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1 **GRASP55 is dispensable for normal hematopoiesis but necessary for Myc-dependent**
2 **leukemic growth.**

3 Anne-Laure Bailly*, Julien M.P. Grenier*, Amandine Cartier-Michaud*, Florence Bardin*,
4 Marielle Balzano*, Armelle Goubard*, Jean-Claude Lissitzky*, Maria De Grandis†, Stéphane
5 J.C. Mancini*, Arnauld Serge* and Michel Aurrand-Lions*#

6

7 **Running title:** Grasp55 is dispensable for normal hematopoiesis

8

9 *Aix Marseille University, CNRS, INSERM, Institut Paoli-Calmettes, CRCM, Marseille,
10 France. Equipe Labellisée Ligue Contre le Cancer.

11 †EFS PACA Corse, « Biologie des Groupes Sanguins », UMR 7268, Aix Marseille
12 Université, CNRS

13

14 #Address correspondence to: MAL Cancerology Research Center of Marseille, CRCM,

15 27 Boulevard Leï Roure CS3005913273 Marseille Cedex 09 France

16 Tel:+33 (0)4 86 97 72 91 Fax:+33 (0)4 86 97 74 99

17 Mail: michel.aurrand-lions@inserm.fr

18

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28 **Abstract**

29 Grasp55 is a ubiquitous Golgi stacking protein involved in autophagy, protein trafficking and
30 glucose deprivation sensing. The function of Grasp55 in protein trafficking has been
31 attributed to its PDZ-mediated interaction with the c-terminal “PDZ binding motifs” of
32 protein cargos. We have recently shown that such an interaction occurs between Grasp55 and
33 the adhesion molecule Jam-C which plays a central role in stemness maintenance of
34 hematopoietic and spermatogenic cells. Accordingly, we have found that *Grasp55*-deficient
35 mice suffer from spermatogenesis defects similar to *Jam-C* knock-out mice. However,
36 whether Grasp55 is involved in the maintenance of immuno-hematopoietic homeostasis
37 through regulation of protein transport and Jam-C expression remains unknown. Here, we
38 show that *Grasp55* deficiency does not affect hematopoietic stem cell differentiation,
39 engraftment or mobilization, which are known to depend on expression of Grasp55-dependent
40 protein cargos. In contrast, using a Myc-dependent leukemic model addicted to autophagy, we
41 show that knock-down of Grasp55 in leukemic cells reduces spleen and bone marrow tumor
42 burden upon intravenous leukemic engraftment. This is not due to reduced homing of
43 Grasp55-deficient cells to these organs but to increased spontaneous apoptosis of Grasp55-
44 deficient leukemic cells correlated with increased sensitivity of the cells to glucose
45 deprivation. These results show that Grasp55 plays a role in Myc-transformed hematopoietic
46 cells but not in normal hematopoietic cells *in vivo*.

47

48 **Key points:**

- 49 - Golgi morphology and *Grasp55* expression are regulated during hematopoiesis
50 - Hematopoiesis is not affected in *Grasp55*-deficient mice
51 - Grasp55 regulates Myc-transformed leukemic cell survival

52

53

54

55 **Introduction**

56 Hematopoiesis and spermatogenesis are two differentiation processes in which adult stem
57 cells produce their progeny in a sequentially ordered manner. It has been shown previously
58 that JAM-C is expressed by hematopoietic stem cells (HSC) and by male germ cells (1-3). In
59 both biological models, JAM-C plays a role in the maintenance of tissue homeostasis through
60 adhesion regulation between developing cells and surrounding stromal cells expressing JAM-
61 B, the high affinity ligand of JAM-C (4, 5). During spermatogenesis, JAM-C regulates
62 polarization of developing spermatids, a critical step in spermatozoa development (3). During
63 hematopoiesis, JAM-C interaction with JAM-B plays a critical role in maintenance of
64 hematopoietic stem cells (HSC) which are at the apex of hematopoietic hierarchy (6). HSC
65 differentiate into more mature multipotent progenitors (MPPs) which have decreased self-
66 renewal capacity and progressively engage in megakaryocyte (MPP2), myeloid biased
67 (MPP3) or lymphoid biased (MPP4) multipotent progenitors (7). In agreement with its
68 function in HSC maintenance, JAM-C is downregulated in MPPs during steady state
69 hematopoiesis (2). JAM-C expression by HSC is also rapidly downregulated after HSC
70 mobilization using Cyclophosphamide and G-CSF (8), suggesting that JAM-C may play a
71 role in the switch from steady state to emergency hematopoiesis. However, down-regulation
72 of JAM-C is not unique to G-CSF-induced mobilization since down-regulation of other bone
73 marrow retention signals such as VCAM-1/VLA-4, CXCL12/CXCR4 or SCF/Kit have also
74 been documented (9-11).

75 We have recently identified the Golgi-reassembly stacking protein of 55kDa (Grasp55) as a
76 PDZ-domain containing protein interacting with the C-terminal PDZ-binding motif of Jam-B
77 and Jam-C in mouse (12). Grasp55, and the related protein Grasp65, are highly conserved
78 throughout evolution and play complementary roles in Golgi cisternal stacking through
79 oligomerization and PDZ-mediated interactions with proteins of the golgin family such as

80 Golgin45, GM130 or p24 (13-17). In addition, GRASP55 depletion results in accelerated
81 protein trafficking through the Golgi apparatus and has striking negative effects on protein
82 glycosylation and sorting (18). More recently, O-GlcNAcylation has been shown to restrict
83 the function of GRASP55 to Golgi stacking, while de-O-GlcNAcylation switches its function
84 as an autophagosome-lysosome tethering molecule (19). However, the function of GRASP55
85 is not limited to Golgi stacking and autophagy regulation since several studies have shown
86 that GRASP55 is involved in protein trafficking through an unconventional secretory pathway
87 triggered by cellular stress or unfolded protein response (UPR) (20-23). TGF- β , MT1-MMP,
88 membrane bound SCF, CD8 α , Frizzled4, CD83 and IL1 β have been shown to be transported
89 in a GRASP55-dependent manner (24-28). In addition, GRASP55 has been involved in MT1-
90 MMP activation which is necessary for various biological processes, including HSC
91 mobilization (29). We have thus explored the function of GRASP55 in adult mammalian
92 normal and pathological hematopoiesis. To this end, we have generated constitutive and
93 inducible knock-out mouse models of *Gorasp2* (encoding Grasp55) and studied HSC self-
94 renewal, engraftment, mobilization and differentiation. To test whether Grasp55 may be
95 involved in pathological hematopoiesis, we also questioned whether Grasp55 controls
96 leukemic development. To this end, we used the E μ -Myc lymphoma model known to be
97 addicted to autophagy activation (30, 31).

98 Our results show that Grasp55 is not involved in the regulation of JAM-C expression in HSC.
99 In addition, Grasp55-deficiency does not affect HSC maintenance or differentiation at steady
100 state or upon hematopoietic stress. We further show that this is not due to compensatory
101 mechanisms since acute depletion of Grasp55 expression using Mx1-Cre inducible system
102 does not result in hematopoietic abnormalities. This is likely due to the inversely regulated
103 expression of Grasp55 and Jam-C during hematopoietic cell differentiation. Indeed, Grasp55
104 transcript and protein are expressed at low levels in the most immature hematopoietic cells

105 (HSC-MPP1) and are upregulated in the following differentiation steps (MPP2 and MPP3-4)
106 in which JAM-C expression is lost. We thus tested whether Myc-induced B cell lymphoid
107 leukemia would depend on Grasp55 expression for their growth. Our results show that
108 Grasp55 is necessary for Myc-dependent lymphoid leukemic development *in vivo*, opening
109 the way for specific Grasp55 targeting in Myc-addicted hematological malignancies.
110

111 **Material and Methods**

112 **Mice**

113 Generation and genotyping of *Gorasp2* constitutive deficient mice were previously described
114 (Cartier-Michaud et al.). Because *Gorasp2* deficient male are sterile, the colony is maintained
115 by interbreeding of heterozygote mice. The colony was backcross on C57/BL6J mice more
116 than 10 generation. Floxed *Gorasp2* lines, obtained before CMV-Cre deleter mice mating
117 during *Gorasp2* knockout generation, are maintained by intercrossing and backcrossing on
118 C57/BL6 background more than 7 generation. Conditional inducible *Grasp55* (*Gorasp2*)
119 deficient mice were obtained by two successive mating: in a first time *Grasp55*^{-/-} female were
120 mated with Mx1-cre^{Pos} male mice to obtained *Grasp55*^{+/-} Mx1-Cre^{Pos} mice, in a second time
121 *Grasp55*^{+/-} Mx1-Cre^{Pos} mice are mated with *Grasp55*^{fl/fl} mice. *Grasp55*^{fl/-} Mx1-Cre^{Neg} and
122 *Grasp55*^{fl/-} Mx1-Cre^{Pos} mice were used for experimentation. Same strategy was used to obtain
123 hematopoietic-specific constitutive *Grasp55*^{fl/-} Vav-Cre mice, *Gorasp2*^{fl/-} Vav-Cre^{Neg}
124 (*Grasp55*^{WT} and *Gorasp2*^{fl/-} Vav-Cre^{Pos} (*Grasp55*^{AHem}) mice were used for experimentation.
125 CD45.1/2 mice used as competitor in competitive assay were obtained by interbreeding of
126 CD45.1 with CD45.2 mice (purchased from JANVIER LABS). Mice were used at age more
127 than 8 weeks old. All animal experiments were performed according to French Guidelines for
128 Animal Handling in CRCM animal facility (Agreement D13 055 04). Experimental protocols
129 were approved by the Ethical Committee #14 under the declaration number #02294.01.

130

131 **Genotyping**

132 Genotyping of *floxed Grasp55* line was achieved by PCR of genomic DNA extracted from
133 mouse tails using the same primers and conditions as constitutive *Gorasp2* mice. But resulting
134 in an amplification of 544-bp fragment for the WT allele and 700-bp fragment for the floxed
135 allele were both obtained using heterozygous mice. Genotyping of Mx1-cre and Vav-cre mice

136 was achieved using the generic Cre and the iVav-Cre genotyping protocols from The Jackson
137 Laboratory.

138

139 **HSC exhaustion after 5-FU treatment**

140 *Grasp55^{WT}* and *Grasp55^{ΔHem}* mice were injected intraperitoneally once a week with 5-Fluoro-
141 uracil (5-FU) at 120 mg/kg during 5 weeks. One day after 5-FU injection, blood paramaters
142 were tested using IDEXX ProCyte Dx® Hematology Analyzer (IDEXX Laboratories). Mice
143 were sacrificed at D35 after the first injection.

144

145 **Inducible deletion with polyI:C and analysis of Grasp55 deletion**

146 *Gorasp2* gene deletion in *Gorasp2^{fl/-} Mx1-Cre* mice was induced by three intraperitoneal
147 injection of 200μg poly(I:C) (Invivogen) on day D0, D2 and D4. *Gorasp2* gene deletion in
148 white blood cells (WBC) was confirmed on day 15 by PCR. Briefly, WBC isolated from
149 100μL of peripheral blood by Ficoll and genomic DNA was extracted by digestion in 250μl
150 lysis buffer (50mM KCl, 10mM Tris pH 8,8, 2,5mM MgCl₂, 0,45% NP40, 0,45% Tween)
151 with 0.4mg/ml Proteinase K (Invitrogen) for 30min at 56°C, then PCR was achieved using
152 same protocol as genotyping. Mice were sacrificed for analysis one month after the first
153 poly(I:C) injection.

154

155 **BM Mobilization Assays**

156 Mobilization was induced using cyclophosphamide (CY, 4 mg i.p. d0) and rhG-CSF (5 mg
157 s.c. at d1, 2, and 3) injections as previously described (8). Mice were sacrificed on day 5 for
158 analysis.

