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- 1 Using a mouse model harboring a WHIM Syndrome-linked gain-of-function CXCR4 mutation and
- 2 bone marrow samples from WHIM patients, the authors show that proper CXCR4 signaling
- 3 termination is essential for bone tissue homeostasis.

1 **WHIM Syndrome-linked *CXCR4* mutations drive osteoporosis**

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26

27 **Abbreviations:** BMSC: Bone marrow stromal cell; C-tail: Carboxyl-terminal tail; HSPC:  
28 Hematopoietic stem and progenitor cell; MFI: Mean fluorescence intensity; OBL: Osteoblast;  
29 OCL: Osteoclast; Ocn: Osteocalcin; OPC: Osteoblastic progenitor; Opn: Osteopontin; Osx:  
30 Osterix; SLAM: Signaling lymphocyte activation molecule; SSC: Skeletal stromal/stem cell;  
31 WS: Warts, Hypogammaglobulinemia, Infections and Myelokathexis Syndrome.

32

33 **ABSTRACT**

34 WHIM Syndrome is a rare immunodeficiency caused by gain-of-function *CXCR4* mutations.  
35 Here we report a decrease in bone mineral density in 25% of WHIM patients and bone defects  
36 leading to osteoporosis in a WHIM mouse model. Imbalanced bone tissue is observed in mutant  
37 mice combining reduced osteoprogenitor cells and increased osteoclast numbers.  
38 Mechanistically, impaired CXCR4 desensitization disrupts cell cycle progression and  
39 osteogenic commitment of skeletal stromal/stem cells, while increasing their pro-  
40 osteoclastogenic capacities. Impaired osteogenic differentiation is evidenced in primary bone  
41 marrow stromal cells from WHIM patients. In mice, chronic treatment with the CXCR4  
42 antagonist AMD3100 normalizes *in vitro* osteogenic fate of mutant skeletal stromal/stem cells  
43 and reverses *in vivo* the loss of skeletal cells, demonstrating that proper CXCR4 desensitization  
44 is required for the osteogenic specification of skeletal stromal/stem cells. Our study provides  
45 mechanistic insights into how CXCR4 signaling regulates the osteogenic fate of skeletal cells  
46 and the balance between bone formation and resorption.

47

48 **Keywords:** Skeletal stromal/stem cell; Bone marrow; CXCR4 signaling; Osteogenesis; WHIM  
49 Syndrome; Osteoporosis.

50

51

52

53 **INTRODUCTION**

54 The bone marrow (BM) is a complex structural and primary immune organ whose development  
55 and maintenance depend on multiple cell types including cells of the hematopoietic lineage like  
56 hematopoietic stem and progenitor cells (HSPCs), but also vascular cells and numerous skeletal  
57 cells encompassing BM stromal cells (BMSCs), skeletal progenitor/precursor cells as well as  
58 bone-making osteoblasts (OBLs)<sup>1,2</sup>. Together these cells compose specialized micro-  
59 anatomical structures called “niches” that sustain their survival and differentiation<sup>3-9</sup>. For  
60 instance, the HSPC niches are thought to be composed of perivascular stromal units associated  
61 with sinusoids and arterioles<sup>10-15</sup>. Bone and adipose cells are thought to derive from subsets of  
62 BMSCs that are located near blood vessels and function as skeletal stromal/stem cells (SSCs)<sup>16-</sup>  
63 <sup>19</sup>. However, the exact localization, composition and crossover of these niches in relation with  
64 bone function are not yet established. Bone tissue homeostasis relies on the balance between  
65 formation and resorption of bone matrix mediated by effector cells that derive from SSCs and  
66 HSPCs respectively. Disequilibrium of this balance can lead to diseases such as osteoporosis  
67 or osteopetrosis. In such a landscape, SSCs are key players: not only they give rise to OBLs but  
68 they also contribute to perivascular structures important for HSPCs<sup>20-28</sup>. Understanding how  
69 SSCs maintain their identity, achieve plasticity and support hematopoiesis in adult BM is thus  
70 an important emerging field<sup>3,6,9,12,29</sup>. Recently, Ambrosi and coll. showed that intrinsic ageing  
71 of SSCs skews skeletal and hematopoietic lineage outputs, leading to fragile bones<sup>30</sup>. Moreover,  
72 Jeffery and coll. reported how BM and periosteal SSCs contribute to bone maintenance and  
73 repair<sup>31</sup>. However, both extrinsic and intrinsic mechanisms regulating their fate remain  
74 incompletely understood.

75 In adult BM, signaling by the G protein-coupled receptor CXCR4 on HSPCs in response  
76 to stimulation by the chemokine CXCL12/Stromal cell-derived factor-1, produced by BMSCs  
77 constitutes a key pathway through which the stromal niches and HSPCs communicate<sup>32-37</sup>.

78 Conditional ablation of *Cxcl12* from perivascular stromal cells or OBLs demonstrated that  
79 HSCs occupy a perivascular but not an endosteal niche<sup>21,38</sup>, whereas targeted deletion of *Cxcl12*  
80 from BM stromal cells has allowed the identification of specialized niches supporting leukemia  
81 stem cell maintenance<sup>39</sup>. Both *Cxcr4* and *Cxcl12* are broadly expressed by non-hematopoietic  
82 tissues and cell types and have multifunctional roles beyond hematopoiesis. Since mice  
83 deficient for *Cxcr4* or *Cxcl12* die perinatally, our understanding of the role of the *Cxcl12/Cxcr4*  
84 axis in regulating the BM ecosystem is mostly based on relatively selective loss-of-function  
85 models<sup>21,40-44</sup>. Conditional inactivation of *Cxcl12* or *Cxcr4* in paired-related homeobox gene 1  
86 (*Prx1*)- or osterix (*Osx*)-expressing cells, *i.e.* respectively multipotent mesenchymal  
87 progenitors or osteoprogenitor cells (OPCs) and descendant OBLs, was associated with reduced  
88 postnatal bone formation, suggesting a positive regulatory role of this pair in OBL development  
89 and/or function<sup>21,43,44</sup>. Single cell transcriptomics recently suggested heterogeneity within adult  
90 *Cxcl12*- or Leptin-receptor (*LepR*)-expressing mesenchymal cells poised to undergo either  
91 adipogenic or osteogenic specification<sup>45,46</sup>. However, it is still unclear whether and how *Cxcr4*  
92 signaling regulates intrinsically osteogenic specification of skeletal cells.

93 Here, we addressed this point using as a paradigm the WHIM Syndrome (WS), a rare  
94 immunodeficiency caused by viable inherited heterozygous gain-of-function mutations in  
95 *CXCR4* affecting homologous desensitization of the receptor, thus resulting in enhanced  
96 signaling following CXCL12 stimulation, defective lymphoid differentiation of HSPCs and  
97 reduced blood leukocyte numbers<sup>47-50</sup>. Taking advantage of a mouse strain that harbors the  
98 naturally occurring WS-linked heterozygous *CXCR4*<sup>S338X</sup> mutation (*Cxcr4*<sup>+/1013</sup>, +/1013)<sup>51-54</sup>,  
99 and of human BM samples from WS donors and clinical data from 19 WS patients, we  
100 investigated whether WS mutations affect the SSC landscape with a particular attention given  
101 to the bone fraction. WS-linked *CXCR4* mutations were associated with reduced bone mass in  
102 mice and humans. In mice, this relied on impaired CXCR4 desensitization that disrupts cell

103 cycle progression and osteogenic commitment of SSCs, while paradoxically increasing the pro-  
104 osteoclastogenic capacities of these cells. Impairment in osteogenic potential was also  
105 evidenced in BMSCs from WS patients. Thus, proper *CXCR4* desensitization is required for  
106 the osteogenic specification of bone SSCs.

107



108 **RESULTS**109 **WS-linked CXCR4 mutations are associated with reduced bone mass in mice and humans**

110 Following CXCL12 stimulation,  $\beta$ -arrestins are recruited to the carboxyl-terminal tail (C-tail)  
111 domain of CXCR4, precluding further G-protein activation (*i.e.* desensitization) and leading to  
112 receptor internalization<sup>55</sup>. Both processes are dysregulated in WS most often due to autosomal-  
113 dominant gain-of-function mutations that result in the distal truncation of the C-tail of CXCR4  
114 and a desensitization-resistant, hyperactive receptor<sup>56</sup>. Although the impact of these WS  
115 mutations on immune cells is currently being understood<sup>51-54,57</sup>, nothing is known about their  
116 impact on the skeletal landscape. Bone mineral density (BMD) values were measured in 19  
117 patients with WS for lumbar spine and femoral neck by total body dual-energy X-ray  
118 absorptiometry. BMD T- and Z-scores were found to be low at least in one site in five patients  
119 (Table 1). Likewise, this was evidenced in adult (8-12-week-old) *Cxcr4*<sup>1013</sup>-bearing (*i.e.*  
120 heterozygous [+ /1013] and homozygous [1013/1013]) mice, as compared to *Cxcr4*<sup>+/+</sup> (WT)  
121 mice. Analyses of lumbar spine revealed decreased BMD values in mutant mice, in a *Cxcr4*<sup>1013</sup>  
122 allele dose-dependent manner (Fig. 1A). Micro-computed tomography ( $\mu$ CT) analyses further  
123 unraveled a reduction in trabecular and cortical bone in mutant mice (Fig. 1B). In mutant  
124 femurs, there was a reduction in the trabecular bone density that followed a *Cxcr4*<sup>1013</sup> allele  
125 copy number-dependent pattern. This was characterized by a significant decrease in bone  
126 volume and trabecular numbers, while the trabecular separation was increased compared to WT  
127 mice (Fig. 1C). The cortical bone volume and thickness were also affected (Fig. 1B and 1D).  
128 This gene-dependent reduction was observed among both female and male mutant mice.  
129 Histomorphometric analyses confirmed decreased bone volume and trabecular numbers in  
130 mutant mice as shown by toluidine blue and osteopontin (Opn) staining (Fig. 1E and 1F).  
131 Strikingly, staining for Alcian Blue and perilipin that are used for chondrocyte and adipocyte  
132 identification respectively, were unaltered in mutant bone (Fig. 1G and 1H). Consistently, the

133 thickness of the growth plate as well as the adipocyte content were similar in mice carrying the  
134 *Cxcr4* mutation compared to WT ones (Fig. 1I and 1J), thus suggesting that *Cxcr4* specifically  
135 regulates the osteogenic fate in adults. Moreover, adult mutant mice did not exhibit significant  
136 changes of body size or weight (Fig. 1K), indicating that the *Cxcr4* mutation likely does not  
137 alter the skeletal growth. Overall, these findings revealed an osteopenic skeleton in *Cxcr4*<sup>1013</sup>-  
138 bearing mice and low BMD in 25% of WS patients.

139

#### 140 **Reduction of skeletal stromal cells in *Cxcr4*<sup>1013</sup>-bearing mice**

141 We then evaluated by flow cytometry the bone composition of WT and mutant mice with a  
142 focus on skeletal cells that encompass notably SSCs, OPCs and OBLs<sup>58</sup>. Long bones were  
143 flushed and then digested. Total stromal cells in the bone fraction were identified as negative  
144 for CD45, Lineage (including Ter119), c-Kit and CD71 expression as previously reported<sup>12,59</sup>.  
145 Endothelial cells were excluded based on CD31 expression. Two distinct CD51<sup>+</sup> stromal cell  
146 subsets were identified based on Sca-1 and PDGFR $\alpha$ : SSCs (Sca-1<sup>+</sup>PDGFR $\alpha$ <sup>+</sup>) and more  
147 differentiated OPCs (Sca-1<sup>-</sup>PDGFR $\alpha$ <sup>+/+</sup>) (Fig. 2A). We consider Sca-1<sup>+</sup>PDGFR $\alpha$ <sup>+</sup> cells at the  
148 top of the hierarchy based on previous works that indicated a highest CFU-F clonogenic  
149 potential of these cells as compared to Sca-1<sup>-</sup>PDGFR $\alpha$ <sup>+</sup> cells<sup>12,28,31,60</sup>. We observed a significant  
150 decrease of the numbers of OPCs, and to a lesser extent of SSCs, that followed a *Cxcr4*<sup>1013</sup>  
151 allele dose-dependent pattern (Fig. 2B), thus reinforcing that the landscape of the bone stroma  
152 is altered in *Cxcr4*<sup>1013</sup>-bearing mice.

153 We next examined *in vitro* the function of the signaling trio formed by Cxcl12 and its  
154 two receptors *Cxcr4* and *Ackr3* in skeletal cells. Membrane expression of *Cxcr4* and *Ackr3* was  
155 similar between WT and mutant skeletal cells including SSCs (Fig. S1A and S1B). However,  
156 +/1013 and 1013/1013 SSCs displayed both impaired *Cxcr4* internalization following Cxcl12  
157 stimulation as well as increased Cxcl12-mediated chemotaxis that was abolished by the specific

158 Cxcr4 antagonist AMD3100 (Fig. S1C and S1D). These dysfunctions likely relied on the  
159 enhanced signaling properties of the truncated Cxcr4 receptor as revealed by Erk PhosphoFlow  
160 analyses (Fig. S1E). Combined with the apparent preserved capacity of Ackr3 to bind and  
161 internalize Cxcl12 *in vitro* (Fig. S1F and S1G), these findings indicated a functional expression  
162 of the desensitization-resistant C-tail-truncated Cxcr4<sup>1013</sup> receptor on SSCs. Abnormal Cxcr4  
163 signaling was not associated with changes in apoptosis of SSCs (Fig. S1H).

164 To determine whether reduction of bone content in *Cxcr4*<sup>1013</sup>-bearing mice resulted  
165 from defects intrinsic to skeletal cells and/or an alteration of the hematopoietic (or another non-  
166 stromal) system, we performed reciprocal long (16 weeks)- and short (3 weeks)-term BM  
167 reconstitution experiments. First, BM cells from WT CD45.1<sup>+</sup> mice were transplanted into  
168 lethally irradiated 8-wk old CD45.2<sup>+</sup> WT or mutant (+/1013 and 1013/1013) mice (Fig. 2C).  
169 Sixteen weeks later, mutant recipients exhibited CD45.1<sup>+</sup> chimerism in hematopoietic  
170 compartments similar to those of WT recipients (Fig. 2D) but displayed reduced numbers of  
171 skeletal cells including SSCs and OPCs (Fig. 2E). Confocal imaging and  $\mu$ CT analyses  
172 confirmed that transplantation of WT BM was not sufficient to rescue the trabecular network  
173 in mutant recipients (Fig. 2F-I). This was also evidenced three weeks after WT BM  
174 transplantation (Fig. 2G). Although we cannot formally exclude that WT hematopoietic cells  
175 may be able to rescue the reduced trabecular bone content in mutant mice earlier in  
176 development, these results suggested that skeletal cell-autonomous Cxcr4 regulation  
177 contributes to the persistent bone defects in adult *Cxcr4*<sup>1013</sup>-bearing mice. We then performed  
178 reverse chimeras in which irradiated 8-wk old CD45.1<sup>+</sup> WT mice were reconstituted with WT,  
179 +/1013 or 1013/1013 CD45.2<sup>+</sup> BM (Fig. 3A). Sixteen weeks later, CD45.2<sup>+</sup> chimerism of LT-  
180 HSCs and leukocytes were decreased respectively in BM and blood of CD45.1<sup>+</sup> WT recipients  
181 engrafted with mutant BM, confirming the impaired reconstitution capacity of mutant HSCs  
182 (Fig. 3B and <sup>49</sup>). There were significantly lower numbers of skeletal cells and defective

183 trabecular bone content in *Cxcr4*<sup>1013</sup>-bearing BM-chimeric mice compared to WT chimeras as  
184 early as 3 weeks post-transplantation (Fig. 3C-G), thereby indicating cell-extrinsic Cxcr4-  
185 mediated regulation of the skeletal landscape. Likewise, cortical bone content was slightly  
186 affected (Fig. 3H and 3I). Altogether, these findings suggest that impaired Cxcr4 desensitization  
187 in both skeletal and hematopoietic cells have combinatorial effects on bone landscape  
188 dysregulation in adult *Cxcr4*<sup>1013</sup>-bearing mice.

189

### 190 **Increased bone resorption and reduced bone formation in *Cxcr4*<sup>1013</sup>-bearing mice**

191 Bone is maintained by coupled activities of bone-forming OBLs and bone-resorbing osteoclasts  
192 (OCLs). Alterations in bone balance can result in pathologic bone loss and osteoporosis. This  
193 led us to investigate whether the gain-of-*Cxcr4*-function mutation modulates the OBL/OCL  
194 balance. First, we analyzed bone resorption by quantifying OCL numbers in mice using Tartrate  
195 Resistant Acid Phosphatase (TRAP) staining<sup>61</sup>. We observed increased OCL surface (Oc.S/BS)  
196 and number (Oc.N/BV) in mutant mice compared to WT ones (Fig. 4A and 4B). To determine  
197 whether the increased bone resorption in mutant mice resulted from OCL-intrinsic defects, we  
198 performed *in vitro* OCL differentiation from BM cells in the presence of M-Csf and Rank-L  
199 and tested their bone resorption capacity. Similar OCL numbers and bone matrix resorption  
200 activities were observed among WT and mutant cultures (Fig. S2A and S2B), suggesting  
201 preserved intrinsic capacities of mutant BM myeloid cells to differentiate *in vitro* into functional  
202 OCLs. Congruent with this, we observed no changes in expression levels of osteoclastogenic  
203 genes in mutant cultures compared to WT ones (Fig. S2C). These findings indicate that the  
204 *Cxcr4* mutation does not affect *in vitro* OCL differentiation and function but suggest that  
205 osteoclastogenesis and increased bone resorption in mutant mice may be promoted by the BM  
206 environment.

