MOZ-Mediated Repression of $p16^{INK4a}$ Is Critical for the Self-Renewal of Neural and Hematopoietic Stem Cells

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ABSTRACT

Although inhibition of $p16^{INK4a}$ expression is critical to preserve the proliferative capacity of stem cells, the molecular mechanisms responsible for silencing $p16^{INK4a}$ expression remain poorly characterized. Here, we show that the histone acetyltransferase (HAT) monocytic leukemia zinc finger protein (MOZ) controls the proliferation of both hematopoietic and neural stem cells by modulating the transcriptional repression of $p16^{INK4a}$. In the absence of the HAT activity of MOZ, expression of $p16^{INK4a}$ is upregulated in progenitor and stem cells, inducing an early entrance into replicative senescence. Genetic deletion of $p16^{INK4a}$ reverses the proliferative defect in both Moz$^{HAT{+/-}}$ hematopoietic and neural progenitors. Our results suggest a critical requirement for MOZ HAT activity to silence $p16^{INK4a}$ expression and to protect stem cells from early entrance into replicative senescence. Stem Cells 2014;32:1591–1601

INTRODUCTION

Self-renewal of stem cells is vital for maintaining tissues homeostasis throughout the life span of an organism. Because of their high-mitotic activity, stem cells have to put in place inherent cellular defense mechanisms, such as senescence and apoptosis, to avoid the expansion of potentially malignant cells resulting from the accumulation of oncogenic mutations. Cells undergoing senescence display dramatic changes in chromatin structure, which contribute to the irreversible nature of the senescent state. These changes are regulated by the activities of chromatin modifying enzymes; however, the nature of these specific enzymes and their role in the control of senescence remains mostly unknown.

The histone acetyltransferase monocytic leukemia zinc finger protein (MOZ; MYST3 or KAT6A) is a key regulator of hematopoiesis recurrently found translocated in acute myeloid leukemia [1–5]. Both MOZ null mouse embryos and mice carrying a G657E mutation, which renders the protein catalytically inactive (Moz$^{HAT{-/-}}$ hereafter), have severe defects in the generation and maintenance of hematopoietic stem cells (HSCs) [6–8]. In the absence of MOZ histone acetyltransferase (HAT) activity, the proliferative capacity of hematopoietic progenitors is dramatically impaired, with many cells withdrawing from the cell cycle during the G1 phase [8].

In this study, we establish that the proliferative defect observed in the absence of the HAT activity of MOZ is not limited to the hematopoietic compartment, but also extends to neural stem cells and progenitors (NSC/Ps). We show that this proliferative defect is caused by the upregulation of $p16^{INK4a}$ expression leading to a premature entry into replicative senescence and that the senescent phenotype can be rescued by genetic deletion of $p16^{INK4a}$. We further demonstrate that MOZ binds directly to the promoter of $p16^{INK4a}$ indicating that this tumor suppressor is a direct target of MOZ. Our findings suggest that these two stem cell types, HSCs and NSCs, use the same novel mechanism involving MOZ-driven acetylation to maintain their capacity to proliferate and avoid senescence. Altogether, these results provide new insights into the control of stem and progenitor cell proliferation and identify an unexpected role of MOZ-mediated acetylation in the regulation of $p16^{INK4a}$ expression. This finding also suggests that a potential reinforcement of the repressive activity of MOZ on $p16^{INK4a}$ expression could be an important mechanism supporting the development of acute myeloid leukemia following MOZ translocations.
Cell Culture and Growth Curves

Differentiation of embryonic stem cells (ESCs) into embryoid bodies (EBs) was carried out as described previously [8, 9]. Serum-free conditions that sustain the proliferation of hematopoietic precursors in liquid culture were described previously [10]. For neurospheres culture, we used the “NeuroCult Proliferation Kit” (Stem Cell Technologies, www.stemcell.com). To test the self-renewal capacity of neurospheres, cells were isolated from primary spheres using a NeuroCult Chemical Dissociation Kit (Stem Cell Technologies, www.stemcell.com). Self-renewal was quantified as number of secondary neurospheres generated per primary neurosphere. For proliferation studies, 10 μM 5-bromo-2-deoxyuridine (BrdU) was added to the cultures for 12 hours at 37°C.

