

1           **Loss of *Nupr1* promotes engraftment by tuning the dormancy threshold of**  
2           **hematopoietic stem cell repository via regulating p53-checkpoint pathway**

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20   **Running head:** *Nupr1* regulates the dormancy threshold of HSCs

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35

36 **Abstract**

37 Hematopoietic stem cells (HSCs) are dominantly quiescent under homeostasis, which  
38 is a key mechanism of maintaining the HSC pool for life-long hematopoiesis.  
39 Dormant HSCs poised to be immediately activated on urgent conditions and can return  
40 to dormancy after regaining homeostasis. To date, the molecular networks of  
41 regulating the threshold of HSC dormancy, if exist, remain largely unknown. Here, we  
42 unveiled that deletion of *Nupr1*, a gene preferentially expressed in HSCs, activated  
43 the dormant HSCs under homeostatic status, which conferred engraftment competitive  
44 advantage on HSCs without compromising their stemness and multi-lineage  
45 differentiation abilities in serial transplantation settings. Following an expansion  
46 protocol, the *Nupr1*<sup>-/-</sup> HSCs proliferate more robustly than their wild type counterparts  
47 *in vitro*. *Nupr1* inhibits the expression of p53 via an unknown mechanism and the  
48 rescue of which offsets the engraftment advantage. Our data unveil the *de novo* role of  
49 *Nupr1* as an HSC dormancy-regulator, which provides insights into accelerating the  
50 engraftment efficacy of HSC transplantation by targeting the HSC  
51 dormancy-controlling network.

52

53 **Introduction**

54 Hematopoietic stem cells (HSCs), the seeds of adult blood system, generate all the  
55 blood lineages via hierarchical hematopoiesis. Under steady-state, the majority of  
56 HSCs are maintained in dormancy to reserve the HSC pool for life-long  
57 hematopoiesis<sup>1</sup>. However, the dormant HSCs can be rapidly activated for stress  
58 hematopoiesis on emergency conditions, such as excessive blood loss, radiation injury,  
59 and chemotherapy damage<sup>2</sup>. Mounting evidence point to the existence of intrinsic  
60 molecular machinery of regulating HSC dormancy. In haploinsufficient *Gata2*<sup>+/-</sup> mice,  
61 HSCs show mildly increase of quiescent cells on homeostasis condition<sup>3</sup>. JunB  
62 inactivation deregulates the cell-cycle machinery and reduces quiescent HSCs<sup>4</sup>.  
63 *Hif-1*  $\alpha$ -deficient HSCs also show decreased dormant HSCs<sup>5</sup>. CDK6, a protein not  
64 expressed in long-term HSCs but short-term HSCs, regulates the quiescence exit in  
65 human hematopoietic stem cells, and overexpression of which promotes engraftment<sup>6</sup>.  
66 To date, the underlying signaling regulatory network of HSC quiescence remains  
67 largely unknown.

68 NUPR1 (Nuclear protein transcription regulator 1) is a member of the high-mobility  
69 group of proteins, which was first discovered in the rat pancreas during the acute  
70 phase of pancreatitis and was initially called p8<sup>7</sup>. The same gene was discovered in  
71 breast cancer and was named as Com1<sup>8</sup>. NUPR1 demonstrates various roles involving  
72 apoptosis, stress response, and cancer progression, which depends on distinct cellular  
73 context. In certain cancers, such as breast cancer, NUPR1 inhibits tumor cell  
74 apoptosis, induces tumor establishment and progression<sup>9-12</sup>. On the contrary, in

75 prostate cancer and pancreatic cancer, NUPR1 shows tumor-growth inhibitory effect<sup>13</sup>,  
76 <sup>14</sup>. Accumulated studies reveal that NUPR1 is a stress-induced protein: interference of  
77 NUPR1 can upregulate the sensitivity astrocytes to oxidative stress<sup>15</sup>; loss of it can  
78 promote resistance of fibroblasts to adriamycin-induced apoptosis<sup>16</sup>; NUPR1 mediates  
79 cannabinoid-induced apoptosis of tumor cells<sup>17</sup>; overexpression of *NUPR1* can  
80 negatively regulate MSL1-dependent HAT activity in HeLa cells, which induces  
81 chromatin remodeling and relaxation allowing access to DNA of the repair  
82 machinery<sup>18</sup>. Nonetheless, the potential roles of *Nupr1*, which is preferentially  
83 expressed in HSC among the HSPC, in hematopoiesis remain elusive.

84 NUPR1 interacts with p53 to regulate cell cycle and apoptosis responding to stress in  
85 breast epithelial cells<sup>16, 19</sup>. p53 plays several roles in homeostasis, proliferation, stress,  
86 apoptosis, and aging of hematopoietic cells<sup>20-24</sup>. Deletion of p53 upregulates HSC  
87 self-renewal but impairs their repopulating ability and leads to tumors<sup>25</sup>. Hyperactive  
88 expression of p53 in HSCs decreased the HSC pool size, reduced engraftment and  
89 deep quiescence<sup>26-28</sup>. These reports support the essential check-point role of p53 in  
90 regulating HSC fate. Nonetheless, it is unknown whether NUPR1 and p53  
91 coordinately regulate the quiescence of HSCs.

92 Here, we used a *Nupr1* conditional knockout model to investigate the consequences of  
93 loss of function of *Nupr1* in HSC context. *Nupr1*-deletion in HSCs led to their  
94 dormancy withdrawal under homeostasis. In a competitive repopulation setting,  
95 *Nupr1*-deleted HSCs robustly proliferated and showed dominant engraftment over  
96 wild type counterparts. Besides, *Nupr1*-deleted HSCs expanded abundantly and

97 preserved their stemness in vitro in comparison with wild type HSCs. The rescued  
98 expression of p53 by *Mdm2*<sup>+/-</sup> offset the effects introduced by loss of *Nupr1* in HSCs.  
99 Our studies reveal the *de novo* role and signaling mechanism of *Nupr1* in regulating  
100 the quiescence of HSCs.

## 101 **Results**

### 102 **Loss of *Nupr1* accelerates the turn-over rates of HSCs under homeostasis**

103 A majority of long-term HSCs are quiescent under homeostasis, which is a key  
104 mechanism for maintaining the HSC pool for life-long steady hematopoiesis. We  
105 hypothesize that among those genes, preferentially expressed in HSCs but  
106 immediately down regulated in MPPs, might form an intrinsic regulatory network for  
107 maintaining the HSC dormancy. To test our hypothesis, we explored such factor  
108 candidates by RNA-Seq analysis of the sorted HSCs (Hematopoietic stem cells, Lin<sup>-</sup>  
109 CD48<sup>-</sup> Sca1<sup>+</sup> c-kit<sup>+</sup> CD150<sup>+</sup>) and MPPs (Multipotent stem cells, Lin<sup>-</sup> CD48<sup>-</sup> Sca1<sup>+</sup>  
110 c-kit<sup>+</sup> CD150<sup>-</sup>). Differential expression gene analysis showed a pattern of  
111 HSC-preferential transcription factors, including *Rorc*, *Hoxb5*, *Rarb*, *Gfi1b*, *Mllt3*,  
112 and *Nupr1*. By literature search, we found that most of the candidate genes were  
113 reportedly not involved in regulating HSC homeostasis. Thus, we focus on the *Nupr1*  
114 gene, the role of which in hematopoiesis has not been reported. The expression of  
115 *Nupr1* in HSCs is significantly higher (> 25-fold, p = 0.002) than MPPs (Figure 1A,  
116 left). Real-Time PCR further confirmed the same expression pattern (p <0.001),  
117 implicating an unknown role of *Nupr1* in HSCs (Figure 1A, right).

