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# Perturbed hematopoiesis in mice lacking ATMIN

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## Key points:

- ATMIN deletion using Vav-Cre causes chronic leukopenia, with fewer B cells and common myeloid progenitors
- Long-term hematopoietic stem cells in ATMIN-deficient mice show increased cell cycling and are more prone to exhaustion under stress

## Abstract

The ATM-interacting protein ATMIN mediates non-canonical ATM signaling in response to oxidative and replicative stress conditions. Like ATM, ATMIN can function as a tumor suppressor in the hematopoietic system: deletion of *Atmin* under the control of CD19-Cre results in B cell lymphomas in aging mice. ATM signaling is essential for lymphopoiesis and hematopoietic stem cell (HSC) function; however, little is known about the role of ATMIN in hematopoiesis. We thus sought to investigate if the absence of ATMIN would affect primitive hematopoietic cells in an ATM-dependent or -independent manner. Apart from its role in B cell development, we show that ATMIN has an ATM-independent function in the common myeloid progenitors (CMPs) by deletion of *Atmin* in the entire hematopoietic system using Vav-Cre. Despite the lack of lymphoma formation, ATMIN-deficient mice developed chronic leukopenia as a result of high levels of apoptosis in B cells and CMPs and induced a compensatory mechanism in which HSCs displayed enhanced cycling. Consequently, ATMIN-deficient HSCs showed impaired regeneration ability, with the induction of the DNA oxidative stress response, especially when aged. ATMIN therefore has multiple roles in different cell types and its absence results in perturbed hematopoiesis, especially during stress conditions and aging.

## Introduction

ATM (ataxia telangiectasia mutated) coordinates cell-cycle checkpoints with DNA repair in response to DNA damage<sup>1</sup>. ATM can be activated by the MRN complex (MRE11/RAD50/NBS1) via interaction with NBS1<sup>2</sup> but can also be activated by the ATM interactor ATMIN<sup>3</sup>, also known as ASCIZ (McNees et al, 2005 EMBO J). ATMIN has a complementary function to NBS1: NBS1 is required for ionizing radiation-induced ATM signaling, whereas ATMIN is required for ATM activation following hypotonic or replication stress<sup>3-5</sup>.

ATMIN has been shown to function in conditions of oxidative stress and aging<sup>6</sup> and also as a transcription factor<sup>7</sup>. We have previously shown that ATMIN-deleted B cells (induced by CD19-Cre) have impaired class switch recombination and increased genomic instability, leading to B cell lymphomas<sup>8</sup>. In contrast, mice conditionally deleted of *Atmin* using Mx1-Cre developed B cell lymphopenia<sup>9</sup>. It is unclear whether the lack of B cell lymphoma formation in this mouse model is due to a B cell developmental defect or to other deficiencies in primitive hematopoietic cells that prevent the accumulation of genetically unstable cells. Since the role of ATMIN in hematopoietic stem/progenitor cells is currently unknown, we sought to investigate a possible role for ATMIN in primitive hematopoietic cells and whether this would be ATM-dependent or -independent.

## Materials and Methods

### **Vav-*Atmin*<sup>Δ/Δ</sup> mice**

*Atmin*<sup>f/f</sup> mice (described previously<sup>6,8</sup>) were crossed with heterozygous Vav-Cre mice to generate Vav-*Atmin*<sup>Δ/Δ</sup> mice. Gene deletion efficiency and genotyping was determined by PCR using primers specific for the floxed exon 4, deleted exon 4, WT *Atmin* alleles and for Vav-Cre and immunoblotting (Figures S1A and S1B and Table S1).

### **Intracellular immunostaining**

Briefly<sup>10</sup>, cells were incubated with antibodies against extracellular antigens then fixed in PBS with 2% methanol-free formaldehyde at room temperature (RT) (for Ki-67, BD Biosciences) or at 37°C (for pS824-Kap1, Bethyl Laboratories; Bim, Cell Signaling; pS139-γH2AX, Abcam) for 10 min. Cells were permeabilized with PBS containing 0.1% Triton-X-100 (Sigma) for 10 min at RT, blocked using PBS containing 5% serum for 15 min and incubated with primary antibodies at 4°C for 1 hour, followed by appropriate secondary antibodies in the same conditions. Cells were resuspended with PBS/2% FBS containing DAPI and pulse processing was used to exclude any unstained, apoptotic and clumped cells.

Please see Supplementary Information for more detailed Methods.

## Results and discussion

*Vav-Atmin*<sup>ΔΔ</sup> mice were born healthy and were not anemic but were leukopenic at 8-12 weeks (Figure S1C-D). Unlike the CD19-Cre model<sup>8</sup>, mice did not develop lymphoma even in aged mice, and instead numbers of splenic B cells were reduced (Figure S2A-B) which resulted in a complete absence of germinal centers (GC) (Figure S2C). B cell apoptosis was increased, and while ROS levels were unaffected there was a pronounced reduction in *Dynll1* expression, as previously described<sup>9</sup> (Figure S2D-F). Additionally, ATMIN-deficient B cells displayed a significant increase in the DNA damage signaling, consistent with an ATM-dependent competitive function of ATMIN<sup>3,4</sup> (Figure S2G-H). Numbers of pre-B cells in the bone marrow (BM) were also significantly lower (Figure S2I-K). These data suggest an early developmental defect with a concomitant high level of apoptosis that does not allow the accumulation of damaged B cells in the Mx-Cre model<sup>9</sup> and the *Vav-Atmin*<sup>ΔΔ</sup> mice. We then analyzed the primitive hematopoietic cell compartment in more detail.

