



Published in final edited form as:

Cell Rep. 2014 December 24; 9(6): 2084–2097. doi:10.1016/j.celrep.2014.11.031.

p62 is required to retain short-term repopulating and myeloid progenitor cells through inhibition of IKK/NF- κ B/Ccl4 signaling at the bone marrow macrophage-osteoblast niche

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Abstract

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K.H.C., A.S., M.D.-M., J.M. and J.A.C. designed research. K.H.C. and A.S. performed majority of the experiments with the help from A.D., S.J.L., D.G.-N., S.R.H. and A.M.W. R.C.N. and K.H.C. performed confocal image analysis. R.P. performed micro CT analysis of femurs. D.T.S. contributed with reagents and experimental design in NF κ B activation experiments. M.W. and R.C. contributed to bone histomorphometry analysis and bone experimental design and interpretation. B.J.A. performed bioinformatics analysis of Wt and p62-deficient Ob. K.H.C., A.S. and J.A.C. analyzed data; and K.H.C., A.S. and J.A.C. wrote the paper.

The authors declare not to have any relevant conflicts of interest.

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In the bone marrow (BM), hematopoietic progenitors (HP) reside in specific anatomical niches near osteoblasts (Ob), macrophages (MΦ) and other cells forming the BM microenvironment. A connection between immunosurveillance and traffic of HP has been demonstrated but the regulatory signals that instruct immune regulation on HP circulation are unknown. We discovered that the BM microenvironment deficiency of p62, an autophagy regulator and signal organizer, results in loss of autophagic repression of macrophage contact-dependent activation of Ob NF-κB signaling. Consequently, Ob p62-deficient mice lose bone, Ob Ccl4 expression and HP chemotaxis towards Cxcl12 resulting in egress of short-term hematopoietic stem cells and myeloid progenitors. Finally, Ccl4 expression and myeloid progenitor egress are reversed by the deficiency of the p62 PB1 binding partner Nbr1. A functional 'MΦ-Ob niche' is required for myeloid progenitor/short-term stem cell retention, in which Ob p62 is required to maintain NF-κB signaling repression, osteogenesis and BM progenitor retention.

INTRODUCTION

Steady-state blood formation during most adulthood depends on long-lived hematopoietic progenitors (HP) (Sun, 2014). Constitutive egress of bone marrow (BM)-resident HP into the blood is a well-established phenomenon. Circulating HP can survey peripheral organs and foster the local production of tissue-resident innate immune cells under both steady-state conditions and in response to inflammatory signals (Baldrige et al., 2010; Essers et al., 2009; Massberg et al., 2007). Dysregulation of stromal components of the HP niches within the BM, such as changes in the levels of chemokines from osteoblasts (Ob) and other mesenchymal cells, has been associated with HP egress (Ding and Morrison, 2013; Greenbaum et al., 2013; Mendez-Ferrer et al., 2010; Omatsu et al., 2010; Petit et al., 2002; Sugiyama et al., 2006; Visnjic et al., 2004). Specifically, the deletion of the major hematopoietic stem cell and progenitor (HSC/P) traffic regulator Cxcl12 (Peled et al., 1999; Peled et al., 2000) from Cxcl12-abundant reticular cells and Ob, results in constitutive HP mobilization and a loss of B-lymphoid progenitors, while their HSC function is normal (Greenbaum et al., 2013). Physiological regulation of these mesenchymal components modulates HP trafficking, and is afforded by several mechanisms, including signals derived from BM-resident macrophages (MΦ) (Casanova-Acebes et al., 2013; Chow et al., 2011; Christopher et al., 2011; Winkler et al., 2010). Cellular cross-talk between MΦ and Ob in the HP niche may critically regulate the response of HP to cytokines and chemokines.