Expression Analysis

Total RNA was extracted with an RNAeasy kit, treated with RNase-free DNase (QIAGEN, www.qiagen.com), and reverse-transcribed into cDNA with random hexamers by use of an Omniscript RT kit (QIAGEN, www.qiagen.com). Real-time polymerase chain reaction (PCR) was performed on an ABI 7900 system (Applied Biosystems, www.lifetechnologies.com) using the Exiqon universal probe library and primer designer (Roche, www.roche.com). All expression data were calculated relative to β-actin as 2−ΔCt. Data are presented as ΔCt values from triplicates normalized to β-actin. Primer sequences are available upon request.

Flow Cytometry

EBs were trypsinized (TrypE; Life technologies, www.lifetechnologies.com) for 3 minutes. Bone marrow of transplanted NOD Scid Gamma NSG mice was isolated by flushing the femurs with phosphate-buffered saline containing 2% fetal bovine serum. Single-cell suspensions were analyzed on a FACScan or a FACScanibur flow cytometer (Becton Dickinson, www.bd.com) or sorted on a FACS Vantage cell sorter (Becton Dickinson). The antibodies used were as follows: Mac1 (biotinylated), Sca-1 labelled with fluorescein isothiocyanate (FITC), and c-Kit antibody, were used for the HSC analysis; For the isolation of Lin−cKit− or CD45.2−Lin−cKit− population from bone marrow, we used CD45.2 (Biotin) and cKit labelled with allophycocyanin (APC) antibody together with a mix of antibodies recognizing lineage specific antigens Gr1, Mac1, B220, CD3, and Ter119 (PE). Staining with CD34 (Biotin) and cKit (APC) was performed to isolate hematopoietic progenitors from day 6 EBs. For the isolation of HSCs, we also included a combination of antibodies recognizing members of the SLAM markers CD150 (PE), CD48 (APC), and CD224.2 (FITC). All the antibodies were from BD Pharmingen or ebioscience. For cell cycle analysis, BrdU incorporation (BrdU Flow kit, BD Pharmingen, www.bdbiosciences.com) was performed according to the manufacturer’s instructions.

Senescence Analysis

Senescence associated β-galactosidase (SA β-gal) assay was performed using a senescence β-galactosidase staining kit (Cell Signaling, www.cellsingal.com).

Fetal Liver Transplantation and 5FU Treatment

NSG recipients (CD45.1) of 8- to 12-week-old were lethally irradiated with 250cGy in two doses of 125 cGy 3 hours apart and injected with donor (CD45.2) fetal liver cells. To determine the repopulating level of donor cells, peripheral blood was collected and stained with anti-CD45.1 and anti-CD45.2. For analysis of HSC proliferation in vivo, wild type (WT) and MOZ−/− mice were intravenously administered 5-fluorouracil (5FU; Mayne Pharma PLC, Warwick, UK) at a single dose of 150 mg/kg body weight. Six days after 5-FU treatment, bone marrow cells were isolated and analyzed for p16Ink4a expression by immunostaining. Sorted 6-day 5FU cells were grown in liquid culture in round-bottom microtiter plates (10 cells per well). After 10 days of incubation, cell number per well was scored using an inverted light microscope.

Competitive Repopulation Assays

Experimental conditions for this assay were published previously [11]. Repopulating units (RUs) from each donor were calculated according to the method described by Harrison and Astle [12], where numbers of RUs are calculated from the percentage donor cells. In brief, the calculations are based in the formula RU = %C/(100 − %), where the number of fresh competitor marrow cells used per 105 equals C and percentage corresponds to the obtained percentage of donor cells.

Transgenic Mice and Embryo Generation

All animal work was performed under regulations governed by the Home Office Legislation under the Animal Scientific Procedures Act of 1986. Ink4a−/− mice were obtained from Dr. O. Samson with the consent of Dr. M. Serrano.

ChIP Assays

Chromatin immunoprecipitation was performed using the Red ChIP Kit (Diagenode, www.diagenode.com) following the instructions of the manufacturer. Crosslinked cells were sonicated for 15 cycles (30s on/30s off) with the Bioruptor (Diagenode, www.diagenode.com). Antibodies used were RNA Polymerase (H-224 from Santa Cruz Biotechnology, www.scbt.com) and anti-HAT MYST3 antibody (Ab41718 from Abcam, www.abcam.com). Ten million cells were used for each immunoprecipitation with the anti-Moz antibody. Eluted chromatin was quantified by qualitative PCR (qPCR). Data for ChIP were obtained by subtracting IgG control values to the corresponding antibody values. Graphs represent fold increase over control IgG.