In young ATMIN-deficient mice (8-12 weeks), a ~40% reduction in BM cellularity was observed across all cell types, with a prominent reduction in the frequency of common myeloid progenitors (CMPs) (Figure S3A-B). The very modest oxidative stress detected in the CMPs did not seem to play an evident role in reducing this cellular fraction in ATMIN-deficient mice (Figure 1A-C). We observed neither an increased DNA damage response (Figure 1D-E) nor evidence of developmental defects (Figure 1F) in *Vav-Atmin*<sup>ΔΔ</sup> CMPs compared with control cells. However, a significant increase in late apoptosis was detected in *Vav-Atmin*<sup>ΔΔ</sup> CMPs (Figure 1G) but not in other primitive

compartments (not shown) and this was accompanied by a ~33-fold decrease in *Dynll1* expression (a direct transcriptional target of ATMIN<sup>7</sup>, Figure 1H). Because Dynll1 sequesters pro-apoptotic Bim away from mitochondria<sup>11</sup>, reduced Dynll1 levels in *Vav-Atmin*<sup>ΔΔ</sup> CMPs should free Bim to translocate to mitochondria, triggering higher apoptosis. Consistent with this, immunofluorescence revealed significant co-staining of Bim with MitoTracker dye in *Vav-Atmin*<sup>ΔΔ</sup> cells (Figure 1I-K).

As two major cell compartments downstream of LT-HSC were severely affected, we hypothesized that LT-HSC function could be compromised. Indeed, sub-populations upstream of CMPs, particularly LT-HSCs, consisted of more cells that had exited quiescence (Figures 2A and S3C), supported by reduced expression of *Cdkn1c* (*p57*<sup>Kip2</sup>) and *Rbl2* (p130) (Figure 2B). Additionally, ATMIN-deficient primitive cells had a 2-3-fold reduction in the frequency of CAFCs and LTC-IC-derived colonies compared with control cells (Figure 2C-D). Importantly, the regenerative capacity of *Vav-Atmin*<sup>ΔΔ</sup> LT-HSCs 16 weeks post-transplantation was reduced 16-fold compared with control cells, affecting all three lineages (Figures 2E and S3D) and this was not due to homing defects (Figure 2F). *Vav-Atmin*<sup>ΔΔ</sup> LT-HSCs were mostly unable to regenerate hematopoiesis when re-transplanted (Figure 2E), despite the apparent lack of oxidative stress, increased apoptosis (Figure S3E-F), or active DNA damage response in young *Vav-Atmin*<sup>ΔΔ</sup> cells at steady state (Figure 2G-H). These results suggest that stresses induced during regeneration disturbed LT-HSC function.

Consistent with disturbed LT-HSC function, aged (55-65-week-old) *Vav-Atmin*<sup>ΔΔ</sup> mice continued to have reduced BM cellularity (Figure S3G) with

similar defects in aged ATMIN-deficient B cells as reported<sup>8</sup> (data not shown) and a reduced CMP compartment (Figure S3H). Although the numbers of short-term (ST)-HSCs and multipotent progenitors (MPPs) were not altered in aged ATMIN-deficient mice, the proportion of these sub-populations was increased, perhaps due to increased cell cycling in younger but not in aged mice (Figures 2I-J and S3H-I). As in young mice, ATMIN-deficient primitive cells from aged mice had a reduced capacity to produce CAFs and LTC-IC colonies (Figure 2K-L) and LT-HSCs lacking ATMIN were unable to provide long-term reconstitution (Figure 2M), although BM homing was unaffected (Figure 2N). There were no apparent signs of senescence (such as increased *p16<sup>Ink4a</sup>* and *p19<sup>Arf</sup>* expression) in any of the aged ATMIN-deficient primitive sub-populations (data not shown) and no increase in ROS production in ATMIN-deficient LT-HSCs was observed (Figure S3J), unlike in *Atm*<sup>-/-</sup> mice<sup>12,13</sup>. Contrary to young cells, we observed a significant increase in expression of DNA oxidation repair genes in aged ATMIN-deficient LT-HSCs, as well as an increase in pKap1, suggesting that both aging and ATMIN deficiency are required to stimulate this response (Figure 2O-P). Regeneration of PB hematopoiesis after myelo-suppressive 5-FU treatment was much weaker in ATMIN-deficient compared with control mice (Figure 2Q). ATMIN-deficient LT-HSCs cycled less and showed an increased DNA damage response compared with control cells at day 7 post-5-FU treatment (Figure 2R-S). *Vav-Atmin*<sup>ΔΔ</sup> mice succumbed to multiple doses of 5-FU faster than *Atmin*<sup>ff</sup> mice (Figure 2T).

We reveal ATMIN as a novel player with multiple functions in hematopoiesis, that are both ATM-dependent and -independent. We have



uncovered an important ATM-independent function of ATMIN in the CMP compartment. ATMIN-deficient mice also have impaired LT-HSC function during regeneration and aging, most likely due to exhaustion from compensating for the reduced/damaged lymphoid and myeloid compartments over time. It would be interesting to dissect other possible roles that ATMIN might have in myeloid development with more specific lineage gene deletion approaches, as myeloid cells can differentiate without passing the CMP stage<sup>14-16</sup>.

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### **Author contributions**

F.A-A: conceived, designed and performed experiments, and wrote the manuscript. J.I.L: provided mice and conceived and performed some experiments. A.Br. performed some experiments. S.P. optimized, performed and analyzed data related to ImageStream experiments. N.K: provided mice and helped designing experiments; C.D-C: maintained and re-derived mouse colony; D.B. and A.Be. conceived the study.

## **Conflict of interest**

There is no conflict of interest to declare.