207 *Cxcr4*<sup>1013</sup>-bearing mice exhibited similar bone formation as revealed by osteoid surface  
208 (OS/BS) and osteoblast surface (Obl.S/BS) compared to WT mice (Fig. 4C). Dynamic  
209 parameters of *in vivo* bone formation were also assessed by quantifying bone surfaces labelled  
210 with tetracycline and calcein. Total and double labelled surfaces were lower in mutant than WT  
211 mice (Fig. 4D), whilst mineral apposition rate (MAR) were similar in WT and *Cxcr4*<sup>1013</sup>-  
212 bearing mice (Fig. 4E). Overall, bone formation rate (BFR/BS) was significantly reduced in  
213 mutant mice (Fig. 4E). This suggests a decrease in bone formation related to a lower number  
214 of OPCs and OBLs with maintained activity of individual OBL. In line with preserved intrinsic  
215 bone formation capacities of active osteoblastic lineage cells in mutant mice, high-throughput  
216 RNA sequencing (RNA-seq) and qPCR analyses of OPC bulk-sorted by flow cytometry from  
217 the bone fraction on the basis of the CD51 and Sca-1 markers (Fig. 2A) highlighted a gene  
218 signature with preserved mineralized matrix potential in mutant OPCs (Fig. 4F-H and S2D-F).  
219 In agreement, sorted OPCs from mutant mice were as efficient as WT ones *in vitro* at producing  
220 differentiated OBLs and mineralized nodules after 14- or 21-days culture in osteogenic medium  
221 as determined by Alkaline phosphatase (Alp) and Alizarin Red (AR) staining  
222 respectively<sup>24,60,62,63</sup> (Fig. 4I and S2G). This was confirmed by qPCR analyses with no changes  
223 in expression of genes encoding osteogenic regulators in mutant cultures (Fig. S2H). These  
224 findings suggest reduced osteogenic lineage commitment in *Cxcr4*<sup>1013</sup>-bearing mice. Given that  
225 osteogenic cells support osteoclastogenesis through the production of factors such as Rank-L  
226 (*Tnfsf11*)<sup>64</sup>, we questioned our RNAseq data on the related gene expression profile in mutant  
227 and WT OPCs. No major changes in expression levels of pro-osteoclastogenic or anti-resorptive  
228 genes were revealed in mutant OPCs (Fig. 4J and 4K).

229 To investigate whether increase in OCLs in mutant mice is linked to an altered stromal  
230 BM environment, we sought to set up co-culture experiments between *in vitro* expanded  
231 osteogenic cells carrying or not the *Cxcr4* mutation and WT OCL precursors, *ie.*, BM CD11b<sup>+</sup>

232 myeloid cells as reported<sup>65</sup>. We showed that mutant osteogenic cells promoted exacerbated  
233 OCL differentiation compared to WT cells (Fig. 4L). Soluble factors seem not to be sufficient  
234 as the supernatants of such stimulated expanded osteogenic cells (WT or mutant) did not induce  
235 OCL differentiation. Additionally, transcriptomic analyses of stimulated osteogenic cells  
236 carrying or not the *Cxcr4* mutation did not reveal any major changes in expression levels of  
237 master genes regulating osteoclastogenesis (Fig. 4M). These findings suggest a juxtacrine  
238 function of osteogenic cells toward OCL differentiation that likely relies on direct interactions  
239 between both cell types and involves the Cxcl12/Cxcr4 axis.

240 Taken as a whole, our findings suggest that the hematopoietic contribution to bone loss  
241 in *Cxcr4*<sup>1013</sup>-bearing mice likely involves dysregulation of the OCL compartment regardless of  
242 their activity. The overall decrease in bone mass in mutant mice may involve modulation of  
243 osteogenic and osteoclastogenic components with decreased bone formation and increased  
244 bone resorption. Bone remodeling involves a tight regulation of opposite processes leading to  
245 bone resorption and bone formation<sup>66,67</sup>. The observation that immature and mature osteogenic  
246 cells, *i.e.*, OPCs and OBLs, displayed preserved intrinsic functions led us to study the early  
247 differentiation process of the osteolineage.

248

#### 249 **Impaired osteogenic specification of *Cxcr4*<sup>1013</sup>-bearing skeletal stromal cells**

250 We thus investigated the intrinsic characteristics of SSCs carrying the *Cxcr4* mutation.  
251 Undifferentiated stem cells are characterized by their slow cell cycle progression in unperturbed  
252 conditions<sup>9,68</sup>. This led us to interrogate by flow cytometry the cycling status of SSCs from the  
253 bone fractions of *Cxcr4*<sup>1013</sup>-bearing mice by performing DAPI/Ki-67 staining. A slight but  
254 significant increase in the frequency of cells in the quiescent G0 state (DAPI<sup>low</sup>Ki-67<sup>-</sup>) was  
255 observed among 1013/1013 SSCs but not in the more differentiated osteoblastic pool (Fig. 5A).  
256 The turnover of those cells was then studied by performing a 12-day BrdU pulse-chase assay

257 *in vivo* (Fig. 5B). Consistent with previous studies<sup>28,69</sup>, the fraction of BrdU<sup>+</sup> cells in WT SSCs  
258 reached ~5%, while we observed a *Cxcr4*<sup>1013</sup> allele copy number-dependent reduction in BrdU  
259 incorporation within mutant SSCs. No changes were observed among mutant OPCs compared  
260 to WT ones. Combined to reduced SSC and OPC numbers in mutant bones (Fig. 2B), these  
261 findings are suggestive of reduced cycling and osteogenic differentiation capacities of  
262 *Cxcr4*<sup>1013</sup>-bearing SSCs.

263 To gain further mechanistic insights, we investigated the impact of the gain-of-*Cxcr4*-  
264 function on the molecular identity of SSCs by performing RNA-seq analyses of bulk-sorted  
265 SSCs from the bone fraction of WT and mutant mice. Biological processes related to cell cycle  
266 and osteogenic differentiation were significantly modulated in 1013/1013 SSCs as determined  
267 by Gene set enrichment analysis (GSEA) (Fig. 5C). The signature for genes related to cell cycle  
268 progression and regulation was reduced in 1013/1013 SSCs (Fig. S3A and S3B). Likewise,  
269 genes related to osteogenic differentiation appeared to be decreased in mutant SSCs (Fig. 5D  
270 and 5E). In contrast, key genes involved in both adipogenesis and chondrogenesis were not  
271 differentially expressed in mutant SSCs (Fig. S3C). These results were confirmed by  
272 microfluidic-based multiplex gene expression analyses (Fig. 5F and 5G and Fig. S3D), thus  
273 suggesting that proper *Cxcr4* signaling is required for regulating osteogenic specification of  
274 SSCs. No changes in expression levels of pro-osteoclastogenic genes were detected in mutant  
275 SSCs compared to WT ones (Fig. 5H-J). Therefore, these results unravel a *Cxcr4*-mediated  
276 transcriptional signature in *Cxcr4*<sup>1013</sup>-bearing SSCs suggestive of impaired cell cycle  
277 progression and defective osteogenic specification.

278 In adult BM, the majority of OBLs derives from OPCs identified by markers such as  
279 osterix (*Osx*)<sup>3,9,17,28,70-72</sup>. They are predominantly found close to the growth plate cartilage along  
280 trabecular bone of the primary spongiosa, and along the metaphyseal cortical bone<sup>70,71,73</sup>. We  
281 thus examined whether the gain-of-*Cxcr4*-function mutation alters the number of *Osx*-positive

282 OPCs by immunodetection on bone sections. We found fewer Osx-positive OPCs in mutant  
283 bones compared to WT (Fig. 5K and 5L). This decrease was confirmed by flow cytometry in  
284 the flushed stromal marrow fraction that encompasses Sca-1-negative and PDGFR $\alpha$ -positive  
285 early OPCs with multipotent adipo/osteogenic potential (Fig. 5M)<sup>28,45,70</sup>. Together, these data  
286 suggest that the decrease in early and committed OPCs in mutant mice may arise from a defect  
287 in osteogenic specification of BM-residing SSCs.

288

289 **Cxcr4 desensitization intrinsically regulates *in vitro* the osteogenic differentiation of**  
290 **skeletal stromal cells**

291 To assess whether Cxcr4 desensitization could regulate SSC fate toward the osteogenic lineage  
292 in a cell-intrinsic manner, we first compared *in vitro* the clonogenic capacities of WT and  
293 mutant total skeletal cells. There was a significant decrease in the number of colony-forming  
294 units-fibroblast (CFU-Fs) in mutant bone cell cultures that followed a *Cxcr4*<sup>1013</sup> allele copy  
295 number-dependent pattern (Fig. 6A). These results suggested that impaired Cxcr4 signaling  
296 might affect *in vitro* overall SSC numbers as well as their proliferation. To test this, we  
297 evaluated by flow cytometry cell cycle and proliferation of SSCs expanded *in vitro* using BrdU,  
298 Cell Trace Violet (CTV) and DAPI/Ki-67 staining. By day 5 after BrdU pulse, we observed a  
299 *Cxcr4*<sup>1013</sup> allele dose-dependent reduction in BrdU incorporation within mutant SSCs as  
300 compared to WT (Fig. 6B, left panel). Consistently, the fraction of proliferating CTV<sup>low</sup> cells  
301 was reduced among mutant SSCs three days after loading (Fig. 6B, right panel). This altered  
302 proliferative capacity of *Cxcr4*<sup>1013</sup>-bearing SSCs was associated with a slight but significant  
303 increase in proportions of SSCs in the quiescent G0 state (DAPI<sup>low</sup>Ki-67<sup>-</sup>), whereas no changes  
304 in apoptosis level were observed (Fig. 6C). This might account for the increased doubling time  
305 of mutant SSCs as well as their overall reduced number during the culture (Fig. 6D). Altogether,



306 these findings suggest that *Cxcr4* desensitization is required *in vitro* for appropriate SSC  
307 proliferation, expansion and likely maintenance.

308         Next, we investigated *in vitro* the osteogenic potential capacities of *Cxcr4*<sup>1013</sup>-bearing  
309 SSCs<sup>62,63</sup>. Staining of *in vitro* differentiated osteogenic cells and mineralization capacities by  
310 Alp and AR was significantly reduced in cultures from mutant SSCs in an allele dose-dependent  
311 manner (Fig. 6E and 6F, upper panels). Real-time PCR analysis revealed decreased expression  
312 of genes encoding osteogenic regulators in mutant cultures (Fig. 6G, upper panels). This was  
313 more marked for early osteogenic genes downstream the master regulator Runx2 such as *Osx*  
314 and particularly evident in the culture of 1013/1013 SSCs, thus suggesting defects at very early  
315 stages in the osteogenic differentiation process. In line with this, higher mRNA expression of  
316 *Scal* and *Pdgfra* was observed in mutant cultures three weeks after pro-osteogenic condition  
317 initiation (Fig. S4A, left panel). Consistent with the results obtained with Alcian blue and  
318 Perilipin staining on bone sections (Fig. 1G and 1H), *Cxcr4*<sup>1013</sup>-bearing SSCs differentiated into  
319 adipocytes or chondrocytes similarly to WT SSCs when cultured *in vitro* with adipogenic or  
320 chondrogenic media respectively (Fig. S4B and S4C). Collectively, these data reveal *in vitro* a  
321 selective reduction of the osteogenic differentiation capacity of mutant SSCs, and further  
322 confirm a pivotal role for *Cxcr4* desensitization in regulating this process at very early stages.

323

#### 324 **Normalization of *Cxcr4* signaling rescues the osteogenic properties of *Cxcr4*<sup>1013</sup>-bearing** 325 **mouse skeletal cells**

326 We then determined whether targeting *Cxcr4* signaling would counteract the defective  
327 osteogenic fate of mutant SSCs. First, we assessed *in vitro* the impact of adding AMD3100  
328 every 2 days on the osteogenic capacities of SSCs. AMD3100-mediated inhibition of *Cxcr4*  
329 signaling in WT SSCs led to slight changes including decreased numbers of osteogenic cells  
330 (Fig. 6E and 6F, lower panels). By contrast, mutant cultures were highly sensitive to AMD3100

331 treatment as shown by the normalization of Alp and AR colorations 14 and 21 days after  
332 differentiation respectively (Fig. 6E and 6F) and of the expression of *Scal* and *Pdgfr $\alpha$*  (Fig.  
333 S4A). Moreover, AMD3100-mediated reversion of defective osteogenesis within *Cxcr4*<sup>1013</sup>-  
334 bearing SSC cultures was associated with normalized gene expression of osteogenic master  
335 regulators (Fig. 6G, lower panels), thus unravelling that Cxcr4 desensitization intrinsically  
336 regulates *in vitro* the osteogenic differentiation of SSCs.

337 Then, we assessed the impact of daily intraperitoneal injections for 3 weeks of 5 mg/kg  
338 AMD3100 on the bone landscape in adult WT and mutant mice (Fig. 7A). Cxcr4 inhibition  
339 decreased slightly the number of WT skeletal cells, and notably OPCs, in the bone fraction (Fig.  
340 7B). In line with this, Opn-stained femoral sections revealed minor alterations in the  
341 architecture of WT mice trabecular microstructures upon treatment (Fig. 7C). This was  
342 extended to lumbar spine that displayed roughly normal BMD values in treated vs untreated  
343 WT mice (Fig. 7D). In 1013/1013 mice, chronic AMD3100 treatment reversed the quantitative  
344 defect in skeletal cells by normalizing the numbers of SSCs and OPCs (Fig. 7B). This was not  
345 evidenced in +/-1013 mice nor associated with a rescue of the trabecular or the cortical network  
346 (Fig. 7C and 7E). However, AMD3100 treatment ameliorated slightly but significantly BMD  
347 values of lumbar spine in mutant mice (Fig. 7D), suggesting a correcting effect of Cxcr4-  
348 dependent signaling dampening on other cell types and mechanisms such as OCLs or LepR-  
349 positive BM SSCs as recently reported<sup>31</sup>. Therefore, these data suggest that integrity of Cxcr4  
350 signaling is required for maintaining the osteogenic properties of skeletal cells.

351

### 352 **BM stromal cells from WS patients displayed *in vitro* impaired osteogenic capacities**

353 Finally, we sought to investigate if CXCR4 desensitization was mechanistically involved in  
354 regulating *in vitro* the multilineage differentiation capacities of human primary BMSCs that  
355 constitute a heterogeneous population containing skeletal progenitors<sup>18</sup>. To this end, we

356 analyzed BM samples from two unrelated patients with WS and carrying the heterozygous  
357 *CXCR4*<sup>R334X</sup> mutation. In parallel, we expanded *in vitro* BMSCs from BM aspirates of seven  
358 independent healthy donors. All culture-expanded BMSCs were negative for the CD45  
359 hematopoietic marker lineage but positive for CD73, CD90 and CD105, a combination of  
360 markers that are indicative of stromal/fibroblastic cells (Fig. S5A). Both healthy and WS  
361 BMSCs were spindle shaped and fibroblast-like cells and had the ability to form stromal  
362 colonies as shown by CFU-F assay (Fig. S5B and S5C). CXCL12 and its two receptors CXCR4  
363 and ACKR3 were readily detectable and found at similar levels between cultured healthy and  
364 WS BMSCs (Fig. S5D-F). However, real-time PCR analyses revealed decreased expression of  
365 genes encoding early and late osteogenic master regulators in WS BMSC cultures compared to  
366 healthy controls (Fig. 8A). In line with this, when equal numbers of cells were plated at the start  
367 of the assay, WS BMSCs exhibited defective capacities to generate *in vitro* osteogenic progeny  
368 in contrast to BMSCs harvested from healthy donors (Fig. 8B). In contrast, WS BMSCs were  
369 as efficient as control cells to generate adipocytes in appropriate culture media condition (Fig.  
370 8C). Therefore, these findings suggest that *in vitro* osteogenic differentiation of human primary  
371 BMSCs requires proper CXCR4 signaling regulation.