Immunoblotting and Immunocytochemistry

To analyze protein expression levels, cells were solubilized in Radio-Immunoprecipitation Assay (RIPA) lysis buffer containing a cocktail of protease inhibitors (Sigma Aldrich). Electrophoresis was carried out using commercial reagents (Novex; Life Technologies, www.lifetechnologies.com). For immunoblot, proteins were transferred to a nitrocellulose membrane using the iBlot gel transfer apparatus (Life Technologies, www.lifetechnologies.com). Nonspecific binding was blocked by incubation in blocking buffer; Tris-buffered saline (TBST; 0.1% Tween-20) containing 5% skimmed milk. After incubation with the corresponding secondary antibodies, signal was developed using the Enhanced Chemiluminescence Plus kit (ECL-Plus kit; GE-Healthcare Bio-Sciences, www.gelifesciences.com). For p16Ink4a and HP1-γ immunostainings cells were cytocentrifuged, fixed and stained with the corresponding antibody. Antibodies used were HP1-γ (07332 from Millipore,
RESULTS

HSC/Ps Undergo Early Entrance into Replicative Senescence in the Absence of MOZ HAT Activity

We established previously that HSCs and blood precursors carrying the mutated G657E MOZ protein lacking HAT activity have a profound proliferative deficiency, with many cells arresting in the G1 phase of the cell cycle [8]. A cell leaving the cell cycle during the G1 phase may encounter different fates: it can differentiate, become quiescent, senescent, or undergo apoptosis. No signs of increased apoptosis or defects in differentiation were observed in MozHAT−/− hematopoietic progenitors [8] suggesting quiescence or senescence as the most likely fates. Therefore, we evaluated whether the reported G1 arrest previously observed in MozHAT−/− CD34+ cKit+ hematopoietic progenitors is related to the acquisition of a senescent phenotype. Consistent with this hypothesis, a higher frequency of cells positive for the presence of senescence-associated heterochromatin foci (SAHF) [13], marking the accumulation of the nuclear Heterochromatin Protein 1-gamma (HP1γ) protein was detected in MozHAT−/− CD34+ cKit+ hematopoietic progenitors generated by in vitro ESC differentiation than in their WT counterparts (Fig. 1A). A significantly higher percentage of the hematopoietic progenitors also expressed the senescence-associated β-galactosidase (SA β-gal) in the absence of the HAT activity of MOZ (Fig. 1B). The progression through the G1 phase of the cell cycle in stem cells has been shown to be controlled by different cyclin-dependent kinase (CDK) inhibitors, such as p16INK4a [14–16], p21(CIP1) [16–20], p27 (Kip1) [21, 22], and p57(Kip2) [23, 24]. We analyzed the transcription levels of these CDK inhibitors to evaluate whether changes in their expression could be linked to the observed phenotype in MozHAT−/− hematopoietic progenitors. Only transcriptional levels of the tumor suppressor p16INK4a were significantly altered in MozHAT−/− cells (Fig. 1C). To further investigate whether this upregulation of p16INK4a was reflecting changes in the transcription levels of known regulators of this tumor suppressor, such as Bmi1 [15, 25], EzH1 [26], EzH2 [27, 28], and SuZ12 [29], we analyzed the expression of these genes by qPCR. We found no significant difference in the expression levels of proteins known to control p16INK4a transcription (Fig. 1C). We then verified that the transcription levels of p16INK4a were rapidly upregulated in MozHAT−/− hematopoietic progenitors upon culture conditions that promote their proliferation (Fig. 1D). Higher levels of p16INK4a protein were also detected in these cells by immunoblotting and immunostaining (Fig. 1E, 1F). Similarly, to the in vitro ESC-derived blood cells, MozHAT−/− cells isolated from embryonic fetal liver and highly enriched for HSCs (Lin−Sca+ cKit+ CD150+ CD48−) [30] had a limited proliferative capacity (Fig. 2A). This proliferative defect was reflected by a significantly lower percentage of cells in the S-phase of the cell cycle, and the accumulation of cells in the G1 phase as shown by BrdU incorporation analysis (Fig. 2B). qPCR analysis also revealed that MozHAT−/− fetal liver HSCs displayed increased expression levels of p16INK4a upon culture (Fig. 2C). To confirm our findings with adult hematopoietic progenitors and circumvent the limiting perinatal lethality of MozHAT−/− mice, we transplanted WT or MozHAT−/− fetal liver cells (CD45.2+) into irradiated immunodeficient NSG (CD45.1+) mice. Analyses of peripheral blood chimerism in transplanted animals 4 weeks after transplantation indicated that baselines of engraftment by CD45.2+ cells were higher than 90% and similar between mice repopulated by either WT or MozHAT−/− fetal liver cells (Supporting Information Fig. S1A). We then isolated adult CD45.2+Lin−cKit+ hematopoietic progenitors from the bone marrow of the reconstituted mice for analysis. The MozHAT−/− cells displayed again proliferative defects associated with an increase in p16INK4a protein levels upon ex vivo culture in proliferation media (Supporting Information Fig. S1B–S1F). To confirm these ex vivo findings and assess proliferation in vivo, cohorts of reconstituted mice were treated with 5FU to induce HSC entry into cell cycle [31, 32]. The p16INK4a protein was detected by immunostaining in CD45.2+Lin−cKit+ bone marrow cells isolated from 5FU treated MozHAT−/− mice 6 days after treatment (Fig. 2D), whereas no positive staining was observed in 5FU-treated WT controls. In addition, 7–10 days after treatment, a high percentage of 5FU-treated mice reconstituted with MozHAT−/− had to be euthanized (Fig. 2E) due to excessive weight loss (Supporting Information Fig. S1G) associated with the development of low blood counts (Fig. 2F). These findings are consistent with the profound long-term repopulation potential defect of MozHAT−/− HSCs in serial transplantation experiments as documented previously [8]. Altogether, these experiments demonstrate that the absence of the HAT activity of MOZ either in ESCs derived, fetal or adult hematopoietic progenitors results in cell autonomous proliferative defects triggered by a premature entry into replicative senescence.

