SUPPLEMENTAL INFORMATION

Hematopoietic Stem Cells Preferentially Traffic Misfolded Proteins to Aggresomes and Depend on Aggrephagy to Maintain Protein Homeostasis

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Figure S1 (Related to Figure 1)

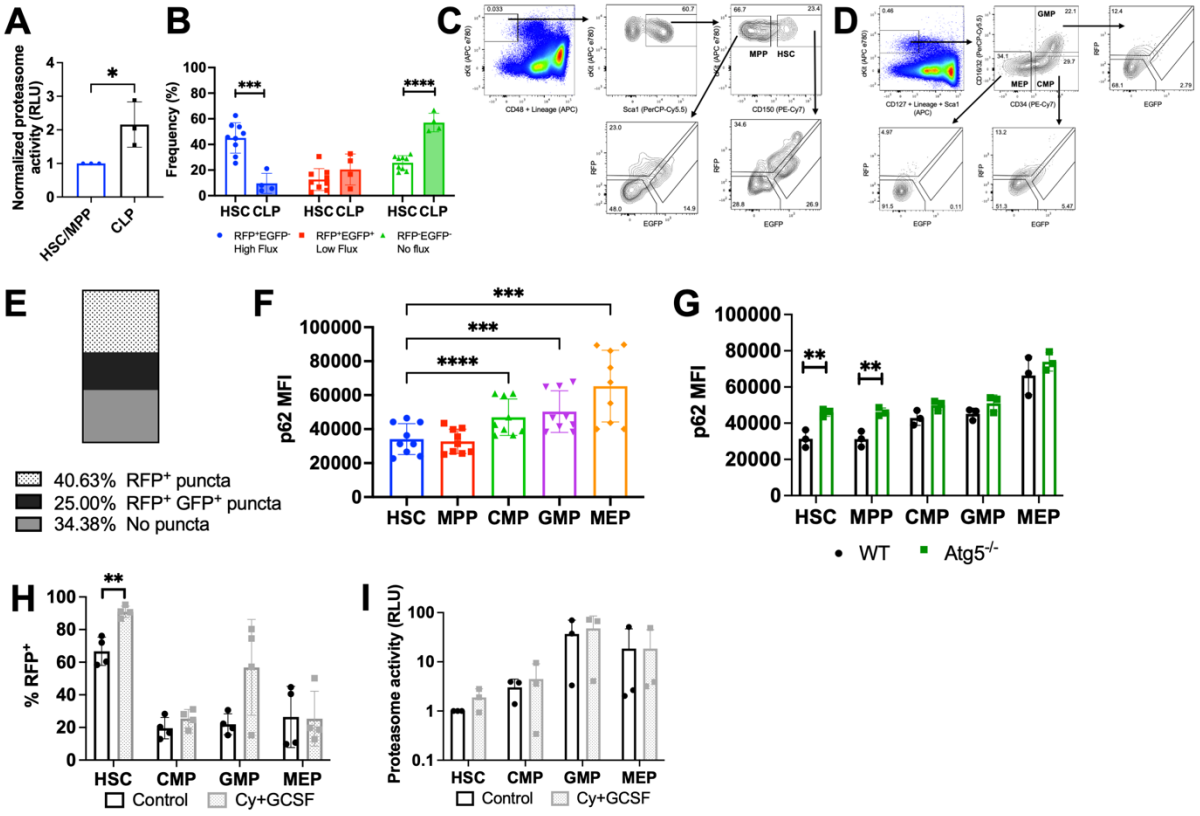

Figure S1. Identification of autophagic HSCs and progenitors using flow cytometry. Related to Figure 1.

A Proteasome activity in CD48$^-$LSK HSC/MPPs as compared to CLPs. Data show technical replicates and mean ± SD; n=3. Data were assessed using a student's t-test.

B Frequency of HSCs and CLPs exhibiting high, low, and no autophagic flux. Data show technical replicates and mean ± SD; n=4-9. Data were assessed using a student's t-test.

C-D. Gating strategy to identify HSCs and MPPs (C) and myeloid progenitors (D) in CAG-RFP-EGFP-LC3 mice.

E Frequency of HSCs exhibiting RFP$^+$, RFP$^+$EGFP$^+$, and no puncta as analyzed by confocal microscopy. Individual HSCs were assessed; n=30.

F p62 (mean fluorescence intensity; MFI) in HSCs and progenitors at steady state in vivo. Data show individual mice and mean ± SD; n=9. Data were assessed by one-way ANOVA with Dunnett's test relative to HSCs.