372

373 **DISCUSSION**

374 In this study, we investigated the regulatory role of CXCR4 signaling termination in the self-  
375 renewal and differentiation capacities of adult BM-residing SSCs. We used a knock-in mouse  
376 model expressing a naturally occurring WS-linked heterozygous gain-of-function *Cxcr4*  
377 mutation as well as human BM samples and clinical data from healthy and WS donors. We  
378 demonstrated a mutated allele dose-dependent effect of the WS-linked *Cxcr4*<sup>1013</sup> mutation on  
379 trabecular bone microstructures mimicking an osteoporotic-like syndrome, evidenced as well  
380 in one quarter of WS patients. This *Cxcr4*-mediated reduction in bone content involved both  
381 cell-autonomous and cell-extrinsic defects in SSCs (Fig. 8D). Indeed, we provided  
382 unanticipated evidence that *Cxcr4* desensitization is intrinsically required for regulating *in vitro*  
383 the quiescence/cycling balance of SSCs and preserving their osteogenic potential, while it was  
384 found to be dispensable for their adipogenic and chondrogenic differentiation. Some other BM  
385 cellular components contributed to the dysregulation of the bone phenotype. We observed in  
386 trabecular area an increase in OCL number. However, the osteoclastogenic differentiation  
387 potential of OCL precursors and the resorptive function of differentiated OCLs were not  
388 affected *in vitro* by the *Cxcr4*<sup>1013</sup> mutation. Therefore, the osteopenia might proceed from a  
389 deregulated bone matrix resorption that might not be compensated enough by bone-forming  
390 cells, enlightening further the strong entanglement between both actors of bone remodeling.  
391 Unravelling the mechanisms leading to such an alteration of the skeletal landscape with a focus  
392 on the defective osteogenic capacities of SSCs driving the observed excessive bone defect will  
393 require further investigations using specific conditional mouse models. Importantly, defective  
394 osteogenic capacities were also evidenced *in vitro* in BMSCs from WS patients. These  
395 anomalies establish the C-tail of CXCR4 as an important regulatory domain of the receptor  
396 function in BM stromal cell biology in both mice and humans. In light of previous  
397 works<sup>40,41,43,44</sup>, our results also suggest that both increased and decreased *Cxcr4*-mediated

398 signaling negatively impact skeletal stromal elements, thus indicating that fine-tuning of Cxcr4  
399 signaling is critical for maintenance and osteogenic specification of adult SSCs. Although the  
400 underlying molecular mechanisms remain to be elucidated, Cxcr4 might act as a rheostat  
401 regulating the strength and kinetic of signaling pathways involved in osteogenic fate  
402 specification of SSCs. Interestingly, a recent work using a new mouse model of WS further  
403 illustrated the crucial role of fine-tuned Cxcr4-mediated signaling in mesenchymal  
404 stromal/stem cell (MSC) function<sup>46</sup>. Indeed, the lymphopoiesis process was reduced, as  
405 observed in our model<sup>53</sup>, because of a dysregulated transcriptome in MSCs isolated from the  
406 flushed marrow fraction and characterized by a switch from an adipogenic to an osteolineage-  
407 prone program with limited lymphopoietic activity. This might proceed from reduced  
408 expression of IL-7 and excessive signaling through the lymphotoxin beta receptor in MSCs.  
409 Whether these changes are observed in the BM of our mouse model and how they would  
410 contribute to the bone loss warrant further investigations. Moreover, mice deficient for the gene  
411 encoding the transcription factor Ebf3 display an opposite BM phenotype to the one of  
412 *Cxcr4*<sup>1013</sup>-bearing mice, characterized by osteosclerosis with HSC depletion and reduced  
413 expression of niche factors<sup>69</sup>. This was related to the uncontrolled ability of *Ebf3*-deficient SSCs  
414 to differentiate into OBLs. Further studies are required to address the status of Ebf3 and  
415 downstream target genes that act to modulate osteogenic fate of SSCs in *Cxcr4*<sup>1013</sup>-bearing  
416 mice. A potential crosstalk between distinct SSC subsets, either prone to differentiate into  
417 osteochondro-lineage cells or perivascular and adipocyte lineage cells, has been reported<sup>74</sup>. This  
418 seems to imply ligand-receptor gene pairs such as TGF $\beta$ , WNT or BMP ligands and their  
419 cognate receptors that regulate SSC fate decision. Whether and how the Cxcl12/Cxcr4 signaling  
420 axis contributes to these regulatory mechanisms across SSC types remains also to be explored.

421 We reported that loss of Cxcr4 signaling termination impairs overall number, impedes  
422 cell cycle progression, and limits osteogenic differentiation of SSCs. Indeed, mutant mice have

423 a global alteration of the bone stromal landscape, including decreased numbers of SSCs and  
424 their progeny including early and committed OPCs and impaired architecture of trabecular and  
425 cortical bone microstructures that occurred in a mutated allele copy number-dependent manner.  
426 Altogether, these findings indicate that the gain-of-*Cxcr4*-function mutation promotes a  
427 reduced OBL commitment and differentiation, but does not alter the bone forming activity of  
428 individual OBL. Congruent with this, chronic treatment with AMD3100 normalized *in vitro* the  
429 osteogenic properties of mutant SSCs. Impaired *Cxcr4* desensitization might alter the balance  
430 between quiescence and differentiation of mutant SSCs and reduce the number of osteogenic-  
431 endowed precursors. Currently, the prevailing view is that BM *Cxcl12*-expressing stromal cells  
432 display slow cell cycle progression and constitute an active source of trabecular and cortical  
433 OBLs under physiological conditions, as well as in response to injury<sup>6,9,28,31,69</sup>. We found a  
434 higher proportion of quiescent SSCs in mutant mice that was particularly evident in 1013/1013  
435 SSCs, thus suggesting the importance of *Cxcr4* desensitization in controlling SSC proliferation  
436 and quiescence and likely their capacity to give rise to osteogenic cells.

437       Loss of bone content in mutant mice was accompanied by a higher number of OCLs  
438 within the trabecular bones, possibly reflecting that the *Cxcr4* mutation was intrinsically  
439 perturbing the OCL differentiation process. This seems not to be the case since we showed that  
440 defective *Cxcr4* desensitization did not increase *in vitro* differentiation of OCLs from mutant  
441 BM progenitors, nor their mineral matrix resorbing capacities. BM chimeras leading to a WT  
442 hematopoietic development into a mutant bone environment further ruled out the sole  
443 involvement of an uncontrolled bone resorption due to defective OCLs with excessive activity.  
444 These cells derive from monocytic lineage precursors upon stimulation by RankL and M-  
445 *Csf*<sup>75,76</sup>. In a constant cross interaction between the bone forming and the bone resorbing  
446 pathways, these osteoclastic cytokines are produced by mature and immature stromal cell  
447 populations within the BM<sup>9,77,78</sup>. While we did not observe increased *Rank-L*, *M-Csf* or *Opg*

448 (encoding a Rank-L antagonist) gene expression in sorted committed OPCs from mutant bones,  
449 we cannot exclude that modification of the bone stroma due to osteogenic defects might in turn  
450 modulate the production of osteoclastic factors from the mutant bone environment. Supporting  
451 this assumption, co-cultures of WT OCL progenitors with *in vitro* expanded *Cxcr4*<sup>1013</sup>-bearing  
452 bone-derived stromal cells, stimulated to produce osteoclastogenic factors, led to enhanced  
453 osteoclastogenesis compared to WT stromal cells. No increase in *Rank-L* or *M-Csf* expression  
454 nor decrease in *Opg* level could be detected, suggesting the involvement of other additional  
455 mediators. It has recently been shown that intrinsic aging of SSCs resulted in higher proportion  
456 of stromal lineages producing pro-inflammatory and pro-resorptive factors, promoting myeloid  
457 skewing, and osteoclastic activity<sup>30</sup>. Whether a similar mechanism occurs in *Cxcr4*<sup>1013</sup>-bearing  
458 mutant mice remains to be characterized. Moreover, BM chimeras leading to a mutant  
459 hematopoietic development into a WT bone environment displayed dysregulated bone  
460 landscape, thus emphasizing that transplanted hematopoietic cells can participate in bone loss  
461 through direct and/or indirect actions on osteoclastogenesis. Despite altered hematopoiesis in  
462 such chimeric mice, the myeloid skewing reported elsewhere<sup>53</sup> might account for excessive  
463 OCL number. As well, the reduced lymphopoiesis observed<sup>46,53</sup> still led to generation of mature  
464 B and T lymphocytes that are present in the BM and hence may act as potential actors in bone  
465 erosion as they can produce osteoclastogenic mediators such as Rank-L in non-physiological  
466 settings<sup>79</sup>. Whether such action of mutant hematopoietic donor cells recreates a pro-  
467 osteoclastogenic environment through a remodeling of the myeloid and lymphoid  
468 compartments deserves further investigations. Interestingly, the dysregulated bone landscape  
469 in BM chimeras with a mutant hematopoietic compartment in WT recipients was even stronger  
470 than that observed in mutant mice at steady-state. This observation raises the intriguing  
471 possibility of a pro-osteogenic effect of one or several *Cxcr4*<sup>1013</sup>-bearing radioresistant cell

472 type(s). To test this possibility, further work will be required using mouse model where the  
473 mutation is carried in a cell-specific manner.

474 Osteogenesis is regulated, among different mechanisms, by undifferentiated skeletal  
475 cells and more specified osteolineage cells that express factors promoting or preventing their  
476 own differentiation into OBLs<sup>23,59,69,80</sup>. In BM, HSPC niches constitute critical spatio-temporal  
477 regulatory units composed of multiple cell populations of hematopoietic and non-hematopoietic  
478 origin cross-interacting with each other's in a dynamic setting<sup>1,3,9,81,82</sup>. This implies that immune  
479 and vascular cells among others may influence the osteogenic differentiation process<sup>83</sup>. Again,  
480 in BM chimeras in which *Cxcr4*<sup>1013</sup>-bearing HSPCs were differentiating into a WT bone  
481 environment, we reported a similar bone loss as observed in mutant mice, thus indicating cell-  
482 extrinsic *Cxcr4*-mediated regulation of the skeletal landscape. This also suggests that neither  
483 the epiphyseal cartilage nor any developmental defect contribute to impaired trabecular bone  
484 architecture in adult mutant mice, and further supports the notion that HSPCs, as osteolineage  
485 cells do, express regulating osteogenic factors such as BMP-2, BMP-7 and WNT3a, that are  
486 particularly involved in SSC osteogenesis specification<sup>17</sup>. Whether and how hematopoietic  
487 cells, or other BM components such as vascular cells, participate in the defective osteolineage  
488 specification of SSCs in *Cxcr4*<sup>1013</sup>-bearing mice deserves further investigations.

489 Finally, we reported that five out of nineteen patients with WS and carrying distinct  
490 autosomal-dominant mutations in *CXCR4* exhibit a decrease in BMD at different anatomical  
491 sites. Although this would merit to be extended to a larger cohort before introducing any  
492 potential bone-affecting drugs, these data suggest that accelerated osteopenia/osteoporosis and  
493 increased risk of fractures may constitute a novel feature of WS. Lack of *CXCR4*  
494 desensitization could be mechanistically involved in such anomaly since BMSCs from WS  
495 patients carrying a heterozygous *CXCR4* mutation displayed *in vitro* impaired capacities to  
496 differentiate into osteogenic, but not adipogenic, cells. Strikingly, we observed that chondro-



497 and adipo-genic differentiation of murine mutant SSCs was normal both *in situ* and *in vitro*.  
498 Considering recent studies unraveling human SSCs expressing the CXCL12/CXCR4 axis with  
499 osteoblastogenic and, depending on their tissue origin, adipocytic potential<sup>18,84</sup>, our findings  
500 pave the way for exploring the BM of WS patients in search for potential defect(s) in these  
501 skeletal populations.

502

503

504 **METHODS**505 **Healthy and WS donors and bone mineral density measurements**

506 Investigations of human BM samples were performed in compliance with Good Clinical  
507 Practices and the Declaration of Helsinki. The study was approved by the Ethical Board Ile-de-  
508 France X. Recruited WS patients were not compensated and gave their written informed  
509 consent for participating to the clinical study that has been approved by NIAID Institutional  
510 Review Board (IRB). Cryopreserved BM aspirates from two WS patient (NIH protocol 09-I-  
511 0200) were provided through a NIH Material Transfer Agreement. The samples were  
512 anonymized. BM samples from seven healthy donors that were matched for age and sex and  
513 used as control subjects were isolated from hip replacement surgery samples (Protocol 17-030,  
514 n° ID-RCB: 2017-A01019-44). Primary BMSCs from healthy and WS donors were amplified  
515 and used at passage 1 to 3. For BMD assessment, data were collected from nineteen WS patients  
516 as part of an IRB approved clinical protocol conducted at the NIH (NIAID Protocol #2014-I-  
517 0185, IND # 118767). Patients had a baseline bone density scan as part of a drug treatment trial  
518 (NCT02231879) comparing 1 year of twice daily filgrastim (Neupogen) versus plerixafor  
519 (Mozobil) in a randomized, blinded crossover design. There were 13 women and 6 men with  
520 an average age of 30.5 years (range 10-56). The samples were anonymized. Patients had been  
521 on filgrastim (Neupogen) for an average of 5.7 years prior to enrolling in the trial (range 0-27).  
522 6 of the 19 had not used filgrastim regularly prior to trial enrollment. BMD values expressed as  
523 T- or Z-scores were measured by total body dual-energy X-ray absorptiometry with a Lunar  
524 iDXA densitometer (GE Healthcare). Five WS patients had abnormal screening bone density  
525 by WHO criteria, anonymized at the start of the Phase 3 trial (Table 1), while the other 14  
526 patients had normal bone density.

527

528 **Mice and genotyping**

529 All mice were bred in our animal facility under a 12h light/dark cycle, specific pathogen-free  
530 conditions (EOPS status) and fed *ad libitum*. For breeding, mice were in conventional cages  
531 with filter top. For experimentation, mice were housed in individually ventilated cages. All  
532 experiments were performed in accordance with the European Union guide for the care and use  
533 of laboratory animals and have been reviewed and approved by institutional review committees  
534 (CEEA-26, Animal Care and Use Committee, Villejuif, France and Comité d'Ethique Paris-  
535 Nord/N°121, Paris, France). *Cxcr4*<sup>+/<sup>1013</sup></sup> (+/1013) mice were generated by a knock-in strategy<sup>51</sup>.  
536 Homozygous *Cxcr4*<sup>1013/1013</sup>(1013/1013) mice were obtained by crossing heterozygous +/1013  
537 mice. WT mice were used as controls. Unless specified, all mice were littermates, females and  
538 age-matched (8-12 wk-old). Adult Boy/J (CD45.1) (Charles River) mice were used as BM  
539 donors. Daily observation was performed to ensure that no animal was left in a state of pain or  
540 suffering during experimentation. Euthanasia was performed by increasing gradient of CO<sub>2</sub>.

541

#### 542 **Sample isolation in mice**

543 Mouse SSCs were obtained from bones after centrifugation of intact femurs, tibias and hips to  
544 flush out the BM cells. Flushed long bones were cut into fine pieces before enzymatic digestion  
545 with 2.5 U/mL collagenase type I (Thermofisher) for 45 min at 37°C under agitation. Released  
546 cells were filtered and washed with PBS, 2% FBS (Fetal Bovine Serum). Cell numbers were  
547 standardized as total counts per two legs. Peripheral blood was collected by cardiac puncture.  
548 Freshly isolated cells were either immunophenotyped, incubated at 37°C for 60 min in RPMI  
549 20 mM HEPES 0.5% BSA (Euromedex) prior to chemokine receptor internalization studies, or  
550 expanded in  $\alpha$ MEM medium supplemented with 10% FBS, 1% P/S (penicillin 100 Units/mL,  
551 streptomycin 100 Units/mL, Gibco) and 50  $\mu$ M  $\beta$ -mercaptoethanol (PAN biotech). For BMD  
552 quantification, lumbar spines were fixed overnight in ethanol 70° and analyzed by dual-energy

553 X-ray absorptiometry with an ultrafocus DXA densitometer (Faxitron). Quantifications were  
554 made on a ROI of 2 lumbar spines.

555

556 **Flow-cytometric analyses**

557 Mouse and human staining analyses were carried out on an LSRII Fortessa flow cytometer (BD  
558 Biosciences) using the antibodies (Abs) described in Table S1. A Live/Dead Fixable Aqua Dead  
559 Cell Stain Kit (Biolegend) was used. To assess the compartmentalization of CXCR4 and  
560 ACKR3, human BMSCs were incubated with saturating concentrations of non-conjugated  
561 mouse anti-human CXCR4 or ACKR3 Abs, washed in PBS, fixed and permeabilized using the  
562 BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). BMSCs were  
563 subsequently stained with anti-CXCR4 and -ACKR3 conjugated mAbs, or the corresponding  
564 isotype control, at 4°C for 30 min and then analyzed by flow cytometry. FACS Diva software  
565 version 7 (BD) were used for collecting data. FLOWJO v10.7 (BD) and GraphPad Prism v8.0e  
566 (GraphPad software Inc.) were used for analyzing flow cytometric data.

567

568 ***In vitro* functional assays**

569 Mouse CFU-Fs were performed by plating  $1 \times 10^5$  bone cells at passage 2-3 from WT and mutant  
570 mice. Human CFU-Fs were performed by plating  $0.2 \times 10^3$  BMSCs into a 25 cm<sup>2</sup> flask at  
571 passage 3 from healthy or WS donors. After 7 or 10 days of culture, colonies were fixed with  
572 ethanol 70%, stained with 2% crystal violet (Sigma-Aldrich), and counted with a binocular  
573 magnifying glass. For chemotaxis assays,  $5 \times 10^4$  SSCs were added to the upper chambers of a  
574 24-well plate with 8- $\mu$ m-pore-size Transwell inserts (EMD Millipore) containing or not 1 nM  
575 Cxcl12 (R&D Systems) in the lower chamber. For inhibiting Cxcr4-mediated signaling, 10  $\mu$ M  
576 AMD3100 (Sigma-Aldrich) was added in the upper and lower chambers. After 24h, membranes  
577 were removed and fixed in 4% paraformaldehyde (PFA). The cells that migrated to the lower

578 side of the membrane were stained with 0.1% crystal violet and three fields from each insert  
579 were counted under a light microscope. Cxcr4 and Ackr3 internalization assays were performed  
580 by incubating total bone cells at 37°C for 45 min with 10 nM Cxcl12. Then the reaction was  
581 stopped by adding ice-cold RPMI and quick centrifugation at 4°C. After one wash in acidic  
582 glycine buffer at pH= 4.3, levels of Cxcr4 and Ackr3 membrane expression were determined  
583 by flow cytometry. Cxcr4 or Ackr3 expression was calculated as follows: (Cxcr4 or Ackr3  
584 geometric MFI of treated cells/Cxcr4 or Ackr3 geometric MFI of unstimulated cells) × 100;  
585 100% corresponds to receptor expression at the surface of cells incubated in medium alone. For  
586 the chemokine scavenging assay, cultured SSCs were harvested by trypsinization and placed in  
587 complete medium for 90 min at 37°C and 5% CO<sub>2</sub> to normalize receptor expression. 4x10<sup>6</sup>  
588 cells/mL were pre-incubated with 100 μM CCX733, a functional Ackr3 antagonist or vehicle  
589 in 1% BSA/PBS for 45 min at room temperature (RT). Then, 2x10<sup>6</sup> cells/mL were incubated in  
590 presence of 5 nM AF647-Cxcl12 (Almac) in 1% BSA/PBS during 45-60 min at 37°C to allow  
591 internalization or on ice to inhibit this process. Cells were washed with 1% BSA/PBS and then  
592 either treated with an acidic glycine wash buffer pH= 2.7 for 3 min to dissociate cell-surface-  
593 bound chemokine, or washed with PBS to estimate internalized plus cell-surface-bound control.  
594 AF647 fluorescence (geometric MFI) was determined by flow cytometry. Phosphoflow assays  
595 were performed with the PerFix EXPOSE kit (Beckman coulter) on cultured SSCs and an anti-  
596 phospho Erk (pT202/pY204) was used. Fold change was calculated as follows: (Phospho-Erk  
597 geometric MFI of stimulated cells/Phospho-Erk geometric MFI of unstimulated cells).