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## Figure legends

**Figure 1. ATMIN-deficient mice have a reduced CMP compartment due to increased apoptosis.** Black bars/symbols represent *Atmin*<sup>ff</sup> and white *Vav-Atmin*<sup>ΔΔ</sup> unless otherwise indicated. **(A)** DCFDA stains for ROS detection showing a mild increase in the percentage of ROS-positive cells in ATMIN-deficient CMPs (n=3/genotype). **(B)** Treating total BM cells with the antioxidants NAC or γ-GCE (not shown) was unable to rescue the myeloid colony formation (CFU) defect of *Vav-Atmin*<sup>ΔΔ</sup> cells (n=5/genotype). **(C)** Expression of key anti-oxidant genes was unchanged between ATMIN-deficient and control CMPs. Expression values were determined by qRT-PCR and were relative to the mean (n=3-5/genotype). No significant increase in pKap1 levels (indicative of ATM activation) **(D)**, upregulation of genes required for DNA oxidation repair **(E)**, or changes in the indicated CMP developmental factors **(F)** were detected in ATMIN-deficient compared with control CMPs. pKap1 levels were determined by intracellular flow cytometry analysis (MFI ratios are shown) and qRT-PCR was used to determine the expression of the indicated genes (n=5-6/genotype). **(G)** The frequency of late apoptosis was significantly increased and **(H)** *Dynll1* expression was drastically reduced in CMPs lacking ATMIN compared with control cells (n=4-6/genotype). **(I)** Representative cell images captured by an ImageStream flow cytometer in green and red channels followed by their respective composite images, showing higher co-localization of mitochondria (red) with Bim (green) staining in *Vav-Atmin*<sup>ΔΔ</sup> CMPs. **(J)** Representative MitoTrackerRed-CMXRox and Bim similarity staining score histograms for *Atmin*<sup>ff</sup> (black) and *Vav-Atmin*<sup>ΔΔ</sup> (open)-derived CMPs respectively. A score above value 2 (R1) indicates translocation of Bim into mitochondria. **(K)** Percentage of Bim translocation in CMPs (n=3/genotype). The different BM hematopoietic stem

and progenitor populations were defined as follows: LT-HSC ((L<sup>-</sup>S<sup>+</sup>K<sup>+</sup> (Lineage<sup>-</sup>Sca-1<sup>+</sup>cKit<sup>+</sup>) CD34<sup>-/lo</sup>Flt3<sup>-</sup>); ST-HSC (L<sup>-</sup>S<sup>+</sup>K<sup>+</sup> CD34<sup>+</sup>Flt3<sup>-</sup>); MPP (L<sup>-</sup>S<sup>+</sup>K<sup>+</sup>CD34<sup>+</sup>Flt3<sup>+</sup>); CMP (L<sup>-</sup>S<sup>-</sup>K<sup>+</sup>IL7R<sup>-</sup>CD34<sup>+</sup>FcyR<sup>-</sup>); GMP (granulocyte-monocyte progenitor, L<sup>-</sup>S<sup>-</sup>K<sup>+</sup>IL7R<sup>-</sup>CD34<sup>+</sup>FcyR<sup>+</sup>); MEP (megakaryocytic-erythroid progenitor; L<sup>-</sup>S<sup>-</sup>K<sup>+</sup>IL7R<sup>-</sup>CD34<sup>-</sup>FcyR<sup>-</sup>); CLP (L<sup>-</sup>S<sup>-</sup>K<sup>lo</sup>IL7R<sup>+</sup>). Mean (SD) values are shown. \*, P<0.03; \*\*\*, P<0.0003.

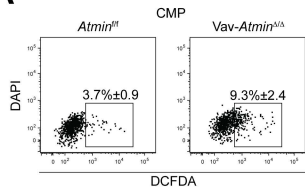
**Figure 2. Vav-Atmin<sup>ΔΔ</sup> LT-HSCs have impaired functions during regeneration and aging.** Experiments were performed with young (**A-H** and **Q-T**) and aged (**I-P**) BM cells. Black bars/symbols and lines represent *Atmin*<sup>ff</sup> and white bars/symbols and grey lines *Vav-Atmin*<sup>ΔΔ</sup> unless otherwise indicated. (**A, I**) Cell cycle distribution was determined using Ki67/DAPI stains in BM LT-HSCs (n=4/genotype). (**B, J**) qRT-PCR for the expression of *Cdkn1c* and *Rbl2* in LT-HSCs. Expression values are relative to the mean of control (n=3-5/genotype). Other cell cycle regulators were unchanged between ATMIN-deficient and control cells (data not shown). (**C, K**) To estimate the stem/progenitor cell capacity of the more primitive cells, the frequency of L<sup>-</sup>S<sup>+</sup>K<sup>+</sup> cells to form cobblestone area-forming cells (CAFCs) was determined by limiting dilution 5 wks after seeding on MS5 stroma. Five week long-term culture-initiating cell (LTC-IC) -derived colonies were also determined (**D, L**; n=3/genotype). (**E**) To evaluate the regeneration capacity of LT-HSCs, peripheral blood (PB) chimerism was determined at different time points from sub-lethally irradiated NSG mice (non-obese diabetic/severe combined immunodeficiency disease/interleukin-2 receptor γ-chain-null; CD45.1 due to the mixed background) that were transplanted with 15 young LT-HSCs from either *Atmin*<sup>ff</sup> or *Vav-Atmin*<sup>ΔΔ</sup> mice (CD45.2). After 16 wks, 15 LT-HSCs were sorted by flow cytometry from primary mice and re-transplanted into separate secondary recipients and PB chimerism was determined at different time points. (**M**) PB chimerism was also determined from sub-lethally irradiated NSG mice transplanted with 15 aged LT-HSCs from either *Atmin*<sup>ff</sup> or *Vav-Atmin*<sup>ΔΔ</sup> mice. (**F, N**) Percentage of engrafted LT-HSCs 16h after intravenous injection of total *Atmin*<sup>ff</sup> or *Vav-Atmin*<sup>ΔΔ</sup> BM mononuclear cells (n=2 independent experiments). Each symbol indicates a