159

160 **Colony-Forming Assay**

161 Peripheral blood and BM white blood cells were isolated by Ficoll. 200.000 blood cells were
162 seeded per well in methylcellulose medium following the manufacturer's instructions (Stem
163 Cell Technologies, Grenoble, France, www.stemcell.com, ref 3434).

164

165 **Competitive Bone Marrow Transplantation**

166 Lethally irradiated (8 grays) CD45.1 mice were transplanted with 2.10^6 of total bone marrow
167 cell from donors (*Gorasp2*^{+/+} or *Gorasp2*^{-/-} CD45.2) and competitor (CD45.1/2) cells in a 1:1
168 ratio by retroorbital sinus injection. Mice chimerism was assessed monthly by monitoring of
169 host (CD45.1), competitor (CD45.1/2) and donor (CD45.2) cells in peripheral blood by flow
170 cytometry. Chimerism and hematopoietic phenotype in bone marrow were evaluated 18
171 weeks after transplantation.

172

173 **Thioglycollate induced-peritonitis in chimeric mice**

174 Lethally irradiated (8 grays) CD45.1 mice were transplanted with 2.10^6 of total bone marrow
175 cell from donors (*Gorasp2*^{+/+} or *Gorasp2*^{-/-} CD45.2) by retro-orbital sinus injection. 18 weeks
176 after transplantation, chimeric mice were injected intraperitoneally with 3ml of thioglycollate
177 3% (w/v; Sigma). 18 hours after injection, mice were sacrificed and peritoneal exudates were
178 collected by three successive peritoneal washes with 10 ml cold phosphate buffer saline
179 (PBS). Cells were counted and analyzed using IDEXX ProCyte Dx® Hematology Analyzer
180 (IDEXX Laboratories).

181

182 **EμMyc cells engraftment in mice**

183 Mouse primary Eμ-Myc lymphoma cells were isolated as previously described (32) and
184 expanded through passaging in irradiated recipient mice. 500.000 GRASP55^{KD} or Grasp55^{WT}

185 Eμ-Myc cells are injected into caudal tail vein of Ly5.1 mice. Mice were sacrificed at D9
186 after injection. Tumoral invasion in blood, spleen and bone marrow was assessed by flow
187 cytometry.

188

189 **Cytometry and cell sorting**

190 Single cells suspensions were prepared from bone marrow (one leg: femur and tibia) and red
191 blood cells were lysed with ACK (ammonium-chloride-potassium) buffer (Gibco). Cells were
192 incubated with mAbs in PBS-0,5% BSA for 30 min at 4°C. For blood samples, RBC lysis
193 was done after staining with BD FACS Lysing buffer (BD Bioscience). For hematopoietic
194 cells identification, following antibodies were used: biotin anti-CD3, CD4, CD8, CD19,
195 CD11c, DX5, Ter119, CD11b, B220, Gr1 (Lineage cocktail, Biolegend), CD16-32-PE (clone
196 2.4G2, BD Pharmingen), CD45.1-PECF594 (clone A20, BD Horizon), CD45.2-eF450
197 (clone104, eBioscience), CD48-PE-Cy7 (clone HM48-1, Biolegend), CD150-eF647 (clone
198 TC15-12F12.2, Biolegend), CD117-APC-e780 (clone 2B8, eBioscience), CD127-PE-Cy5
199 (clone A7R34, eBioscience), CD135-PECF594 (clone A2F10.1, BD Horizon), Sca-1-BV421
200 (clone D7, BD Horizon) **CD4-FITC (clone RAM4-5, eBioscience), CD8-PE (clone 53-6.7,**
201 **eBioscience), B220-AF700 (clone RA3-6B2, eBiosciences), CD11b-APC (M1/70,**
202 **eBioscience)** and BV510-Streptavidin (BD Horizon). EμMyc cells were phenotyped using :
203 CD19-BV500 (clone 1D3, BD Biosciences), CD23-BV711 (clone B3B4, BD Biosciences),
204 CD25-PE-Cy5 (clone PC61, eBioscience), CD45.2-PE-Cy5.5 (clone 104, eBioscience),
205 B220-APC-Cy7 (clone RA3-6B2, BD Biosciences), CD71-BV605 (clone C2, BD
206 Biosciences), CD93-PE-Cy7 (clone AA4.1, eBioscience), BP1-biotin (clone BP1, BD
207 Biosciences), streptavidin-AF594 (Biolegend). In all experiments a viability marker was used
208 depending of the panels: SYTOX Green nucleic acid stain (Life Technologies), Fixable
209 Viability Dye eF506 (eBioscience), DRAQ7 (Biostatus). JAM-B and JAM-C staining were

210 done using staining polyclonal rabbit anti-mouse JAM-B (pAb829) and JAM-C (pAb 501)
211 produced in laboratory and R-Phycoerythrin conjugated goat-anti-rabbit IgG(H+L) antibody
212 (1/200, Jackson Immunoresearch Laboratories) as secondary antibody. Grasp55 intracellular
213 staining was done using anti-Grasp55 antibody (Protein-tech) and BD Cytfix/Cytoperm
214 solution following the manufacture's instruction.

215 For hematopoietic stem and progenitor cells sorting, bone marrow cells were depleted using
216 Lineage cell depletion Kit from Miltenyi and AutoMACS Pro. The Lin- cells were stained as
217 described in preceding paragraph.

218 For Annexin-PI staining cells were stained with APC-AnnexinV (BD Bioscience) and
219 propidium iodide solution (Sigma Aldrich) in Annexin binding buffer (Life Technologies) for
220 15 minutes at room temperature.

221 Stained cells were analyzed by a BD LSR-II FACS (lasers 405, 488, 561, and 633nm) and
222 sorted by Aria III SORP sorter (BDBiosciences). Results were analyzed with BD-Diva
223 version 8.0.1 software or FlowJo version 10.0.7 software (Treestar).

224

225 **Cell culture**

226 Eμ-Myc clone 504 cells were provided JE. Ricci. EμMyc cells were cultured in DMEM
227 Glutamax, supplemented with 10% FBS, 1% non-essential amino acids, 1% L-glutamin, 1%
228 Hepes 1mM, 1%PS, 0,1% β-mercaptoethanol (all cell culture products were purchased from
229 Gibco). For cell proliferation, cells were plated at a density of 10.000 cells/well into 96-wells
230 plate and cell numbers were quantified after 24, 48, 72, 96 hours using Cell Titer Glow Assay
231 (Promega). *Grasp55^{+/+}* and *Grasp55^{-/-}* mouse embryonic fibroblasts (MEFs) were obtained as
232 previously described (12). Chloroquine treatment was performed at a concentration of half
233 IC50 (5μM for EμMyc cells and 17.5μM for MEFs) during 24 hours. For EμMyc glucose

234 starvation, cells were washed twice in PBS, incubated in DMEM without glucose (Gibco),
235 10% FCS, 1%PS and cells were quantified 24 hours later using Cell Titer Glow (Promega).

236

237 **Lentiviral transduction**

238 For lentiviral transduction, 1.10^6 cells were plated in 96-wells plates in 190 μ L complete
239 medium with 8 μ g/ml polybrene and 10 μ l of lentiviral particles expressing Grasp55 shRNA-
240 GFP (GRASP55^{KD}) or Control GFP (GRASP55^{WT}) vectors (TRIPdU3 vector). Cells were
241 centrifuged at 3000 rpm without brake during 2h at 32°C and incubated overnight. Next day
242 medium was replaced by complete medium and cell fluorescence was checked by microscopy
243 (AMG EVOS LED fluorescence microscope). One week after transduction, transduced cells
244 were sorted by flow cytometry based on GFP expression. Correct GRASP55 silencing was
245 tested by Western blot.

246

247 **Gene expression analysis**

248 500 for each population (HSC-MPP1, MPP2, and MPP3-4) from WT CD45.1 mice were
249 sorted using the autoclone module on an Aria III SORP sorter (BD Biosciences) into 96-well
250 plates in the RT-STA Master Mix Solution. Cell lysis, cDNA synthesis, and amplification
251 were performed according to Fluidigm protocols. Selected TaqMan Gene Expression assays
252 (original magnification $\times 20$) were pooled and diluted with water 100-fold so that each assay
253 is at a final concentration of 0.2 \times in the pooled assay mix. Five microliters of CellsDirect 2 \times
254 Reaction Mix (Invitrogen), 2.5 μ l pooled assay mix, 0.2 μ l SuperScript III RT/Platinum
255 Taqmax mix, and 2.3 μ l water were combined to a final volume of 10 μ l in 1 well of a 96-well
256 qPCR plate for the sort. cDNA samples were amplified using the following program (1):
257 50°C, 10 min (2); 95°C, 2 min (3); 95°C, 15 s (4); 60°C, 4 min; repeat steps (3) and (4) 18
258 times. Preamplified products were diluted 5-fold before analysis with Universal PCR Master

259 Mix and inventoried TaqMan gene expression assays in 96.96 Dynamic Arrays on a BioMark
260 System (Fluidigm). For each gene, the relative expression was defined as the mean value of
261 40 minus cycle threshold for the gene of interest divided by 40 minus cycle threshold for
262 actin. Results were represented as an heat map using Morpheus software
263 (<https://software.broadinstitute.org/morpheus/>).

264

265 **Western blot**

266 GRASP-55 expression by hematopoietic cells was tested using a total of 10000-sorted cells
267 for each population (HSC-MPP1, MPP2, MPP3-4) re-suspended in 50µl of RIPA buffer
268 (50mM Tris HCl pH7.5, 150mM NaCl, 1% Triton, 0.1% SDS, 1% Na desoxylate) and 10µl of
269 6x sample buffer. Western blots for LC3 were performed on 20µg of proteins. Whole protein
270 extracts were separated by SDS-PAGE and transferred onto nitrocellulose membrane.
271 Saturated membranes were exposed to rabbit anti-GRASP55 antibody (1/500e ProteinTech),
272 rabbit anti-LC3 antibody (1/1000e, MBL international corporation), mouse anti- α -tubulin
273 antibody (1/1000, T6074 Sigma) in PBS-1% non-fat milk, 0,1% tween at 4°C overnight.
274 Membranes were incubated with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated
275 goat anti-mouse IgG antibody (1/5000, Jackson Immunoresearch Laboratories) at RT one
276 hour. Signal was revealed using SuperSignal West Pico Chemiluminescent substrate
277 (ThermoFischer Scientific).

278

279 **Immunofluorescence**

280 1000 sorted cells were incubated in a 50µl PBS-0,5% BSA drop on poly L-lysine-coated slide
281 in a humidified chamber at 37°C during 2 hours, buffer was carefully removed by aspiration
282 and replaced by saturation mix (PBS-2%BSA).

283 Primary antibody anti-GRASP55 (ProteinTech, 1/250) was incubated overnight at 4°C in
284 PBS-0,5% BSA. After soft washes, samples were incubated one hour at room temperature
285 with donkey anti-rabbit-eF594-conjugated secondary probe purchased from Jackson
286 Immunoresearch. DAPI nuclear staining was done with the second final wash. Coverslip was
287 mounted using prolong gold antifade reagent (Invitrogen).

288 Images were obtained using a Zeiss LMS880 Meta confocal. Images were analyzed using
289 Image J. Three dimensions Golgi volume quantification was performed as previously
290 described (12).

291

292 **Caspase activity assay**

293 Cells were plated at a density of 10.000 cells/wells into a white walled 96-wells plate.
294 Caspase activity was determined using the Caspase-Glo® 3/7 kit following the
295 manufacturer's instructions (G811A from Promega®). Luminescence was measured using a
296 POLARstar omega from BMG LABTECH.

297

298 **In vivo homing**

299 Eμ-myc GRASP55^{KD} were labeled with calcein violet (violet) and GFP (green) was used for
300 Eμ-myc GRASP55^{WT} and injected at 1:1 ratio into mice (5.10⁶ cells/200μL NaCl per mice).
301 Cells in each organ were analyzed for expression of green or violet fluorescence by flow
302 cytometry. Stained cells were analyzed by an LSRII FACS (lasers 405, 488, 561, and 633nm
303 BDBiosciences). Results were analyzed with BD-Diva version 8.0.1 software.