118 To study whether *Nupr1* has any potential impact on the hematopoiesis of HSCs, we

119 constructed the *Nupr1* conditional knockout mice by introducing two loxp elements  
120 flanking the exon 1 and 2 of *Nupr1* locus using a C57BL/6 background mESC line  
121 (Figure 1B). The generated *Nupr1<sup>fl/fl</sup>* mice were further crossed to Vav-Cre mice to  
122 generate *Nupr1<sup>fl/fl</sup>*; Vav-Cre compound mice (*Nupr1<sup>-/-</sup>* mice). Adult *Nupr1<sup>-/-</sup>* mice  
123 (8-10-week-old) had a normal percentage of blood lineage cells in peripheral blood,  
124 including CD11b<sup>+</sup> myeloid, CD19<sup>+</sup> B, and CD3<sup>+</sup> T lineage cells (Supplementary Fig  
125 1). We further investigated the potential alterations of HSC hemostasis in the absence  
126 of *Nupr1*. Flow cytometry analysis demonstrated that *Nupr1<sup>-/-</sup>* HSC pool was  
127 comparable to wild type counterparts in terms of ratios and absolute numbers  
128 (Supplementary Fig 2). Subsequently, we examined the cell cycle status of *Nupr1<sup>-/-</sup>*  
129 HSCs using the proliferation marker Ki-67 and DAPI staining and found that the ratio  
130 of *Nupr1<sup>-/-</sup>* HSCs in G0-status was reduced significantly ( $p = 0.009$ ). Compared with  
131 those of WT HSCs (median value: *Nupr1<sup>-/-</sup>* HSCs = 68.1%, WT HSCs = 81.2%), more  
132 *Nupr1<sup>-/-</sup>* HSCs entered G1-S-S2 and M phase (Figure 1C, D). To further confirmed  
133 this novel phenotype, we performed BrdU incorporation assay, which is  
134 conventionally used for assessing the turn-over rates of blood cells *in vivo*<sup>29</sup>. The  
135 8-week-old *Nupr1<sup>-/-</sup>* mice and littermates were injected intraperitoneally with 1mg  
136 BrdU on day 0, followed by administration of BrdU via water feeding (0.8 mg/ml) for  
137 up to 5 days (Figure 1E). After three days of BrdU labeling, ~50% of *Nupr1<sup>-/-</sup>* HSCs  
138 became BrdU<sup>+</sup> compared with ~35% of WT HSCs. Kinetic analysis with BrdU  
139 incorporation from day 3 to day 5 revealed that *Nupr1<sup>-/-</sup>* HSCs contained a 1.5-fold  
140 higher BrdU<sup>+</sup> population over WT HSCs (Figure 1F, G). Collectively, these data

141 indicate that the *Nupr1*-deletion drives HSCs entering cell cycle and accelerates their  
142 turn-over rates on homeostasis.

143 ***Nupr1*<sup>-/-</sup> HSCs show repopulating advantage without compromising multi-lineage**  
144 **differentiation capacity**

145 To confirm whether *Nupr1*<sup>-/-</sup> HSCs have repopulating advantage or disadvantage in  
146 *vivo*, we performed typical HSC-competitive repopulation assay. Two hundred and  
147 fifty thousand whole bone marrow nucleated cells (BMNCs) from *Nupr1*<sup>-/-</sup> mice  
148 (CD45.2) were transplanted into lethally irradiated recipients (CD45.1) along with  
149 equivalent WT (CD45.1) competitors. Sixteen weeks later, one million BMNCs of the  
150 primary recipients were transplanted into lethally irradiated recipients for assessing  
151 long-term engraftment (Figure 2A). We observed that donor *Nupr1*<sup>-/-</sup> cells took about  
152 60%-70% in the primary recipients. *Nupr1*<sup>-/-</sup> cells gradually dominated in peripheral  
153 blood of recipients over time after transplantation (Figure 2B). In the chimerism,  
154 ~70% of myeloid cells and B lymphocytes were *Nupr1*<sup>-/-</sup> donor-derived cells, while  
155 ~60% of T lymphocytes were CD45.1 competitive cells (Figure 2C). To further  
156 explore whether *Nupr1*<sup>-/-</sup> HSCs dominate in chimerism, we sacrificed the chimerism  
157 and analyzed the HSCs 16 weeks after transplantation. Compared with the  
158 competitive HSCs, the proportion and absolute number of *Nupr1*<sup>-/-</sup> HSCs were  
159 significantly more (~3 folds) than the CD45.1<sup>+</sup> HSC competitors in primary recipients  
160 (Figure 2D, E). Previous research reported that HSCs proliferated rapidly at the  
161 expense of their long-term repopulating ability<sup>30-34</sup>. Interestingly, consistent with the  
162 dominating trend in primary transplantation, *Nupr1*<sup>-/-</sup> cells continuously dominated in



163 secondary recipients (Figure 3A). *Nupr1*<sup>-/-</sup> HSCs occupied up to 90% of the total  
164 HSCs in the bone marrow (BM) of secondary recipients (Figure 3B, C). In aggregate,  
165 these results indicate that the deletion of *Nupr1* promotes the repopulating ability of  
166 HSCs without impairing their long-term engraftment ability.

### 167 ***Nupr1*-deleted HSCs expand robustly *in vitro***

168 We next examined whether the deletion of *Nupr1* could enhance HSC expansion *in*  
169 *vitro*. Fifty HSCs sorted from WT and *Nupr1*<sup>-/-</sup> mice were cultured *in vitro* for 10 days  
170 as previously described<sup>35</sup> (Figure 4A). After 10-day-culture, the wild type input cells  
171 achieved a yield of more than  $2 \times 10^4$  cells, while *Nupr1*<sup>-/-</sup> HSCs produced  
172 approximately  $5 \times 10^4$  total cells ( $p < 0.001$ , Figure 4B). The colonies derived from  
173 *Nupr1*<sup>-/-</sup> HSCs were much larger than WT HSCs (Figure 4C). Furthermore, we  
174 analyzed the phenotypic HSC populations in the expanded cells and found that the  
175 absolute number of phenotypic HSC in individual *Nupr1*<sup>-/-</sup> colonies were 3 times more  
176 than WT HSCs ( $p = 0.005$ , Figure 4D, E). To determine whether the quantitative  
177 expansion of phenotypic HSC contains net proliferation of functional HSCs, we  
178 performed competitive repopulating unit (CRU) assays<sup>36</sup>, using the serial doses of  
179 limiting dilutions of the *in vitro* expanded cells. The WT HSC frequency in the  
180 10-day expanded cells is 1 in 326 cells, which is equivalent to 62 functional HSCs.  
181 While the *Nupr1*<sup>-/-</sup> HSC frequency in the 10-day expanded cells is 1 in 251 cells  
182 (Figure 4F)<sup>37</sup>, which is equivalent to 200 functional HSCs. Therefore, the deletion of  
183 *Nupr1* induced around three-fold expansion in functional HSC number over the WT  
184 HSCs. Deletion of *Nupr1* enhances the expansion ability of HSCs *in vitro*.