NSCs Self-Renewal Relies on MOZ HAT Dependent Silencing of p16INK4a

MOZ and its close homologue MORF (MOZ related factor, MYST4 or KAT6B) have been assigned specific roles in either hematopoietic or neural development, respectively [33, 34]. However, our previous observation that MozHAT−/− ESCs, unlike their WT counterparts, did not extensively contribute to the formation of the brain in chimeric mice [8] suggests that MOZ, through its HAT activity, might also play a role in regulating the proliferation of NCS/Ps. To test this hypothesis, we first cultured cells isolated from the telencephalon of WT and MozHAT−/− E14.5 embryos under clonogenic conditions to compare their potential to generate self-renewing neurospheres, a measurement of the number of cells with neural stem-like properties [35, 36]. MozHAT−/− embryos generated three times less neurospheres than WT controls (Fig. 3A) suggesting that there are significantly fewer NSCs in the telencephalon of MozHAT−/− embryos. In addition, MozHAT−/− neurospheres displayed reduced expansion kinetics, producing fewer neurospheres at each passage, with an expansion index fivefold lower than WT neurospheres (Fig. 3B), suggesting a reduced self-renewal potential. In fact, no neurospheres could be generated from the MOZHAT−/− cells after the third passage. In addition to this

www.millipore.com and p16INK4a (M-156 from Santa Cruz Biotechnologies, www.scbt.com)