mouse and horizontal lines represent median reconstitution levels. **(G, O)** qRT-PCR for the expression of the indicated DNA oxidation repair genes in LT-HSCs was also evaluated. Expression values were relative to the mean of each control sub-population (n=3/genotype). **(H, P)** pKap1 expression in LT-HSCs was determined by intracellular flow cytometry analysis. Grey and open histograms represent isotype-matched control and pKap1 stains respectively (n=3/genotype). **(Q)** Hematopoietic reconstitution was monitored by serial PB counts in mice injected with a single dose of 5-FU. Total WBC counts are shown (n=5-6/genotype). Cell cycle status **(R)** and pKap1 expression **(S)** in ATMIN-deficient and control LT-HSCs at day 7 after a single dose of 5-FU administration (n=4-5/genotype). **(T)** Kaplan-Meier survival curve of *Atmin*<sup>ff</sup> and *Vav-Atmin*<sup>Δ/Δ</sup> mice injected 3 times (arrows) with 5-FU (n=7-8/genotype). Unless stated, mean (SD) values are shown.

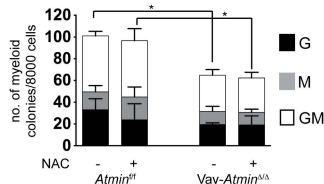
\*, P<0.03; \*\*, P<0.003; \*\*\*, P<0.0003.

# Figure 1

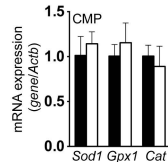
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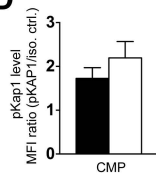
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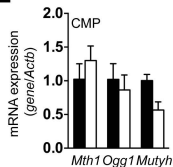
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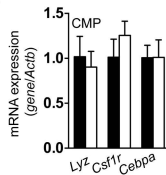
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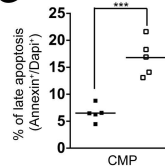
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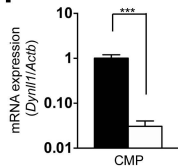
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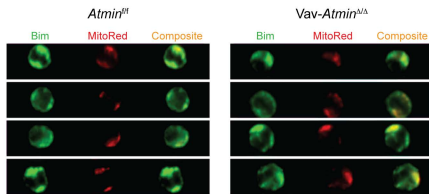
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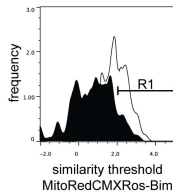
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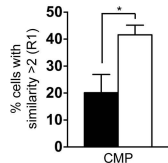
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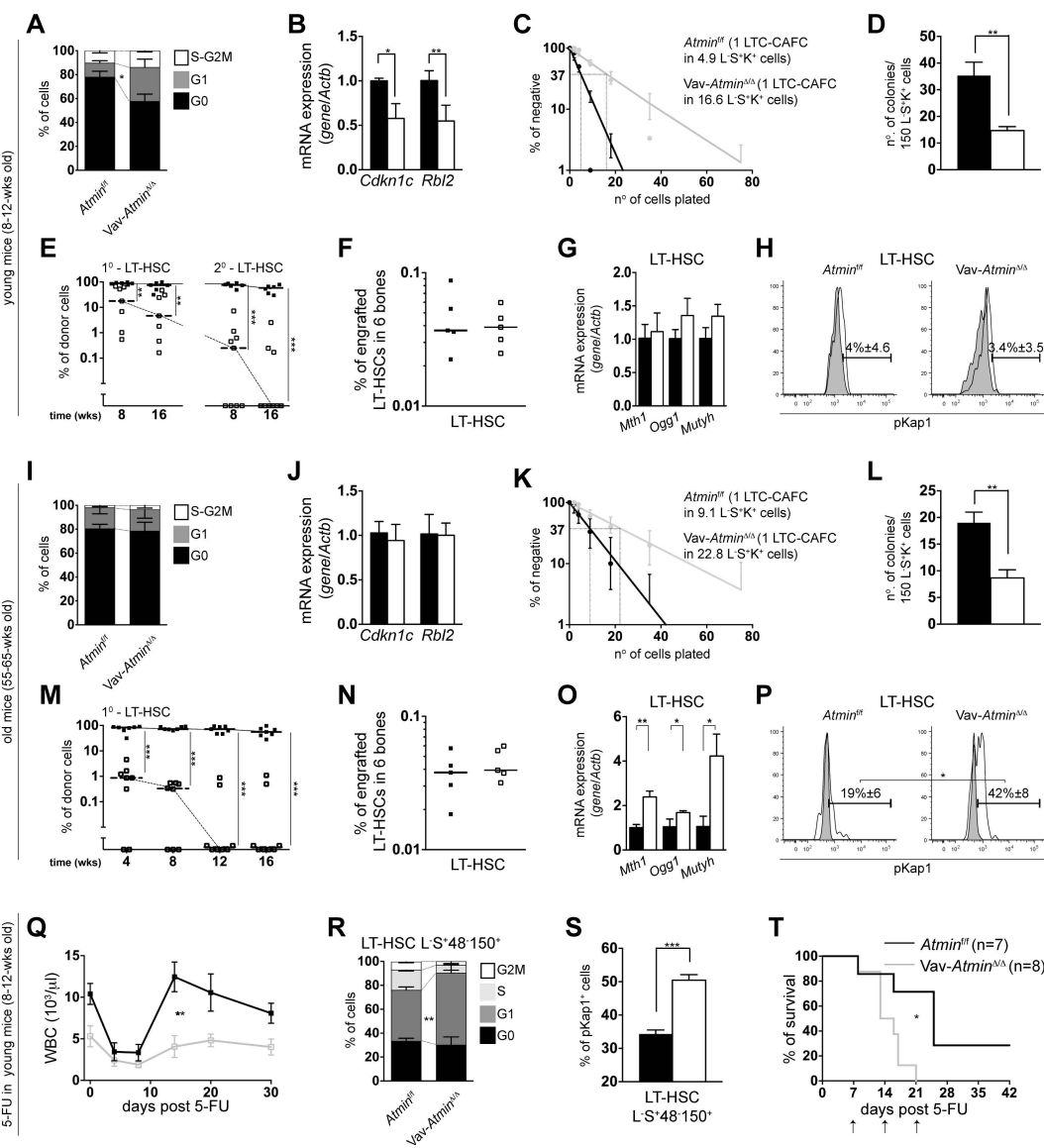