The transcription factor NF-κB has a key role in inflammation and immune responses (Ghosh and Karin, 2002; Silverman and Maniatis, 2001; Sun et al., 2013), and has been recently shown to play a role in the response of myeloid progenitors to stress hematopoiesis (Zhao et al., 2014). NF-κB can also control mesenchymal derived osteogenesis, and mice with a loss of function of NF-κB signaling show osteopetrosis (Iotsova et al., 1997). IκB kinase (IKK)-dependent NF-κB activation is essential for the bone remodeling function of osteoclasts (Ruocco et al., 2005) and the restoration of NF-κB in IKK-deficient mice prevents Ob differentiation (Chang et al., 2009). However, the mechanisms and regulatory pathways that control NF-κB activation in the BM Ob niche, and the putative effect of NF-κB signaling on HP activity in the BM remain unknown.

p62 (also called Sqstm-1) is a master regulator of ubiquitinated protein turnover via autophagy and the ubiquitin-proteasome system (Moscat and Diaz-Meco, 2009). p62 also has a central role in osteoclastogenesis. It controls the receptor activator of NF- κ B (RANK) signaling by interacting with TRAF6 and activating NF- κ B through atypical protein kinase C (aPKC)-mediated activation of IKK in osteoclasts (Duran et al., 2008; Duran et al., 2004). The loss of p62 signaling is implicated in osteolytic lesions in multiple myeloma and adipogenesis (Hiruma et al., 2009; Rodriguez et al., 2006), while gain-of-function mutations of p62 are associated with aberrant and excessive bone turnover in Paget disease (Rodriguez et al., 2006). p62 has also been implicated in the selective autophagy of components of the NF- κ B signaling pathway (Chang et al., 2013), however, the specific cellular and molecular roles of p62 in Ob, and its role in the Ob control of HP activity, have not yet been elucidated. In this manuscript, we reveal that upstream BM-M Φ signaling and cell-to-cell interaction is required for Ob differentiation and the expression of the chemokine Ccl4 (M Φ inflammatory protein-1 β , Mip-1 β). While the cell autonomous deficiency of p62 does not translate into significant HP activity defects, the deficiency of p62 in the non-hematopoietic compartment of BM results in osteopenia due to defective Ob differentiation and HP egress. Mechanistically, the p62 within Ob attenuates NF- κ B signaling through the downregulation of phospho-focal adhesion kinase (p-FAK), NF- κ B and p-I κ B α , thus impairing NF- κ B activation, M Φ -dependent Ob differentiation and Ccl4 production.

RESULTS

Deficiency of p62 induces non-cell-autonomous HP egress in vivo

p62^{-/-} mice exhibit egress of myeloid HP (Fig. 1A) and short-term (ST) repopulating stem cells (Fig. 1B–C) to the peripheral blood (PB), but not long- or medium-term repopulating hematopoietic stem cells (Fig. S1A–C), common lymphoid progenitors Fig. S1D), or B-cell lineage populations (Fig. S1E). This egress of myeloid progenitors was also observed in the spleen (Fig. 1D). However, p62-deficiency was not associated with expansion of the BM content of cells (data not shown), repopulating HSC (Fig. S1F–H), myeloid or common lymphoid progenitors, or B-cell lineage populations (Fig. S1I–K), or changes in the hematopoietic regenerative response to 5-fluorouracil administration (Fig. S1L–M).

To determine whether the myeloid progenitor egress is hematopoietic intrinsic or if it depends on the hematopoietic microenvironment (HM), we generated full chimeric animals of wild-type hematopoiesis (H-Wt) and p62-deficient hematopoiesis (H-p62^{-/-}) by BM transplantation into CD45.1⁺ animals (Fig. S2A), or through reverse transplantation of Wt HSC into lethally irradiated CD45.2⁺ Wt (Wt HM) or p62^{-/-} animals (p62^{-/-} HM) (Fig. 1E). We observed that the effect of p62 on HP traffic is non-cell-autonomous since H-p62^{-/-} HP did not recapitulate the increased egress of HP (Fig. S2B). Conversely, mice lacking p62 in the HM (p62^{-/-} HM) did phenocopy the HP egress of primary mice (Fig. 1F), and increased HP egress of p62^{-/-} HM mice was rescued by secondary transplantation into Wt recipients (Fig. 1G–H). Altogether these data indicate that the effect of p62-deficiency on HP egress is non-cell-autonomous.