Statistics

Statistical comparisons of data sets were performed with the two-tailed Student’s t-test.
reduced expansion rate, MozHAT−/− neurospheres were, on average, smaller than their Wt counterparts (Fig. 3C), which could be indicative of cell-cycle arrest. BrdU incorporation analysis of MozHAT−/− secondary neurospheres revealed a reduced percentage of cells in the S-phase of the cell cycle and accumulation of cells at the G1 phase, similar to the phenotype observed for MozHAT−/− hematopoietic progenitors (Fig. 3D). Consistent with the acquisition of a senescent phenotype, cells from secondary MozHAT−/− neurospheres also displayed SA β-gal activity and higher p16INK4a expression levels than Wt controls (Fig. 3E, 3F). These in vitro findings were further substantiated in vivo by the observation that a lower percentage of cells expressing aldehyde dehydrogenase (ALDH), a marker of cells with stem-like properties [37, 38], was detected in brains of E14.5 MozHAT−/−/2 embryos.
Figure 2. Impaired proliferation and p16INK4a upregulation in MozHAT−/− hematopoietic stem cell (HSC)/Ps in vivo. (A): Individual HSCs (Lin−, Sca1+, cKit+, CD150−, CD48−) isolated from the fetal liver of Wt and MozHAT−/− embryos were sorted into 96-well plates containing proliferation media. Cell number was scored after 10 days. (B): Cell cycle status of Lin−, Sca1+, cKit+, CD150−, CD48− fetal liver cells isolated from E14.5 embryos. Pregnant females were injected with 5-bromo-2-deoxyuridine 1 hour before harvesting of embryos and cell cycle was analyzed by flow cytometry. (C): Qualitative polymerase chain reaction analysis of p16INK4a expression in CD45.2+ Lin−, Sca1+, cKit+, CD150−, CD48− cells isolated from the bone marrow of reconstituted mice after 24 hours of culture in proliferation media. (D): Reconstituted mice were treated with 5FU. Bone marrow was harvested 6 days later and CD45.2+ Lin−, cKit+ cells were immunostained with a p16INK4a antibody. MOZHAT−/− bone marrow cells expressing p16INK4a are shown in the picture (magnification ×40). No cells positive for the p16INK4a staining were detected in the bone marrow of Wt reconstituted mice (data not shown). (E): Kaplan-Meier graph showing the survival of reconstituted NSG mice after 5FU injection. Control mice were not injected. Wt (n = 6), MozHAT−/− (n = 8), histone acetyltransferase HAT−− (n = 8) and HAT−/− 5FU (n = 8). (F): Low white and red blood cell counts and reduced bone marrow cellularity are detected in NSG mice reconstituted with MozHAT−/− fetal liver cells 7 days after injection with 5FU. Abbreviations: HAT, histone acetyltransferase; HSC, hematopoietic stem cell; MOZ, monocytic leukemia zinc finger protein; NSG, NOD Scid Gamma; Wt, wild type.
embryos compared with Wt embryos (Fig. 4A). Furthermore, a reduction in the number of cells expressing the proliferation marker Ki67 (Fig. 4B) and cells incorporating BrdU (Fig. 4C) was also observed in the brains of E14.5 MozHAT2/2 embryos. Finally, qPCR analysis of E14.5 telencephalons revealed an increased p16INK4a expression in the telencephalon of MozHAT2/2 embryos (Fig. 4D). Altogether, these data demonstrate that, similarly to the hematopoietic system, MozHAT2/2 NSC/Ps display proliferative defects ex vivo and in vivo, upregulate the expression of p16INK4a and readily enter into replicative senescence.

Genetic Deletion of p16INK4a Largely Restores the Proliferative Capacity of HSC/Ps and NSC/Ps

As p16INK4a upregulation could be exacerbated in culture, we decided to directly evaluate to which extent the proliferative defects observed in MozHAT−/− mice are associated to an entry into replicative senescence induced by p16INK4a upregulation in vivo. To this end, we crossed heterozygote mice for the HAT mutation with p16INK4a/p19ARF knockout mice (hereafter INK4a−/−) [39] to generate double-knockout mice as well as heterozygotes and Wt control littermates. In the absence of INK4a, the embryonic and perinatal lethality of MozHAT−/− mice was clearly diminished resulting in increased frequency of MozHAT2/2 mice at weaning (Fig. 5A). In addition, in the absence of p16INK4a, MozHAT−/− mice displayed an overall improved health status and a recovery of the runt phenotype reported previously for these mice [8]. We next investigated whether the decreased frequency in HSC population observed in the fetal liver of MozHAT−/− embryos [8] was restored to normal level by deletion of p16INK4. Indeed INK4a−/−/MozHAT−/− (Wt/Ko) embryos displayed an overall improved health status and a recovery of the runt phenotype reported previously for these mice [8]. We next investigated whether the decreased frequency in HSC population observed in the fetal liver of MozHAT−/− embryos [8] was restored to normal level by deletion of p16INK4. Indeed INK4a−/−/MozHAT−/− (Wt/Ko) embryos displayed an overall improved health status and a recovery of the runt phenotype reported previously for these mice [8]. We next investigated whether the decreased frequency in HSC population observed in the fetal liver of MozHAT−/− embryos [8] was restored to normal level by deletion of p16INK4. Indeed INK4a−/−/MozHAT−/− (Wt/Ko) embryos displayed an overall improved health status and a recovery of the runt phenotype reported previously for these mice [8]. We next investigated whether the decreased frequency in HSC population observed in the fetal liver of MozHAT−/− embryos [8] was restored to normal level by deletion of p16INK4. Indeed INK4a−/−/MozHAT−/− (Wt/Ko) embryos displayed an overall improved health status and a recovery of the runt phenotype reported previously for these mice [8].
INK4a backgrounds to repopulate the bone marrow of lethally irradiated recipients. *INK4a*<sup>1/1</sup>/MozHAT<sup>1/1</sup> (Wt/Wt), *INK4a*<sup>1/1</sup>/MozHAT<sup>2/2</sup> (Wt/Ko) and *INK4a*<sup>2/2</sup>/MozHAT<sup>2/2</sup> (Ko/Ko) E14.5 fetal liver cells were transplanted into lethally irradiated congenic CD45.1<sup>1</sup> mice together with competitor CD45.1<sup>1</sup>/CD45.2<sup>1</sup> cells. *INK4a*<sup>2/2</sup>/MozHAT<sup>2/2</sup> fetal liver cells showed a significantly higher capacity to repopulate the bone marrow of recipient mice than *INK4a*<sup>1/1</sup>/MozHAT<sup>2/2</sup> (Wt/Ko) cells (Fig. 5C) indicating that the defective self-renewal capacity of MozHAT<sup>2/2</sup> HSCs could be rescued, at least partially, by the deletion of p16<sup>INK4a</sup>. These results indicate that p16<sup>INK4a</sup> upregulation in MozHAT<sup>2/2</sup> cells inhibit NSC self-renewal driving cells into replicative senescence in a similar fashion to HSC/Ps lacking MOZ HAT activity.