**K**



■ *Atmin*<sup>fl/fl</sup>

□ *Vav-Atmin*<sup>Δ/Δ</sup>

**Figure 2**



## Supplementary Figures

**Figure S1. ATMIN-deficient mice are leukopenic.** (A) Genotyping of *Atmin* WT, floxed and  $\Delta$  alleles by PCR from tail, thymus, spleen and bone marrow (BM) of *Atmin*<sup>ff</sup>, *Atmin*<sup>fl/+</sup> and *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice. (B) Representative western blot using *Atmin*<sup>ff</sup> and *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  bone marrow lysates. Adult *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice are leukopenic but not anemic as shown by (C) the reduced absolute peripheral blood leukocyte (CD45<sup>+</sup>), B cell, T cell, granulocyte and (D) red blood cell counts respectively (n=6/genotype; 8-12-wks old). Mean (SD) values are shown. \*, P<0.03; \*\*\*, P<0.0003.

**Figure S2. ATMIN-deficient mice have B cell lymphopenia.** (A) Analysis of total B cell (B220<sup>+</sup>) numbers in the spleens of *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice and *Atmin*<sup>ff</sup> litter-mates (8-12-wk-old; n=5/genotype) showing a ~75% reduction in total B cell numbers in *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice compared with control mice. This was accompanied by a significant reduction in (B) the frequency of immature and re-circulating B cells (n=5/genotype) and (C) loss of germinal centre (GC) and red pulp regions (RP; representative H&E stainings of spleens are shown). (D) No increase in intracellular reactive oxygen species (ROS) was detected in the different *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  B cell sub-populations (n=3/genotype). (E) A significant 2-fold increase in early apoptosis was observed in all the different B cell sub-sets analyzed (each dot represents a mouse). (F) Expression of *Dynl1* (a direct ATMIN transcriptional target<sup>6</sup> responsible for sequestering the pro-apoptotic protein Bim<sup>12</sup>) was reduced in *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  cells. Expression values were determined by qRT-PCR and were relative to the mean of each control population (n=3-5/genotype). (G, H) *Atmin*-deleted B cells show an increase in pKap1 (an ATM substrate) and  $\gamma$ H2AX compared with control B cells. Expressions were determined by intracellular flow cytometry analysis and mean fluorescence intensity (MFI) ratios are shown. (I) Total B cell numbers are significantly reduced in the BM of *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice compared with *Atmin*<sup>ff</sup> littermates (from two tibiae, two femurs and two iliac crests; n=6-7/genotype). Pre-B cell counts are reduced by 50%. (J) No accumulation of other primitive B cell sub-sets was observed in the absence of ATMIN. (K) BM

cells were unable to efficiently generate pre-B cell colonies *in vitro* (n=3/genotype; in triplicate each). Mean (SD) values are shown.

\*, P<0.03; \*\*, P<0.003; \*\*\*, P<0.0003.

**Figure S3. Vav-*Atmin*<sup>Δ/Δ</sup> LT-HSCs have impaired functions during aging and regeneration after transplantation or 5-FU treatment.** (A, G) Total cellularity and absolute numbers of the indicated sub-populations were determined in the BM of *Atmin*<sup>fl/fl</sup> and Vav-*Atmin*<sup>Δ/Δ</sup> mice (from two tibiae, two femurs and two iliac crests; n=5-6/genotype). (B, H) Frequencies of the different primitive BM stem/progenitor sub-populations (n=6-8/genotype) were also analyzed. Horizontal bars indicate the mean. (C, I) Cell cycle distribution was determined using Ki67/DAPI stains in the indicated BM stem/progenitor sub-populations (n=4/genotype). (D) Contribution of the transplanted primary LT-HSCs (as shown in Figure 2E) to the B, T and granulocyte cell lineages. Each symbol indicates a mouse and horizontal lines represent median reconstitution levels. (E, J) As *Atm*<sup>-/-</sup> LT-HSCs have 10-fold elevated ROS compared to WT cells<sup>10</sup>, we investigated ROS production in *Atmin*<sup>fl/fl</sup> or Vav-*Atmin*<sup>Δ/Δ</sup> LT-HSCs. DCFDA stains for ROS detection are shown. Values indicate the percentage of ROS-positive cells (n=3/genotype). (F) Frequency of late apoptosis in young LT-HSCs (n=4-6/genotype). Mean (SD) values are shown. \*, P<0.03; \*\*, P<0.003.