185 **Reversion of p53 expression offsets the competitiveness of *Nupr1*<sup>-/-</sup> HSCs**

186 To further investigated the underlying molecular mechanisms of *Nupr1* in regulating  
187 HSCs, we performed RNA-Seq analysis of *Nupr1*<sup>-/-</sup> HSCs from 8-week-old *Nupr1*<sup>-/-</sup>  
188 mice. Gene set enrichment analysis (GSEA) illustrated that p53 pathways feedback  
189 loops-related genes, including *Trp53*, *Ccng1*, *Ctnnb1*, *Pten*, and *Pik3c2b*, were  
190 enriched in WT HSCs (Figure 5A). p53 pathway regulates a series of target genes  
191 involving cell cycle arrest, apoptosis, senescence, DNA repair, and metabolism<sup>38</sup>.  
192 Interestingly, the expression of p53 was significantly ( $p < 0.001$ ) reduced to 1/3 of  
193 control in *Nupr1*<sup>-/-</sup> HSCs (Figure 5B). Therefore, we hypothesized that  
194 down-regulation of p53 in *Nupr1*<sup>-/-</sup> HSCs might account for the competitive advantage  
195 of the HSCs. MDM2 is a ubiquitin ligase E3 for p53, which is a key repressive  
196 regulator of p53 signaling<sup>39</sup>. *Mdm2* deficient mice showed active p53 levels, which is  
197 an ideal substitute model of up-regulating p53 since direct overexpressing p53 leading  
198 to cell death and blood malignancies in mice<sup>24,40</sup>. The *Nupr1*<sup>-/-</sup> mice were crossed to  
199 the *Mdm2*<sup>+/-</sup> mice to achieve up-regulation of p53 expression in *Nupr1*<sup>-/-</sup> HSCs. The  
200 expression level of p53 in *Nupr1*<sup>-/-</sup> and *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> HSC. The expression level of  
201 p53 protein in *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> HSCs is comparable with WT HSCs, which is  
202 significantly higher than *Nupr1*<sup>-/-</sup> HSCs when measured by indirect  
203 immunofluorescence assay (Figure 5C, D). Next, we examined the phenotypic HSC  
204 of the *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> mice. Flow cytometry analysis demonstrated that  
205 *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> HSC pool was indistinguishable with wild type and *Nupr1*<sup>-/-</sup>  
206 counterparts in terms of ratios and absolute numbers (Figure 6A, B). Further, we

207 tested the competitiveness of *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> HSCs in parallel with *Nupr1*<sup>-/-</sup> HSCs.  
208 Two hundred and fifty thousand whole bone marrow nucleated cells from *Nupr1*<sup>-/-</sup>  
209 *Mdm2*<sup>+/-</sup> mice (CD45.2) or *Nupr1*<sup>-/-</sup> mice (CD45.2) were transplanted into lethally  
210 irradiated recipients (CD45.1) along with equivalent WT (CD45.1) whole bone  
211 marrow nucleated cells. In the recipients of *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> donor cells, the  
212 contribution of *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> cells was significantly ( $p < 0.001$ ) reduced to ~20%,  
213 which was far below the percentage of *Nupr1*<sup>-/-</sup> cells in recipients of *Nupr1*<sup>-/-</sup> donor  
214 cells (Figure 6C). Sixteen weeks after transplantation, we also analyzed the  
215 *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> HSCs in the chimerism. Surprisingly, the *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> HSCs  
216 almost disappeared in the HSC pool of the recipients, while the *Nupr1*<sup>-/-</sup> HSCs  
217 dominantly occupied in the HSC pool (Figure 6D, E). Altogether, the reversion of p53  
218 expression offsets the competitiveness advantage of *Nupr1*<sup>-/-</sup> HSCs.

## 219 **Discussion**

220 The intrinsic networks of regulating the quiescence of HSCs are largely unknown. In  
221 this study, loss of *Nupr1* (p8), a gene preferentially expressed in long-term HSCs,  
222 tunes the dormancy threshold of HSCs on homeostasis condition without  
223 compromising their key functions in hematopoiesis. *Nupr1* coordinates with p53 to  
224 form a signaling machinery regulating HSC quiescence and turnover rate. For the first  
225 time, we unveil the *de novo* role of *Nupr1* in controlling HSC dormancy.

226 *Nupr1*<sup>-/-</sup> HSCs replenished faster than WT HSCs under homeostasis. However, the  
227 size of *Nupr1*<sup>-/-</sup> HSC pool was not altered. These data implicate that despite the  
228 existence of intrinsic machinery of controlling HSC dormancy, the scale of HSC-pool

229 is restricted by extrinsic bone marrow microenvironment<sup>41</sup>. Conventionally,  
230 molecules activating HSCs showed transiently phenotypic proliferation of HSCs but  
231 eventually led to their functional exhaustion and even tumors<sup>30-34</sup>. Interestingly, *Nupr1*  
232 signaling seemingly plays a unique role in regulating HSC dormancy and turnover  
233 rates, as deletion of *Nupr1* maintains the hematopoiesis features of HSCs.  
234 Consistently, enforced CDK6 expression in HSCs confers competitive advantage  
235 without impairing their stemness and multi-lineage potential<sup>6</sup>. These evidence  
236 supports the concept that targeting the intrinsic machinery of balancing HSC  
237 dormancy threshold might safely promoting engraftment.

238 Loss of *Nupr1* in HSCs resulted in engraftment advantage. Under the transplantation  
239 stress settings, the HSC niche occupied by WT HSCs was ablated, providing niche  
240 vacuum for donor *Nupr1*<sup>-/-</sup> HSC entrance. The dominance of *Nupr1*<sup>-/-</sup> HSCs is a  
241 consequence of fast turnover rate of these cells over WT counterparts. In the previous  
242 research, loss of *Dnmt3a* also leads to clonal dominance of HSCs, however,  
243 accompanied with hematopoiesis failure due to differentiation block<sup>42, 43</sup>. Thus, the  
244 engraftment advantage caused by loss of *Nupr1* might have prospective translational  
245 implications for hematopoietic stem cells transplantation (HSCT), since a faster  
246 recovery of hematopoiesis in transplanted host definitely reduces infection risks in  
247 patients<sup>44, 45</sup>.

248 In our models, *Nupr1* regulated hematopoietic homeostasis via targeting p53 pathway.  
249 Consistently, p53 is essential in regulating hematopoietic homeostasis<sup>24</sup>. Whether  
250 NUPR1 directly interacts with p53 in HSC context remain unknown, as currently

251 antibodies suitable for protein-protein interaction assays are not available. NUPR1  
252 and p53 directly interacted in human breast epithelial cells<sup>19</sup>. Knocking out p53 in  
253 HSCs can promote HSC expansion, but directly targeting p53 caused HSC apoptosis  
254 and tumorigenesis<sup>46</sup>. Thus, *Nupr1* might behave as an upstream regulator of p53  
255 signaling and uniquely regulate cell dormancy in HSC context.

256 In conclusion, loss of *Nupr1* in HSCs promotes engraftment by tuning the dormancy  
257 threshold of HSCs via regulating p53 check-point pathway. Our study unveils the  
258 prospect of shortening the engraftment time of HSCT by targeting the intrinsic  
259 machinery of controlling HSC dormancy.

## 260 **Materials and Methods**

### 261 **Mice**

262 Animals were housed in the animal facility of the Guangzhou Institutes of  
263 Biomedicine and Health (GIBH). *Nupr1*<sup>fl/fl</sup> mice were constructed by Beijing  
264 Biocytogen Co., Ltd. CD45.1, Vav-cre, *Mdm2*<sup>+/-</sup> mice were purchased from the  
265 Jackson laboratory. All the mouse lines were maintained on a pure C57BL/6 genetic  
266 background. All experiments were conducted in accordance with experimental  
267 protocols approved by the Animal Ethics Committee of GIBH.

### 268 **Flow cytometry analysis**

269 For HSC and MPP staining, total BM cells were stained with antibodies against  
270 CD2/CD3/CD4/CD8/CD11b/Gr-1/B220/Ter119/CD48-FITC, c-Kit-APC-eFluor®  
271 780, Sca1-Percp-cy5.5, and CD150-PE-cy7. Cells were analyzed by LSR Fortessa (BD  
272 Bioscience). For lineage analysis of peripheral blood, the white blood cells were

273 stained with antibodies of anti-CD45.1-FITC, anti-CD45.2-percp-cy5.5,  
274 anti-CD90.2-APC, anti-CD19-PE, anti-CD11b-PE-cy7, anti-Gr-1-APC-eFlour® 780.

