks have been calculated for the respective histone modifications according to the cumulative coverage (red: FDR < 0.05, yellow FDR < 0.2, grey: FDR > 0.2). Transcription start sites are indicated by arrows.
Figure S5: Differentiation of CD34+/Lin- HSPCs into the myeloid lineage.

CD34+/Lin- HSPCs were isolated from CB and transduced with either the empty control vector or the EED-overexpression vector. After one week, eGFP positive cells expressing or not expressing CD34 were sorted by flow cytometry and differentiated into the myeloid lineage as described in materials and methods. Different colony types were counted in a light microscope after 17 days of culture (A, B). BFU-E = Burstforming units-erythrocyte, CFU-GEMM = Colony forming units-granulocyte, erythrocyte, monocyte, megakaryocyte, CFU-GM = Colony forming units- granulocyte, monocyte. (C) After counting, the colonies were manually picked and separated according to their red or white color. Flow cytometrical analysis of these colonies revealed that at the most 50% of the cells retained eGFP-expression after 17 days while most of the cells were viable as demonstrated by Draq5 staining.
Figure S6: H3K27me3 is increased at “age-PGCTs” in HSPCs.

Teschendorff et al. [3] defined a group of 64 so-called “age-PGCTs”, which are associated with PRC2 in hematopoietic stem cells and acquire DNA methylation during aging. 46 of these genes were present on our array. For each promoter the sum of H3K27me3 peaks as obtained by ChIP-on-chip analyses was calculated for CB (white bars) and G-CSF mobilized PB (grey bars). Overall 93.5% of the analyzed “age-PGCTs”, had higher H3K27me3 levels in adults than in newborns.

Supplementary Literature:


Supplementary Methods

Methods S1: Histone Isolation
Isolation of histones was performed as described by Young et al. [1]. In short, cell pellets were homogenized in H2 buffer (5 mM Hepes, pH 7.6, 0.5 mM EDTA, 0.1 mM DTT, 0.1 M sucrose with protease inhibitors) and washed twice with ice cold H2O. The remaining pellet contained the nuclei which were lysed for 5 hours in 0.8 M H2SO4, at 4°C. The nuclei were pelleted at 3,000 x g for 15 min at 4°C. Histones were precipitated with 10 volumes of cold acetone overnight at 4°C, washed and air-dried. Subsequently histone H3 was purified by fast protein liquid chromatography (FPLC), on an Äkta explorer (900 series, Amersham Biosciences). For separation, histones were resuspended in 0.3% (w/v) trifluoroacetic acid and loaded onto a reversed-phase HPLC column (Jupiter C18, 5µ, 300 Å, 250 x 10 mm, Phenomenex) preequilibrated with 0.3% trifluoroacetic acid, 35% acetonitrile in water. Histones were eluted by a linear gradient from 35% acetonitrile in water to 70% acetonitrile in water over 60 minutes. Protein was measured continuously by absorption at 214 nm. The H3 fraction was air-dried and stored at -80°C until further use.

Methods S2: Analysis of ChIP-on-chip data
For genome wide analyses peak scores were calculated by the NimbleScan software. Only reads with a FDR < 0.2 were considered significant. Subsequently, peaks were mapped to tiles using the SignalMap software (Roche, NimbleGen). The threshold for peak calculation was set at 4. Peaks were matched with promoters according to HG18. Peak scores mapping to a single tiled region were summed up and assigned to the corresponding promoter. For Gene Ontology (GO) analyses summed up peak scores were analyzed on the DAVID platform [2,3]. Promoters were included in these analyses, when only one of the histone marks could be mapped unambiguously to just one distinct promoter. Furthermore, we excluded all promoters with a ratio of 0.8 to 1.2 histone mark occupancy between adults and newborns, for this indicates only minor differences.

Supplementary Literature:

**Supplementary Table S1**: Antibodies for Western Blot (WB) and Chromatin-Immunoprecipitation (ChIP)

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### Supplementary Table S2: Antibodies for flow cytometry

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