598

### 599 ***In vivo* functional assays**

600 For BM transplantation experiments, 1.5x10<sup>6</sup> total marrow cells from young CD45.1<sup>+</sup> WT mice  
601 were injected i.v. into lethally irradiated (two rounds of 5.5 Gy separated by 3 h) young CD45.2<sup>+</sup>  
602 WT, +/-1013 or 1013/1013 recipient mice. For reverse experiments, 1.5x10<sup>6</sup> total marrow cells

603 from CD45.2<sup>+</sup> WT, +/-1013 or 1013/1013 mice were injected into lethally irradiated CD45.1<sup>+</sup>  
604 WT recipient mice. Chimerism was analyzed 3 or 16 weeks after transplantation. For Cxcr4  
605 blockade experiments, mice were daily injected intraperitoneally with 5 mg/kg AMD3100 or  
606 PBS during 3 weeks. BM were harvested 2 h after the last injection and analyzed by flow  
607 cytometry and imaging.

608

609 **ELISA**

610 Supernatants of culture-expanded human BMSCs were analyzed using a standardized ELISA  
611 for human Cxcl12 (Quantikine; R&D Systems).

612

613 **Bone immunostaining and histomorphometry**

614 Mouse bones were fixed in 4% PFA overnight followed by one-week decalcification in EDTA  
615 (0.5 M) at pH 7.4 under agitation. Bones were incubated in PBS with 20% sucrose and 2%  
616 polyvinylpyrrolidone (PVP) (Sigma) at 4°C overnight and then embedded in PBS with 20%  
617 sucrose, 2% PVP and 8% gelatin (Sigma) before storage at -80°C. Sections of 30 µm-thick  
618 were rehydrated in PBS 1X, incubated 20 min at RT in PBS with 0.3% triton X-100, saturated  
619 in blocking solution (PBS with 5% BSA) and finally incubated with primary Abs (Table S2).  
620 After washing, secondary Abs were incubated for 1h at RT with DAPI for nuclear staining and  
621 mounting using Permafluor mounting medium (Thermofisher). Images were acquired using  
622 TCS SP8 confocal microscope and processed using Fiji software. For alcian blue and perilipin  
623 A staining, fixed and decalcified femur bones were embedded in paraffin, sectioned (7 µm-  
624 thick) and deparaffinized with xylene. Staining of cartilage tissues was performed with a 1 %  
625 alcian blue solution for 30 min. Images were acquired using a LEICA DM4000B microscope  
626 equipped with a DFC425C camera and processed with the Leica Application Suite V3.8  
627 software. For perilipin A staining, heat induced epitope retrieval was performed in citrate

628 sodium buffer solution. Sections were saturated for 1h in PBS 1% BSA at RT, washed in PBS  
629 0.2% BSA and 0.1% Triton X-100, and incubated with anti-perilipin A Ab in PBS BSA 1%  
630 overnight at 4°C. After washing, sections were incubated with TRITC-coupled rabbit anti-  
631 guinea pig Ab in PBS 1% BSA for 45 min and counterstained with DAPI. For Osx staining, 16  
632 µm frozen sections were permeabilized in TBS-0.3% Triton X-100 for 10 min and blocked in  
633 TBS-2.5% BSA-2.5% Donkey Serum for 1h at RT. Sections were incubated with anti-Osx Ab  
634 (rabbit, Santa Cruz SC-22536R) in blocking solution overnight at 4°C. After washing with  
635 TBS+0.025% Triton X-100, sections were incubated in donkey anti-rabbit secondary Ab  
636 dylight 550 (SA5-10039, invitrogen) in blocking solution. After washing, sections were  
637 incubated 15 min at RT in DAPI at 0.1µg/mL prior to mounting in GB-Mount (Diagomics).  
638 Image acquisitions were done using the ApoTome optical sectioning system (Zeiss) with an  
639 inverted microscope (Zeiss Axio Observer Z1). Osx quantification was performed using the  
640 ICY software. For human BMSC immunofluorescence studies, cells were plated on coverslips  
641 and fixed with 4% PFA in PBS. Fixed cells were permeabilized with Triton X 0.3% for 10 min,  
642 blocked with PBS 5% BSA, 5% goat serum and incubated with unlabeled primary CXCL12  
643 mAb overnight at 4°C followed by secondary AF633-coupled goat anti-mouse polyclonal Ab  
644 (Invitrogen) and the nuclear dye Hoechst 33342. Images were obtained with a Plan-  
645 Apochromatic objective using the LSM800 confocal microscope (Carl Zeiss). Sections were  
646 acquired as serial z stacks (0.39 µm apart) and were subjected to three-dimensional  
647 reconstruction (Zen 2.3 System).

648 Bone histomorphometry was performed in plastic samples, allowing the measurements  
649 of bone formation and resorption parameters. Mouse femurs were fixed in ethanol 70°,  
650 dehydrated and embedded in methyl methacrylate resin. Five micrometer-thick coronal sections  
651 were cut parallel to the long axis of the femur using an SM2500S microtome (Leica, Germany).  
652 Sections were deplastified, rehydrated and stained with toluidine blue or with naphthol 3-

653 hydroxy-2-naphthoic acid 4-chloro-2-methylanilide (ASTR phosphate, Sigma, St Louis,  
654 France) for detecting mature osteoclasts with TRAP staining. Quantifications were made on a  
655 polarizing microscope (Nikon) using a software package (Bonolab) developed for bone  
656 histomorphometry (Microvision, France). To allow the measure of dynamic parameters of bone  
657 formation, mice were intraperitoneally injected with tetracycline (20 mg/kg) and calcein  
658 (10 mg/kg; Sigma) 5 days and 1 day respectively before being killed. Two 12- $\mu$ m-thick  
659 unstained sections were taken for measurement of the dynamic parameters under UV light. The  
660 matrix apposition rate (MAR) was measured using the Microvision image analyzer by a  
661 semiautomatic method using tetracycline and calcein double-labeled bone surfaces. The  
662 mineralizing surfaces (MS/BS) were measured in the same areas using the objective eyepiece  
663 Leitz integrate plate II. When specified, the cortical thickness was measured in paraffin-  
664 embedded sections stained with Toluidine Blue. The 2 cortices were measured using  
665 histomorphometry software and expressed as mean of both cortices for each sample. All the  
666 histomorphometric parameters were recorded in compliance with the recommendation of the  
667 American Society for Bone and Mineral Research Histomorphometry Nomenclature  
668 Committee. Five to six animals per genotype were analyzed by two different investigators.

669

#### 670 **Bone structure analysis by micro-computed tomography**

671 Femurs were collected for bone microarchitecture analysis after fixation and before  
672 decalcification. They were analyzed with high-resolution microcomputed tomography (micro-  
673 CT) using a Skyscan 1272 microCT (SkyScan, Kontich, Belgium). Measurements were made  
674 on the femurs using the following acquisition parameters: voltage 60kV, pixel size 6 $\mu$ m, Filter  
675 Alu + 0.5mm. After 3-dimensional images reconstruction with NRecon<sup>®</sup>, analyses were  
676 performed on the trabecular and cortical region (1.72 mm and 0.43 mm thickness, respectively).  
677 Morphometric parameters such as Bone Volume/Tissue volume (BV/TV, %), Trabecular



678 number (Tb.Nb, 1/mm), Trabecular Separation (Tb.Sp, mm), Cortical Bone Volume (Ct.BV,  
679 mm<sup>3</sup>) and Cortical Thickness (Ct.Th, mm) were assessed.

680

### 681 **Cell culture and differentiation**

682 Mouse osteoblastic differentiation was performed for 3 weeks in  $\alpha$ -MEM medium with 10%  
683 FBS, 1% P/S, 50  $\mu$ M  $\beta$ -mercaptoethanol supplemented with 50  $\mu$ g/mL L-ascorbic acid and 10  
684 mM glycerophosphate (Sigma) either from SSCs or sorted OPCs. Alkaline phosphatase staining  
685 was performed after 14 days of differentiation according to the Alkaline phosphatase Kit  
686 (Sigma). At day 21, cultures were fixed with 4% PFA, stained with alizarin red and quantified  
687 using the Osteogenesis assay kit (Millipore). When specified, AMD3100 (*versus* vehicle) was  
688 added into the osteogenic medium every 2 days at 10  $\mu$ M respectively. Chondro- and adipo-  
689 genic differentiations of SSCs were performed according to the StemPro-Chondrogenesis or -  
690 Adipogenic Differentiation Kits (ThermoFisher) for 2 weeks. After fixation, cells were treated  
691 with either Alcian Blue 1% (Sigma) to stain chondrocyte matrix or Oil Red O solution (Sigma)  
692 to reveal lipid droplets. For *in vitro* human osteogenic differentiation assays, expanded BMSCs  
693 were seeded at  $3 \times 10^3$  *per* cm<sup>2</sup> in  $\alpha$ -MEM supplemented with 10% FBS and 1% antibiotics.  
694 After cell adhesion, medium was replaced by  $\alpha$ -MEM supplemented with 10% FBS, 1%  
695 antibiotics and 0.1  $\mu$ M dexamethasone, 0.05 mM L-ascorbic acid-2-phosphate and 10 mM  $\beta$ -  
696 glycerophosphate. Medium was changed every 2 days during 3 weeks. Quantification of  
697 mineralization was performed after Alizarin Red S staining<sup>85</sup>. Human adipogenic  
698 differentiation assays were performed as described for the murine ones.

699

### 700 **Osteoclast differentiation and functional analysis**

701 OCLs were differentiated *in vitro*<sup>86</sup>. Briefly,  $2.3 \times 10^5$  BM cells/cm<sup>2</sup> were plated in MEM-alpha  
702 (ThermoFisher) complemented with 5% serum (Hyclone, GE Healthcare), 1% P/S, 50  $\mu$ M 2-

703 mercaptoethanol, 25 ng/ml M-CSf and 30 ng/ml Rank-L (R&D Systems). OCL differentiation  
704 (multinucleated TRAP<sup>+</sup> cells) was quantified at day 5 after TRAP coloration using the  
705 leukocyte acid phosphatase kit (Sigma). Matrix dissolution activity was evaluated by seeding a  
706 total of 2x10<sup>4</sup> differentiated OCLs on 96-well osteoassay plates (Corning) in  $\alpha$ -MEM  
707 containing 10% FBS and 30 ng/ml Rank-L. After 3 days, medium was removed and cells were  
708 detached by the addition of water. Resorbed areas were quantified using Fiji/ImageJ software<sup>87</sup>.

709

#### 710 **Co-culture assays between osteogenic cells and osteoclast precursors**

711 Mouse osteogenic cells were isolated from the bone fraction of WT or mutant mice as indicated  
712 above. They were expanded in  $\alpha$ -MEM medium with 10% FBS, 1% P/S, 50  $\mu$ M  $\beta$ -  
713 mercaptoethanol for 2 weeks, passaged and plated in 96-well plates (2 x 10<sup>4</sup> cells/well).  
714 Osteoclast precursors were obtained from CD11b<sup>+</sup>-enriched cells (with CD11b-Microbeads,  
715 Milteniy Biotec, France) from the flushed marrow fraction of WT mice as previously  
716 described<sup>86</sup>. They were added, in co-culture, to WT or mutant expanded osteogenic cells (5 x  
717 10<sup>4</sup> cell/well) and stimulated with 1,25-dihydroxy vitamin D3 (vitD3, 10 nM), prostaglandin  
718 E2 (PGE2, 1  $\mu$ M) and Dexamethasone (Dex, 50 nM) as previously described<sup>65</sup> in order to  
719 stimulate osteogenic cells to produce RankL/MCSF and to inhibit OPG production<sup>88,89</sup>. OCL  
720 differentiation (multinucleated TRAP<sup>+</sup> cells) was quantified at day 8 after TRAP coloration as  
721 indicated above.

722

#### 723 **Cell cycle, viability, survival and proliferation assays**

724 For flow cytometry-based cell cycle analyses, bone cells were permeabilized, fixed with the  
725 FOXP3 permeabilization kit (Foxp3/Transcription Factor Staining Buffer Set; eBioscience) and  
726 labelled with a Ki67 Ab and DAPI. For BrdU assays, mice were injected intraperitoneally with  
727 180  $\mu$ g BrdU (Sigma) and maintained with drinking water containing 800  $\mu$ g/ml BrdU and 1%

728 glucose over 12 days. The BrdU labelling was analyzed by flow cytometry using the BrdU-  
729 FITC labeling kit (BD Biosciences). For *in vitro* BrdU incorporation, 3 µg/ml of BrdU was  
730 added to the culture and after five days the percentage of incorporation was determined as  
731 above. Apoptosis was measured using the Annexin V detection kit (BD Biosciences) with DAPI  
732 staining. For *in vitro* proliferation assays, SSCs were detached with 0.5% trypsin and loaded at  
733  $3 \times 10^4$  cells/well with cell trace violet (CTV, ThermoFisher) for 15 min at 37°C. CTV dilution  
734 was assessed by flow cytometry. To estimate the doubling time values, SSCs were seeded at  
735  $3 \times 10^3$  cells/cm<sup>2</sup> and counted after 3 days of culture. The doubling time was calculated as  
736 follows: (time of culture x log(2))/(log(final number of SSC)-log(initial number of SSC)).

737

### 738 **Quantitative real time-PCR**

739 For mouse gene expression, total RNA was isolated from cultured or sorted SSCs and OPCs  
740 using the RNeasy Plus Mini or Micro Kit (Qiagen) and reverse transcribed with oligo(dT) and  
741 SuperScript II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR reactions were  
742 performed on a Light Cycler instrument (LC480, Roche Diagnostics) with the LightCycler 480  
743 SYBR Green detection kit (Roche Diagnostics) using primers reported in Table S3. For human  
744 gene expression, total RNA was isolated from cultured BMSCs using Trizol Reagent  
745 (ThermoFisher). Reverse transcription was performed using SuperScriptVilo IV  
746 (ThermoFisher). When required, total RNA from WS BMSCs and their related controls were  
747 extracted from  $0.2 \times 10^3$  BMSCs and pre-amplified using CellsDirect One-Step qRT-PCR kit  
748 (Invitrogen). PCR reactions were performed using primers reported in Table S3 with Power  
749 SYBRGreen (Applied Biosystems) on a 7500 FAST apparatus (Applied Biosystems). Mouse  
750 *β-actin* and *36b4* and human *β-ACTIN* and *GAPDH* were used as standards for normalization.  
751 Relative quantification was determined by the comparative delta-Ct ( $2^{-\Delta Ct}$ ) method (fold

752 changes calculated relative to house-keeping genes) or delta-delta-Ct ( $2^{-\Delta\Delta CT}$ ) method (fold  
753 changes calculated by setting the mean values obtained from WT cells as one).

754

755 **Multiplex qPCR**

756 Multiplex qPCR was performed using the microfluidic Biomark system. One hundred SSCs  
757 were sorted into PCR tubes containing 5  $\mu$ l of reverse transcription/pre-amplification mix  
758 containing 2X reaction buffer, SuperScriptIII from the CellsDirect One-Step qRT-PCR kit and  
759 0.2X Taqman assay (Life technologies) (Table S4). cDNA pre-amplification was performed  
760 during 22 cycles and pre-amplified product was diluted 1:5 in TE buffer before processing with  
761 Dynamic Array protocol (Fluidigm). Cells expressing  *$\beta$ -actin* and control genes (*Runx2*, *Coll  $\alpha$* ,  
762 *Alp* and *Ibsp*) and not *Pax5* and/or *Cd3* (negative controls) were considered for analyses.  
763 Expression of  *$\beta$ -actin* was used for normalization. Heatmaps were generated with  
764 <http://www.heatmapper.ca> using Z scores.