It has been reported that over-expression of AAAGUGC seed-containing micro-RNA promotes cell expansion, replating capacity, and signaling in hematopoietic cells, by

interfering with p62-regulated pathways in myeloid cell lines, and that these changes may reflect the effect of p62-deficiency on HSC and myeloid progenitor mobilization (Meenhuis et al., 2011). To identify whether p62 regulates *in vivo* hematopoietic cell proliferation, we analyzed the cell cycle status of primary Wt and p62-deficient as well as Wt hematopoietic cells engrafted in full chimeric WtHM and p62^{-/-} HM mice. We found no significant differences in the cell cycle status of BM long-term (LT)-HSC (Fig. S2C), ST-HSC (Fig. S2D) and Lin⁻/c-kit⁺/Sca-1⁻ (LK) (Fig. S2E) cells from primary mice or in Wt HM or p62^{-/-} HM mice (Figs. S2F–H). Our data using models of primary loss-of-function of p62 did not support the existence of a cell-autonomous or microenvironment-dependent role of p62 on HSC/P cell cycle regulation.

To determine whether a defect in the homing of circulating HP is responsible for HP egress in p62-deficient HM, we measured the ability of Wt HSC and HP to home into non-myeloablated Wt HM or p62^{-/-} HM. As compared to Wt HM recipients, there was a 50–60% reduction in the homing of immunophenotypically defined BM HSC (Lin⁻/c-kit⁺/Sca-1⁺/CD34⁻/CD135⁻ cells) to p62^{-/-} HM recipient mice (Fig. 1I), and the progenitor containing population of Lin⁻/c-kit⁺/Sca-1⁺ (LSK) BM cells (Fig. 1J), as well as LK BM cells (Fig. 1K). This homing defect suggested a significant impairment in the ability of the p62^{-/-} HM to lodge HSC/P upon transplantation into an unmanipulated host. Nevertheless, this difference in HSC/P homing ability disappeared when the recipient mice were myeloablated following lethal irradiation (Figs. S2I–K), suggesting the existence of an accessory contribution from a radiosensitive cell population which can be regenerated by Wt HSC/P transplantation.

Ob deficiency of p62 is responsible for osteogenic defects and HP egress

The mammalian BM microenvironment consists of multiple cell types, including mesenchymal progenitors, CXCL12-expressing adventitial reticular cells, Ob lineage cells, endothelial cells, pericytes, fibroblasts and adipocytes, among others. Functional studies support a role for Ob-lineage cells to maintain HP in the BM *in vivo*. Long-term HSC are connected to Ob cells (Zhang et al., 2003). Ob in the BM contribute to HP expansion through activation of Notch signaling (Calvi et al., 2003), whereas targeted ablation of Ob *in vivo* results in a loss of HP from the BM (Visnjic et al., 2004). To understand whether osteogenesis was impaired in p62^{-/-} HM mice, and to delineate the role of p62 in the non-hematopoietic HP niche, we examined the bone architecture of p62^{-/-} HM chimeric mice. Bone histomorphometric analysis using micro-computed tomography (micro-CT, Fig. 2A) revealed significantly decreased femoral trabecular bone volume (Fig. 2B) and trabecular number (Fig. 2C) in p62^{-/-} HM mice as compared to Wt HM mice. In p62-deficient femoral sections, histological analysis of endosteal Col1 α 1-expressing Ob demonstrated a flatter appearance (Fig. 2D–E) and a decreased number of cortical osteocytes (Fig. 2F). However, the frequency (and content) of mesenchymal progenitors (colony-forming unit fibroblasts, CFU-F, Fig. 2G) and osteoprogenitors (CFU-Ob, Fig. 2H) did not change, suggesting that p62 controls Ob maturation and terminal differentiation, but not mesenchymal progenitor differentiation. Finally, to confirm whether the presence of p62 in Ob is responsible for HP mobilization, we enumerated the number of circulating HP in mice with a specific deletion of p62 in Ob. We crossed Col1(I)-Cre mice with p62^{fl/fl} mice to obtain Ob-specific