MOZ Binds to the Promoter of the p16<sup>INK4a</sup> Tumor Suppressor

In the absence of significant changes in the expression levels of known p16<sup>INK4a</sup> regulators, we decided to evaluate next by chromatin immunoprecipitation whether MOZ could directly bind to the p16<sup>INK4a</sup> promoter. The use of hematopoietic progenitors for these experiments would involve the isolation of very large numbers of cells difficult to obtain. To overcome this limitation, we decided to check whether the senescent phenotype was conserved in MozHAT<sup>2/2</sup> mouse embryonic fibroblasts (MEFs), which would provide a source of large numbers of cells needed for this study. We observed in clonogenic assays that the number of large proliferative colonies formed by individual MozHAT<sup>2/2</sup> MEFs was almost three times less than those formed by Wt

Figure 4. Impaired proliferation and p16<sup>INK4a</sup> upregulation in MozHAT<sup>2/2</sup> neural stem cells and progenitors in vivo. (A): Flow cytometry plots of ALDH activity in Wt and MozHAT<sup>2/2</sup> telencephalon cells. Cells isolated from E14.5 telencephalons were incubated with the ALDH substrate aldefluor in the presence or absence of the ALDH inhibitor diethylaminobenzaldehyde. Bar graph reflects the average percentage of ALDH positive cells. (B): Coronal sections of Wt and MozHAT<sup>2/2</sup> E14.5 telencephalonstained with anti-Ki67 antibody. Scale bar = 0.2 mm. (C): Sections of Wt and MozHAT<sup>2/2</sup> E14.5 telencephalons stained with anti-BrdU antibody. Bar graph represents the percentage of cells stained with BrdU. (D): Analysis of p16<sup>INK4a</sup> transcript levels in telencephalon tissue (n = 24) isolated from Wt and MozHAT<sup>2/2</sup> E14.5 embryos. Scale bar = 0.2 mm. Abbreviations: ALDH, aldehyde dehydrogenase; BrdU, 5-bromo-2-deoxyuridine; DEAB, diethylaminobenzaldehyde; HAT, histone acetyltransferase; SSC, Side Scatter; Wt, wild type.