### 275 **HSC cell cycle analysis**

276 We first labeled the HSCs with (CD2, CD3, CD4, CD8, Ter119, B220, Gr1,  
277 CD48)-FITC, Sca1-Percp-cy5.5, c-kit-PE-cy7, and CD150-PE. Then the cells were  
278 fixed using 4% PFA. After washing, the fixed cells were permeabilized with 0.1%  
279 saponin in PBS together with the Ki-67-APC staining for 45 minutes. Finally, the  
280 cells were resuspended in DAPI solution for staining 1 hour. The data were analyzed  
281 using Flowjo software (FlowJo).

### 282 **BrdU incorporation assay**

283 *Nupr1*<sup>-/-</sup> mice and WT littermate mice were injected with 1 mg BrdU on Day 0. Then  
284 they were fed with water containing BrdU (0.8 mg/mL). On Day 3, 4, 5 after the  
285 injection of BrdU, four mice of each group were sacrificed. The incorporation rates of  
286 BrdU were analyzed by flow cytometry according to the BD Pharmingen™ APC  
287 BrdU Flow Kit instructions.

### 288 **HSC culture**

289 The HSC culture protocol is as described<sup>35</sup>. Briefly, fifty HSCs were sorted into  
290 fibronectin (Sigma)-coated 96-well U-bottom plate directly and were cultured in  
291 medium composed of F12 medium (Life Technologies), 1%  
292 insulin–transferrin–selenium–ethanolamine (ITSX; Life Technologies), 10 mM  
293 HEPES (Life Technologies), 1% penicillin/streptomycin/glutamine (P/S/G; Life  
294 Technologies), 100 ng/ml mouse TPO, 10 ng/ml mouse SCF and 0.1% PVA (P8136).

295 Complete medium changes were made every 2–3 days, by manually removing  
296 medium by pipetting and replacing fresh medium as indicated.

### 297 **Limiting dilution assay**

298 For limiting dilution assays<sup>36</sup>, the 10-day cultured cells were transplanted into lethally  
299 irradiated C57BL/6-CD45.1 recipient mice, together with  $2 \times 10^5$  CD45.1  
300 bone-marrow competitor cells. Donor chimerism was analyzed as above. Limiting  
301 dilution analysis was performed using ELDA software<sup>37</sup>, based on a 1%  
302 peripheral-blood multilineage chimerism as the threshold for positive engraftment.

### 303 **Indirect Immunofluorescence Assay**

304 Sorted HSCs were directly pipetted onto the poly-lysine coated slides (100-500 cells  
305 in 5 $\mu$ l) and incubated at room temperature for 10 min. Upon the solution was  
306 completely dry, the cells were fixed with 4%PFA for 10 min following with  
307 0.15%Triton X-100 permeabilization for 2 min at room temperature. To avoid  
308 non-specific antibody binding, the cells were blocked in 1% BSA/PBS for 1-2h at  
309 room temperature and then incubated with the primary p53 antibody in 1% BSA in  
310 PBS overnight at 4°C (Abcam, ab16465). Slides were washed three times in PBS and  
311 incubated with secondary antibodies for 1h at room temperature in 1% BSA in PBS  
312 (donkey anti-mouse Alexa Fluor® 488, Abcam, ab150105). After washing the slides,  
313 the cells were incubated with DAPI solution for 10 min. Confocal analysis was  
314 performed at high resolution with a Zeiss laser scanning confocal microscope,  
315 LSM-800. The images were processed with ZEN 2012 software (blue edition).

316 **RNA-Seq and data analysis.** For HSC library preparation, HSCs  
317 (Lin<sup>-</sup>CD48<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD150<sup>+</sup>) were sorted from 8-10 weeks old *Nupr1*<sup>-/-</sup> mice and  
318 wild type mice. HSCs were sorted from four mice of each group. 1000 target cells per  
319 sample were sorted into 500 µl DPBS-BSA buffer (0.5%BSA) using 1.5ml EP tube and  
320 transferred into 250 µl tube to spin down with 500 g. The cDNA of sorted 1000-cell  
321 aliquots were generated and amplified as described previously<sup>47</sup>. The qualities of the  
322 amplified cDNA were examined by Q-PCR analysis of housekeeping genes (*B2m*, *Actb*,  
323 *Gapdh*, *Ecf1a1*). Samples passed quality control were used for sequencing library  
324 preparation by illumina Nextera XT DNA Sample Preparation Kit (FC-131-1096).  
325 For data analysis, all libraries were sequenced by illumina sequencers NextSeq 500.  
326 The fastq files of sequencing raw data samples were generated using illumina bcl2fastq  
327 software (version: 2.16.0.10) and were uploaded to Gene Expression Omnibus public  
328 database (GSE131071). Raw reads were aligned to mouse genome (mm10) by  
329 HISAT2<sup>48</sup> (version: 2.1.0) as reported. And raw counts were calculated by  
330 featureCounts of subread<sup>49</sup> (version 1.6.0). Differential gene expression analysis was  
331 performed by DESeq2<sup>50</sup> (R package version: 1.18.1). Heatmaps were plotted using  
332 gplots (R package, version 3.01). GSEA was performed as described<sup>51</sup>. The gene set  
333 (p53 pathway feedback loop) for GSEA were from PANTHER pathways dataset.

#### 334 **Quantitative real-time PCR**

335 Total RNA was extracted from ten thousand purified HSCs and MPPs with an RNeasy  
336 micro kit (QIAGEN). Then, 2 ng of RNA was used for linear amplification according  
337 to the manufacturer's instructions (3302-12, Ovation Pico WTA System V2, NuGEN



338 Technologies, Inc.). The RNA was diluted and 10ng RNA was used as the template  
339 for quantitative real-time PCR (CFX-96, Bio-Rad). The forward primer of *Nupr1* is  
340 5'-CCCTTCCCAGCAACCTCTAA-3' and the reverse primer is  
341 5'-AGCTTCTCTCTTGGTCCGAC-3'. Fold expression relative to the reference gene  
342 was calculated using the comparative method  $2^{-\Delta\Delta C_t}$ , and the values were normalized  
343 to 1 for comparison.

#### 344 **Bone marrow competitive repopulation assay**

345 One day before bone marrow transplantation, adult C57BL/6 (CD45.1, 8-10 weeks  
346 old) recipient mice were irradiated with 2 doses of 4.5Gy (RS 2000, Rad Source) for a  
347 4-hour interval. Two hundred and fifty thousand BMNCs from *Nupr1*<sup>-/-</sup> mice (CD45.2)  
348 and equivalent WT (CD45.1) counterparts were mixed and injected into irradiated  
349 CD45.1 recipients by the retro-orbital injection. *Mdm2*<sup>+/-</sup>*Nupr1*<sup>-/-</sup> BMNCs (CD45.2)  
350 were also mixed with equivalent competitors (CD45.1) and transplanted into  
351 recipients. The transplanted mice were maintained on  
352 trimethoprim-sulfamethoxazole-treated water for 2 weeks. For secondary  
353 transplantation, BMNCs of primary competitive transplanted recipients were obtained.  
354 One million of total BMNCs were injected into irradiated CD45.1 recipients (2 doses  
355 of 4.5Gy, one day before transplantation). Donor-derived cells and hematopoietic  
356 lineages in PB were assessed monthly by flow cytometry.

#### 357 **Statistic analysis**

358 The data were represented as mean  $\pm$  SD. Two-tailed independent Student's t-tests  
359 were performed for comparison of two groups of data (SPSS v.23, IBM Corp.,

360 Armonk, NY, USA). P values of less than 0.05 were considered statistically  
361 significant (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

362

363 **Author contributions:** T.J.W. and C.X.X. performed research, analyzed data and  
364 wrote the paper; Y.D. and Q.T.W. analyzed RNA-Seq data; H.C., S.H., F.D., K.T.W.,  
365 X.F.L., L.J.L., Y.G., and Y.X.G. performed experiments; J.D. and T.C. discussed the  
366 manuscript; J.Y.W. designed research, and wrote the manuscript.