Col1(I)-Cre;WT and Col1(I)-Cre;p62^{f/f} mice. Interestingly, Col1(I)-Cre;p62^{f/f} mice phenocopied increased HP egress as observed in p62^{-/-} and p62^{-/-} HM mice (Fig. 2I–J), suggesting that the loss of p62 in Ob is responsible for non-cell-autonomous HP egress in vivo.

p62 regulates NF- κ B-dependent Ob differentiation in presence of M Φ

The homing defect which is associated with a deficiency of p62 in non-conditioned HM (Fig. 1H, S1L–M) is not found in mice with irradiated HM, this may suggest that radiosensitive hematopoietic cells are required as effectors to retain HP in the BM. Myeloid mononuclear accessory cells, in addition to Ob, have been proposed to form a myeloid signaling network responsible for HP homing and engraftment in the BM (Katsumoto et al., 2005). Specifically, M Φ have been known to mediate HP retention in the BM (Casanova-Acebes et al., 2013; Chow et al., 2011). It has been shown that depletion of BM M Φ , but not other lineage-related cells such as osteoclasts (Miyamoto et al., 2011), is adequate to suppress endosteal Ob, inhibit the expression of HP-supportive cytokines at the endosteum, and elicit HP mobilization into the PB (Winkler et al., 2010). The content of M Φ in contact with Ob in the trabecular and endosteal lining of BM from Wt HM and p62^{-/-} HM mice was similar (Figs. S3A–B). Similar to untreated mice with global deficiency of p62 (Duran et al., 2004), the loss of p62 in osteoblasts did not modify significantly the bone osteoclast content (Figs. S3C–D). Together, these data suggested that the content of osteogenic M Φ or osteoclasts was not causative of the loss of BM retention observed in HM- or Ob- p62-deficient mice.

To identify whether M Φ are required for p62-mediated Ob activity, we isolated and expanded F4/80⁺/CD68⁺/CD115⁺/CD169⁻ M Φ derived from the BM of ubiquitin C-EGFP mice (Fig. S3E) and cocultured with Ob in vitro at a 1:1 ratio for 24 hours in the absence of M Φ or osteogenic differentiation factors, and then analyzed their biochemical and expression effect on downstream signaling. Notably, the expression levels of Ob differentiation genes are significantly reduced in p62^{-/-}Ob that have been cocultured with Wt M Φ (Fig. 3A), implying that M Φ induce specific signaling that interfere with Ob differentiation. Ob focal adhesion kinase (FAK) phosphorylation and activation has been shown to depend on M Φ β_2 -integrin binding which regulates Ob differentiation (Kim et al., 2007). By using phospho-flow cytometry analysis, we found that M Φ are capable of activating FAK in Wt or p62^{-/-} Ob (Fig. S3F). The absence of p62 expression in Ob resulted in a modest reduction of M Φ -dependent Ob FAK activation, suggesting that the moderate decrease in FAK activation in p62^{-/-} Ob may not be responsible for the dramatic loss of p62^{-/-} Ob differentiation.