765

766 **RNA sequencing**

767 Pools of 3 x 10<sup>3</sup> SSCs or OPCs were sorted from the bone fraction into RLT buffer (Qiagen)  
768 with 1% of  $\beta$ -mercaptoethanol. RNA was isolated using RNeasy Micro Kit. cDNAs were  
769 generated from 400 to 1,000 pg of total RNA using Clontech SMART-Seq v4 Ultra Low Input  
770 RNA kit for Sequencing (Takara Bio Europe) and amplified with 12 cycles of PCR by Seq-  
771 Amp polymerase. For Tn5 transposon tagmentation, 600 pg of pre-amplified cDNAs were used  
772 by the Nextera XT DNA Library Preparation Kit (96 samples) (Illumina) followed by library  
773 amplification of 12 cycles. Purification was performed with Agencourt AMPure XP and  
774 SPRIselect beads (Beckman-Coulter). Sequencing reads were generated, in Paired-End mode,  
775 on the GenomEast platform (Illumina). FastQC program was used to evaluate the quality of the  
776 raw sequencing data and reads shorter than 50 bp were removed. Reads were aligned to the *Mus*

777 *musculus* genome (mm10 build) using the Star tool<sup>90</sup>. Gene expression quantification was  
778 obtained using read counting software Htseq<sup>91</sup>. Normalization and differential analysis were  
779 carried out with DESeq2 package by applying the Benjamini-Hochberg FDR correction ( $p <$   
780  $0.05$ ; 1.5-fold) for comparison between samples. Heatmaps and volcano plots were obtained  
781 using the web server Heatmapper and EnhancedVolcano packages respectively.

782

### 783 **Statistics**

784 All statistical analyses were conducted using Prism software (GraphPad Software). A Kruskal-  
785 Wallis test was used to determine the significance of the difference between means of WT,  
786 +/-1013 and 1013/1013 groups. Unless specified, the unpaired two-tailed Student *t* test was used  
787 to compare means among two groups.

788

### 789 **DATA AVAILABILITY**

790 RNA-seq data that support the findings of this study have been deposited in the Gene  
791 Expression Omnibus repository with the accession code GSE217422  
792 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE217422>). The data that support the  
793 findings of this study are available from the corresponding author upon request. Source data  
794 are provided with this paper.

795

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1081 **AUTHOR CONTRIBUTIONS**

1082 A.A., J.N. and Z.A-N. designed and performed experiments, analyzed data and contributed to  
1083 manuscript writing; V.R., A.Bon., M.K., A.Bou., J.L., V.B., J.K., L.S., A.P. and A.C. performed  
1084 experiments and analyzed data; S.P., N.D., M.A-L., S.J.C.M., G.L., F.G., C.B-W. and M.C-S.  
1085 performed experiments, contributed to data analyses and reviewed the manuscript; D.H.M. and  
1086 P.M.M provided WS samples and clinical data and reviewed the manuscript; M.E. and M.R.  
1087 helped with the study design, performed experiments, contributed to data analyses and reviewed  
1088 the manuscript; K.B. conceived, designed and supervised the study, contributed to data  
1089 analyses, found funding for the study, and wrote the manuscript.

1090

1091 **COMPETING INTERESTS**

1092 The authors declare no competing interests.

1093

1094 **TABLES**

1095

	<b>Gender</b>	<b>Chronic treatment</b>	<b>Lumbar spine</b>	<b>Femoral neck</b>
<b>P1</b>	Female	No	-3.1 <sup>(5)</sup>	0
<b>P2</b>	Female	No	-1.1	-1.8
<b>P3<sup>(1)</sup></b>	Male	Yes <sup>(2)</sup>	-1.8	-2.3
<b>P4</b>	Female	Yes <sup>(3)</sup>	-2.7	-1.3
<b>P5<sup>(1)</sup></b>	Male	Yes <sup>(4)</sup>	-1.8	-2.2

1096

1097 **Table 1: Abnormal bone mineral density values in WS patients.** Characteristics of each  
 1098 patient with low BMD value are shown. Four patients carry the *CXCR4*<sup>R334X</sup> mutation and one  
 1099 displays the *CXCR4*<sup>S338X</sup> mutation. There were 3 women and 2 men with an average age of 33.2  
 1100 years (range 13-52). T-scores for lumbar spine (L1-L4) and femoral neck have been evaluated.  
 1101 According to World Health Organization (WHO) criteria, values classify patients as osteopenic  
 1102 with a T-score between -1.0 and -2.5 or osteoporotic with a T-score at or below -2.5.

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1108 <sup>(1)</sup>For patients 3 and 5, because of their young age, Z-scores are given with a value at or below  
 1109 -2.0 considered as abnormal; <sup>(2)</sup>G-CSF since age of 2; <sup>(3)</sup>G-CSF several years at the time of  
 1110 scan; <sup>(4)</sup>G-CSF for 6 months at the time of scan; <sup>(5)</sup>Values outside the normal range defined by  
 1111 WHO are italicized.

1112 **FIGURE LEGENDS**

1113 **Figure 1: WS-linked *Cxcr4* mutations are associated with reduced bone mass in mice. (A)**

1114 The bone mineral density (BMD) of lumbar spine of WT, +/-1013 and 1013/1013 mice was  
 1115 measured through Dual-energy x-ray absorptiometry. Results represent means  $\pm$  SEM with 5  
 1116 mice per group examined over two independent experiments. Statistics were calculated with  
 1117 the nonparametric Kruskal–Wallis H test ( $^{##}p=0.0042$ ) and the nonparametric Mann–Whitney  
 1118 test, two-sided, +/-1013  $p=0.0556$ , 1013/1013  $^{**}p=0.0079$ . **(B–D)** 3D representative images of  
 1119 trabecular and cortical composites (B) and quantitative  $\mu$ CT analyses of trabecular (C) and  
 1120 cortical (D) parameters of femurs from WT and mutant mice. BV = bone volume; TV =  
 1121 trabecular volume; Tb.Nb = trabecular number; Tb.Sp = trabecular separation; Ct.BV = cortical  
 1122 bone volume; Ct.Th = cortical thickness. Data (means  $\pm$  SEM) are from three independent  
 1123 experiments with  $n= 15, 15,$  and 7 mice in total for WT, +/-1013 and 1013/1013 groups,  
 1124 respectively. Statistics were calculated with the nonparametric Kruskal–Wallis H test  
 1125 ( $^{###}p=0.0007$ , BV/TV;  $p=0.0001$ , Tb.Nb;  $p<0.0001$ , Tb.Sp; Ct.BV,  $^{##}p=0.0072$ ;  $^{\#}p=0.0241$ ) and  
 1126 the unpaired two-tailed Student’s t test (+/1013 vs WT  $^{**}p=0.0556$ , 1013/1013 vs WT  
 1127  $^{***}p=0.0005$ , +/-1013 vs 1013/1013  $^{\$}p=0.032$  for BV/TV;  $^{***}p=0.0005$ ,  $^{***}p<0.0001$ ,  
 1128  $^{\$\$}p=0.008$  for Tb.Nb;  $^{***}p=0.0038$ ,  $^{***}p<0.0001$ ,  $^{\$ \$ \$}p<0.0001$  for Tb.Sp;  $^{**}p=0.0072$ ,  
 1129  $^{**}p=0.0078$  for Ct.BV;  $^*p=0.0147$ ,  $^*p=0.02$  for Ct.Th). **(E)** BM sections were stained with  
 1130 toluidine blue. Larger images show 2X inserts in trabecular areas. Bars: 200  $\mu$ m. Images are  
 1131 representative of at least three independent determinations. **(F)** BM sections were  
 1132 immunostained for osteopontin (Opn) in association with DAPI. Trabeculae are indicated by  
 1133 white arrows. Bars: 250  $\mu$ m. Images are representative of five independent determinations. **(G**  
 1134 **and H)** BM sections were stained for chondrocyte (alcian blue, G) or adipocyte (perilipin, H)  
 1135 markers. Bars: 500 (G) or 20 (H)  $\mu$ m. Images are representative of at least six independent  
 1136 determinations. **(I)** Cartilaginous growth plates were evaluated based on overall growth plate

1137 thickness measured on  $\mu$ CT scans. Data (means  $\pm$  SEM) are from 2 independent experiments  
1138 with n= 7, 8, and 7 mice in total for WT, +/1013 and 1013/1013 groups, respectively. **(J)**  
1139 Adipocyte counts were evaluated on perilipin-stained BM sections. Six fields of 3 mm<sup>2</sup> were  
1140 analyzed per section. Results (means  $\pm$  SEM) are from 4 independent experiments with 13 mice  
1141 in total per group. **(K)** Size (left) and weight (right) of WT and mutant mice. Results (means  $\pm$   
1142 SEM) are from five independent experiments with n= 6, 9, and 10 mice in total for WT, +/1013  
1143 and 1013/1013 groups, respectively. Mice were littermates, females and age-matched (8-12 wk-  
1144 old) in A-J and at 8 weeks of age in K. Source data are provided as a Source Data file.

1145

1146 **Figure 2: Reduction of skeletal stromal cells in *Cxcr4*<sup>1013</sup>-bearing mice.** **(A)** Representative  
1147 dot-plots showing the flow cytometric gating strategies used to sort stroma cells (CD45<sup>-</sup>  
1148 TER119<sup>-</sup>), differentiated osteoblast progenitor cells (OPCs, CD45<sup>-</sup>TER119<sup>-</sup>CD31<sup>-</sup>Sca-1<sup>-</sup>  
1149 CD51<sup>+</sup>PDGFR $\alpha$ <sup>+/+</sup>) and SSCs (CD45<sup>-</sup>TER119<sup>-</sup>CD31<sup>-</sup>Sca-1<sup>+</sup>CD51<sup>+</sup>PDGFR $\alpha$ <sup>+</sup>) in the mouse  
1150 bone fraction. **(B)** Absolute numbers of the indicated stroma cell subsets from bone fractions  
1151 were determined by flow cytometry. Data (means  $\pm$  SEM) are from at least six independent  
1152 experiments with n= 31, 31, and 17 mice in total for WT, +/1013 and 1013/1013 groups,  
1153 respectively. Statistics were calculated with the nonparametric Kruskal–Wallis H test  
1154 (####p<0.0001, OPC) and the unpaired two-tailed Student's t test (1013/1013 vs WT \*p=0.0206  
1155 for SSC; +/1013 vs WT \*\*p=0.0031, 1013/1013 vs WT \*\*\*p<0.0001, +/1013 vs 1013/1013  
1156 §p=0.0217 for OPC). **(C)** Schematic diagram for the generation of CD45.1 $\rightarrow$ CD45.2 short (3  
1157 wks)- or long (16 wks)-term BM chimeras. **(D)** Proportions of WT donor CD45.1<sup>+</sup> LSK SLAMF6<sup>+</sup>  
1158 and leukocytes (Leuko.) recovered from the BM and blood of BM chimeras in CD45.2<sup>+</sup> WT or  
1159 mutant recipients 16 weeks after transplantation. **(E)** Absolute numbers of SSCs and OPCs  
1160 determined by flow cytometry in bone fractions of BM chimeras in CD45.2<sup>+</sup> recipients. Data  
1161 (means  $\pm$  SEM) in D and E are from three independent experiments with n= 10 (D) or 11 (E),

1162 9, and 5 mice in total for WT, +/-1013 and 1013/1013 recipient groups, respectively. Statistics  
1163 were calculated with the nonparametric Kruskal–Wallis H test ( $^{##}p=0.0064$ ) and the  
1164 nonparametric Mann–Whitney test, two-sided (+/1013 vs WT  $^{**}p=0.0042$ , 1013/1013 vs WT  
1165  $^{*}p=0.0126$ , for SSC; +/-1013 vs WT  $^{**}p=0.0031$ , 1013/1013 vs WT  $^{*}p=0.0398$ , for OPC). **(F)**  
1166 Sixteen weeks after transplantation, BM sections from WT or mutant CD45.2<sup>+</sup> recipient mice  
1167 were immuno-stained for Opn in association with DAPI (bars: 250  $\mu$ m). Trabeculae are  
1168 indicated by white arrows. Images are representative of at least three independent  
1169 determinations. **(G)** Left: Proportions of WT donor CD45.1<sup>+</sup> LSK SLAM and leukocytes  
1170 recovered from the BM and blood of BM chimeras in CD45.2<sup>+</sup> WT or mutant recipients 3 weeks  
1171 after transplantation. Right: Absolute numbers of SSCs and OPCs. Data (means  $\pm$  SEM) are  
1172 from three independent experiments with n= 9, 9, and 5 mice in total for WT, +/-1013 and  
1173 1013/1013 recipient groups, respectively, except for blood chimerism analysis (5 mice per  
1174 group). Statistics were calculated with the nonparametric Kruskal–Wallis H test ( $^{#}p=0.0327$ )  
1175 and the nonparametric Mann–Whitney test, two-sided (1013/1013 vs WT  $^{*}p=0.019$ , +/-1013 vs  
1176 1013/1013  $^{§}p=0.019$  for SSC; 1013/1013 vs WT  $^{*}p=0.017$  for OPC). **(H and I)** 3D  
1177 representative images of trabecular composites (H) and  $\mu$ CT analyses of trabecular parameters  
1178 (I) of femurs from WT BM-chimeric CD45.2<sup>+</sup> WT or mutant recipients 4 months after  
1179 transplantation. Data (means  $\pm$  SEM) are from three independent experiments with n= 8, 5, and  
1180 5 mice in total for WT, +/-1013 and 1013/1013 recipient groups, respectively. Statistics were  
1181 calculated with the unpaired two-tailed Student’s t test (+/1013 vs WT  $^{*}p=0.033$ , 1013/1013 vs  
1182 WT  $^{*}p=0.024$  for BV/TV). WT and mutant mice were littermates, females and age-matched  
1183 (8-12 wk-old) and adult Boy/J (CD45.1) WT mice at 8 weeks of age were used as BM donors.  
1184 Source data are provided as a Source Data file.  
1185

1186 **Figure 3: Cell-extrinsic Cxcr4-mediated regulation of the skeletal landscape. (A)**  
 1187 Schematic diagram for the generation of CD45.2→CD45.1 short (3 wks)- or long (16 wks)-  
 1188 term BM chimeras. **(B)** Proportions of WT or mutant donor CD45.2<sup>+</sup> LSK SLAM and  
 1189 leukocytes recovered from the BM and blood of BM chimeras in CD45.1<sup>+</sup> WT recipients 16  
 1190 weeks after transplantation. Statistics were calculated with the nonparametric Kruskal–Wallis  
 1191 H test (###p=0.0008 for SLAM and <0.0001 for Leukocytes) and the unpaired two-tailed  
 1192 Student’s t test (+/1013 vs WT \*\*p=0.0036, 1013/1013 vs WT \*\*\*p=0.0004, for SLAM;  
 1193 +/1013 vs WT \*\*\*p=0.0003, 1013/1013 vs WT \*\*\*p<0.0001, for Leukocytes). **(C)** Absolute  
 1194 numbers of SSCs and OPCs in bone fractions of BM chimeras in CD45.1<sup>+</sup> recipients. Statistics  
 1195 were calculated with the nonparametric Kruskal–Wallis H test (##p=0.009 for SSC and ###p  
 1196 =0.0006) and the unpaired two-tailed Student’s t test (1013/1013 vs WT \*\*p=0.0012, +/1013  
 1197 vs 1013/1013 §§p=0.0076, for SSC; +/1013 vs WT \*p=0.029, 1013/1013 vs WT \*\*\*p<0.0001,  
 1198 +/1013 vs 1013/1013 §p=0.022, for OPC). Data (means ± SEM) in B and C are from three  
 1199 independent experiments with n= 14 mice in total for WT, +/1013 and 1013/1013 donor groups,  
 1200 respectively, except for blood chimerism analysis (n= 11, 9, and 8 mice in total for WT, +/1013  
 1201 and 1013/1013 donor groups, respectively). **(D)** Sixteen weeks after transplantation, BM  
 1202 sections from WT CD45.1<sup>+</sup> recipient mice were immunostained for Opn in association with  
 1203 DAPI (bars: 250 μm). Trabeculae are indicated by white arrows. Images are representative of  
 1204 at least three independent determinations. **(E)** Left: Proportions of WT or mutant donor  
 1205 CD45.2<sup>+</sup> LSK SLAM and leukocytes recovered from the BM and blood of BM chimeras in  
 1206 CD45.1<sup>+</sup> WT recipients 3 weeks after transplantation. Right: Absolute numbers of SSCs and  
 1207 OPCs. Data (means ± SEM) are from three independent experiments with n= 6 (SLAM) or 7  
 1208 (SSC and OPC), 10, and 8 mice in total for WT, +/1013 and 1013/1013 donor groups,  
 1209 respectively, except for blood chimerism analysis (n= 6, 5, and 4 mice in total for WT, +/1013  
 1210 and 1013/1013 donor groups, respectively). Statistics were calculated with the nonparametric

1211 Kruskal–Wallis H test (<sup>##</sup>p=0.0083 for leukocytes; <sup>#</sup>p=0.047 for SSC; <sup>#</sup>p=0.018 for OPC) and  
1212 the unpaired two-tailed Student’s t test (+/1013 vs WT \*p=0.021, 1013/1013 vs WT  
1213 \*\*p=0.0014, for leukocytes; +/1013 vs WT \*p=0.046, 1013/1013 vs WT \*p=0.029, for SSC;  
1214 +/1013 vs WT \*p=0.034, 1013/1013 vs WT \*p=0.01, for OPC). (F–I) 3D representative images  
1215 of trabecular or cortical composites (F and H) and  $\mu$ CT analyses of trabecular or cortical  
1216 parameters (G and I) of femurs from WT or mutant BM-chimeric CD45.1<sup>+</sup> WT recipients 4  
1217 months after transplantation. Ct.BV = cortical bone volume; Ct.Th = cortical thickness. Data  
1218 (means  $\pm$  SEM) in G and I are from two independent experiments with n= 5, 5, and 4 mice in  
1219 total for WT, +/1013 and 1013/1013 donor groups, respectively. Statistics were calculated with  
1220 the nonparametric Kruskal–Wallis H test (<sup>##</sup>p=0.0085 for BV/TV; <sup>##</sup>p=0.0069 for Tb.Nb;  
1221 <sup>###</sup>p=0.0001 for Tb.Sp; <sup>#</sup>p=0.033 for Ct.BV) and the unpaired two-tailed Student’s t test  
1222 (1013/1013 vs WT \*p=0.028, +/1013 vs 1013/1013 <sup>§</sup>p=0.028 for BV/TV; 1013/1013 vs WT  
1223 \*p=0.011, +/1013 vs 1013/1013 <sup>§§</sup>p=0.0088 for Tb.Nb; +/1013 vs WT \*p=0.044, 1013/1013 vs  
1224 WT \*\*\*p<0.0001, +/1013 vs 1013/1013 <sup>§§§</sup>p=0.0006 for Tb.Sp; 1013/1013 vs WT \*p=0.014,  
1225 +/1013 vs 1013/1013 <sup>§</sup>p=0.016 for Ct.BV). Donor WT and mutant mice and Boy/J (CD45.1)  
1226 WT recipient mice were females at 8 weeks of age. Source data are provided as a Source Data  
1227 file.