367

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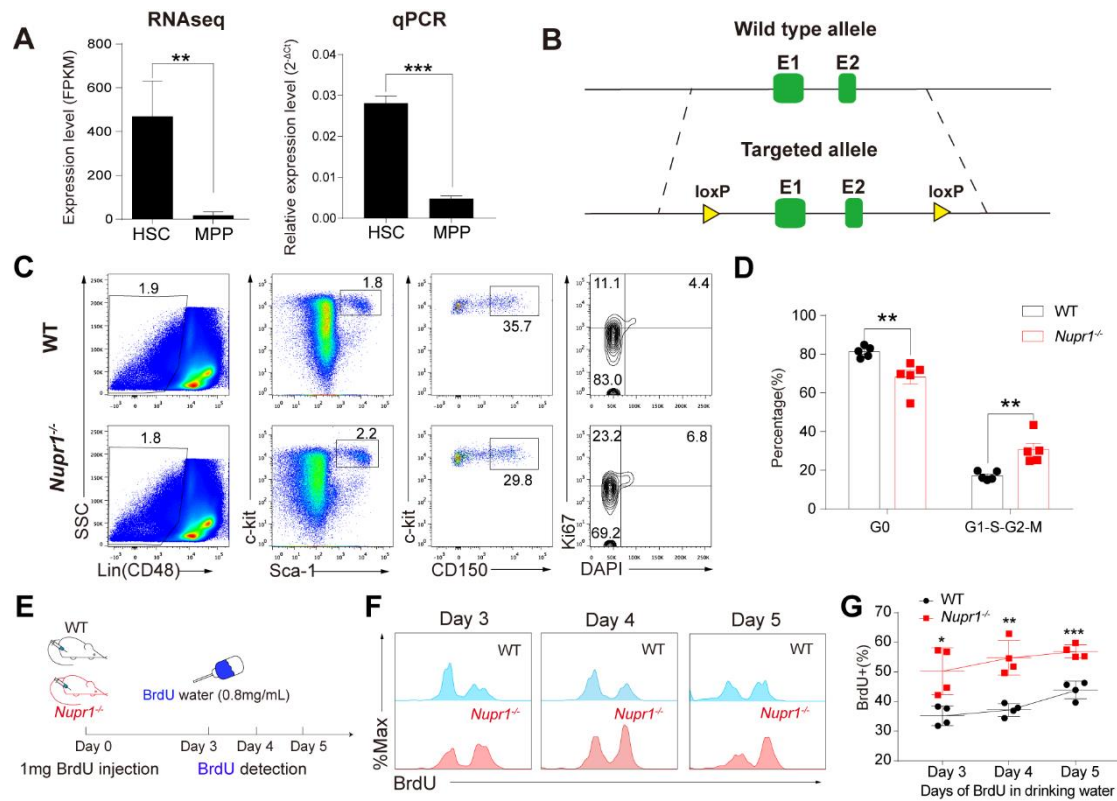
484

#### 485 **Conflict of Interest Disclosures**

486 The authors declare no competing financial interests.

487

488 **Figures and Figure legends**



489

490 **Fig 1. Loss of *Nupr1* activates dormant HSCs under homeostasis**

491 (A) Expression pattern of *Nupr1* in hematopoietic stem cells (HSCs) and multipotent  
 492 progenitors (MPPs) examined by RNA-sequencing and Real-Time PCR. One  
 493 thousand HSC or MPP cells from bone marrow of wild type mice were sorted as  
 494 individual samples for RNA-sequencing (n=4). HSCs are defined as Lin (CD2, CD3,  
 495 CD4, CD8, Mac1, Gr1, Ter119, B220)<sup>-</sup>, CD48<sup>-</sup>, Sca1<sup>+</sup>, c-kit<sup>+</sup>, and CD150<sup>+</sup>. MPPs are  
 496 defined as Lin (CD2, CD3, CD4, CD8, Mac1, Gr1, Ter119, B220)<sup>-</sup>, CD48<sup>-</sup>, Sca1<sup>+</sup>,  
 497 c-kit<sup>+</sup>, and CD150<sup>-</sup>. Data are analyzed by unpaired Student's t-test (two-tailed). \*\*p  
 498 < 0.01, \*\*\*p<0.001. Data are represented as mean ± SD (qPCR, n = 3 mice for each  
 499 group).

500 (B) Targeting strategy of knockout of *Nupr1* gene in mouse. Wild type *Nupr1* exons 1,



501 and 2 are shown as green boxes. Two loxp elements flanking exon 1 and exon 2 were  
502 inserted.

503 (C) Cell cycle analysis of *Nupr1*<sup>-/-</sup> HSCs under homeostasis. Representative plots of  
504 cell cycle from representative WT and *Nupr1*<sup>-/-</sup> mice (8-week-old). WT littermates  
505 (8-week-old) were used as control. HSCs (Lin<sup>-</sup> (CD2<sup>-</sup> CD3<sup>-</sup> CD4<sup>-</sup> CD8<sup>-</sup> B220<sup>-</sup> Gr1<sup>-</sup>  
506 CD11b<sup>-</sup> Ter119<sup>-</sup>) CD48<sup>-</sup> Sca1<sup>+</sup> c-kit<sup>+</sup> CD150<sup>+</sup>) were analyzed by DNA content (DAPI)  
507 versus Ki-67. G0 (Ki-67<sup>low</sup>DAPI<sup>2N</sup>), G1 (Ki-67<sup>high</sup>DAPI<sup>2N</sup>), G2-S-M  
508 (Ki-67<sup>high</sup>DAPI<sup>>2N-4N</sup>).

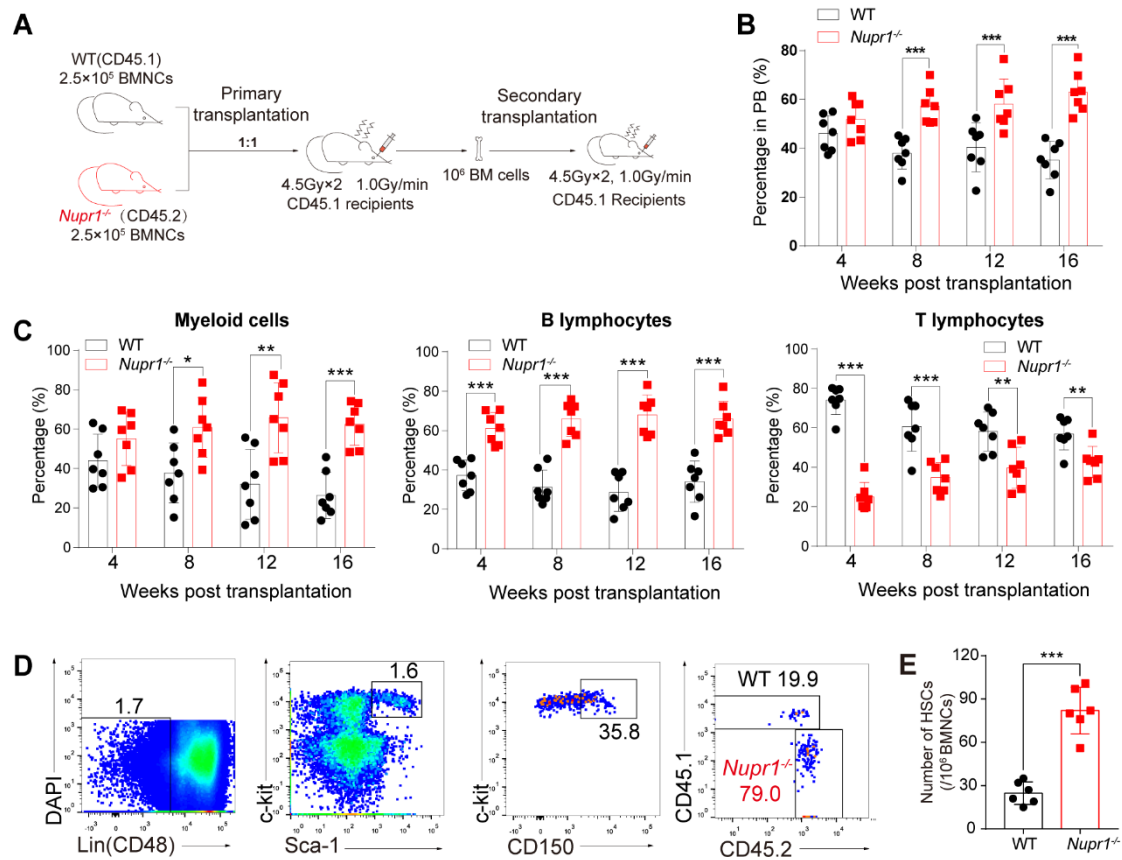
509 (D) Statistical analysis of HSC cell cycle. The percentages (%) of HSCs in G0,  
510 G1-G2-S-M stages were analyzed. Data are analyzed by unpaired Student's t-test  
511 (two-tailed). \*\*p < 0.01. Data are represented as mean ± SD (n = 5 mice for each  
512 group).