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Figure 5. Impaired proliferation of MozHAT<sup>−/−</sup> hematopoietic stem cell (HSC), neural stem cell, and progenitors is rescued by genetic deletion of p16<sup>INK4a</sup>. (A): Genotype segregation of live mice produced by intercrossing of heterozygotes mice for the p16<sup>INK4a</sup>/ARF and MozHAT mutations (Ink4a<sup>+/−</sup>, MozHAT<sup>+/−</sup>). Numbers indicate the frequencies of the mice for each genotype. (B): Analysis of E14.5 fetal liver HSCs frequencies in Ink4a<sup>+/−</sup>MozHAT<sup>+/−</sup> (Ko/Wt), Ink4a<sup>+/−</sup>MozHAT<sup>−/−</sup> (Wt/Wt), Ink4a<sup>+/−</sup>MozHAT<sup>−/−</sup> (Wt/Ko), and Ink4a<sup>−/−</sup>MozHAT<sup>−/−</sup> (Ko/Ko) littermates. Fluorescence-activated cell sorting plots show the percentage of Lin<sup>−</sup>Sca<sup>−</sup>cKit<sup>+</sup>CD150<sup>−</sup>CD48<sup>−</sup> cells of representative individuals in each group. Bar graph represents the average percentage of Lin<sup>−</sup>Sca<sup>−</sup>cKit<sup>−</sup>CD150<sup>−</sup>CD48<sup>−</sup> cells in each phenotype. (C): Competitive repopulation assay of irradiated mice. Data are expressed as Repopulating units per 10e6 cells. (D): Bar graph shows the size of the neurospheres formed by telencephalon cells isolated from Ink4a<sup>+/−</sup>MozHAT<sup>−/−</sup> (Wt/Wt), Ink4a<sup>−/−</sup>MozHAT<sup>−/−</sup> (Ko/Ko), Ink4a<sup>−/−</sup>MozHAT<sup>−/−</sup> (Wt/Ko), and Ink4a<sup>−/−</sup>MozHAT<sup>−/−</sup> (Ko/Ko) littermates. *p < .05; **p < .01. Abbreviations: HSC, hematopoietic stem cell; Ko, knockout; MOZ, monocytic leukemia zinc finger protein; RU, repopulating unit; Wt, wild type.
MEFs (Supporting Information Fig. S3A). Additionally, growth curves and cell cycle analyses using BrdU revealed a significant decline in the proliferation rate of the MozHAT2/2 population over time (Supporting Information Fig. S3B) as well as a defect in the progression into the S-phase of the cell cycle (Supporting Information Fig. S3C).

MozHAT2/2 MEFs at passage five showed a high proportion of flattened cells containing SA-b-gal (Supporting Information Fig. S3D) and qPCR analysis of p16INK4a expression revealed that transcript levels were upregulated on average threefold in MozHAT2/2 MEFs compared with Wt (Supporting Information Fig. S3E). Accordingly, protein levels of p16INK4a were clearly higher in MozHAT2/2 MEFs than in the Wt and heterozygous MozHAT+/− MEFs (Supporting Information Fig. S3F).

Together these results clearly indicate that the senescent phenotype mediated by the upregulation of p16INK4a was also observed in MozHAT2/2 MEFs.

To determine whether the transcriptional upregulation of p16INK4a in the MozHAT−/− cells could be mediated by direct binding of MOZ to this locus, we performed ChIP analyses using MEFs isolated from E13.5 embryos. We detected MOZ binding to the p16INK4a promoter in Wt and MozHAT+/− MEFs. Binding of RNA Polymerase II was used as a positive control. Samples were prepared from passage 3 MEFs. The promoter of the CSF1R gene, not expressed in MEFs, was used as a negative control. *p < .05; **p < .01.

**Figure 6.** Monocytic leukemia zinc finger protein (MOZ) binding to the p16INK4a promoter at Wt and MOZHAT−/− Mouse embryonic fibroblasts. (A): Upper panel depicts p16INK4a promoter, exons, and introns. Numbers indicate amplified regions. Lower panel shows the ChIP analysis of MOZ binding at the p16INK4a promoter in Wt and MozHAT+/− MEFs. Binding of RNA Polymerase II was used as a positive control. Samples were prepared from passage 3 MEFs. The promoter of the CSF1R gene, not expressed in MEFs, was used as a negative control. *p < .05; **p < .01. Abbreviations: MOZ, monocytic leukemia zinc finger protein; TSS, Transcription Start Site.

**Figure 7.** Monocytic leukemia zinc finger protein (MOZ) histone acetyltransferase (HAT) activity regulates the proliferation of different types of stem cells. The histone acetyltransferase activity of MOZ prevents entry into early replicative senescence by regulating the expression of the tumor suppressor p16INK4a. In the absence of MOZ HAT activity, the levels of p16INK4a are significantly increased in both hematopoietic and neural stem and progenitor cell compartments. These cells then leave the cell cycle to become senescent, therefore resulting in severely impaired hematopoietic stem cells and neural stem cells self-renewal. Abbreviations: BM, bone marrow; HAT, histone acetyltransferase; HSC, hematopoietic stem cell; MOZ, monocytic leukemia zinc finger protein; NSC, neural stem cell.
the p16INK4a promoter in the absence of HAT activity was consistently observed and might reflect a compensatory mechanism. Altogether, these results indicate that MOZ directly binds to the p16INK4a promoter. This might through acetylation of histones or potentially other interacting proteins, repress the transcription of this locus.