G p62 (MFI) in WT and *Atg5*$^{-/-}$ cells at steady state in vivo. Data show individual mice and mean ± SD; n=3. Data were assessed using a paired student's t-test.

H Frequency of cells exhibiting autophagic activity (RFP^+) in CAG-RFP-EGFP-LC3 mice treated with vehicle (PBS; control) or cyclophosphamide and GCSF. Data show individual mice and mean ± SD; n=4. Data were assessed using a paired student's t-test.

I Proteasome activity in hematopoietic stem and progenitor cells from mice treated with vehicle (PBS; control) or cyclophosphamide and GCSF. Data show technical replicates and mean ± SD; n=3. Data were assessed using a student's t-test.

*P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001.

Figure S2 (Related to Figure 2)

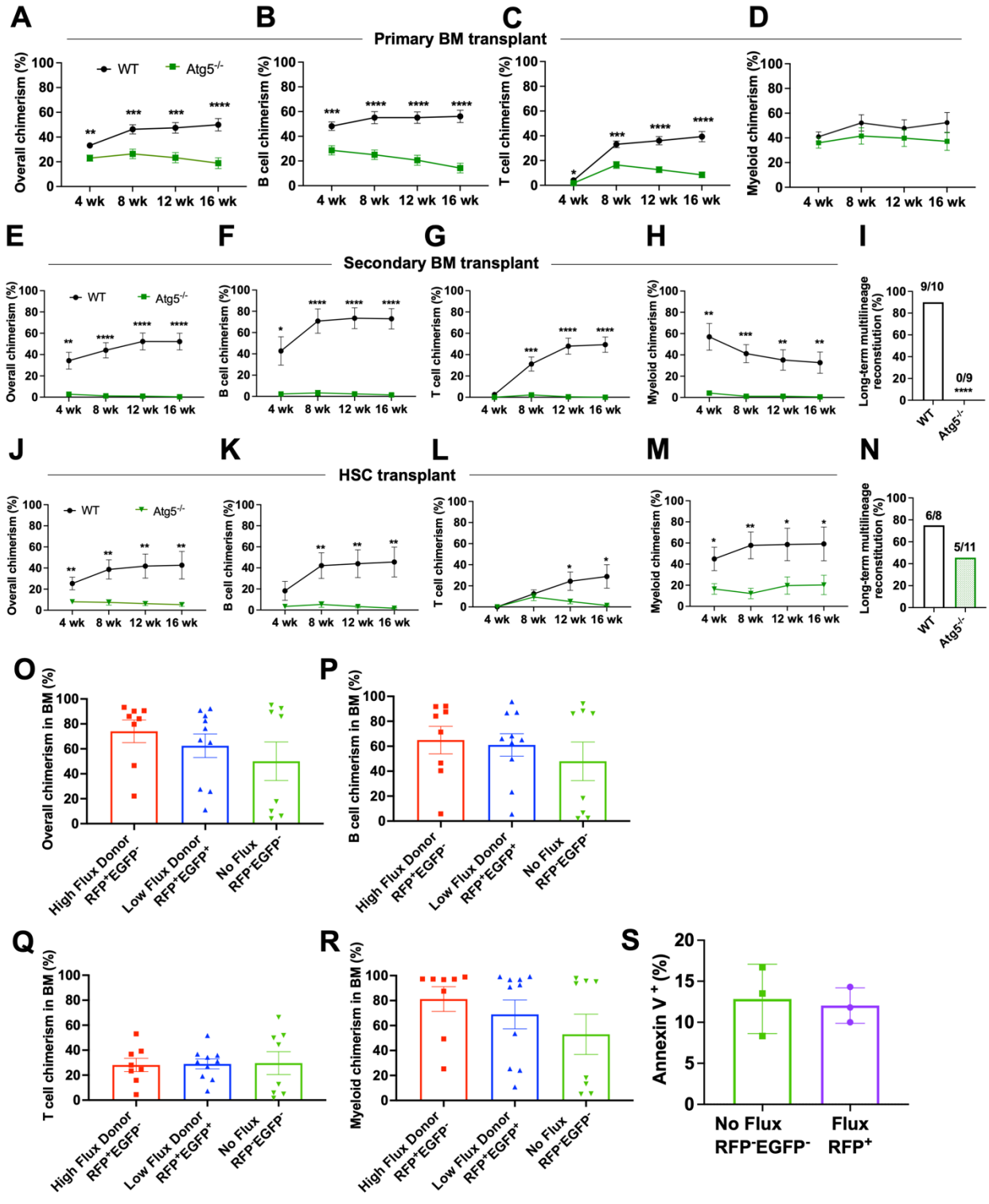

Figure S2. HSCs are not fixed in their autophagic potential. Related to Figure 2.