513 (E) The strategy of BrdU incorporation assay. The 8-week-old *Nupr1*<sup>-/-</sup> mice and  
514 littermates were injected intraperitoneally with 1mg BrdU on day 0. Then the mice  
515 were continuously fed with BrdU (0.8mg/ml) water until analyzed on day 3, 4, and 5.

516 (F) Dynamic tendency analysis of BrdU<sup>+</sup> HSCs after BrdU administration by flow  
517 cytometry on day 3, 4, and 5.

518 (G) Ratio kinetics of BrdU<sup>+</sup> HSCs. Data are analyzed by unpaired Student's t-test  
519 (two-tailed). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. Data are represented as mean ± SD  
520 (n = 4 mice for each group).

521



522

523 **Fig 2. *Nupr1*<sup>-/-</sup> HSCs show repopulating advantage in competitive transplantation**

524 (A) Schematic diagram of competitive transplantation assay. 2.5 × 10<sup>5</sup> *Nupr1*<sup>-/-</sup>

525 BMNCs (CD45.2) and equivalent WT (CD45.1) counterparts were mixed and injected

526 into individual lethally irradiated recipients (CD45.1). Four months later, the

527 recipients were sacrificed. One million BMNCs from primary transplanted recipients

528 were transplanted to lethally irradiated secondary recipients.

529 (B) Kinetic analysis of donor chimerism (CD45.2<sup>+</sup>) in peripheral blood. Data are

530 analyzed by paired Student's t-test (two-tailed). \*\*\*p < 0.001. Data are represented as

531 mean ± SD (n = 7 mice).

532 (C) Kinetic analysis of of donor-derived lineage chimerism in peripheral blood,

533 including myeloid cells (CD11b<sup>+</sup>) (left), B lymphocytes (CD19<sup>+</sup>) (middle), and T

534 lymphocytes (CD90.2<sup>+</sup>) (right) in peripheral blood. Data are analyzed by paired

535 Student's t-test (two-tailed). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Data are represented

536 as mean  $\pm$ SD (n = 7 mice).

537 (D) Flow cytometry analysis of HSC compartment in primary recipients four months

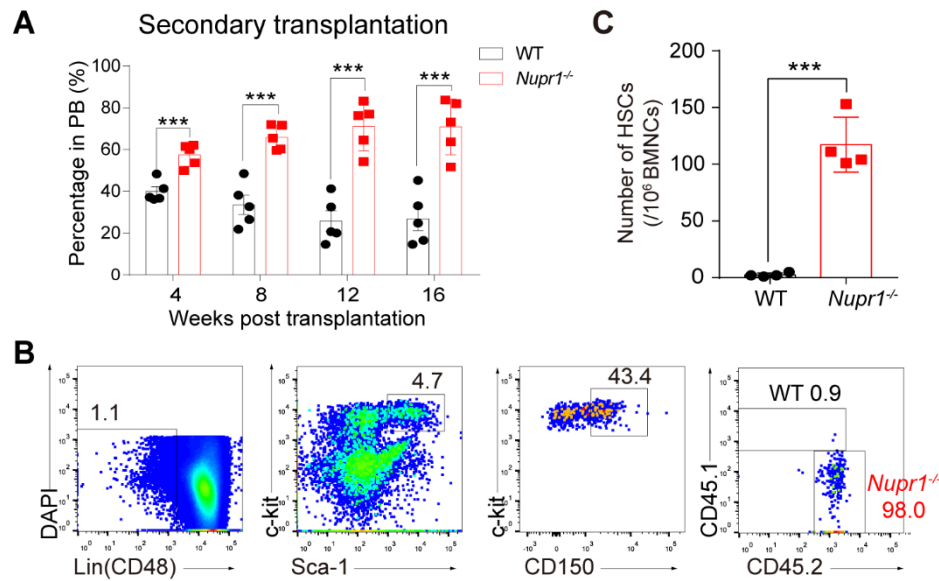
538 after transplantation. Representative plots from one recipient mouse are shown.

539 (E) Cell number of donor-derived HSCs in primary recipients four months after

540 competitive transplantation. Data are analyzed by paired Student's t-test (two-tailed).

541 \*\*\* $p < 0.001$ . Data are represented as mean  $\pm$ SD (n = 6 mice).

542



543

544 **Fig 3. *Nupr1*<sup>-/-</sup> HSCs continuously show competitive advantage without losing**  
545 **their long-term self-renew ability in secondary transplantation**

546 (A) Kinetic analysis of donor chimerism (CD45.2<sup>+</sup>) in peripheral blood of secondary  
547 transplanted recipients. Data are analyzed by paired Student's t-test (two-tailed). \*\*\*p  
548 < 0.001. Data are represented as mean ± SD (n = 5 mice).