1228

1229 **Figure 4: Increased bone resorption and reduced bone formation in *Cxcr4*<sup>1013</sup>-bearing**  
1230 **mice. (A)** Bone sections were stained for Tartrate Resistant Acid Phosphatase (TRAP) activity  
1231 (bars: 100  $\mu$ m). OCLs are visualized as brown-stained TRAP-positive cells attached to bone  
1232 trabeculae and are indicated by arrows (representative images). **(B)** OCLs were quantified  
1233 (Oc.S/BS) and (Oc.N/BV). Results represent means  $\pm$  SEM with 6 mice in total per group over  
1234 3 independent experiments. Statistics were calculated with the nonparametric Kruskal–Wallis  
1235 H test (<sup>###</sup>p=0.0006) and the unpaired two-tailed Student’s t test (1013/1013 vs WT \*p=0.012



1236 for Oc.S/BS; 1013/1013 vs WT \* $p=0.049$  for Oc.N/BV). **(C and D)** Dynamic  
1237 histomorphometric measures of bone formation. OS/BS = Osteoid number / Bone surface;  
1238 Obl.S/BS = Osteoblast surface / Bone surface; MS/BS = Mineralized surface / Bone surface;  
1239 Dbl/BS = Double labelled surface / Bone surface. Data (means  $\pm$  SEM) are from 3 independent  
1240 experiments with  $n=6, 6,$  and 5 mice in total for WT, +/1013 and 1013/1013 groups respectively  
1241 in C, and  $n=6, 6,$  and 5 (for MS/BS) or 6 (for Dbl/BS) mice in total for WT, +/1013 and  
1242 1013/1013 groups respectively in D. Statistics were calculated with the nonparametric Kruskal–  
1243 Wallis H test ( $\#p=0.038$ ) and the unpaired two-tailed Student’s t test (1013/1013 vs WT  
1244 \*\* $p=0.0097$  for MS/BS; 1013/1013 vs WT \* $p=0.035$  for Dbl/BS). **(E)** The mineral apposition  
1245 rate (MAR) and bone formation rate (BFR/BS) were determined. Results (means  $\pm$  SEM) are  
1246 from 3 independent experiments with  $n=6, 6,$  and 5 (for MAR) or 6 (for BFR/BS) mice in total  
1247 for WT, +/1013 and 1013/1013 groups respectively. Statistics were calculated with the  
1248 nonparametric Kruskal–Wallis H test ( $\#p=0.039$ ) and the unpaired two-tailed Student’s t test  
1249 (1013/1013 vs WT \* $p=0.03,$  +/1013 vs 1013/1013  $\$p=0.044$ ). **(F)** Volcano plot analysis of  
1250 differentially expressed genes obtained by RNA-seq between WT and 1013/1013 OPCs  
1251 ( $p<0.05;$   $FC\geq 2$ ) performed on three biological replicates per group with one replicate  
1252 representing the pool of 3 mice. Data represent analysis of cpm estimates with a log of fold  
1253 change of more than 1.5 fold and  $p<0.05$  using enhanced Volcano package. **(G and J)** Heatmap  
1254 representing the relative expression levels of selected genes (osteogenic, G and  
1255 osteoclastogenic, J) expressed by sorted OPCs. **(H and K)** Normalized counts of osteogenic  
1256 (H) and osteoclastogenic (K) genes using the DESeq2 method. Data are represented as floating  
1257 bars (min to max and line equal median) of the three biological replicates per group. For  
1258 significance testing, DESeq2 uses a Wald test (p values). The Wald test P values from the subset  
1259 of genes that pass an independent filtering step, are adjusted for multiple testing using the  
1260 procedure of Benjamini and Hochberg (padj values). **(I)** *In vitro* osteoblastic differentiation of

1261 sorted OPCs evaluated at day 21 post-culture by Alizarin Red S coloration. The images are  
 1262 representative of 3 independent cultures. The quantification (means  $\pm$  SEM) from three  
 1263 independent experiments with 6 mice in total per group is shown. **(L)** *In vitro* expanded  
 1264 osteogenic cells from bone fractions were cultured with WT CD11b<sup>+</sup> osteoclast progenitors and  
 1265 stimulated with PGE2/Vitamin D3 (VitD3)/Dexamethasone (Dex) for 8 days. OCLs (TRAP-  
 1266 positive) were identified (left, representative images, bars: 100  $\mu$ m) and quantified (right). Data  
 1267 (means  $\pm$  SEM) are from 2 independent experiments with 6 mice in total per group. Statistics  
 1268 were calculated with the nonparametric Kruskal–Wallis H test (<sup>#</sup>p=0.033) and the unpaired two-  
 1269 tailed Student’s t test (+/1013 vs WT \*p=0.02 and 1013/1013 vs WT \*p=0.014). **(M)** The  
 1270 relative expression levels of osteoclastogenic genes were determined by quantitative PCR in  
 1271 stimulated osteogenic cells (3 mice per group). Each individual sample was run in triplicate and  
 1272 has been standardized for *36B4* expression levels. All mice were littermates, females and age-  
 1273 matched (8-12 wk-old). Source data are provided as a Source Data file.

1274

1275 **Figure 5: Impaired osteogenic specification of *Cxcr4*<sup>1013</sup>-bearing skeletal stromal/stem**  
 1276 **cells. (A)** Ki-67 and DAPI co-staining to analyze by flow cytometry the cell cycle status of  
 1277 SSCs and OPCs from bone fractions. Bar graphs show the percentage of cells (DAPI<sup>low</sup>Ki-67<sup>-</sup>)  
 1278 in the quiescent G0 phase. Data (means  $\pm$  SEM) are from three independent experiments with  
 1279 n= 9, 6, and 6 mice in total for WT, +/-1013 and 1013/1013 groups, respectively. Statistics were  
 1280 calculated with the nonparametric Kruskal–Wallis H test (<sup>#</sup>p=0.029) and the unpaired two-  
 1281 tailed Student’s t test (1013/1013 vs WT \*\*p=0.0091). **(B)** Flow-cytometric detection of BrdU  
 1282 staining in SSCs (left). Percentages of BrdU<sup>+</sup> bone SSCs and OPCs after a 12-day labelling  
 1283 period (right). Data (means  $\pm$  SEM) are from three independent experiments with six mice in  
 1284 total per group. Statistics were calculated with the nonparametric Kruskal–Wallis H test  
 1285 (<sup>##</sup>p=0.0021) and the unpaired two-tailed Student’s t test (+/1013 vs WT \*p=0.016, 1013/1013

1286 vs WT \*\* $p=0.0011$ ). **(C)** Characterization of some biological processes displaying differential  
1287 gene expression signatures in sorted SSCs as defined by GSEA and obtained by RNA-seq on 2  
1288 (1013/1013) or 3 (WT and +/1013) biological replicates per group with one replicate  
1289 representing the pool of 3 mice. For significance testing, DESeq2 uses a Wald test ( $p$  values).  
1290 The Wald test  $P$  values from the subset of genes that pass an independent filtering step, are  
1291 adjusted for multiple testing using the procedure of Benjamini and Hochberg ( $padj$  values). **(D)**  
1292 RNA-seq-based heatmap representing the relative expression levels of osteogenic genes. **(E)**  
1293 Normalized counts of selected osteogenic genes using the DESeq2 method. Data are  
1294 represented as floating bars (min to max and line equal median) of the 2 or 3 biological  
1295 replicates per group. For significance testing, DESeq2 uses a Wald test ( $p$  values). **(F)** The  
1296 heatmap shows the relative expression levels (RQ) normalized for  $\beta$ -actin expression levels in  
1297 each sample of selected genes involved in SSC differentiation towards the osteogenic lineage  
1298 (6 pools of 100 cells *per* condition) by quantitative PCR. **(G)** RQ of the most regulated genes  
1299 involved in differentiation and cell cycle of SSCs. Data (means  $\pm$  SEM) are from two  
1300 independent experiments with 6 mice in total per group. Statistics were calculated with the  
1301 nonparametric Kruskal–Wallis H test ( $\#p=0.028$  for *Runx2*;  $\#p=0.011$  for *Ccnd3*) and the  
1302 unpaired two-tailed Student's t test (1013/1013 vs WT \*\* $p=0.0063$  for *Runx2*; 1013/1013 vs  
1303 WT \* $p=0.048$  for *Coll $\alpha$* ; +/1013 vs WT \* $p=0.022$  and 1013/1013 vs WT \* $p=0.02$  for *Ccnd3*).  
1304 **(H)** RNA-seq-based heatmap representing the relative expression levels of osteoclastogenic  
1305 genes expressed by sorted SSCs. **(I)** Normalized counts of selected osteoclastogenic genes  
1306 using the DESeq2 method. Data are represented as floating bars (min to max and line equal  
1307 median) of the 2 or 3 biological replicates per group. For significance testing, DESeq2 uses a  
1308 Wald test ( $p$  values). **(J)** Relative expression of osteoclastogenic genes in SSCs by quantitative  
1309 PCR. Each individual sample was run in triplicate and has been standardized for  $\beta$ -actin  
1310 expression levels and presented as relative expression to WT. Data (means  $\pm$  SEM) are from

1311 two independent experiments with 5 mice in total per group. **(K)** Immunofluorescence showing  
 1312 in red Osterix (Osx)-positive cells and in blue DAPI-stained nuclei in WT and mutant mice  
 1313 femurs (bars: 100  $\mu$ m). Dashed lines indicate the limit between the cartilage growth plate (above  
 1314 the line) and the bone (below the line). Images are representative of at least 3 independent  
 1315 determinations. **(L)** Quantification of Osx<sup>+</sup> cells per mm<sup>2</sup> below the growth plate. Data (means  
 1316  $\pm$  SEM) are from 5, 5, and 3 independent mice in total for WT, +/1013 and 1013/1013 groups  
 1317 respectively. Statistics were calculated with the unpaired two-tailed Student's t test (1013/1013  
 1318 vs WT \*p=0.0101). **(M)** Absolute numbers of the indicated stroma cell subsets from marrow  
 1319 fractions determined by flow cytometry. Data (means  $\pm$  SEM) are from four independent  
 1320 experiments with n= 9, 10, and 7 mice in total for WT, +/1013 and 1013/1013 groups,  
 1321 respectively. Statistics were calculated with the nonparametric Kruskal–Wallis H test  
 1322 (###p=0.0004 for stroma; ##p=0.0013 for OPC) and the unpaired two-tailed Student's t test  
 1323 (+/1013 vs WT \*\*p=0.0011, 1013/1013 vs WT \*\*\*p=0.0002, +/1013 vs 1013/1013 \$p=0.033,  
 1324 for stroma; +/1013 vs WT \*\*p=0.0049, 1013/1013 vs WT \*\*\*p=0.0003, for OPC). All mice  
 1325 were littermates, females and age-matched (8-12 wk-old). Source data are provided as a Source  
 1326 Data file.

1327

1328 **Figure 6: Cxcr4 desensitization intrinsically regulates *in vitro* the osteogenic**  
 1329 **differentiation of skeletal stromal cells.** **(A)** Number of colonies formed from bone fractions  
 1330 in CFU-F assays. Data (means  $\pm$  SEM) are from two independent experiments with n= 4, 6,  
 1331 and 4 mice in total for WT, +/1013 and 1013/1013 groups respectively. Statistics were  
 1332 calculated with the nonparametric Kruskal–Wallis H test (###p=0.002) and the unpaired two-  
 1333 tailed Student's t test (+/1013 vs WT \*p=0.029, 1013/1013 vs WT \*\*\*p=0.0008). **(B)** After *in*  
 1334 *vitro* loading with BrdU (5 days) or CTV (3 days), the percentages of BrdU<sup>+</sup> (left) or CTV<sup>low</sup>  
 1335 (right) cells within WT and mutant bone-derived SSCs were determined by flow cytometry.

1336 Data (means  $\pm$  SEM) are from 3 independent experiments with 6 mice in total per group.  
1337 Statistics were calculated with the nonparametric Kruskal–Wallis H test ( $\#p=0.0231$  and  
1338  $\#\#p=0.0047$  for BrdU<sup>+</sup> and CTV<sup>low</sup> respectively) and the unpaired two-tailed Student's t test  
1339 (+/1013 vs WT  $*p=0.031$ , 1013/1013 vs WT  $**p=0.0023$  for BrdU<sup>+</sup>; +/1013 vs WT  $*p=0.015$ ,  
1340 1013/1013 vs WT  $**p=0.0054$  for CTV<sup>low</sup>). (C) Bar graphs show the percentages of cultured  
1341 WT or mutant SSCs in the quiescent G0 phase (DAPI<sup>low</sup>Ki-67<sup>-</sup>, left) or with an apoptotic  
1342 phenotype (Annexin V<sup>+</sup> DAPI<sup>-</sup>, right) as determined by flow cytometry. Data (means  $\pm$  SEM)  
1343 are from three (right panel) or five (left panel) independent SSC cultures per genotype. Statistics  
1344 were calculated using the unpaired two-tailed Student's t test (+/1013 vs WT  $*p=0.032$ ,  
1345 1013/1013 vs WT  $*p=0.021$ ). (D) Doubling time (left) and absolute numbers (right) of WT and  
1346 mutant SSCs after 3 days of culture. Data (means  $\pm$  SEM) are from 8, 6, and 5 independent SSC  
1347 cultures for WT, +/1013 and 1013/1013 groups respectively. Statistics were calculated using  
1348 the unpaired two-tailed Student's t test (+/1013 vs WT  $*p=0.028$ , 1013/1013 vs WT  $*p=0.033$   
1349 for doubling-time; 1013/1013 vs WT  $*p=0.048$  for SSC). (E) Alkaline phosphatase (Alp)  
1350 staining was performed 14 days after initiation of the culture of WT and mutant SSCs in  
1351 osteogenic medium supplemented every two days with 10  $\mu$ M AMD3100 or vehicle (PBS)  
1352 (bars: 100  $\mu$ m). Quantitative analyses (number of Alp<sup>+</sup> cells) were performed under an inverted  
1353 microscope. Data (means  $\pm$  SEM) are from 6 independent cultures per genotype. Statistics were  
1354 calculated with the nonparametric Kruskal–Wallis H test ( $\#\#p=0.0022$ ) and the unpaired two-  
1355 tailed Student's t test (+/1013 vs WT  $**p=0.0075$ , 1013/1013 vs WT  $**p=0.0018$ ). (F) Alizarin  
1356 Red staining was performed 21 days after initiation of the culture. Quantitative analyses (means  
1357  $\pm$  SEM) of staining were performed using the osteogenesis assay kit in 12 (vehicle) or 6  
1358 (AMD3100) independent cultures per genotype. Statistics were calculated with the  
1359 nonparametric Kruskal–Wallis H test ( $\#\#\#p=0.0002$ ) and the unpaired two-tailed Student's t test  
1360 (+/1013 vs WT  $***p=0.0005$ , 1013/1013 vs WT  $***p<0.0001$ ). (G) Expression levels of

1361 osteogenic genes were determined by quantitative PCR in 6 independent WT and mutant SSC  
 1362 cultures 14 and 21 days after initiation of the osteogenic culture in the presence or absence of  
 1363 AMD3100. Each individual sample was run in triplicate and was standardized for  $\beta$ -actin  
 1364 expression levels. Results (means  $\pm$  SEM) are expressed as relative expression compared to  
 1365 WT samples. Statistics were calculated with the nonparametric Kruskal–Wallis H test  
 1366 ( $\#p=0.038$  and  $0.0205$  for Osx days 14 and 21 respectively;  $\#p=0.024$  and  $0.015$  for Alp days 14  
 1367 and 21 respectively;  $\#\#p=0.0063$  for Opn days 21;  $\#p=0.026$  for Ocn days 21) and the unpaired  
 1368 two-tailed Student’s t test (1013/1013 vs WT  $*p=0.0107$  and  $0.013$  for Osx days 14 and 21  
 1369 respectively; 1013/1013 vs WT  $*p=0.035$  and  $***p=0.0001$  for Alp days 14 and 21  
 1370 respectively; +/1013 vs WT  $*p=0.022$  and 1013/1013 vs WT  $**p=0.0056$  for Opn days 21;  
 1371 1013/1013 vs WT  $**p=0.0108$  for Ocn days 21). All mice were littermates, females and age-  
 1372 matched (8-12 wk-old). Source data are provided as a Source Data file.