## Supplementary Materials and Methods

### Mice

**NSG mice:** Nonobese diabetic/severe combined immunodeficiency disease/interleukin-2 receptor  $\gamma$ -chain–null (NOD/SCID IL2R $\gamma^{\text{null}}$ ; NSG) mice were originally obtained from Dr Leonard Schultz (Jackson Laboratory) and bred in the London Research Institute Biological Resources Unit. Mice were kept in micro-isolators and fed sterile food and acidified water. All experimental procedures were approved by London Research Institute ethics committees and conform to the UK Home Office regulations.

### Bone marrow transplantation and 5-FU treatment

NSG mice aged 8 to 12 weeks were irradiated at 3.75 Gy ( $^{137}\text{Cs}$  source) up to 24 hours before tail vein injection of flow cytometry-purified primary cell populations. For secondary transplants, two NSG mice that received LT-HSCs from either *Atmin*<sup>ff</sup> or *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice were randomly pooled and sorted again for LT-HSCs and injected into two sub-lethally irradiated NSG mice. *Atmin*<sup>ff</sup> and *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice aged 8 to 12 weeks received a single dose of 5-FU (150 mg/Kg body weight) and were analyzed for cell cycle distribution, pKap1 expression and different white blood cell counts. Survival of mice was determined after 3 injections of 5-FU. For homing experiments, *Atmin*<sup>ff</sup> or *Vav-Atmin* <sup>$\Delta/\Delta$</sup>  mice were sacrificed and single cell suspensions of the BM were used for transplantation. BM mononuclear cells equivalent to 1000 LT-HSCs were injected through the tail vein into sub-lethally irradiated NSG recipient mice. Sixteen hours after transplant, the recipient mice were sacrificed and the BM cells of the lower limbs and pelvis were harvested and homing of immunophenotypically identified LT-HSC was performed as described.

## **Hematopoietic analyses**

Single-cell suspensions were first obtained from different hematopoietic tissues, then red blood cells were lysed and samples stained for 30 min. at 4°C with the indicated antibodies in PBS supplemented with 2% FCS (PBS/2%). DAPI was added to exclude dead cells in the flow cytometry analysis. For complete blood counts, peripheral blood was collected from the tail vein into tubes containing K<sub>2</sub>EDTA. Twenty-five µl of blood was stained for different leukocyte antigens as described then without washing diluted to 1 ml and analysed in a MACSQuant analyser (Miltenyi Biotec). For red blood cell counts, blood was diluted 1:1000 and red cells were counted in a hemocytometer. The following antibodies (from BD Bioscience and eBioscience) were used: Lineage cocktail, CD45.1 (A20), CD45.2 (104), B220 (RA3-6B2), Ly-6G (Gr1; RB6-8C5), CD3 (17A2), Sca-1 (D7), CD117 (c-Kit; 2B8), CD34 (RAM34), CD16/32 (93), CD43 (S7), IgM (II/41), CD135 (Flt3; A2F10), CD127 (IL7R $\alpha$ ; A7R34) and AnnexinV.

## **Analysis of intracellular ROS and mitochondria.**

Cells were incubated with the ROS detection agent DCFDA (carboxy-H<sub>2</sub>DFFDA C13293; Life Technologies) at 5 µM or MitoTrackerRed-CMXRos (M7512; Life Technologies) at 12.5 nM and incubated at 37°C for 30 min. After this period cells were washed with PBS/2% FBS containing trypan blue to quench nonspecific staining.

## **Gene Expression Analysis**

RNA was extracted using the RNeasy Micro Kit (Qiagen, Inc.) and treated with DNase. Reverse transcription was performed using the SensiScript Kit (Qiagen) according to the manufacturer's instructions. For quantitative real-time polymerase chain reaction (qRT-PCR), SybrGreen master mix reagent (Applied BioSystems) was used and the amplification was done using the ABI Prism 7700 sequence detection system (Applied BioSystems). To avoid possible amplification of contaminating DNA and unprocessed mRNA, where possible, primers were designed to anneal the end parts of two exons. The specificity of the PCR products was verified by

running a 2% agarose gel, in addition to using the dissociation curve software from the qRT-PCR system. Primers used are listed in Table S1.

### **CAFC and LTC-IC-CFC assays**

Briefly, FACS purified L<sup>-</sup>S<sup>+</sup>K<sup>+</sup> cells were co-cultured as bulk or using a limiting dilution analysis (LDA) both on 80% confluent monolayer of MS5 stromal cells with M5300 media (Stemcell Technologies) supplemented with Pen/Strep and hydrocortisone at 10<sup>-6</sup> M. Cells were cultured at 37°C in 5% CO<sub>2</sub> -humidified incubators for 5 wks with weekly half-media change. For the LDA CAFC assay cells were plated in 96-well microplates and wells were scored for the presence of CAFCs. To determine the frequency, LDA was calculated using the extreme limiting dilution analysis from WEHI (Walter and Eliza Hall Institute) bioinformatics website according to the Poisson statistics and method of maximum likelihood. For LTC-IC-derived colonies, cells were recovered after 5 wks of co-culture and plated on standard methylcellulose colony assay media as described below.

### **Colony assays**

Standard methylcellulose colony assay media, MethoCult<sup>®</sup> GF M3534 was used according to the manufacturer's instructions. NAC (N-acetyl cysteine; Sigma) or  $\gamma$ -GCE (glutathione monoethyl ester; Calbiochem) was added to the methylcellulose at 100  $\mu$ M or 2 mM respectively when cells were seeded and then at day 5. Colonies were scored at day 10.