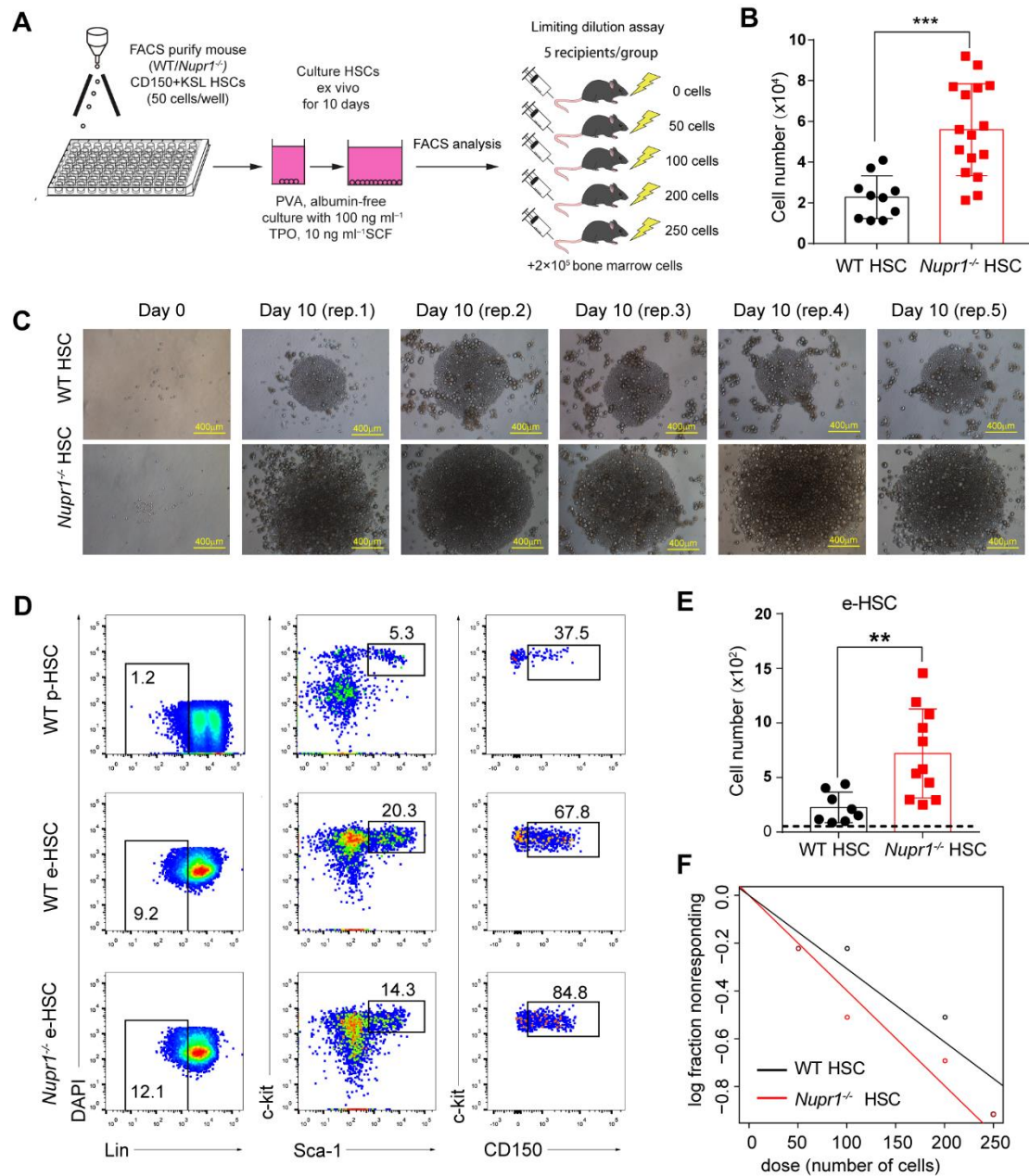
549 (B) Flow cytometry analysis of donor *Nupr1*<sup>-/-</sup> HSCs in secondary recipients four  
550 months after transplantation. Representative plots from each group mice were shown.

551 (C) Cell number of donor-derived HSCs in secondary recipients four months after  
552 competitive transplantation. Data are analyzed by paired Student's t-test (two-tailed).

553 \*\*\*p < 0.001. Data are represented as mean ± SD (n= 4 mice).

554

555



556

557 **Fig 4. Deletion of *Nupr1* promotes HSC expansion in vitro**

558 (A) Schematic diagram of the HSC expansion in vitro. 50 CD150<sup>+</sup>KSL HSCs (from  
 559 WT and *Nupr1*<sup>-/-</sup> mice) were sorted into fibronectin-coated plate wells, containing  
 560 albumin-free F12 medium supplemented with 1 mg/ml PVA, 100 ng/ml TPO and 10  
 561 ng/ml SCF. HSCs were cultured for 10 days and then analyzed by flow cytometry.  
 562 For limiting dilution assay, serial doses were transplanted into lethally irradiated

563 recipients, together with  $2 \times 10^5$  bone-marrow competitor cells.

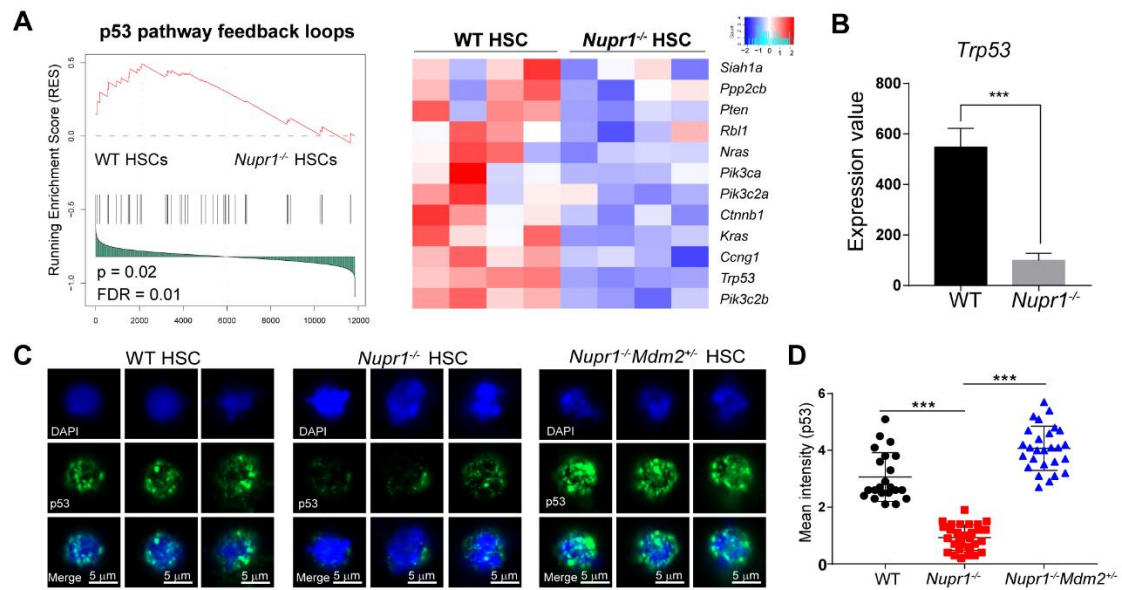
564 (B) Cell number derived from 50 HSCs after a 10-day-long culture *in vitro*. Data are  
565 analyzed by unpaired Student's t-test (two-tailed). \*\*\* $p < 0.001$ . Data are represented  
566 as mean  $\pm$  SD (WT,  $n = 10$ ; *Nupr1*<sup>-/-</sup>,  $n=16$ )

567 (C) Representative images of WT and *Nupr1*<sup>-/-</sup> HSCs from freshly isolated HSCs  
568 (Day 0) and 10-day-long cultures (Day 10). Images of five representative colonies  
569 (biological replicates) are shown.

570 (D) Representative plots of HSC analysis by flow cytometry from cultured WT and  
571 *Nupr1*<sup>-/-</sup> HSCs at day 10. p-HSC indicates primary HSCs from BM. e-HSC indicates  
572 expanded HSCs after 10-day culture *ex vivo*.

573 (E) Cell counts of phenotypic CD150<sup>+</sup>KSL HSCs at day 10 after culture. The dashed  
574 indicates the primary input cell amount. Data are analyzed by unpaired Student's t-test  
575 (two-tailed). \*\* $p < 0.01$ . Data are represented as mean  $\pm$  SD (WT,  $n = 8$ ; *Nupr1*<sup>-/-</sup>,  
576  $n=11$ ).

577 (F) Poisson statistical analysis after limiting-dilution analysis; plots were obtained to  
578 allow estimation of CRU content within each condition ( $n = 5$  mice transplanted at  
579 each dose per condition, total 40 mice). The plot shows the percentage of recipient  
580 mice containing less than 1% CD45.2<sup>+</sup> cells in the peripheral blood at 16 weeks after  
581 transplantation versus the number of cells injected per mouse.



582

583 **Fig 5. Loss of *Nupr1* confers repopulating advantage on HSCs by regulating p53**

584 **check-point signaling**

585 (A) Gene set enrichment analysis (GSEA) of p53 pathway feedback loops in WT

586 HSCs and *Nupr1*<sup>-/-</sup> HSCs. One thousand HSCs from bone marrow of wild type and

587 *Nupr1*<sup>-/-</sup> mice were sorted as individual samples for RNA-sequencing. DESeq2

588 normalized values of the expression data were used for GSEA analysis. Expression of

589 the leading-edge gene subsets was shown. p53 pathway feedback loops-related genes

590 down-regulated in *Nupr1*<sup>-/-</sup> HSCs (a difference in expression over 1.2-fold; adjusted p

591 value, < 0.05 (DESeq2 R package)). WT HSCs, n = 4 cell sample replicates (one per

592 column); *Nupr1*<sup>-/-</sup> HSCs, n = 4 cell sample replicates (one per column).