1373

1374 **Figure 7: Normalization of Cxcr4 signaling rescues the osteogenic properties of *Cxcr4*<sup>1013</sup>-**  
 1375 **bearing mouse skeletal cells. (A)** Schematic diagram for daily AMD3100 intra-peritoneal  
 1376 (*i.p.*) injection for 21 days in WT and mutant mice. **(B)** Absolute numbers of the indicated  
 1377 stroma cell subsets from bone fractions of WT and mutant mice determined by flow cytometry.  
 1378 Data (means  $\pm$  SEM) are from 2 independent experiments with 6 PBS injected mice and 11  
 1379 AMD3100-injected mice in total *per* genotype. Statistics were calculated using the  
 1380 nonparametric Mann–Whitney test, two-sided, 1013/1013 vs WT  $*p=0.041$ , 1013/1013 (AMD)  
 1381 vs 1013/1013 (vehicle)  $\&p=0.01$  for SSC; 1013/1013 vs WT  $**p=0.0022$ , WT (AMD) vs WT  
 1382 (vehicle)  $\&\&p=0.0065$ , 1013/1013 (AMD) vs 1013/1013 (vehicle)  $\&\&\&p=0.0003$  for OPC. **(C)**  
 1383 BM sections from WT and mutant mice treated with vehicle (PBS) or AMD3100 were  
 1384 immunostained for Opn in association with DAPI. Bars: 500 $\mu$ m. Images are representative of  
 1385 3 independent determinations. **(D)** Bone mineral density (BMD) values of lumbar spine from 6

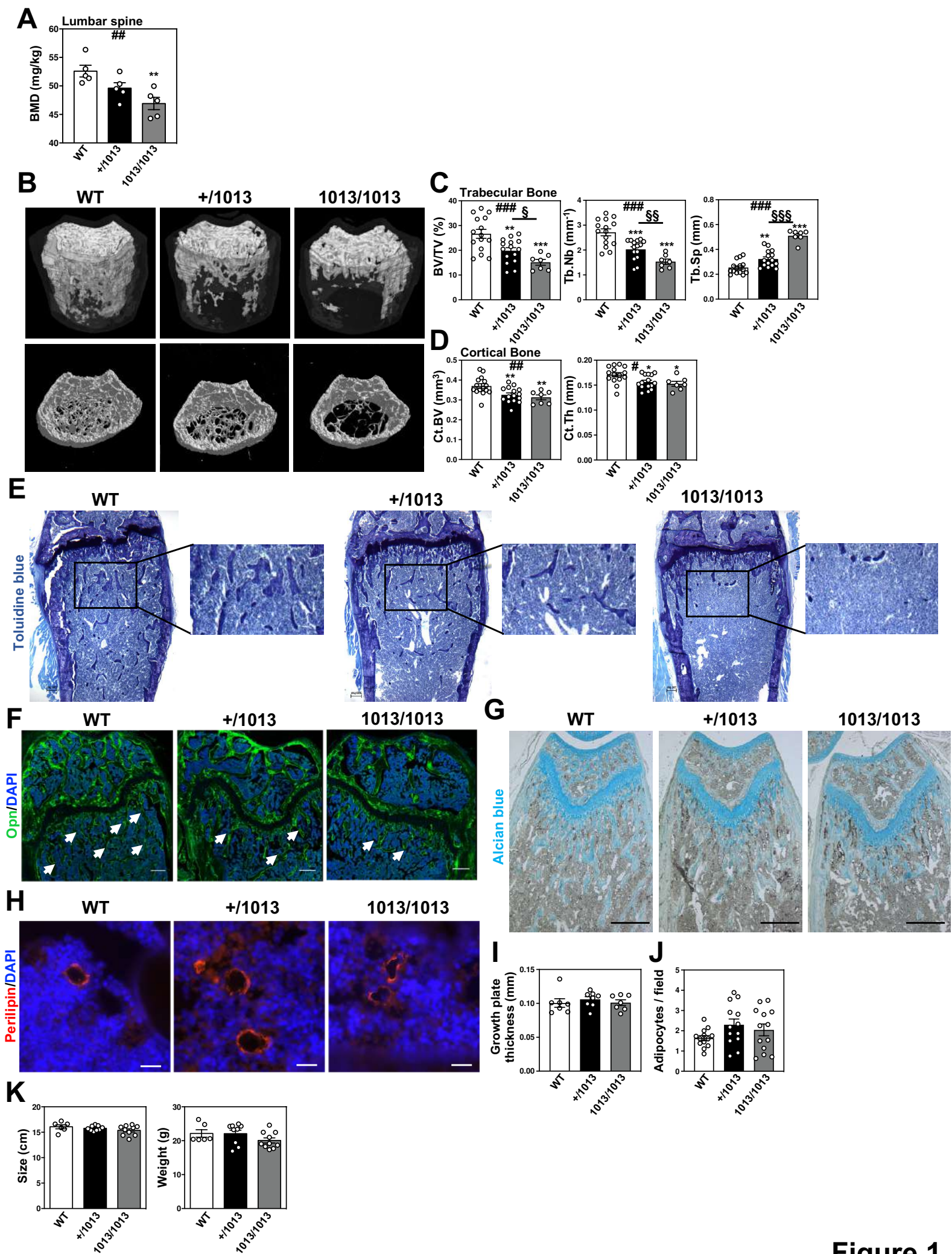
1386 treated mice in total per group are shown. Statistics were calculated using the nonparametric  
1387 Mann–Whitney test, two-sided, +/1013 vs WT \*p=0.041, 1013/1013 vs WT \*\*p=0.0022,  
1388 +/1013 (AMD) vs +/1013 (vehicle) &&p=0.0087, 1013/1013 (AMD) vs 1013/1013 (vehicle)  
1389 &p=0.026. **(E)** Cortical thickness was measured in sections stained with Toluidine Blue. Both  
1390 cortices were measured and data are presented as a mean of both cortices from 6 mice in total  
1391 per group. Statistics were calculated using the nonparametric Mann–Whitney test, two-sided,  
1392 1013/1013 vs WT \*\*p=0.026. Data (means  $\pm$  SEM) displayed in D and E are from 2  
1393 independent experiments. All mice were littermates, females and age-matched (8-12 wk-old).  
1394 Source data are provided as a Source Data file.

1395

1396 **Figure 8: BM stromal cells from WS patients displayed *in vitro* impaired osteogenic**  
1397 **capacities. (A)** Relative expression levels of osteogenic genes were determined by quantitative  
1398 PCR at day 14 in osteogenic-induced cultures of two WS patients-derived BMSCs and 7 healthy  
1399 donors-derived BMSCs. Each individual sample was run in triplicate and was standardized for  
1400 *36B4* expression levels. Results (means  $\pm$  SEM) are expressed as relative expression compared  
1401 to healthy samples (set at 1 and representing the mean of the 7 healthy donors) and are from 2  
1402 independent experiments. Statistics were calculated using the nonparametric Mann–Whitney  
1403 test, two-sided, \*\*p=0.0079 for OSX, RUNX2 and OCN; \*p=0.0179 for OPN. **(B)** Alizarin  
1404 Red staining was performed 21 days after initiation of the culture of  $1.5 \times 10^3$  healthy or WS  
1405 BMSCs in pro-osteogenic medium (left panel). Representative images for healthy (H) and WS  
1406 donors #1 and #2 are shown. Quantitative analyses of staining (means  $\pm$  SEM) were performed  
1407 using the osteogenesis assay kit in 3 independent cultures with 2 WS and 6 healthy donors (right  
1408 panel). Statistics were calculated using the nonparametric Mann–Whitney test, two-sided,  
1409 \*\*p=0.0022 for healthy. **(C)** Oil Red O staining was performed 21 days after initiation of  
1410 cultures of healthy or WS BMSCs in pro-adipogenic differentiation medium. Bars: 200 $\mu$ m. **(D)**

1411 Proposed model: Proper Cxcr4 signaling termination is essential for bone tissue homeostasis.  
1412 Absence of Cxcr4 desensitization leads to imbalance in bone remodeling with decreased OBL-  
1413 mediated bone formation and increased OCL-mediated bone resorption, leading to severe  
1414 trabecular and cortical alterations and a subsequent osteoporotic-like phenotype.  
1415 Mechanistically, impaired Cxcr4 desensitization disrupts the osteogenic commitment of SSCs,  
1416 while strikingly increasing their pro-osteoclastogenic capacities. Source data are provided as a  
1417 Source Data file.