### **Immunohistochemistry**

Tissue was fixed overnight in 10% neutral buffered formalin, briefly washed with PBS and transferred into 70% ethanol, processed and embedded into paraffin. Sections were cut at 4  $\mu$ m for H&E staining.

### **Western blotting**

Cells were extracted in RIPA lysis buffer (NEB) supplemented with protease inhibitors (Sigma). Western blots were performed using standard procedures. Protein samples were separated by SDS-PAGE, and

subsequently transferred onto nitrocellulose membranes. All primary antibodies were used at 1:1000 dilution and secondary antibodies at 1:2000. The following antibodies were used: p-S824-Kap1 (Bethyl Laboratories, Inc); ATMIN, (Millipore);  $\beta$ -actin (Sigma);  $\alpha$ -tubulin (Abcam); HRP-conjugated goat anti-mouse/rabbit IgG (Sigma).

### **ImageStream analysis**

Cells were stained first for extracellular antigens followed by mitochondria staining. Cells were then fixed, permeabilized and stained for Bim as described above. Samples were analyzed on an ASSIST calibrated Dual Camera, 12 Channel ImagestreamX Mark II Imaging Flow Cytometer (Amnis, Merck Millipore) using INSPIRE software (Amnis, Merck Millipore). Images were collected using a 60x objective lens and sample data were acquired on a low speed and high sensitivity setting. Fluorescence was measured from 405nm, 488nm and 642nm lasers. Laser powers were set to 30mW, 50mW and 30mW respectively. A dot-plot of area vs aspect ratio for Brightfield Ch01 was gated to exclude debris during data acquisition. Single stained cells were measured and a compensation matrix created; the acquired data were compensated and analysed using IDEAS software (Amnis, Merck Millipore). Gates were set to include single, focused cells and then gated for cells which were dual stained with MitoTrackerRed-CMXRos (Ch04) and Bim AlexaFluor 488 (Ch02). The “similarity” feature was used to assess the colocalization of the MitotrackerRed-CMXRos and Bim on selected cell populations. The following modified feature and mask was used to measure and define the region where the colocalization of the 2 dyes occurs: Similarity Threshold: Similarity Threshold ((Object (M04, Ch04 MitoCMXRos, Tight), Ch04 MitoCMXRos: 60))\_Ch02 Bim AF488\_Ch04 MitoCMXRos. Cells which scored more than 2 on this feature were assessed to have high Bim/mitochondria colocalization.

**Statistical analysis**

T-tests were used for most analyses. Statistical analysis of the engraftment data was performed using the Mann-Whitney test. The P value for the Kaplan-Meier survival curve was calculated using the Mantel-Cox test.

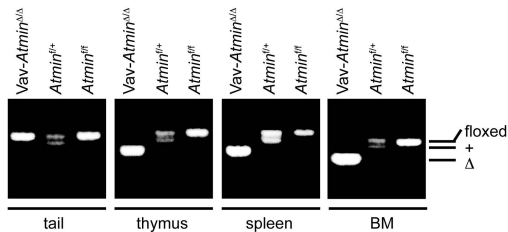
**Table S1. List of primers used in this study**

GENOTYPING	FORWARD	REVERSE
<i>Atmin</i> Lox6133	5'-TCAGCATCTTCTCCAGAGAGACAG-3'	5'-CACATGTGTACAGCACATTCATTG-3'
<i>Atmin</i> Lox6617		5'-CTCAGGGTACACATACTATGCTTGC-3'
<i>Atmin</i> Lox10252		5'-ATCAGCCACACCAGACACAGAGATC-3'
Vav-cre	5'-AGATGCCAGGACATCAGGAACCTG-3'	
qRT-PCR	FORWARD	REVERSE
<i>Cdkn1a</i> (p21)	5'-GTGGCCTTGTCTGCTGCTT-3'	5'-GCGCTTGGAGTGATAGAAATCTG-3'
<i>Cdkn1b</i> (p27)	5'-TGGGTAGCGGAGCAGTGT-3'	5'-TGTTCTGTTGGCCCTTTTGT-3'
<i>Cdkn1c</i> (p57)	5'-CGCAAACGTCTGAGATGAGT-3'	5'-TCCGGTTCCTGCTACATGAA-3'
<i>Cdkn2c</i> (p18)	5'-GCAAAATAATGTAAACGTCAACG -3'	5'-AAATTGGGATTAGCACCTCTGAG-3'
<i>Rbl2</i>	5'-GCTCCTTACACGACGGTCTAGT-3'	5'-GCGGCTAACACGTATTCTTCA-3'
<i>E2F4</i>	5'-TGGCCTACGTGACTCATGAAG-3'	5'-TACTTCTTCTGGCCATTGAGAC-3'
<i>Cdkn2a</i> (p16 <sup>Ink4a</sup> )	5'-AACTCTTTCGGTCGTACCCC-3'	5'-CGAATCTGCACCGTAGTTGAG-3'
<i>Cdkn2a</i> (p19 <sup>Arf</sup> )	5'-GCTCTGGCTTTCGTGAACAT-3'	5'-CGAATCTGCACCGTAGTTGAG-3'
<i>Dynl1</i>	5'-CTCTGCTCCACGGTAACCAT-3'	5'-TGTTGTACTTCTCCAACGCC-3'
<i>Lyz</i>	5'-GGATATTCAGATCAATAGCCGA-3'	5'-CACCTCTTTGCACATTGTATG-3'
<i>Csf1R</i>	5'-AGCACCTTGACCACAAGAAAC-3'	5'-TGTGCCAGCAAATCCAAGA-3'
<i>Cebpa</i>	5'-AACAGCAACGAGTACCGGGTA-3'	5'-TCATTGTCACTGGTCAACTCC-3'
<i>Sod1</i>	5'-GAGACCTGGGCAATGTGACT-3'	5'-CACCTTTGCCCAAGTCATCT-3'
<i>Cat</i>	5'-TGGCACACTTTGACAGAGAGC-3'	5'-CCTTTGCCTTGGAGTATCTGG-3'
<i>Gpx1</i>	5'-GATGAACGATCTGCAGAAAGC-3'	5'-CGGACGTACTTGAGGGAATTC-3'
<i>Mth1</i>	5'-CAGGAAGGAGAGACCATTGAGA-3'	5'-GTCAGCCGAGAAGATATGCAC-3'
<i>Ogg1</i>	5'-CTAGCAGCATGAGACATCGC-3'	5'-ATACTTGATCTGCCAGCACG-3'
<i>Mutyh</i>	5'-GTATGACCAAGAGAAGCGTGAC-3'	5'-GCATAACCTCTGACACCCACAC-3'
<i>Actb</i>	5'-CTGTATCCCCCTCCATCGTG-3'	5'-CGTCCCAGTTGGTAACAATG-3'