304

305 **Statistical Analysis**

306 All experiments were performed at least 3 times. Statistical significance was performed using
307 non-parametric Mann-Whitney U-test, Student t-Test or Two way ANOVA with Bonferroni
308 post-test in Prism software. P-value less than 0.05 was considered as statistically significant.
309

310

311 **Results**

312 **Regulated expression of GRASP55 during HSC differentiation**

313 We first tested if *Gorasp2*, the gene encoding Grasp55, was expressed in hematopoietic stem
314 and progenitor cells (HSPCs) using RT-qPCR on sorted cells from mouse bone marrow
315 according to the gating strategy shown in Figure 1A. Although *Jam3*, the gene encoding Jam-
316 C, was highly expressed on the most immature compartment (HSC-MPP1) and
317 downregulated in more mature cells, *Gorasp2* presented an inverted pattern of expression and
318 was barely detectable in HSC-MPP1 and MPP2 cells (Fig. 1B). Other genes encoding
319 proteins involved in Golgi stacking such as *Blzfl* (encoding Golgin45) or *Golgb1* (encoding
320 Giantin) followed the same pattern of expression as compared to *Gorasp2*, while expression
321 of *Golga2* (encoding GM130) remained stable. Of note, and consistent with publicly available
322 datasets from Immgen consortium (immgen.org), we failed to detect *Gorasp1*, the gene
323 encoding Grasp65 in HSPC (not shown). Grasp55 upregulation between the most immature
324 compartment (HSC-MPP1) and more differentiated multipotent progenitors (MPP2 and
325 MPP3-4) was further confirmed at the protein level by flow cytometry and western-blotting
326 (Fig. 1C-D). Immunostaining for Grasp55 performed on HSC-MPP1, MPP2 and MPP3-4
327 isolated from wild type or *Grasp55*^{-/-} mice confirmed that Grasp55 expression and
328 localization changed during hematopoietic cell differentiation (Fig. 1E). We found that
329 Grasp55 signal was barely detectable in wild type HSC-MPP1 and was increased in MPP2
330 and MPP3-4, as confirmed by staining intensity quantification of confocal Z-stacked pictures
331 (Fig. 1F). In addition, three dimensional quantification of Grasp55 staining revealed that the
332 volume of Grasp55 vesiculo-tubular objects was not significantly different between HSC-
333 MPP1 and MPP2 cells, while there was a significant increase in MPP3-4 cells (Fig. 1G). This
334 indicates that Grasp55 is upregulated and recruited to large Golgi stacks at the transition

335 between MPP2 and MPP3-4 stages of differentiation, suggesting that structural Golgi
336 remodeling occurs during hematopoietic differentiation.

337

338 **Lack of hematopoietic and inflammatory defects in *Grasp55*^{-/-} mice**

339 We thus analyzed early hematopoietic differentiation in *Grasp55*^{-/-} mice and compared to
340 control littermate animals. Total number of bone marrow cells and percentages of
341 Lin^{Neg}Sca^{Pos}Kit^{Pos} (LSK) were identical between *Grasp55*^{+/+} and *Grasp55*^{-/-} (Fig. 2A-B).
342 Results were thus expressed as percentage of the LSK compartment (Fig. 2C) or percentage
343 of total bone marrow (Fig. 2D). Within the LSK compartment, HSC-MPP1, MPP2 or MPP3-4
344 were not affected by the loss of Grasp55 expression (Fig. 2C), nor the more mature common
345 myeloid progenitors (CMPs) or granulocyte/monocyte progenitors (GMP) (Fig. 2D). In
346 addition, downregulation of JAM-C expression during hematopoietic differentiation was not
347 affected suggesting that LT-HSC maintenance remained unaffected in absence of Grasp55
348 expression at steady state (Fig. 2E). We thus tested if Grasp55 may be involved in emergency
349 hematopoiesis using constitutive hematopoietic specific Grasp55 deficient mice
350 (*Grasp55*^{ΔHem}) obtained by crossing *Grasp55*^{fl/-} mice with Vav-Cre transgenic mice.
351 Repeated injection of 5-Fluoro-Uracil (5-FU) resulted in similar decrease in hematocrit values
352 between *Grasp55*^{WT} and *Grasp55*^{ΔHem} animals (Supplementary Fig. 1A-B). The day 35 was
353 defined as the endpoint for analysis of bone marrow composition to avoid death of the
354 animals because of sustained anemia. No difference in absolute cell numbers or frequencies of
355 early hematopoietic subsets was found between *Grasp55*^{ΔHem} and *Grasp55*^{wt} control animals
356 after 4 injections of 5-FU (Supplementary Fig. 1C-F). Although only three mice were
357 analyzed in this pilot experiment, power calculation shows that a tremendous number of mice
358 would have been necessary to get a chance to reach statistical significance. We thus decided
359 to move to another model in which deletion of Grasp55 is induced in an acute manner to not

360 allow for compensatory mechanism to take place. To this end, *Grasp55^{fl/-}* animals crossed
361 with Mx1-cre transgenic mice were injected with poly(I:C) resulting in floxed allele deletion
362 fifteen days upon poly(I:C) treatment (Supplementary Fig. 2A). Analysis of absolute bone
363 marrow cell numbers and frequencies of LSK cells showed no differences between controls
364 and conditional induced knock-out mice poly(I:C)-treated *Grasp55^{fl/-}* Mx1-Cre mice
365 (Supplementary Fig. 2B-C); nor did the analysis of different LSK (HSC-MPP1, MPP2,
366 MPP3-4) and progenitor subsets (CMP, GMP) reveals difference between experimental
367 groups (Supplementary Fig. 2D-E). Finally, since *Grasp55* has also been involved in
368 inflammatory response *in vitro* (23), we also tested whether inflammatory recruitment of
369 leukocytes at effector sites would be affected by *Grasp55* deficiency. Here again, we did not
370 find significant differences in acute inflammatory recruitment of leukocytes to the peritoneal
371 cavity upon thioglycollate challenge (Supplementary Fig. 3). This indicates that *Grasp55* is
372 dispensable for emergency hematopoiesis and inflammatory recruitment *in vivo*.

373

374 **HSC mobilization and engraftment are not affected by *Grasp55* deficiency**

375 Because *Grasp55* has been involved in the regulation of retention signals for HSC in the bone
376 marrow, we next performed HSC mobilization assays using Cyclophosphamide and G-CSF.
377 This experimental setting induces dramatic changes in molecular mechanisms involved in
378 HSC retention within BM niches and results in myeloid biased expansion of HSPCs (33). As
379 expected, increase of LSK cells frequencies was found in mouse bone marrow and blood
380 upon mobilization (Fig. 3A-B). However, such increase was independent of GRASP55
381 expression. When Colony Forming Unit-Cells (CFU-C) assays were performed as a read-out
382 of hematopoietic cell progenitor activity, no differences were found between *Grasp55^{-/-}* mice
383 and control littermate animals (Fig. 3C). To get further insights into the function of GRASP55
384 in controlling cellular crosstalks upon hematopoietic stress, we performed competitive bone

385 marrow engraftment assays. To avoid bias due to the difference in CD45.1; CD45.2 and
386 CD45.1/2 allele expression (34), we designed two experimental groups using CD45.2
387 *Grasp55*^{+/+} or CD45.2 *Grasp55*^{-/-} BM cells admixed at 1:1 ratios with wild-type CD45.1/2
388 bone marrow cells as competitors (Supplementary Fig. 4A). Engraftment was performed in
389 CD45.1 lethally irradiated recipients (Fig. 4A). Such experiments are highly sensitive and
390 have been widely used to highlight subtle defects in hematopoiesis which are not revealed in
391 other experimental settings. Using chimerism measurement in the peripheral blood as follow-
392 up, we found significant decrease in *Grasp55*^{-/-} hematopoietic engraftment as compared to
393 *Grasp55*^{+/+} cells before 12 weeks, while differences disappeared at 16 weeks (Fig. 4B). This
394 was mostly due to a reduction of *Grasp55*-deficient lymphoid cells in the blood circulation
395 during the first four months after engraftment (Fig 4C-F). Such difference was abolished after
396 18 weeks, suggesting that this transient difference revealed subtle changes in the early steps
397 of *Grasp55*-deficient cells commitment toward lymphoid versus myeloid lineage after
398 engraftment. This is consistent with the high expression of *Grasp55* found in MPP3-MPP4s
399 myeloid and lymphoid biased progenitors (Fig.1). When chimerism in the bone marrow was
400 analyzed after 18 weeks, overt engraftment advantage for wild-type competitor cells
401 (CD45.1/2) against donor cells (CD45.2) was found in the LSK compartment irrespective of
402 *Grasp55* genotype (Fig. 4G). No change in bone marrow cellularity was observed
403 (Supplementary Fig. 4B). More interestingly, specific significant decrease in CMP
404 frequencies was observed in mixed chimeric mice engrafted with *Grasp55*^{-/-} and wild-type
405 competitor cells as compared to *Grasp55*^{+/+}/wild-type chimeras while no change in GMP
406 frequencies was detected (Fig. 4H). This was due to a significant decrease in competitor cell
407 frequencies suggesting that loss of *Grasp55* expression in hematopoietic cells may alter
408 indirectly the maintenance of wild-type CMP.

409

410

411

412 **GRASP55 plays a role in Myc-addicted leukemic growth *in vivo***

413 Since we failed to detect Grasp55-dependent alterations of the hematopoietic system at steady
414 state or upon stress, we asked the question whether GRASP55 may contribute to development
415 of Myc-addicted lymphoma/leukemia known to depend on autophagy pathway activation (30,
416 31, 35). One leukemic primary cell clone obtained from E μ -Myc mice harbored a pre-B cell
417 phenotype as demonstrated by positivity for CD19, CD25, CD43, BP1, CD93 (Fig. 5A). In
418 addition, the selected clone expressed JAM-B, a known interacting partner of GRASP55 (Fig.
419 5B & (12)). Two cell lines in which GRASP55 expression was depleted or not were derived
420 upon lentiviral transfection with shRNA directed against GRASP55 or empty vector
421 containing the GFP only (Fig. 5C). Silencing GRASP55 did not affect JAM-B expression (not
422 shown) nor cell proliferation (Fig. 5D). Upon engraftment in C57BL/6 animals, control E μ -
423 Myc cells invaded spleen and bone marrow within nine days, while invasion by E μ -Myc
424 GRASP55 knock-down cells (E μ -Myc GRASP55^{KD}) was greatly reduced (Fig. 5E-F). This
425 was accompanied by a three-fold decrease in peripheral leukemic cell load for E μ -Myc
426 GRASP55^{KD} leukemic cells as compared to control (Fig. 5G). Since such differences may
427 rely on changes in homing or engraftment properties, we tested the tissue homing properties
428 of the two cell lines. To this end, tissue homing was analyzed upon intra-vascular injection of
429 mixed E μ -Myc GRASP55^{WT} and E μ -Myc GRASP55^{KD} cells, respectively labeled by GFP
430 and GFP/Calcein violet reporters (Fig. 6A, left panel). Frequencies of E μ -Myc GRASP55^{WT}
431 cells were significantly increased as compared to E μ -Myc GRASP55^{KD} cells in spleen, bone
432 marrow and blood sixteen hours after injection (Fig 6B). This last result was surprising since
433 reduced short term tissue homing is usually accompanied by increased number of cells
434 remaining in the blood (36).