**Figure 1**

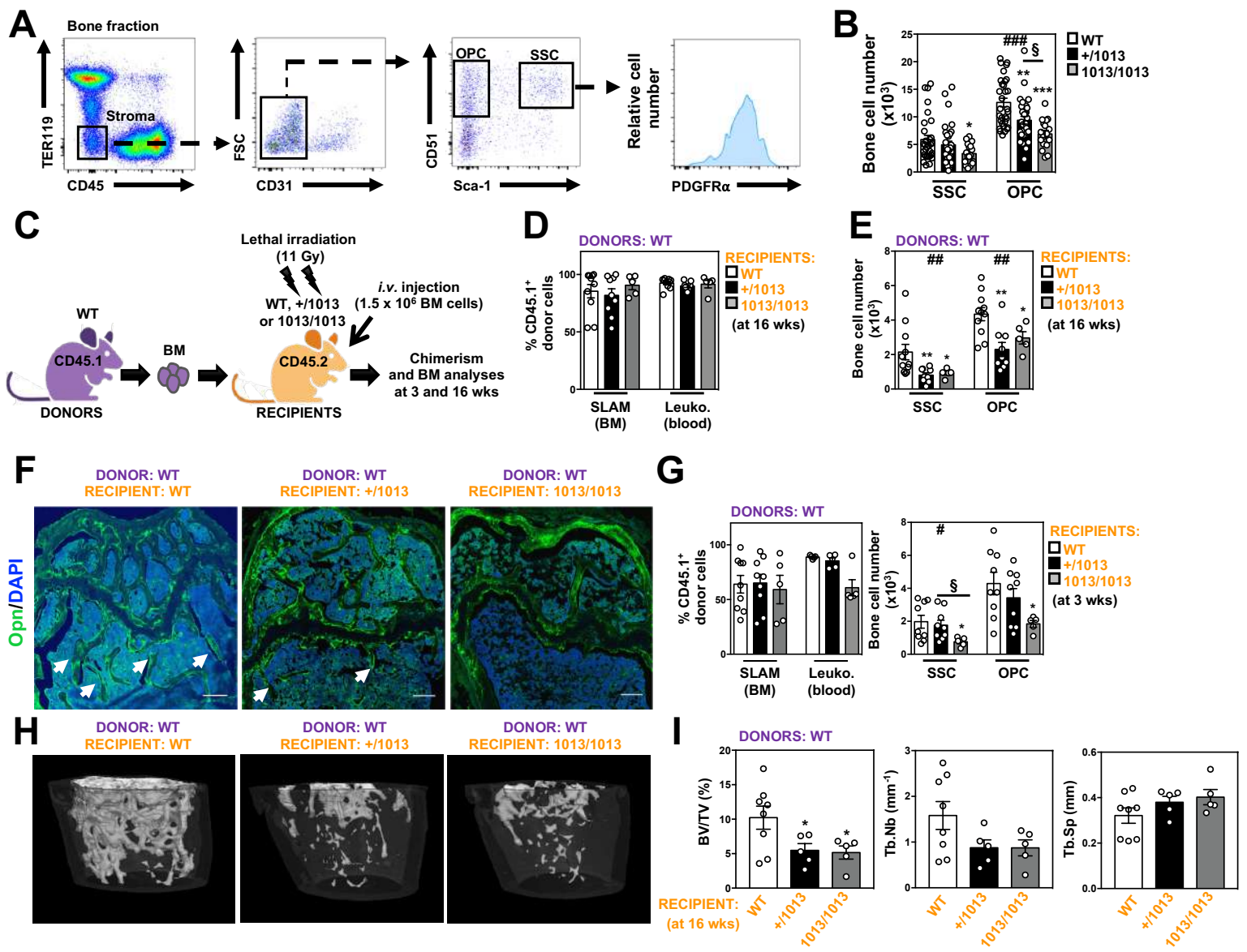


Figure 2

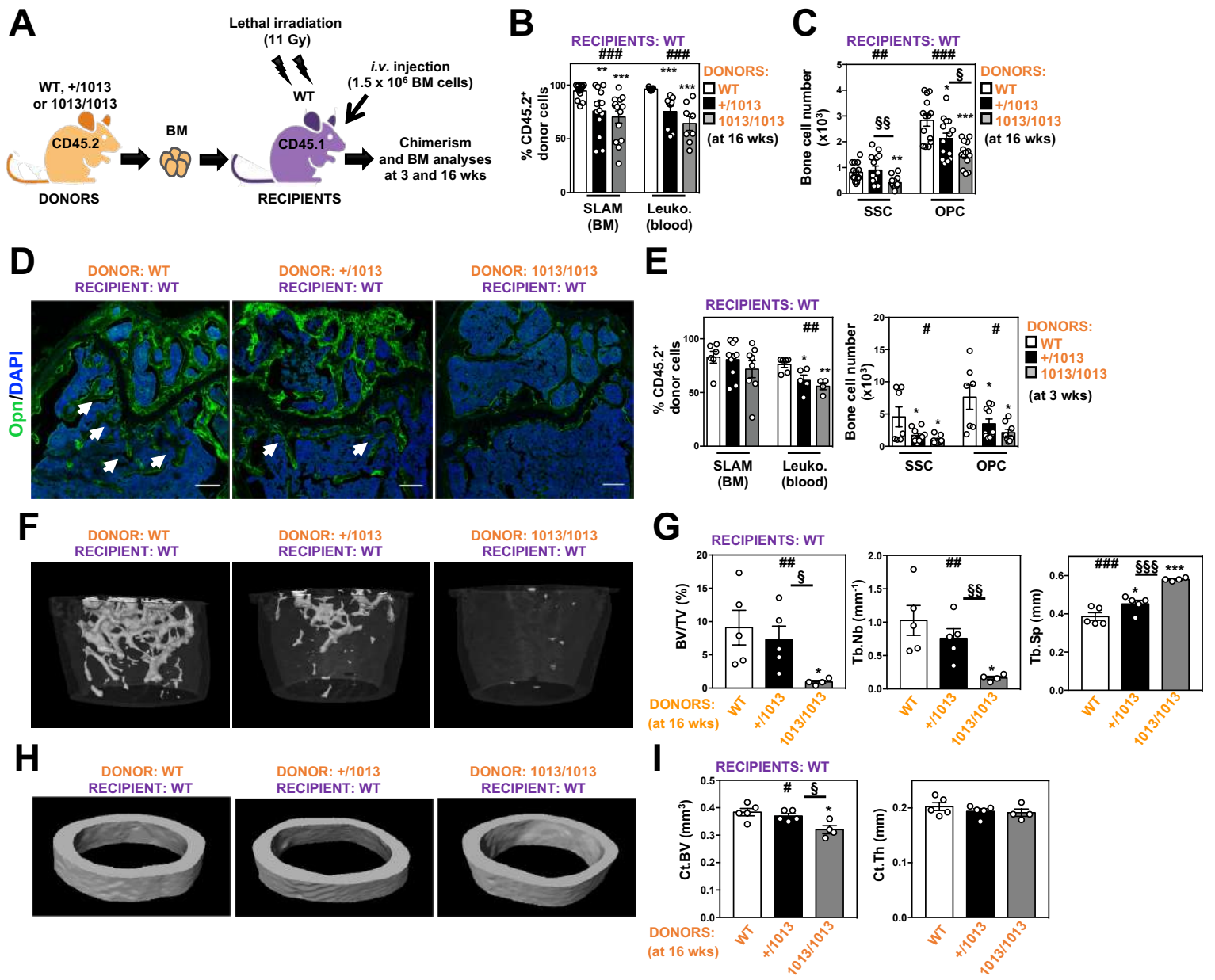
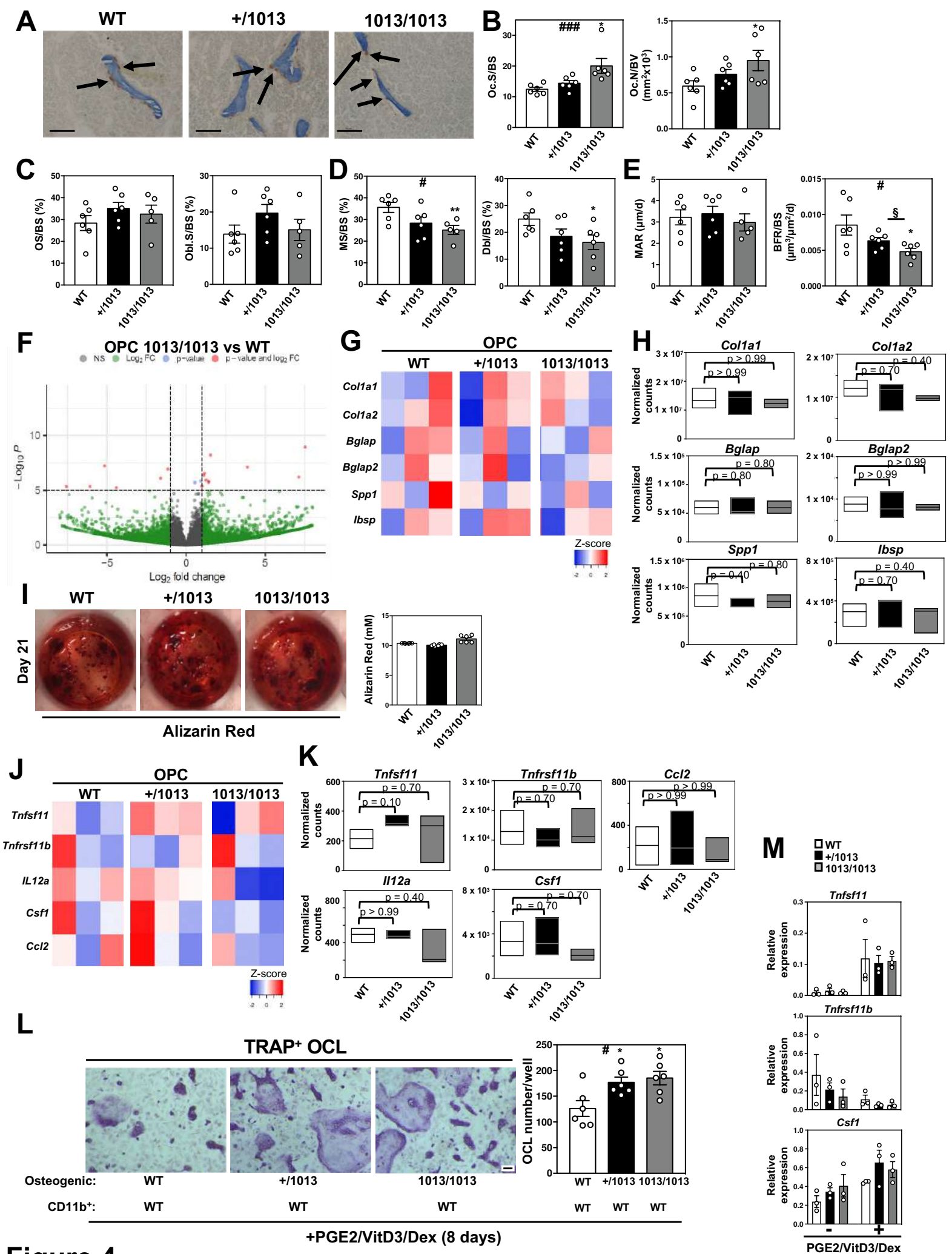
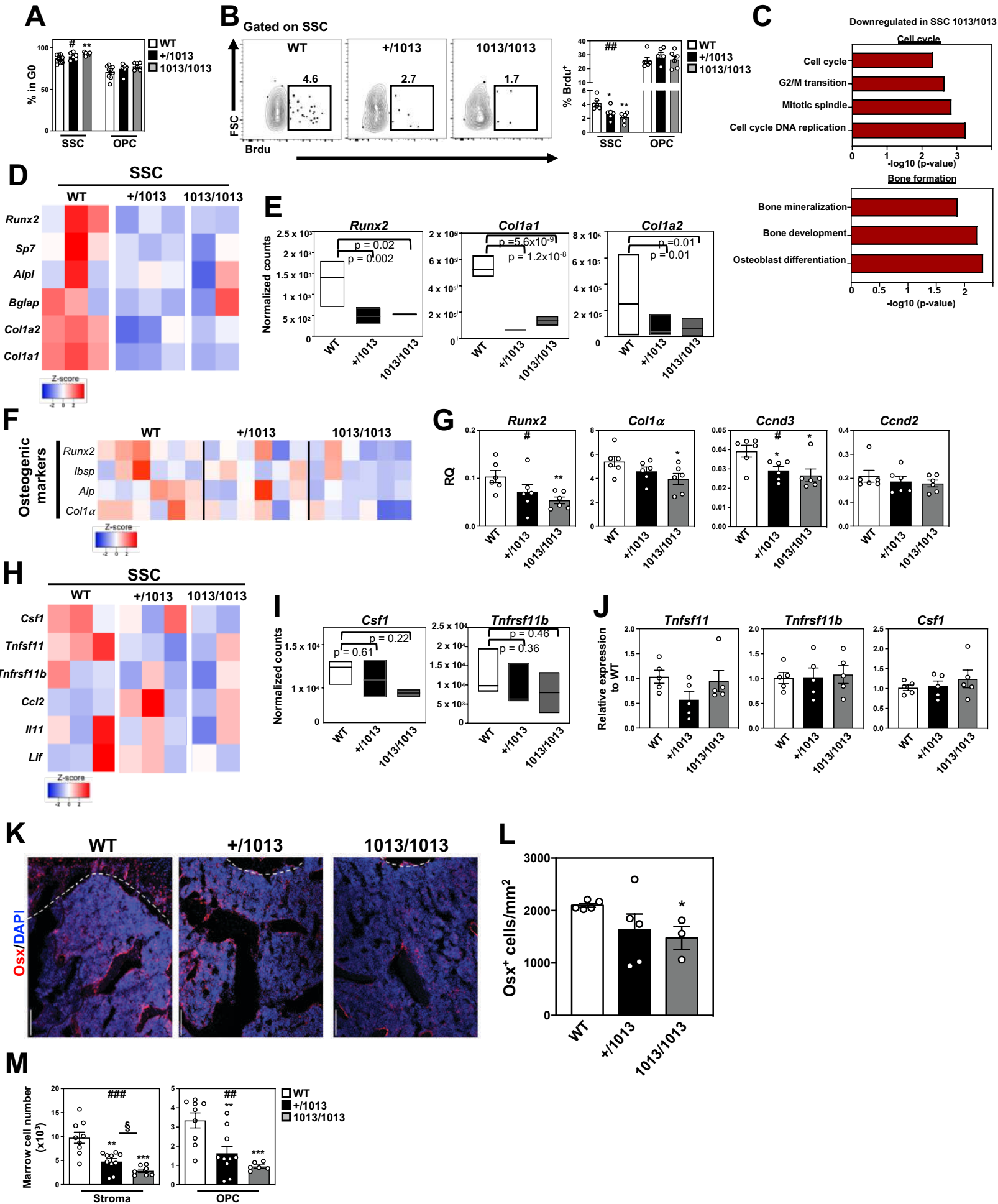


Figure 3



**Figure 4**



**Figure 5**

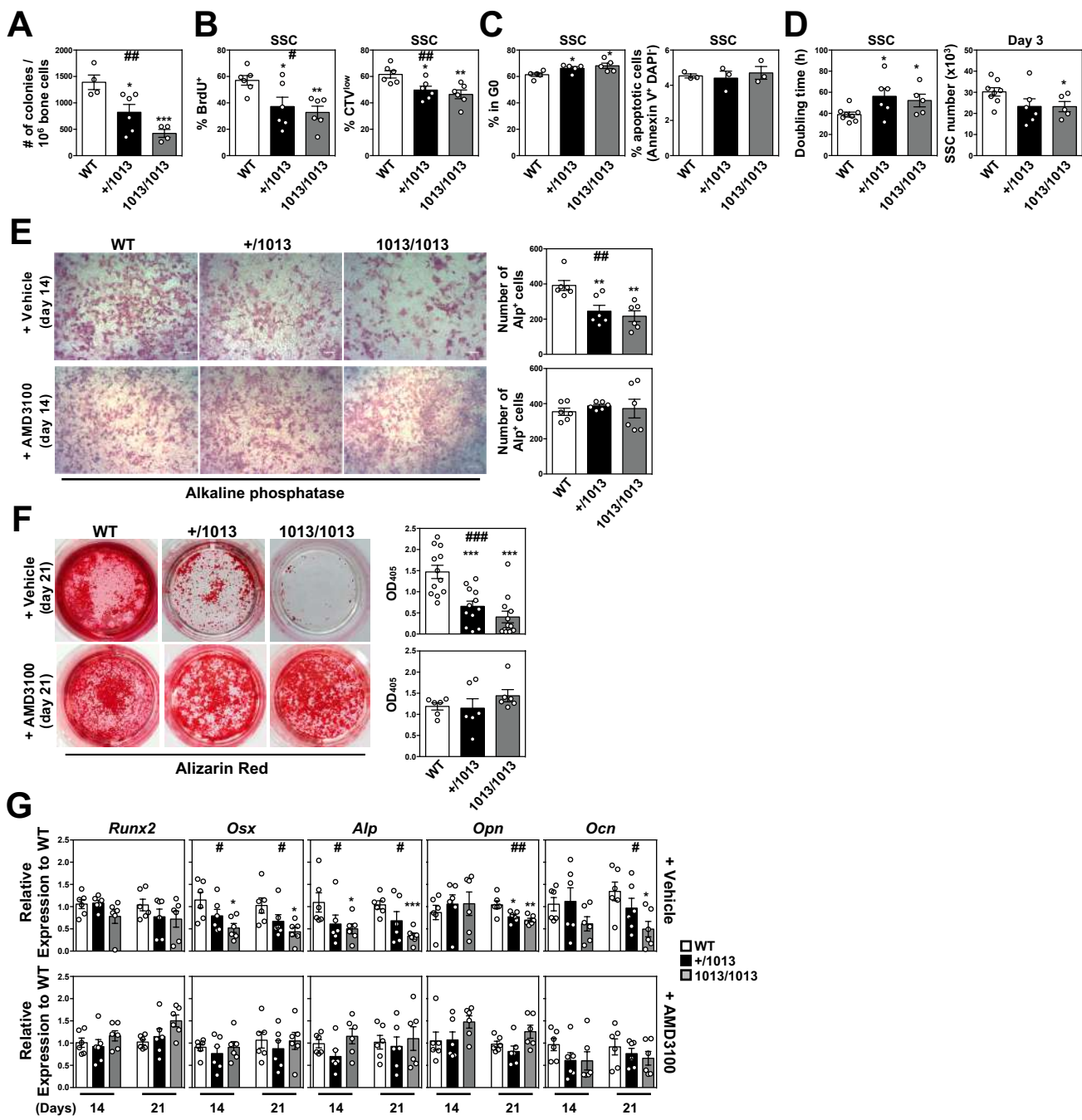


Figure 6

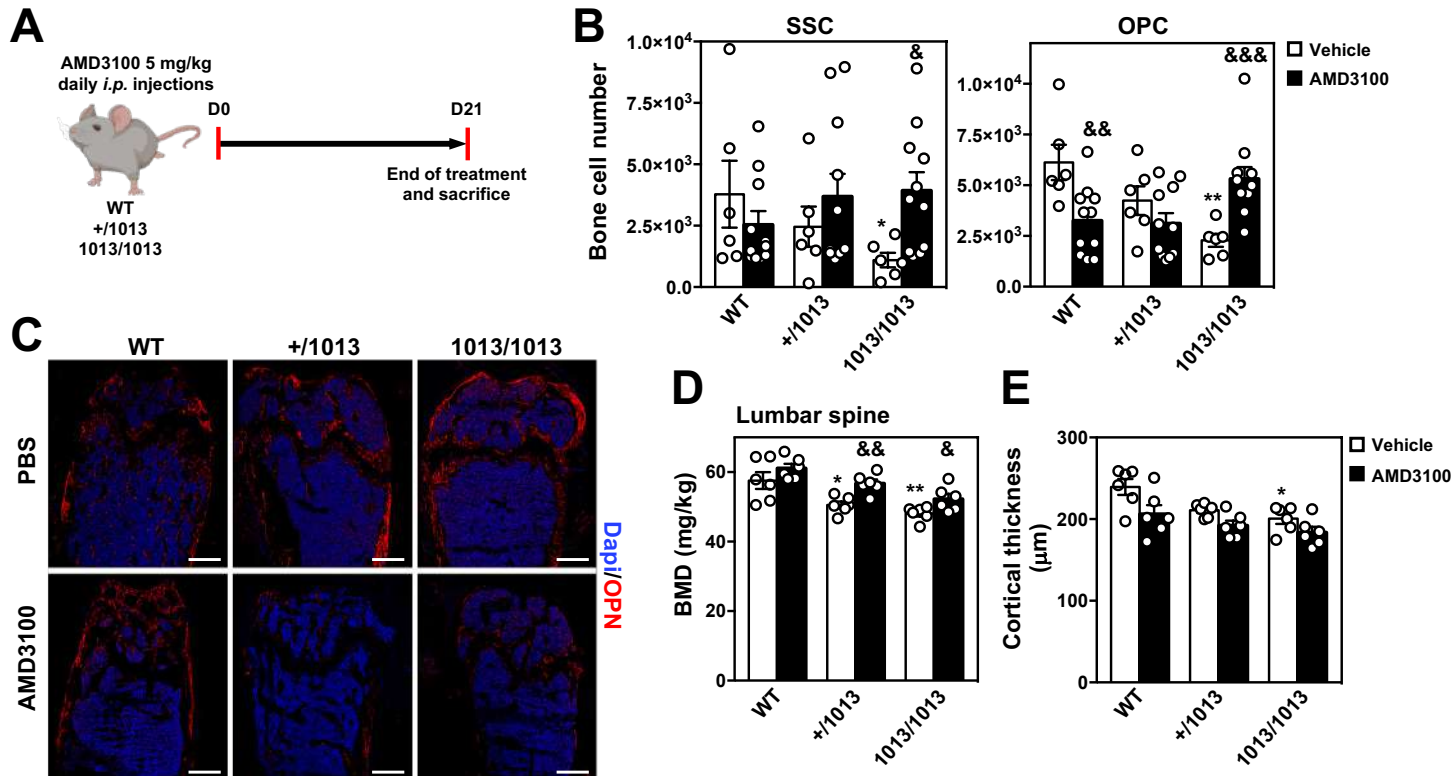


Figure 7

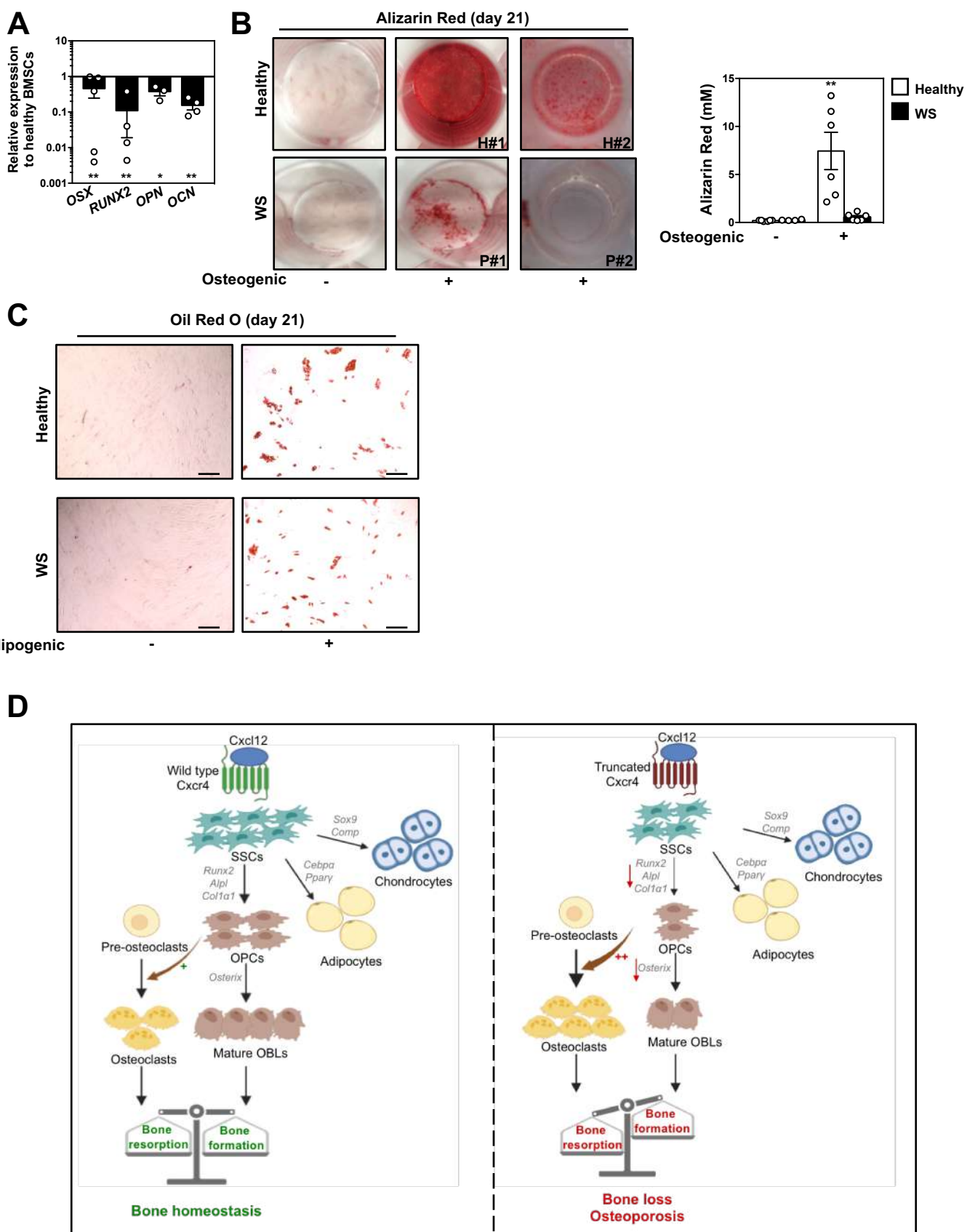


Figure 8