MAPK and NF- κ B transcriptional signatures have been associated with p62 activity. Erk or p38 activation has been shown to be required for Ob differentiation. The Erk or p38 pathway mediates the signal transduction from hormone and growth factors, such as fibroblast growth factor-2 (Xiao et al., 2002) and parathyroid hormone (Chen et al., 2004). They also stimulate Runx-2 phosphorylation and its transcriptional activity (Franceschi et al., 2009; Xiao et al., 2000). In addition, Erk or p38 signaling is required for p62 activity in other mesenchymal lineage BM cells such as adipocytes. It was reported that absence of Erk1 was sufficient to

restore abrogated adipogenesis and energy homeostasis of p62-deficient animals in vivo (Lee et al., 2010). However, while M Φ signaling is associated with increased activation of Erk, but not p38, in FACS sorted Ob (Fig. S3G), the deficiency of p62 resulted in inhibition (Fig. S3G), rather than further upregulation as was reported in adipocytes (Lee et al., 2010; Rodriguez et al., 2006). Furthermore, HP egress was not rescued by deletion of Erk1 (Fig. S3H), strongly suggesting that Erk upregulation is not responsible for HP mobilization in p62-deficient Ob.

NF- κ B signaling has also been shown to be crucial in Ob differentiation. Mice with disrupted NF- κ B signaling show severe osteopetrosis (Iotsova et al., 1997). I κ B kinase (IKK)-dependent NF- κ B activation is essential for the bone remodeling function of osteoclasts (Ruocco et al., 2005), and gain-of-function of NF- κ B activity prevents Ob differentiation as demonstrated in mice deficient in IKK (Chang et al., 2009). Since osteoclasts may be dispensable for HP mobilization in vivo (Miyamoto et al., 2011), we analyzed whether NF- κ B-dependent signaling in Ob correlates with HP egress. Histological analysis of active NF- κ B p65 in endosteal and trabecular Ob showed increased NF- κ B nuclear localization in p62^{-/-} Ob in vivo (Fig. 3B–C). Similar to previous reports in osteoclasts and tumor cells (Duran et al., 2008; Duran et al., 2004), Ob cultured from p62-deficient animals showed decreased levels of the p65 subunit of NF- κ B bound to DNA (Fig. 3D), and I κ B α , the phosphorylated inhibitor of NF- κ B, (p-I κ B α , Fig. 3E) was responsible for degradation. However, cocultures of M Φ with Ob resulted in increased NF- κ B p65 activity, as demonstrated by translocation of NF- κ B to the nucleus (Fig. 3F). In addition, p62^{-/-} Ob isolated after coculture with M Φ showed increased phosphorylation and expression of I κ B α (Fig. 3G). Taken together, these data demonstrate that M Φ reverses p62-dependent NF- κ B signaling in Ob. To ascertain whether NF- κ B is crucial for p62-dependent Ob differentiation, we evaluated the changes of bone specific gene expression after in vitro treatment with BAY 11-7085, an NF- κ B inhibitor. The attenuation of NF- κ B activity was validated by the reduced amount of DNA bound-NF- κ B p65 concentration seen in both Ob and M Φ (Fig. S3I–J). Interestingly, addition of BAY 11-7085 to Ob-M Φ cocultures derepressed Alpl-, Runx2- and Bglap-expression in Wt and p62^{-/-}-sorted Ob (Fig. 3H–J), suggesting that p62-deficiency enhances the ability of IKK/NF- κ B activity to inhibit Ob differentiation. Finally, Cxcl12-driven chemotaxis in the presence of WT M Φ -p62-deficient Ob cocultures was significantly reduced (Fig. 3K), indicating that Ob p62-deficiency results in a loss of sensitivity to Cxcl12 chemotaxis gradients. The inhibition of NF- κ B in WT M Φ -Wt Ob cocultures results in an ~2-fold increase in chemotaxis of Wt HP, and eliminates the effect of p62-deficiency in Ob, suggesting that NF- κ B activity is responsible for the effect of Ob p62 on directed migration towards Cxcl12 gradients (Fig 3K).