A-D Donor hematopoietic (A), B (B), T (C), and myeloid (D) cell engraftment in peripheral blood of mice transplanted with $5x10^5$ WT or *Atg5*$^{-/-}$ bone marrow cells with $5x10^5$ competitor bone marrow cells; n=15 recipient mice/genotype from 3 independent experiments. Data show mean ± SEM and were assessed using a student's t-test.

E-H Donor hematopoietic (E), B (F), T (G), and myeloid (H) cell engraftment after transplantation of $3x10^6$ bone marrow cells from primary recipients of A-D into secondary recipients; n=9-10 recipient mice/genotype from 3 independent experiments. Data show mean ± SEM and were assessed using a student's t-test.

I Frequency of secondary recipients in E-H that exhibited long-term multilineage reconstitution. Data were assessed using a chi-squared test.

J-M Donor hematopoietic (J), B (K), T (L), and myeloid (M) cell engraftment in peripheral blood of mice transplanted with 25 vehicle WT or *Atg5*$^{-/-}$ HSCs together with $3x10^5$ competitor bone marrow cells; n=8-11 recipient mice/genotype from 2 independent experiments. Data show mean ± SEM and were assessed using a student's t-test.

N Frequency of recipients from J-M that exhibited long-term multilineage reconstitution. Data were assessed using a chi-squared test.

O-R Long-term donor hematopoietic (O), B (P), T (Q), and myeloid (R) cell engraftment in bone marrow of mice transplanted with 25 RFP^+EGFP^- high flux, RFP^+EGFP^+ low flux, or RFP^-EGFP^- no flux HSCs together with $3x10^5$ competitor bone marrow cells measured 16 weeks post-transplant. Data show individual recipients and mean ± SEM; n=10-13 recipients/HSC subset from 3 independent experiments (these are individual mice from Fig. 2B-F). Data were assessed by one-way repeated measures ANOVA.

S Frequency of Annexin V^+ cells in non-autophagic (RFP^-EGFP^-) and autophagic (RFP^+) HSCs. Data show individual mice ± SD; n=3. Data were assessed by a paired student's t-test.

*P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001.

Figure S3 (Related to Figure 3)

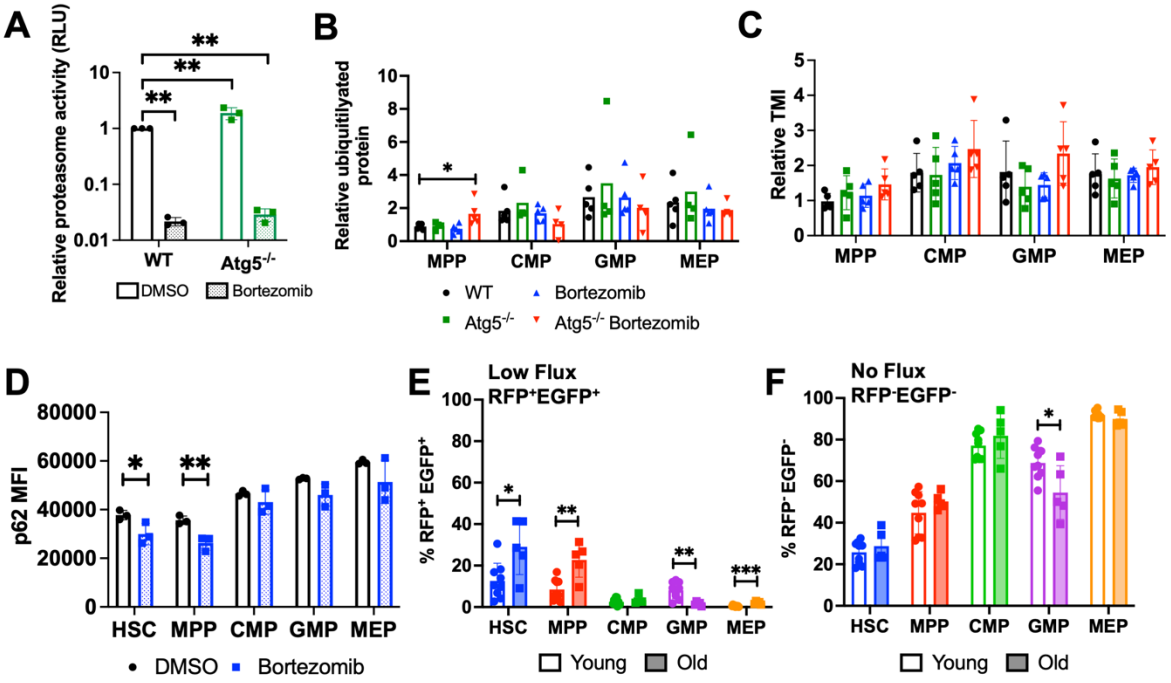

Figure S3. Inhibition of proteasome and autophagy have minimal effects on protein quality control in restricted myeloid progenitors. Related to Figure 3.