435

436

437 **GRASP55 regulates cell survival in an autophagy independent manner**

438 We thus hypothesized that E μ -Myc GRASP55^{KD} cells may present reduced survival during
439 early steps of homing experiments. Spontaneous apoptosis of E μ -Myc GRASP55^{KD} cells was
440 thus compared to E μ -Myc GRASP55^{WT} cells. To this end, caspase activity was measured
441 twelve hours after viable cell replating in fresh medium. A significant increase of caspase 3/7
442 activity was observed in E μ -Myc GRASP55^{KD} cell culture as compared to control (Fig. 7A).
443 Accordingly, we observed an increase in early and late apoptotic cells in E μ -Myc
444 GRASP55^{KD} cell culture as compared to E μ -Myc GRASP55^{WT} (Fig. 7B-C). However, the
445 effect was only marginal and would not totally explain results from *in vivo* homing
446 experiments. Since normal value of glucose level in mouse serum is by far lower than in
447 culture medium (1,4g/l versus 4g/l) and because GRASP55 has been involved in glucose
448 sensing (37), we then reasoned that glucose starvation may be needed to reveal a more
449 pronounced difference. We thus cultured E μ -Myc leukemic cells for 96 hours in complete
450 medium or in absence of glucose. Although more than 67% of E μ -Myc GRASP55^{WT} cells
451 survived glucose starvation, less than 55% of E μ -Myc GRASP55^{KD} cells were still alive as
452 compared to control culture condition in complete medium (Fig 7D). This result is consistent
453 with a recent study showing that GRASP55 sense glucose deprivation to promote
454 autophagosome-lysosome fusion (19). We then questioned whether such a pathway
455 contributes to the better survival of E μ -Myc proficient cells. To this end, we treated cells with
456 chloroquine which is known to induce E μ -Myc cell death through inhibition of lysosome
457 function and autophagosome accumulation (38). We found no differences in cytotoxicity with
458 respect to Grasp55 expression in E μ -Myc cells or in mouse embryonic fibroblasts (MEFs)
459 isolated from *Grasp55*^{+/+} or *Grasp55*^{-/-} mice (Fig. 8A). We confirmed that Myc transformed

460 cells were more sensitive to chloroquine than MEFs as previously reported (Fig. 8B & (38)).
461 However, we found no difference in LC3-II accumulation between Grasp55 proficient and
462 deficient cells at the steady state or upon chloroquine treatment (Fig. 8C-D). Altogether, these
463 results argue for an increased sensitivity to glucose starvation of E μ -Myc Grasp55-deficient
464 cells without dramatic changes in autophagic fluxes.
465

466

467 **Discussion**

468 In the present study, we have addressed the physiological function of Grasp55 in normal,
469 emergency and pathological hematopoiesis using *Grasp55*-deficient mice for which we have
470 previously reported spermatogenesis defects (12). Surprisingly, we found no difference in
471 normal and emergency hematopoiesis between Grasp55-deficient and proficient mice, even
472 though Grasp55 has been involved in the regulation of number of molecules known to be
473 essential for hematopoietic homeostasis. We rule out that this was due to incomplete deletion
474 of the gene since mice presented overt phenotypes reminiscent of *Jam-c*^{-/-} mice such as male
475 infertility (3, 12, 39) which has been confirmed in another *Grasp55*-deficient strain (23).
476 Although the absence of JAM-C deregulation in *Grasp55*^{-/-} hematopoietic cells may be easily
477 explained by the inverted expression pattern of JAM-C and GRASP55 during early
478 hematopoietic cell differentiation (Fig. 1), the lack of overt immuno-hematopoietic phenotype
479 remains puzzling. Indeed, given the roles of GRASP55 in Golgi structure, protein expression
480 and autophagy (19, 23, 40, 41), one could expect dramatic effects due to the loss of GRASP55
481 expression when the hematopoietic system is challenged.

482 Several key regulators of hematopoietic homeostasis such as TGF- β , membrane bound SCF,
483 or MT1-MMP have been shown to interact with GRASP55 (25, 29). TGF- β and membrane
484 bound SCF interact with GRASP55 through canonical c-terminal PDZ binding motifs (25).
485 Interaction of GRASP55 with transmembrane TGF- β was directly correlated to cell surface
486 levels since mutation of the last two hydrophobic amino-acid (aa) reduced surface expression
487 and GRASP55 interaction, while truncation of the last eight aa abolished interaction and
488 expression. However, the reverse experiment consisting in silencing expression of GRASP55
489 and measuring TGF- β or membrane bound SCF expression were not performed in these
490 studies. Thus, we cannot exclude that bone marrow stromal cells use alternative trafficking

491 machinery to express these niche factors. Our results are more difficult to reconcile with the
492 described function of GRASP55 in regulating MT1-MMP activation or IL1 β secretion (23,
493 27, 29). Indeed, MT1-MMP activation by furin has been shown to depend on GRASP55 using
494 dominant negative approaches and overexpression of GRASP55 in HT1080 human
495 fibrosarcoma cell line (29). MT1-MMP plays a central role in maintenance of hematopoietic
496 homeostasis through the control of HIF1 α and MMP2 activation (42, 43). The latter has been
497 shown to activate MMP9 which releases membrane-bound SCF upon HSC mobilization with
498 G-CSF (9). We failed to detect hematopoietic alterations at steady state homeostasis or upon
499 G-CSF-induced HSC mobilization in *Grasp55*^{-/-} mice indicating that alternative pathways
500 regulating MT1-MMP activation must exist in bone marrow stromal cells. Same is true for
501 IL1 β secretion. Indeed a first study has shown that silencing *Grasp55* expression in bone
502 marrow derived macrophages (BMDM) reduces IL1 β secretion induced by nigericin and
503 starvation (27). A more recent study has demonstrated that IL1 β secretion is reduced in
504 BMDM isolated from *Grasp55*-deficient mice due to impaired unfolded protein response
505 signaling (23). However, we found no differences in hematopoietic response upon 5-FU
506 treatment which is known to involve IL1 β secretion by myeloid cells (44). This discrepancy
507 may be due to differences in the pathways involved in IL1 β secretion by BMDM as compared
508 to IL1 β secretion by the whole organism in response to hematopoietic stress. This raises the
509 question as to whether silencing *Grasp55* in isolated cellular models reflects its physiological
510 function. Alternatively, it may well be that redundant unconventional secretory pathways are
511 present in higher eukaryotes and that compensatory mechanisms take place in absence of
512 *Grasp55*. Indeed, a single GRASP isoform is present in *Saccharomyces cerevisiae* and has
513 been involved in autophagic mediated secretion of Acb1 which lacks a signal sequence
514 similar to IL1 β (45). This *Grasp* homologue represents the unique PDZ-containing protein of
515 yeast. This is in sharp contrast with the situation in mammals in which more than 250 PDZ

516 domains are present in more than 150 proteins (46). Therefore, it would be interesting to
517 perform a shRNA screening against genes encoding PDZ containing proteins that could
518 compensate *Gorasp2* deficiency for maintenance of hematopoietic stem cell homeostasis.

519 More interestingly, our results demonstrate that E μ -Myc transformed hematopoietic cells
520 become addict to Grasp55 pathway. Myc-dependent transformation is well known to induce
521 unfolded protein response (UPR) which results in endoplasmic reticulum stress-induced
522 autophagy (31). Autophagy promotes survival of tumor cells through processing cellular
523 contents destined for degradation in order to support bioenergetics (30). Since Grasp55 has
524 been involved in autophagy-driven pathways of unconventional secretion and in
525 autophagosome-lysosome fusion upon glucose deprivation (19, 27, 45, 47), it may well be
526 that the E μ -Myc Grasp55^{KD} cells lose their ability to use autophagy as rescue mechanism and
527 become sensitized to apoptosis. This would be consistent with previous findings showing that
528 caloric restriction sensitizes E μ -Myc cells to inhibitors of the antiapoptotic Bcl-2 protein
529 family (48). It would therefore be interesting to explore effects of combined drug inhibition of
530 Grasp55 and antiapoptotic proteins in additional cancer models known to involve UPR
531 response and autophagy.

532

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537

538 **Author contributions**

539 ALB and JMPG designed and performed experiments, analyzed results, and wrote the
540 manuscript. ACM, FB, MDG, MB and AG performed experiments. JCL performed lentiviral
541 transductions. SJCM designed the flow cytometry panels and wrote the manuscript. AS
542 performed imaging analysis. MAL supervised the study, analyzed results, discussed data and
543 wrote the manuscript. All authors provided valuable inputs on the manuscript.

544

545 **Additional information**

546 The authors declare no competing interests.

547

548 **References**

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711

712 **Legend to Figures**

713 **Figure 1: Regulated expression of GRASP55 during early mouse hematopoietic cell**

714 **differentiation. (A)** Flow-cytometry gating strategy used to define hematopoietic stem and

715 progenitor cells (HSPC). Representative dot-plot for wild-type mouse is shown.

716 Hematopoietic Stem Cells and Multi-Potent Progenitor 1 (HSC-MPP1) are defined as LSK

717 ($\text{Lin}^- \text{Sca-1}^+ \text{c-Kit}^+$) $\text{CD150}^+ \text{CD48}^-$, Multi-Potent Progenitor 2 MPP2 as LSK CD150^+

718 CD48^+ , MMP3-4 as LSK $\text{CD150}^- \text{CD48}^+$. Common Myeloid Progenitors (CMP) and

719 Granulocyte Macrophage Progenitor (GMP) are respectively defined as LK ($\text{Lin}^- \text{Sca-1}^- \text{c-}$

720 Kit^+) $\text{CD127}^- \text{CD16/32}^-$ and LK $\text{CD127}^- \text{CD16/32}^+$. **(B)** Heat-map representation of relative

721 mRNA expression as detected by RT-qPCR on 500 sorted cells. Actin expression levels were

722 used for normalization. **(C)** Histogram showing changes in GRASP55 expression as measured

723 by flow-cytometry. Results are expressed as fold changes relative to mean fluorescence

724 intensity signals obtained for HSC-MPP1 subset. $n=4$ mice, * $p < 0.05$, ** $p < 0.01$. **(D)**

725 Representative western-blot against GRASP55 on cell lysates obtained from 20 000 sorted

726 cells. Ponceau red staining is shown as loading control. **(E)** Representative pictures of

727 GRASP55 immunostaining on indicated cells isolated by flow-cytometry cell sorting. Cells

728 isolated from *Grasp55*^{-/-} mice were used as control. $n= 3$ mice. **(F)** Summed pixel intensities

729 of individual objects found in individual confocal planes of Z stacks acquired on five HSC-

730 MPP1, eight MPP2 and six MPP3-4 cells. *** $p < 0.001$. **(G)** Three dimensional

731 quantification expressed as the volume of the largest vesiculo-tubular structures found in

732 HSC-MPP1, MPP2 and MPP3-4 cells analyzed in (E). A significant enlargement of

733 GRASP55 stained structures was found in MPP3-4. * $p < 0.05$, ** $p < 0.01$.

734

735 **Figure 2: Early hematopoietic differentiation in *Grasp55*^{-/-} mice. (A)** Absolute numbers of

736 bone marrow cells isolated from *Grasp55*^{-/-} and control littermate mice. Results are expressed

737 as mean +/- SEM of cell numbers isolated from two legs per mice (n=6 per group). **(B-D)**
738 Relative frequencies of LSK (Lin^{Neg}Sca^{Pos}Kit^{Pos}) cells (B), HSC-MPP1, MPP2 and MPP3-4
739 cells (C) and CMP and GMP (D) found in the bone marrow of *Grasp55*^{-/-} and control
740 littermate mice. Flow-cytometry gating strategy used to define HSPC in control and *Grasp55*^{-/-}
741 deficient mouse is described in Fig. 1A. **(E)** Graph showing the relative Median Fluorescence
742 Intensity (MFI) detected by flow cytometry for JAM-C staining on HSC-MMP1, MPP2,
743 MPP3-4 cells isolated from *Grasp55*^{-/-} and control littermate mice. Results are expressed as
744 mean +/- SEM. Data are representative of six independent experiments.