593 (B) Expression level of p53 in WT HSCs and *Nupr1*<sup>-/-</sup> HSCs by RNA-seq. Y-axis

594 indicates the expression value (DESeq2 normalized values of the expression data).

595 The expression value (DESeq2 normalized counts) of each gene was illustrated by

596 graphpad. Data are analyzed by unpaired Student's t-test (two-tailed). \*\*\*p < 0.001.

597 Data are represented as mean  $\pm$ SD (n = 4 mice for each group).

598 (C) Immunofluorescence measurement of p53 proteins in single HSCs from the WT,  
599 *Nupr1*<sup>-/-</sup>, *Mdm2*<sup>+/-</sup>*Nupr1*<sup>-/-</sup> mice. Images of three representative single cell of each  
600 group are shown.

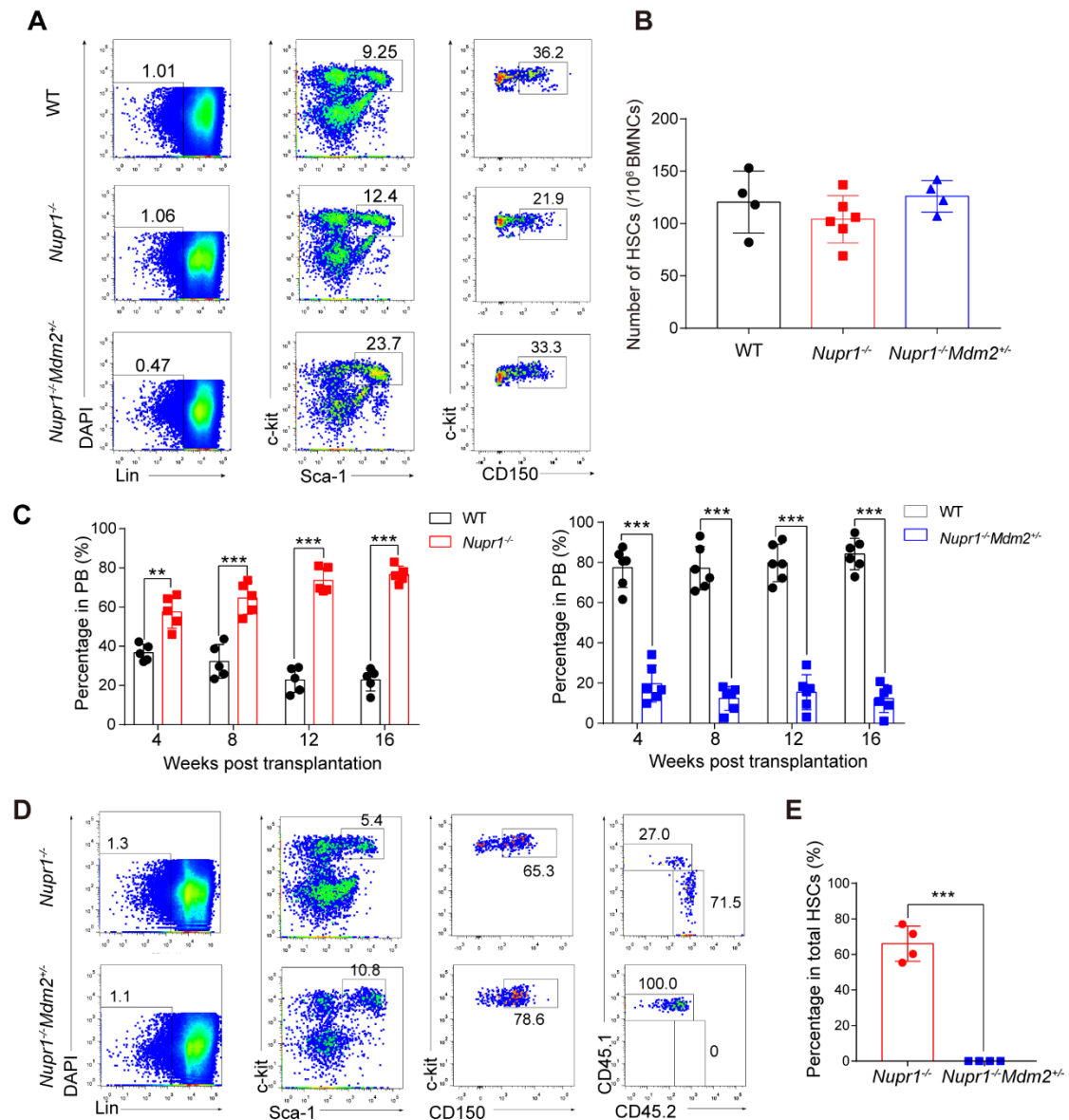
601 (D) Mean intensity of p53 fluorescence in WT, *Nupr1*<sup>-/-</sup>, *Mdm2*<sup>+/-</sup>*Nupr1*<sup>-/-</sup> HSCs. Each  
602 dot represents a single cell. Data are analyzed by One-way ANOVA. \*\*\*p<0.001. WT,  
603 n=22; *Nupr1*<sup>-/-</sup>, n=30; *Mdm2*<sup>+/-</sup>*Nupr1*<sup>-/-</sup>, n=27. Data are represented as mean ± SD.

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607

608 **Fig 6. Reversion of p53 expression by allelic depletion of Mdm2 gene offsets the**  
 609 **repopulating advantage of *Nupr1*<sup>-/-</sup> HSCs.**

610 (A) Representative plots of HSC analysis by flow cytometry from WT, *Nupr1*<sup>-/-</sup> and  
 611 *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> mice bone marrow.

612 (B) Statistical analysis of WT, *Nupr1*<sup>-/-</sup> and *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> HSC number. Data are  
 613 analyzed by One-way ANOVA.  $p > 0.05$ . WT, n=4; *Nupr1*<sup>-/-</sup>, n=6; *Mdm2*<sup>+/-</sup>*Nupr1*<sup>-/-</sup>,

614 n=4.

615 (C) Donor bone marrow cells ( $2.5 \times 10^5$ ) from *Nupr1*<sup>-/-</sup> (left) or *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup> (right)  
616 mice (CD45.2) were transplanted into lethally irradiated recipient mice (CD45.1)  
617 along with  $2.5 \times 10^5$  recipient bone marrow cells. Data are analyzed by paired  
618 Student's t-test (two-tailed). \*\*p < 0.01, \*\*\*p < 0.001. Data are represented as mean ±  
619 SD. *Nupr1*<sup>-/-</sup>, n = 5 mice, *Nupr1*<sup>-/-</sup>*Mdm2*<sup>+/-</sup>, n = 6 mice).

620 (D) Flow cytometry analysis of donor-derived HSCs and recipient HSCs in bone  
621 marrow of recipient mice at four months after transplantation. HSCs were gated as  
622 CD2<sup>-</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>B220<sup>-</sup>Gr1<sup>-</sup>Mac1<sup>-</sup>Ter119<sup>-</sup>(Lin<sup>-</sup>) CD48<sup>-</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup>CD150<sup>+</sup>. Plots  
623 from one representative mice of each group are shown.

624 (E) Statistical analysis of donor-derived HSC percentage in recipient mice at four  
625 months after transplantation. Data are analyzed by unpaired Student's t-test  
626 (two-tailed). \*\*\*p < 0.001. Data are represented as mean ± SD (n = 4 mice for each  
627 group).

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