A Proteasome activity in WT and *Atg5*$^{-/-}$ LSK cells after treatment with DMSO or bortezomib. Data show technical replicates and mean ± SD; n=3. Data were assessed by one-way ANOVA with Dunnett's test relative to WT DMSO.

B Relative ubiquitylated (misfolded) protein in vehicle (DMSO)-treated WT, vehicle-treated *Atg5*$^{-/-}$, bortezomib-treated WT, and bortezomib-treated *Atg5*$^{-/-}$ progenitors. Values are normalized to WT HSCs. Data show individual mice and mean ± SD; n=4-5/condition from 5 experiments. Data were assessed by one-way ANOVA with Dunnett's test relative to WT DMSO controls.

C Relative TMI (unfolded proteins) in vehicle-treated WT, vehicle-treated *Atg5*$^{-/-}$, bortezomib-treated wildtype, and bortezomib-treated *Atg5*$^{-/-}$ progenitors Values are normalized to WT HSCs. Data show individual mice and mean ± SD; n=5 from 5 experiments. Data were assessed by one-way ANOVA with Dunnett's test relative to WT DMSO controls.

D p62 (MFI) in HSCs and progenitors from mice treated with vehicle (DMSO) or bortezomib. Data show individual mice and mean ± SD; n=3. Data were assessed using a paired student's t-test.

E-F Frequency of young (3 month) and old (22-24 month) adult cells exhibiting low (RFP^+EGFP^+) (E) and no (RFP^-EGFP^-) (F) autophagic flux. Data show individual mice and mean ± SD; n=5-9. Data were assessed using a student's t-test.
*P≤ 0.05, **P≤ 0.01, ***P≤ 0.001.

Figure S4 (Related to Figure 4)