745

746 **Figure 3 : HSC mobilization is not affected by *Grasp55* deficiency.**

747 **(A)** Relative frequencies of LSK (Lin^{Neg}Sca^{Pos}Kit^{Pos}) cells found in the bone marrow of
748 *Grasp55*^{-/-} and control littermate mice after HSC mobilization by CY/G-CSF. NaCl treated
749 mice are used as control. **(B)** Relative frequencies of LSK cells found in blood of CY/G-CSF
750 or NaCl injected *Grasp55*^{-/-} and *Grasp*^{+/+} mice. **(C)** Number of CFU-C mobilized into blood
751 from *Grasp55*^{-/-} and control littermate mice after CY/G-CSF. Each symbol represents the
752 average of colony numbers obtained per mice (2.10⁵ cells/well, two wells per mice). * p<
753 0.05, ** p< 0.01, *** p< 0.001. Results from a single experiment with n= 3 to 4 mice are
754 shown.

755

756 **Figure 4 : Competitive engraftment of *Grasp55* deficient bone marrow cells.**

757 **(A)** Experimental design of competitive bone marrow transplantation. The same number of
758 *Grasp55*^{+/+} or *Grasp55*^{-/-} donor (CD45.2) cells were mixed in 1:1 ratio with competitor
759 (CD45.1/2) bone marrow cells (See Sup Fig. 4A) and injected into CD45.1 lethally irradiated
760 recipients. **(B)** Graph showing the frequency of CD45.2 donor cells in the blood of recipient
761 mice engrafted either with *Grasp55*^{+/+} (white dots) or *Grasp55*^{-/-} (filled black dots) donor

762 cells. *p< 0.05 (C-F) Donor specific CD4⁺ T cells (C), CD8⁺ T cells (D), B220⁺ B cells (E)
763 and CD11b⁺ myeloid cells (F) frequencies in the blood of mice transplanted with *Grasp55*^{+/+}
764 (white dots) or *Grasp55*^{-/-} (filled black dots) bone marrow. (G-H) Relative frequencies of
765 donor and competitor LSK (Lin^{Neg}Sca^{Pos}Kit^{Pos}), HSC-MPP1, MPP2, MPP3-4 (G), CMP and
766 GMP (H) cells found in the bone marrow of mixed chimera engrafted with *Grasp55*^{+/+} or
767 *Grasp55*^{-/-} donor cells 18 weeks after cell injection. Results are expressed as mean +/- SEM.
768 Data show results of one representative experiment from two independent experiments.

769

770

771 **Figure 5 : *Grasp55* silencing inhibits Eμ-myc leukemic engraftment**

772 (A) Phenotype of primary Eμ-myc clone assessed by flow cytometry and showing the pre-B
773 cell nature of leukemic cells. (B) JAM-B expression by the primary Eμ-myc clone shown in
774 (A). (C) Western-blot analysis of Eμ-myc cells one week after transfection with Ctl-GFP or
775 shGRASP-55 lentivirus using GRASP55 (upper panel) or actin (lower panel) antibodies. (D)
776 Proliferation of Eμ-Myc GRASP55^{WT} and Eμ-Myc GRASP55^{KD} in complete medium was
777 determined using ATPmetry (Cell Titer Glow, Promega). Results are expressed as mean
778 RLU values +/- SEM obtained from four replicates. Doubling times are 16.20+/-1.468 hours
779 for Eμ-Myc GRASP55^{WT} and 20.10+/-3.869 hours for Eμ-Myc GRASP55^{KD}. (E-G) Tumor
780 invasion of Eμ-myc cells in the indicated organ nine days after injection of 50.000
781 GRASP55^{WT} or GRASP55^{KD} Eμ-myc cells in CD45.1 mice. Tumor invasion in spleen is
782 revealed by spleen weight and percentage of CD45.2⁺GFP⁺ cells (E). Percentages of CD45.2⁺
783 GFP⁺ cells in bone (F) and blood (G) are shown. Results are expressed as mean +/- SEM. n =
784 6 mice per group. One representative experiment from two independent experiments is
785 shown. ** p<0.01.

786

787 **Figure 6 : Grasp55 silencing decreases leukemic cell survival**

788 (A) Left panel: dot plot showing the indicated fluorescence of the mix of GRASP55^{WT}
789 (GFP⁺Calcein violet⁻) and GRASP55^{KD} (GFP⁺Calcein violet⁺) E μ -myc cells before injection.
790 Right panels: dot plots showing the GFP and Calcein violet fluorescence signals 16 hours
791 after injection in the indicated organs. Relative proportions of GRASP55^{WT} (Calcein violet⁻)
792 and GRASP55^{KD} (Calcein violet⁺) cells within the GFP⁺ compartments are indicated. (B)
793 Absolute number of GRASP55^{WT} and GRASP55^{KD} E μ -myc cells in the indicated organs
794 sixteen hours after injection. Each symbol represents an individual mouse. **n = 6 mice per**
795 **group. Results obtained in a single experiment are expressed as mean +/- SD. *** p<0.001.**

796

797 **Figure 7: Grasp55 silencing increases apoptosis**

798 (A) Histograms showing Caspase 3/7 activity of GRASP55^{WT} (white bar) and GRASP55^{KD}
799 E μ -myc cells (black bar) twelve hours after plating and expressed as fold increase over time
800 0. (B) Dot plots showing representative results of AnnexinV and iodinium propide (PI) or
801 cytometry staining of GRASP55^{WT} and GRASP55^{KD} E μ -myc cells twelve hours after plating
802 in fresh medium. (C) Quantification of results shown in (B) showing significant decrease
803 survival of GRASP55^{KD} as compared to GRASP55^{WT} E μ -myc cells twelve hours after plating.
804 Conversely, significant increases in early and late apoptosis are observed * p<0.05, ***
805 p<0.001. (D) Graph showing E μ -myc cell survival upon glucose starvation after 96 hours.
806 Results are expressed as percentage of survival obtained in complete medium. * p<0.05. **One**
807 **representative experiment from three independent experiments is shown.**

808

809 **Figure 8: GRASP55 is not required for autophagy.** (A) Cytotoxicity of chloroquine on
810 GRASP55^{WT} and GRASP55^{KO} E μ Myc and Grasp55^{+/+} and Grasp55^{-/-} MEFs at 24 hours using
811 Cell Titer Glo assay. (B) IC₅₀ of chloroquine on GRASP55^{WT} and GRASP55^{KO} E μ Myc and

812 Grasp55^{+/+} and Grasp55^{-/-} MEFs at 24hours. Results of one representative experiment from
813 three independent experiments are shown. (C-D) Western blot against GRASP55 and LC3 on
814 GRASP55^{WT} and GRASP55^{KO} EμMyc (C) and Grasp55^{+/+} and Grasp55^{-/-} MEFs (D) after 24
815 hours chloroquine treatment. Tubulin was used as loading control. One representative
816 experiment from three independent experiments is shown.
817