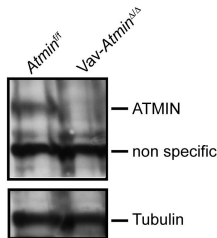


# Figure S1

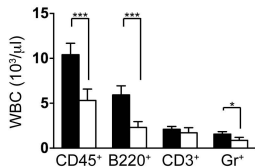
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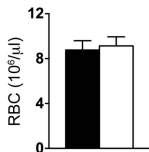
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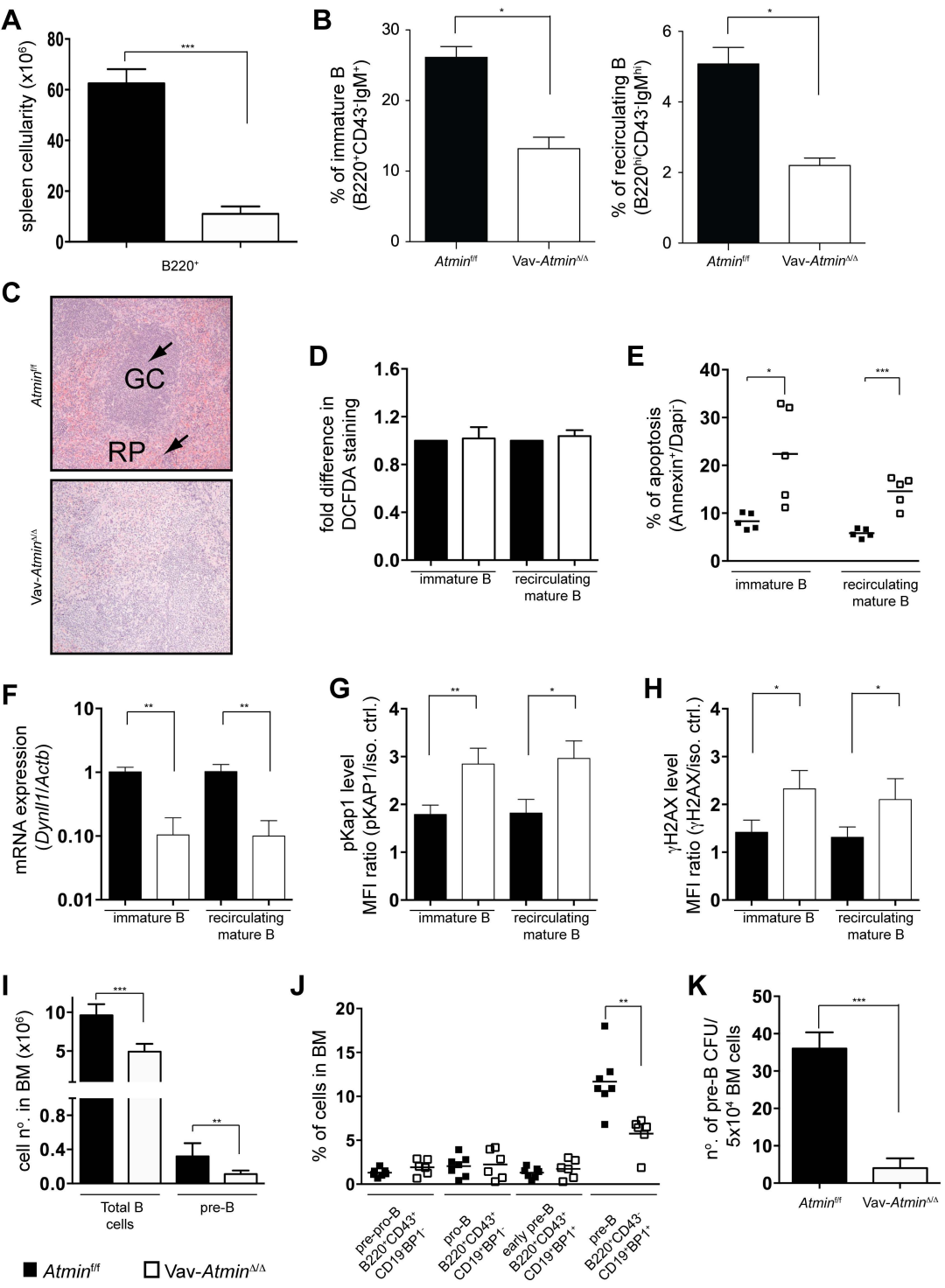


**C**



**D**



**Figure S2**

# Figure S3