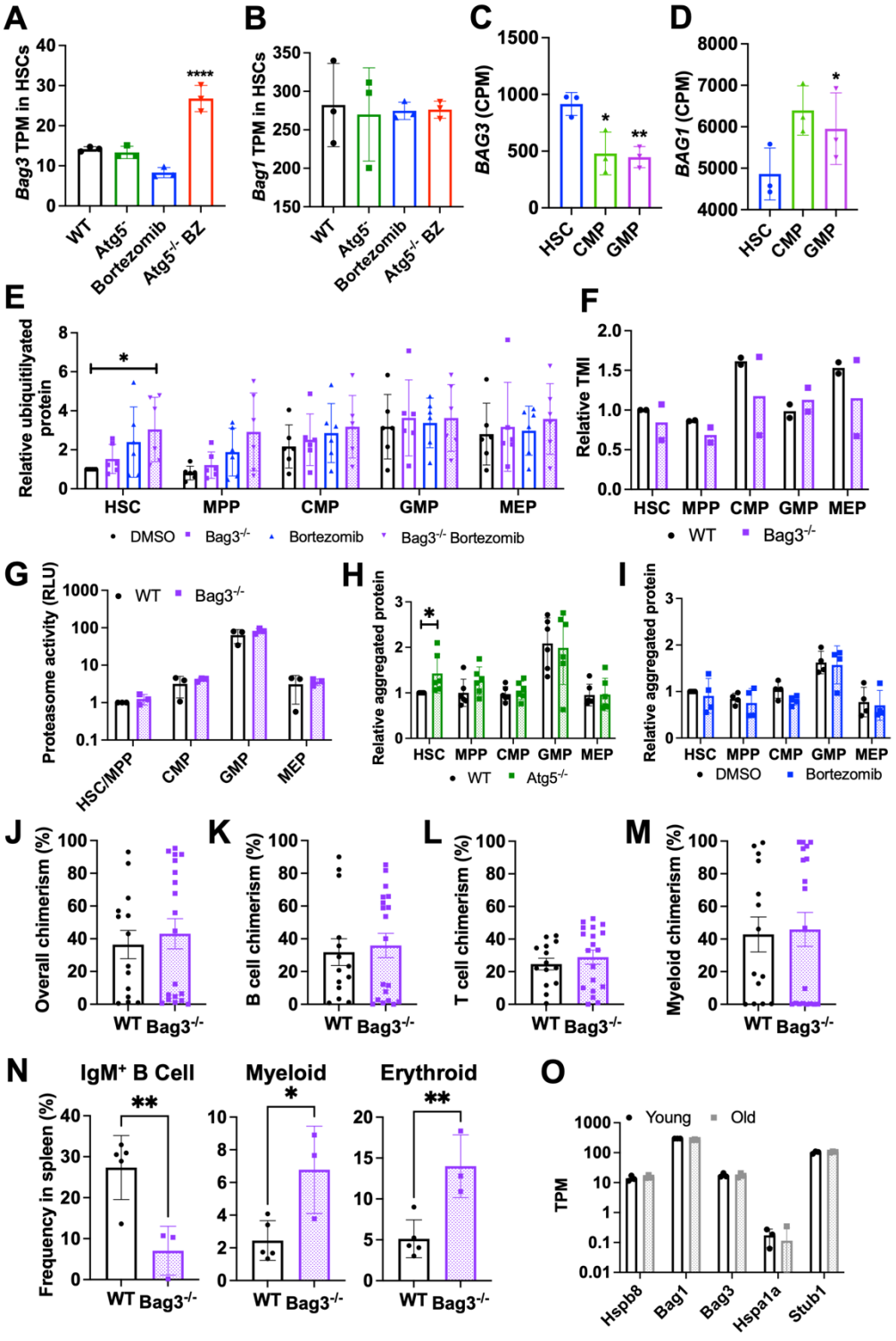

Figure S4. Restricted myeloid progenitors do not rely on Bag3-mediated transport of misfolded proteins into aggresomes for protein quality control. Related to Figure 4.

A-B *Bag3* (A) and *Bag1* (B) expression in HSCs from WT, *Atg5*$^{-/-}$, bortezomib-treated WT, and bortezomib-treated *Atg5*$^{-/-}$ mice. Data show individual replicates and mean transcripts per million (TPM) ± SD; n=3. Data were assessed by one-way ANOVA with Dunnett's test relative to WT.

C *BAG3* expression in human cord blood-derived HSCs, CMPs, and GMPs. Data show individual replicates and mean ± SD; n=3. Data were assessed by one-way ANOVA with Dunnett's test relative to WT.

D *BAG1* expression in human HSCs, CMPs, and GMPs Data show individual replicates and mean counts per million ± SD from Laurenti et al., 2013 and Xie et al., 2019; n=3. Data were assessed by one-way ANOVA with Dunnett's test relative to WT.

E Relative ubiquitinated (misfolded) protein levels in hematopoietic stem and progenitor cells from vehicle (DMSO)- and bortezomib-treated WT and *Bag3*$^{-/-}$ mice. Data show mean ± SD; n=6 from 6 experiments. Data were assessed by one-way ANOVA with Dunnett's test relative to DMSO control.

F Relative unfolded protein (TMI fluorescence) in WT and *Bag3*$^{-/-}$ HSCs and progenitors. Data show individual mice; n=2.

G Proteasome activity in WT and *Bag3*$^{-/-}$ CD48^{-}LSK HSC/MPPs. Data show mean ± SD; n=3. Data were assessed using a student's t-test.

H Relative aggregated protein levels (ProteoStat MFI) in WT and *Atg5*$^{-/-}$ HSCs and progenitors. Data show individual replicates and mean ± SD; n=6. Data were assessed using a student's t-test.

I Relative aggregated protein (ProteoStat MFI) in HSCs and progenitor cells from WT mice treated with vehicle (DMSO) or bortezomib. Data show replicates and mean ± SD; n=4 from 4 independent experiments. Data were assessed using a student's t-test.

J-M Donor hematopoietic (J), B (K), T (L), and myeloid (M) cell chimerism 16 weeks after secondary transplant of 3x10^{6} bone marrow cells from transplant primary recipients of WT or *Bag3*$^{-/-}$ HSCs from Fig. 4M-P. Data show individual mice and means; n=13-19 recipients from 3-4 primary donors. Data were assessed using a student's t-test.

N Frequency of IgM^{+} B cells, myeloid lineage cells, and erythroid lineage cells in the spleen of old (22-25 month) adult WT and *Bag3*$^{-/-}$ mice; n=3-5. Data show individual mice and means ± SD; n=3-5. Data were assessed using a student's t-test.

O Expression of Bag3 and Bag1 complex members in young and old adult HSCs. Data show individual replicates ± SD; n=3. Data were assessed using a paired student's t-test.

*P≤ 0.05, **P≤ 0.01, ****P≤ 0.0001.