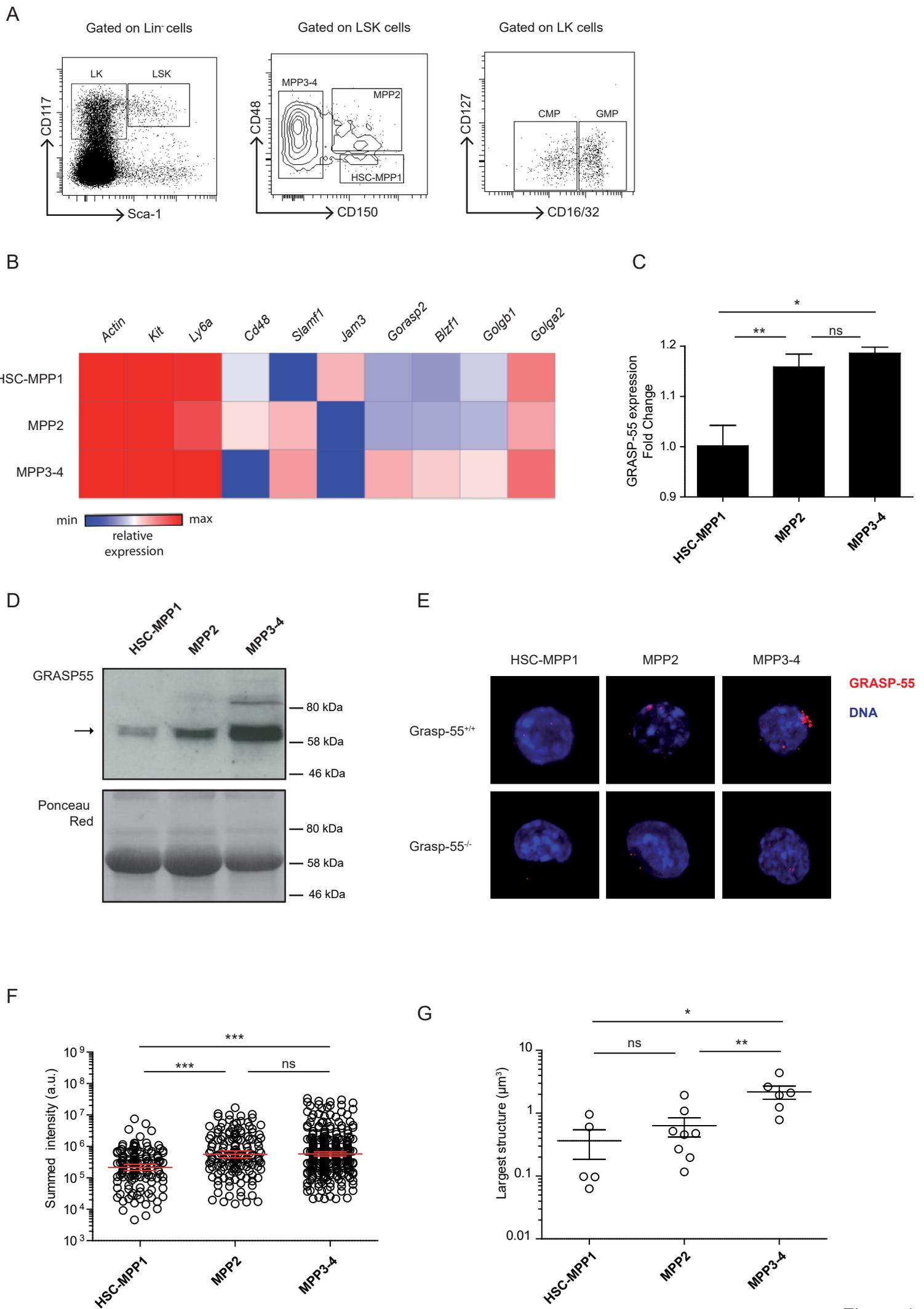


Figure 1

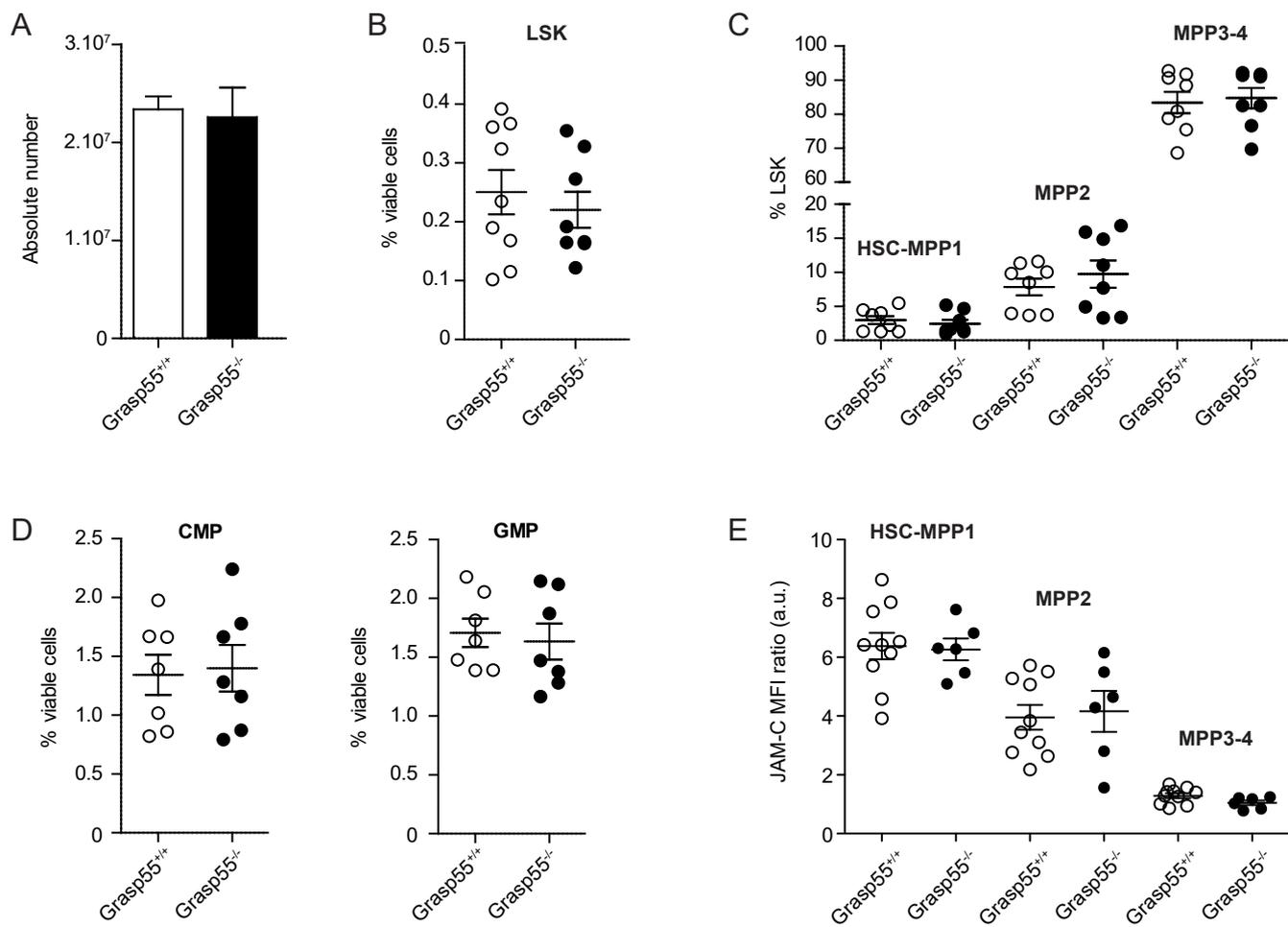
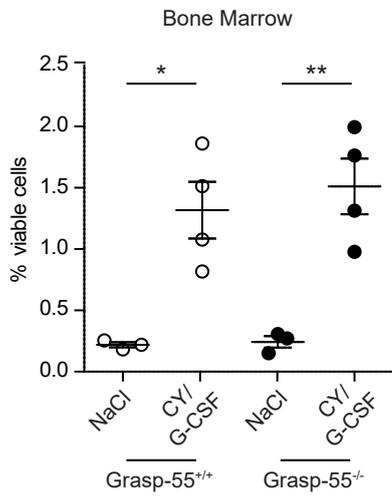
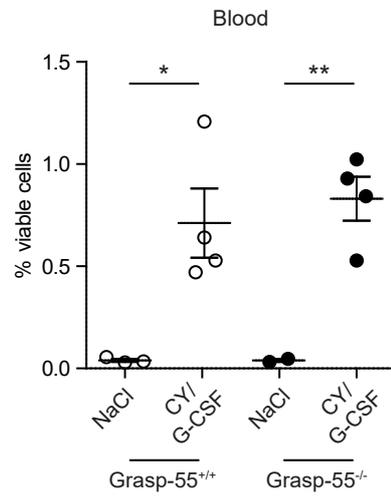
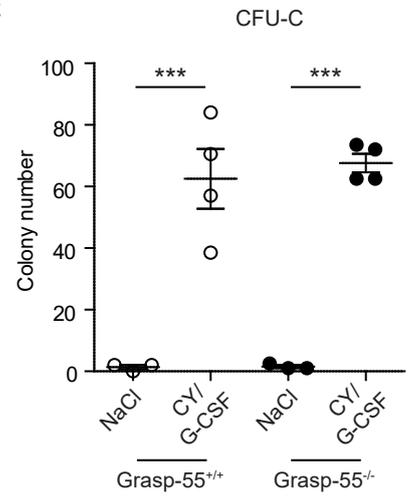