**DISCUSSION**

In this study, we establish that in the absence of the HAT activity of MOZ HSC/Ps readily exit the cell cycle to undergo premature entry into replicative senescence. These findings provide a likely explanation for the reported impairment of HSC self-renewal observed in mice expressing the catalytically inactive version of MOZ [8]. In contrast to previous studies restricting the critical functions of MOZ and its close homologue MORF to hematopoietic and NSCs, respectively [34], we demonstrate here that similar proliferative defects are found in NSC/Ps lacking MOZ HAT activity (model in Fig. 7). Our data reveal that this common phenotype is at least partially caused by a premature upregulation of p16INK4a expression. Accordingly, genetic deletion of p16INK4a rescues to a large extent the proliferative defect. The fact that this phenotype is shared between the hematopoietic and neural compartments suggests that MOZ controls a regulatory mechanism conserved among stem cells from different tissues. This notion is supported by previous results showing that the contribution to specific organs in chimeric mice was consistently lower with MozHAT−/− ESCs than with Wt ESCs. The tissues with different contributions also included gut and liver in addition to the brain and hematopoietic organs [8].

The hematopoietic and neuronal phenotype of the MozHAT−/− mice bear strong similarities with the proliferative defects and premature senescence observed in neuronal and hematopoietic cells in the Bmi1 knock out animals [25, 36, 40–43]. Bmi1 and other polycomb members are well-established negative epigenetic regulators of p16INK4a [44] whereas trithorax proteins [45] and SWI/SNF proteins [46] act positively on its expression. We did not detect a significant change in the level of expression of the p16INK4a regulators analyzed. However, we observed a direct interaction of MOZ to p16INK4a promoter suggesting that MOZ could introduce changes in histone acetylation pattern which, in turn, could alter the binding of transcriptional regulators of p16INK4a harboring bromodomains. Further studies will be required to determine whether the HAT activity of MOZ directly impacts on the binding of regulators of p16INK4a expression or whether MOZ is implicated in a completely novel level of regulation. The Ink4a locus is one of the genomic regions most commonly mutated, deleted or epigenetically silenced in human cancers [47, 48]. It has been proposed that the fusion proteins produced upon translocation of the human MOZ locus with other HAT-encoding genes, such as CBP or p300, support the development of leukemia by altering the regulation of MOZ transcriptional targets. It would be interesting to examine if the repressive activity on p16INK4a expression mediated by MOZ acetylation is further exacerbated in these fusion proteins. As such, these MOZ leukemic fusion proteins might inhibit the triggering of senescence and promote the development of leukemia [49]. Our findings also raise the intriguing possibility that the regulation of p16INK4a expression by MOZ could be used as a molecular target to induce senescence in cancer stem cells.

**CONCLUSION**

The histone acetyltransferase MOZ (Monocytic Leukemia Zinc Finger protein, MYST3, or KAT6A) has a crucial role in controlling hematopoietic stem cells (HSCs) proliferation. In this study, we identified a critical requirement for MOZ-HAT activity to silence p16INK4a expression, to avoid senescence and sustain self-renewal of hematopoietic stem cells. We established that this effect is not limited to the hematopoietic compartment, but extends to neural stem cells and progenitors (NSC/P) suggesting that these two types of cells, HSCs and NSCs use the same mechanism involving MOZ-driven acetylation in order to maintain their capacity to proliferate. We propose that this mechanism could be also be critical for the self-renewal of other types of stem cells.

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**AUTHOR CONTRIBUTIONS**

F.M.P.-C.: conception and design, collection and assembly of data, data analysis, and interpretation, manuscript writing and final approval of manuscript; G.C.: data analysis and interpretation, collection and assembly of data, manuscript writing and final approval of manuscript; M.L.-a-L. and S.S.: data analysis and interpretation; V.K.: conception and design, financial support and final approval of manuscript; G.L.: conception and design, financial support, manuscript writing and final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors declare no potential conflicts of interest.

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