Figure 2

A**B****C**

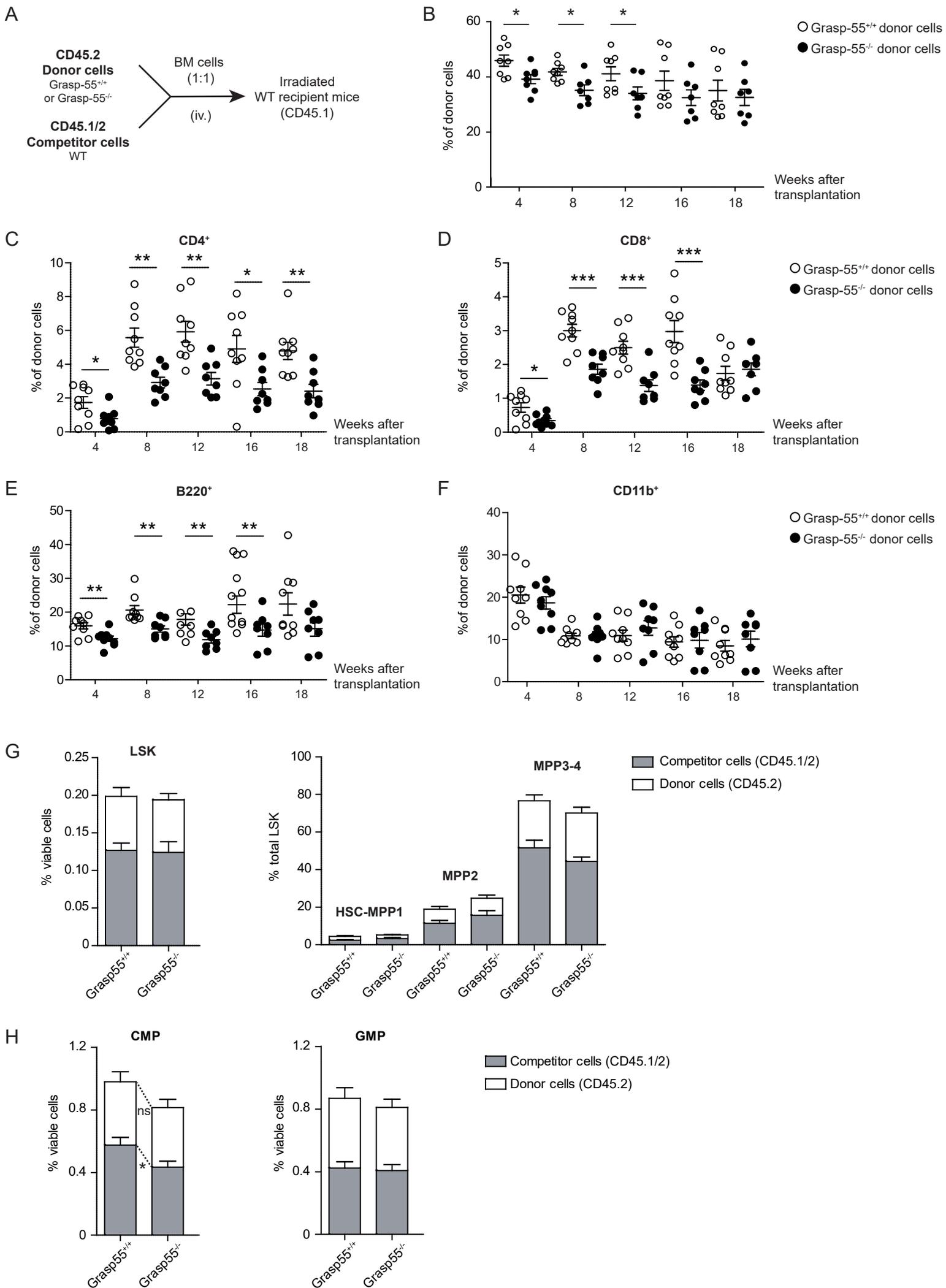


Figure 4

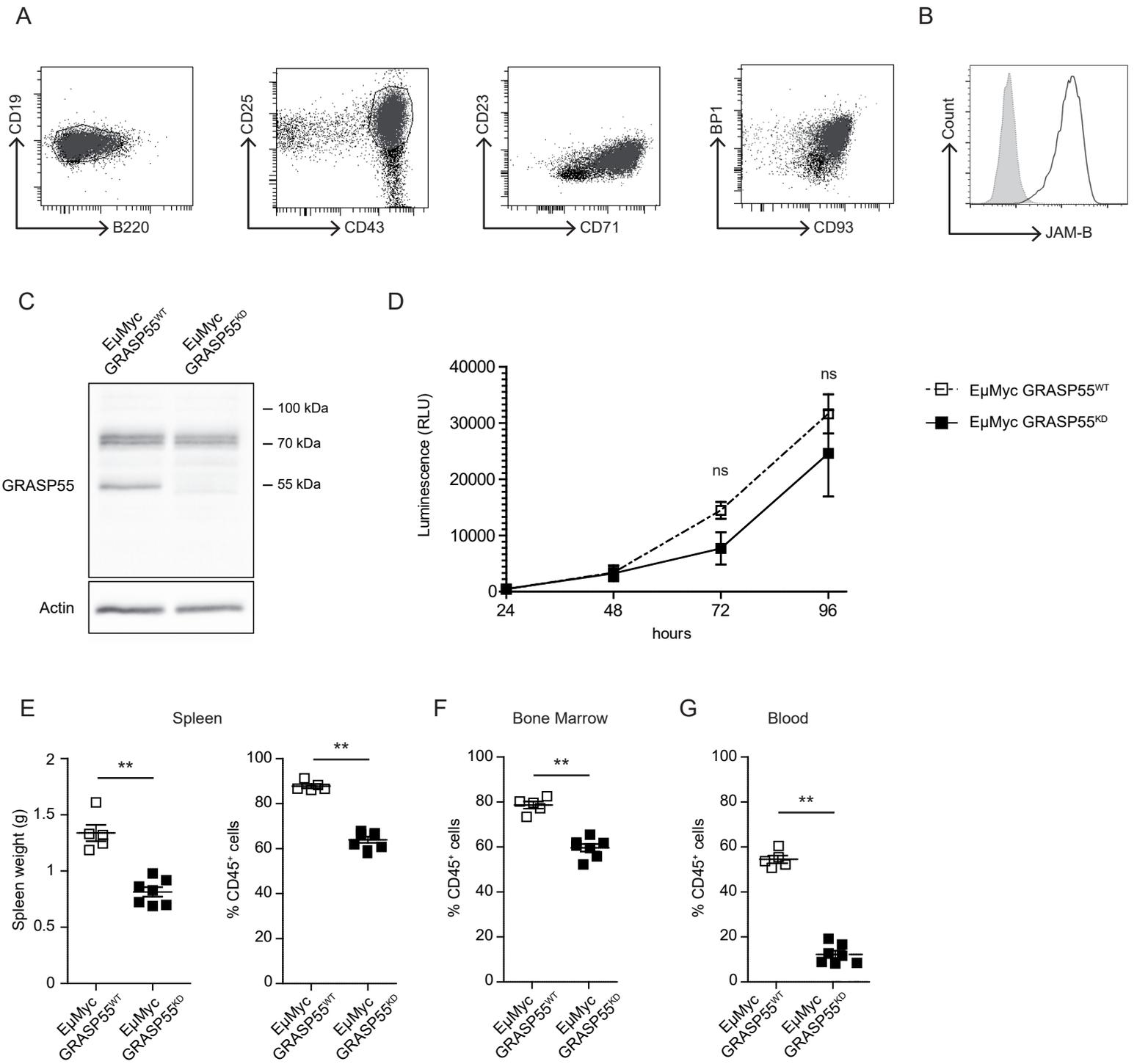


Figure 5

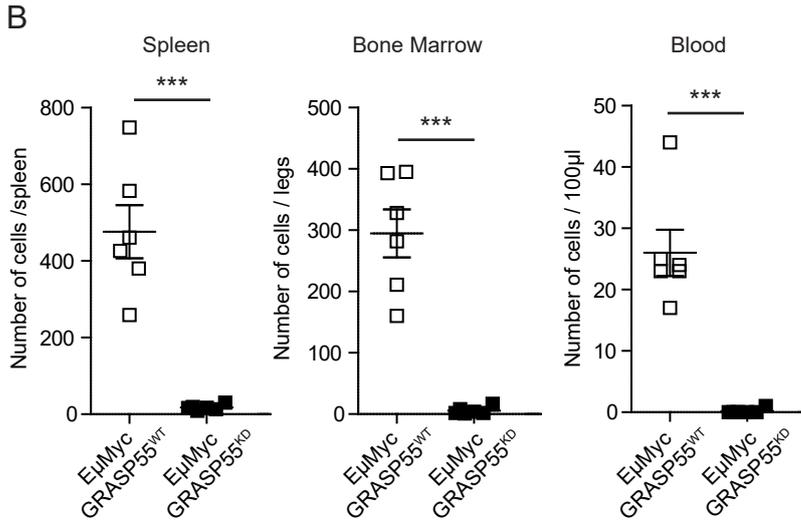
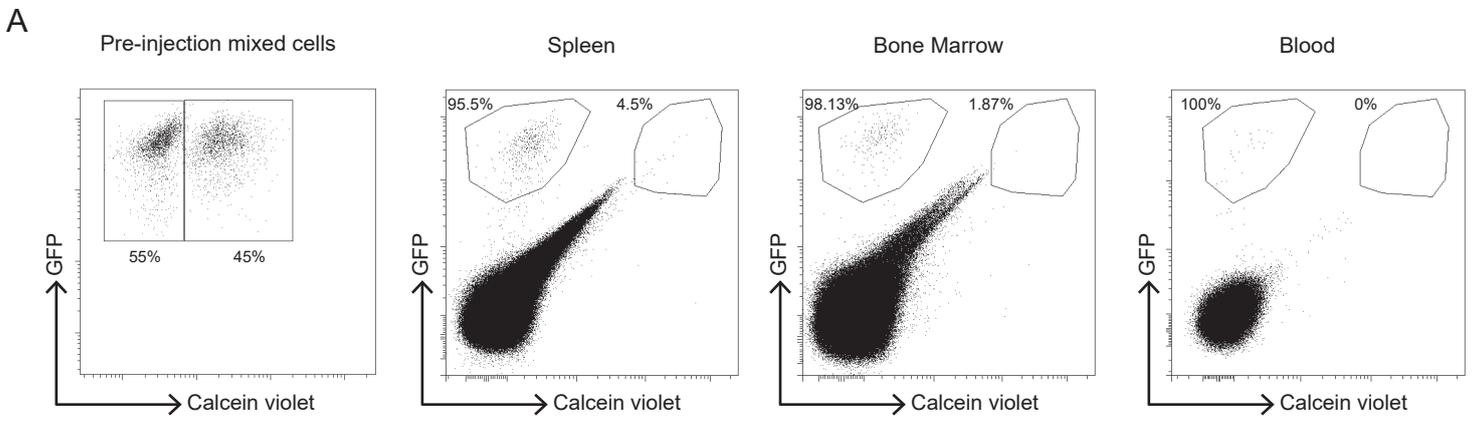
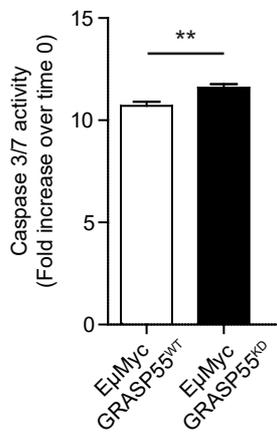
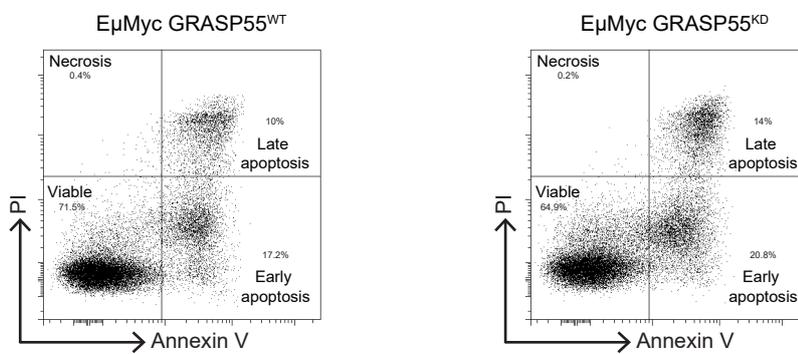


Figure 6

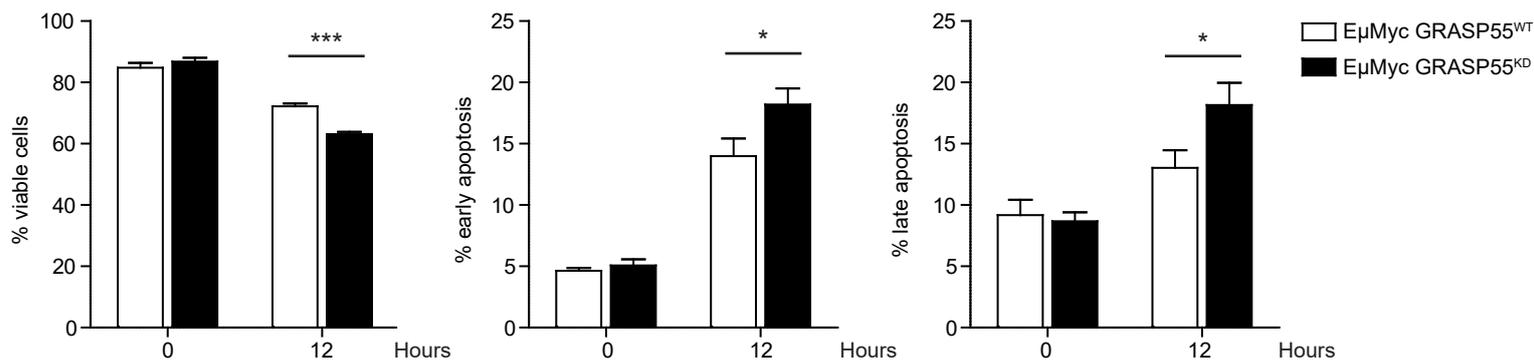
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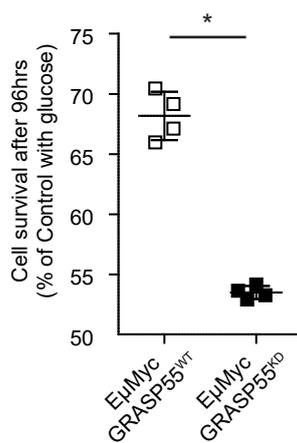
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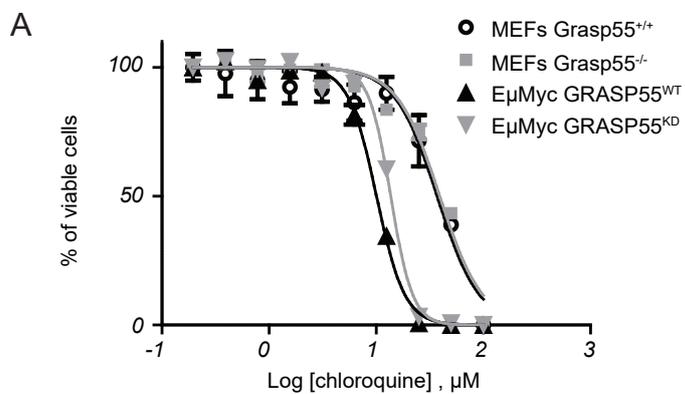


C



D





B

	IC50 (μM)
EμMyc GRASP55 ^{WT}	8.85
EμMyc GRASP55 ^{KD}	11.56
MEFs Grasp-55 ^{+/+}	37.01
MEFs Grasp-55 ^{-/-}	37.53

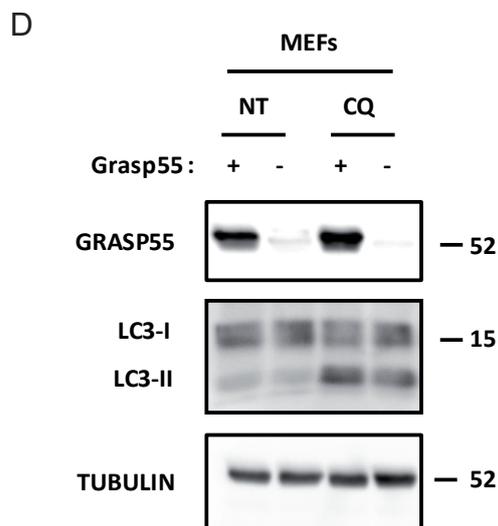
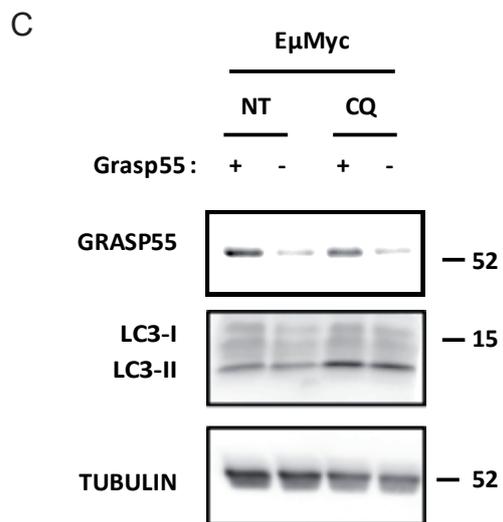
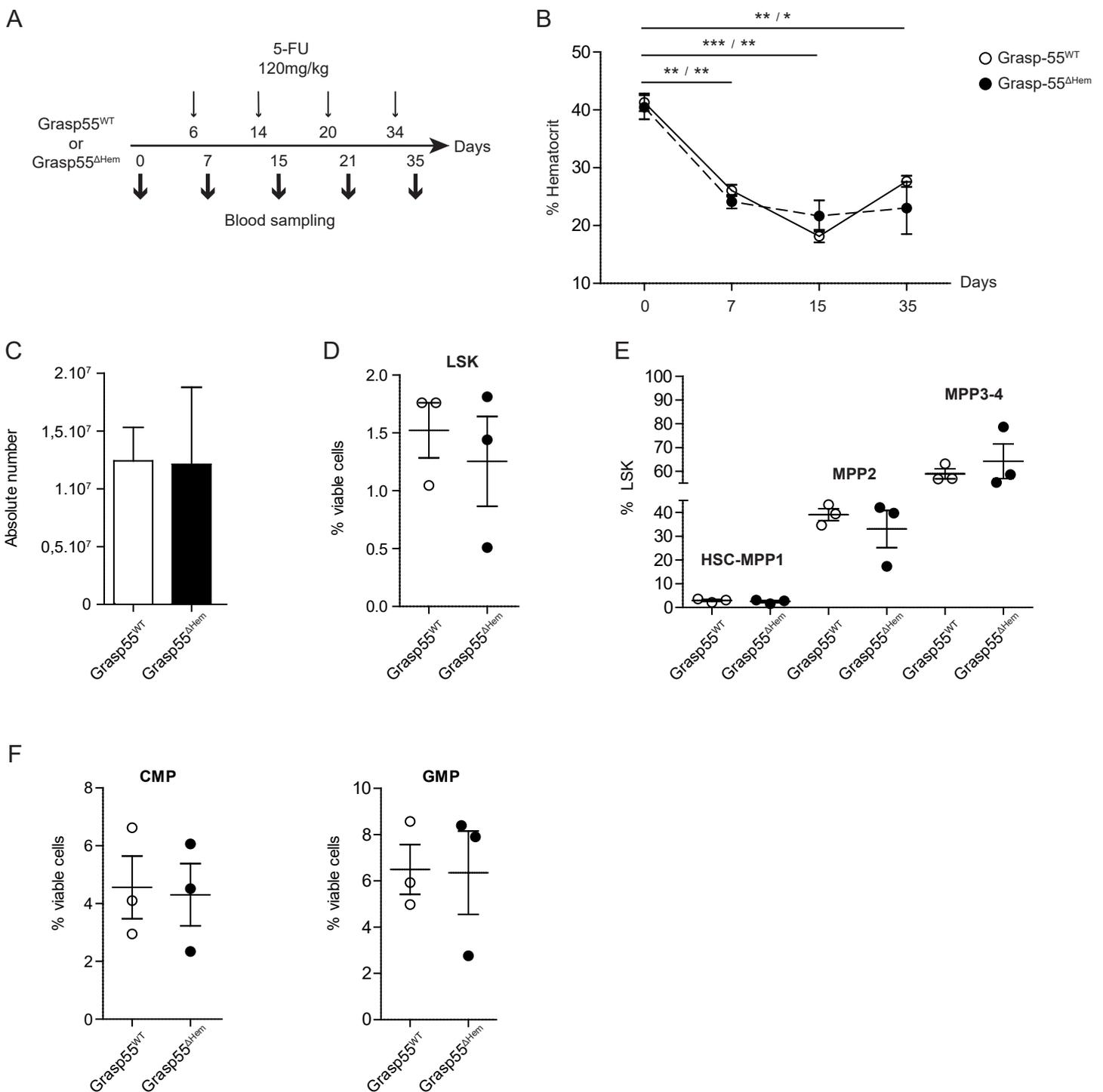


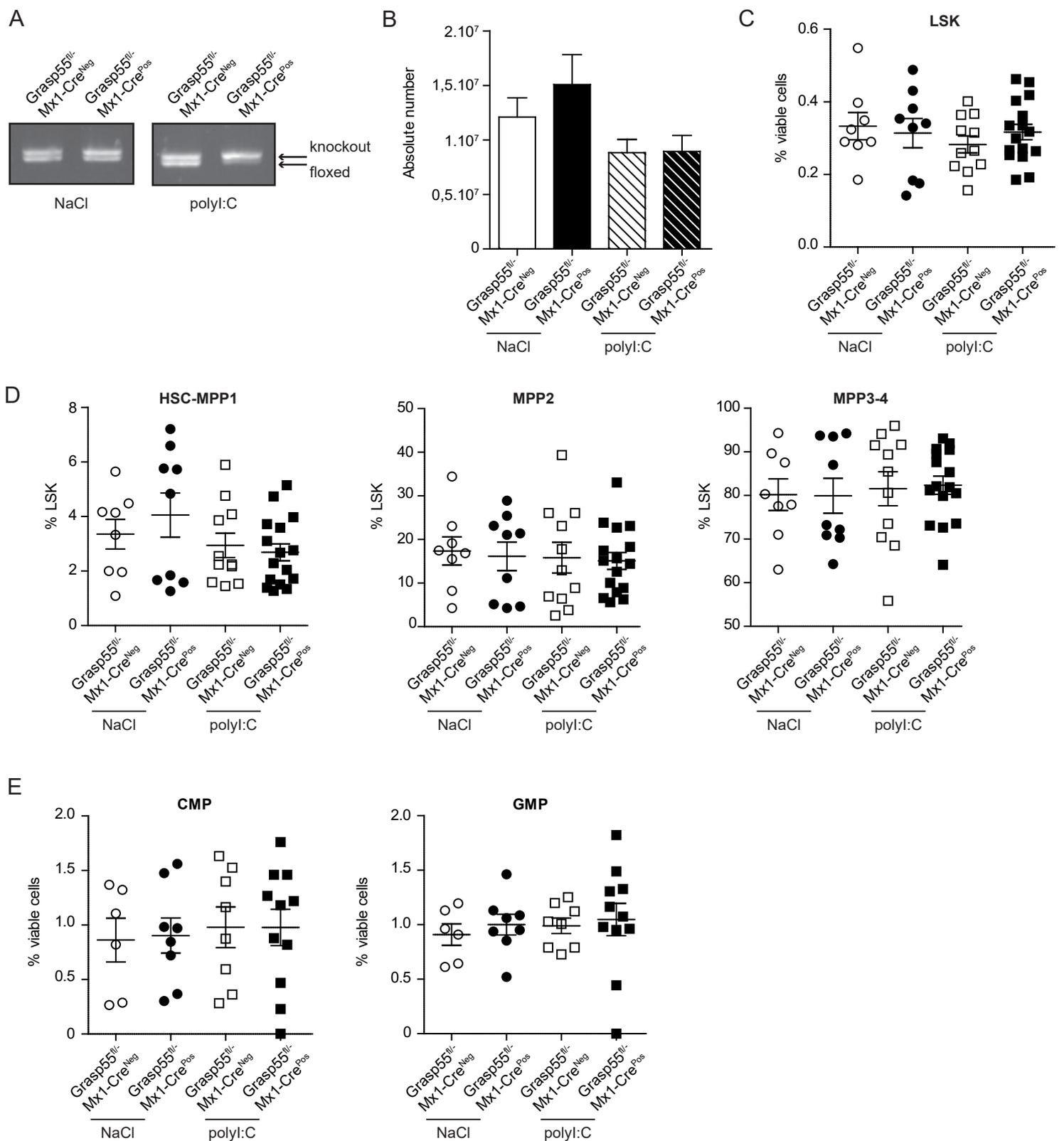
Figure 8

GRASP55 is dispensable for normal hematopoiesis but necessary for Myc-dependent leukemic growth.

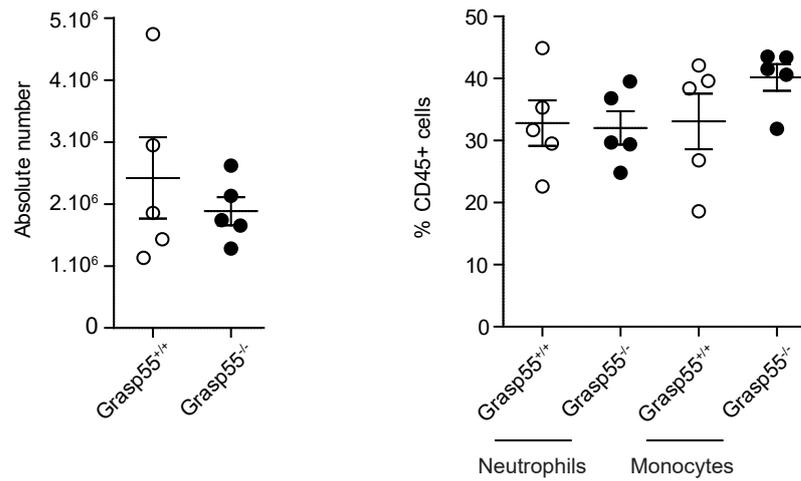
Anne-Laure Bailly^{*}, Julien M.P. Grenier^{*}, Amandine Cartier-Michaud^{*}, Florence Bardin^{*}, Marielle Balzano^{*}, Armelle Goubard^{*}, Jean-Claude Lissitzky^{*}, Maria De Grandis[†], Stéphane J.C. Mancini^{*}, Arnauld Sergé^{*} and Michel Aurrand-Lions^{*#}



Supplementary Figure 1: Hematopoietic stress response of *Grasp55*^{-/-} mice. (A) Experimental design for HSC exhaustion after 5-fluoro-uracil (5-FU) treatment: *Grasp55*^{fl/-} Vav-cre^{Neg} and *Grasp55*^{fl/-} Vav-cre^{Pos} mice were injected weekly with 120mg/kg 5-FU and bled one day later over five weeks. Mice were sacrificed at D35 after first injection. **(B)** Hematocrit levels of *Grasp55*^{fl/-} Vav-cre^{Neg} and *Grasp55*^{fl/-} Vav-cre^{Pos} mice at indicated time points. (n=3 per group). * p<0.05, ** p<0.01, *** p<0.0001. **(C)** Absolute numbers of bone marrow cells isolated from *Grasp55*^{fl/-} Vav-cre^{Neg} and *Grasp55*^{fl/-} Vav-cre^{Pos} animals at sacrifice. **(D-F)** Relative frequencies of LSK (D), HSC-MPP1, MPP2, MPP3-4 (E), CMP and GMP (F) found in the bone marrow of from *Grasp55*^{fl/-} Vav-cre^{Neg} and *Grasp55*^{fl/-} Vav-cre^{Pos} animals after five injections of 5-FU. Results from a single experiment are shown.



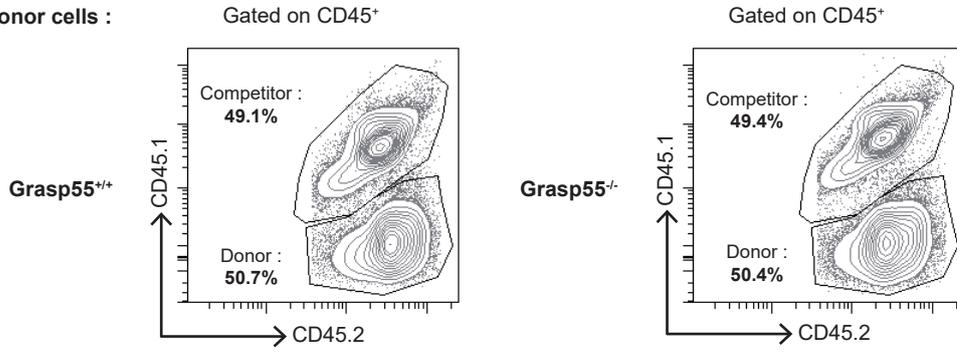
Supplementary Figure 2: Induced deletion of Grasp55 in adult hematopoietic cells does not result in impaired hematopoiesis. **(A)** Agarose gel of the PCR-amplified products obtained from white blood cells isolated from Grasp55^{fl/-} Mx1-Cre^{Neg} and Grasp55^{fl/-} Mx1-Cre^{Pos} fifteen days after injection with polyI:C or NaCl. **(B-E)** Absolute numbers of bone marrow cells (B) and relative frequencies of LSK (C) HSC-MPP1, MPP2, MPP3-4 (D) CMP and GMP (E) found in the bone marrow of from Grasp55^{fl/-} Mx1-Cre^{Neg} and Grasp55^{fl/-} Mx1-Cre^{Pos} one month after Mx1-cre induction with polyI:C. NaCl injected animals and mice lacking Mx1-Cre expression are used as control. No significant difference between experimental groups is observed. Results represent the pool of two independent experiments with n=3 to 5 mice per group.



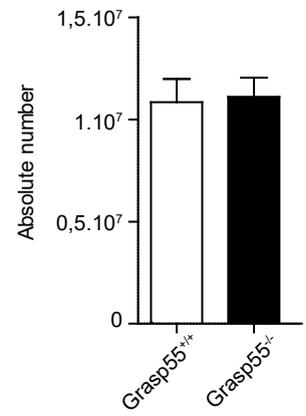
Supplementary Figure 3: GRASP-55 is dispensable for inflammatory cells recruitment in thioglycollate-induced peritonitis. Absolute number of total cells (left panel) and leukocytes frequencies (right panel) in peritoneal lavage fluids of Grasp55^{-/-} and Grasp55^{+/+} chimeric mice 18 hours after thioglycollate i.p. injection. One representative experiment is shown (from three independent experiments).

A

Donor cells :



B



Supplementary Figure 4: Competitive bone marrow transplantation

(A) Representative dot plots showing the 1:1 ratio of donor and competitor cells for the indicated experimental group used in Figure 4 (*Grasp55*^{-/-}, left panel and *Grasp55*^{+/+}, right panel). (B) Absolute number of bone marrow cells in recipient mice 18 weeks after engraftment with *Grasp55*^{+/+} and *Grasp55*^{-/-} donor cells.