HM p62-deficiency impairs M Φ -dependent Ob expression of the chemokine Ccl4 and recapitulates part of the mobilization phenotype associated with BM M Φ activity

In order to determine whether the osteogenic deficiency of p62 in p62^{-/-} HM mice could result in the alteration of the levels of chemokines responsible for HP egress, we performed an in vivo expression screen of a panel of chemokines or cytokines known to be relevant in HP traffic (Figs. S4A–N). We hypothesized that their expression and secretion in plasma

and/or femoral extracellular fluid may depend on Ob activity, which could be regulated by NF- κ B activity.

Interestingly, Ccl4 (M Φ inflammatory protein-1 β , Mip-1 β) was uniquely found to be downregulated in the femoral extracellular fluid of p62-deficient mice (Fig. 4A), and trended to be decreased in plasma as well (Fig. S4A). Ccl4 is a C-C motif chemokine that modulates BM HP chemotactic response to Cxcl12 (Basu and Broxmeyer, 2009). Cxcl12 levels in the plasma and bones of p62^{-/-} HM mice were normal or upregulated (Fig. S5A–B), which was consistent with a null or minimal contribution of mature Ob to systemic or local Cxcl12 levels, as previously reported (Ding and Morrison, 2013; Greenbaum et al., 2013). As expected, Ob from Wt mice did not show any significant expression or secretion of Ccl4, neither did the deficiency of p62 modify its expression or secretion (Fig. 4B–D). Interestingly, the expression and secretion of Wt Ob Ccl4 protein was significantly increased upon coculture with M Φ , whereas the deficiency of p62 in Ob resulted in diminished levels of Ccl4 expression and secretion, similar to the levels of unstimulated Ob (Fig. 4E–G, to be compared with Figs. 4B–D).

Administration of clodronate liposomes has been shown to deplete M Φ and induce HP mobilization (Winker et al., 2010). We compared the effect of M Φ depletion by clodronate with the deficiency of Ob p62, in terms of their effect on HP mobilization. We found that clodronate did result in a depletion of CD11b⁺/F4/80⁺/CD68⁺/CD115⁺ (c-fms)⁺ M Φ in vivo, did not affect other CD11b⁺/F4/80⁺ BM cell populations (Fig S5C). Exhaustion of CD11b⁺/F4/80⁺/CD68⁺/CD115⁺ induced an ~4-fold increase in the number of circulating HP while Ob p62-deficiency resulted in only ~2-fold increase (Fig. S5D). Interestingly, the effect of HM p62-deficiency on HP mobilization was lost in CD11b⁺/F4/80⁺/CD68⁺/CD115⁺ depleted animals (Fig S5D). These results indicate that CD11b⁺/F4/80⁺/CD68⁺/CD115⁺ M Φ signal through Ob p62, and that Ob p62 is, at least partly, responsible for the effect of CD11b⁺/F4/80⁺/CD68⁺/CD115⁺ cell depletion on myeloid progenitor retention in the BM.

We confirmed that direct cell-to-cell interaction between Ob and M Φ was necessary to modulate Ccl4 production, since culture of Ob and M Φ in different chambers of a non-contact transwell culture system failed to reproduce the difference in expression of Ccl4 (Fig. 4H). Moreover, addition of an anti-Ccl4 antibody to cocultures of p62-deficient Ob and Wt M Φ , restored the ability of Wt HP to respond to Cxcl12-driven chemotaxis (Fig 4I). Collectively, our data indicate that p62 prevents activation of IKK and NF- κ B in Ob, and that p62 is required for osteogenesis, Ob differentiation, expression of the chemokine Ccl4, Cxcl12-directed chemotaxis and HP retention within the BM microenvironment.

p62 signaling attenuates IKK/NF- κ B activity through its autophagic activity, facilitating Ob differentiation and Ccl4 expression

To mechanistically ascertain the role of p62 in Ob differentiation and Ob Ccl4 expression, p62^{-/-} Ob were transduced with a retroviral EGFP-expressing bicistronic vector expressing full-length p62 or an empty vector (mock, Fig. S6A). Transduced cells were cocultured (or not) with Wt (unlabeled or ubiquitin C-EGFP transgenic) M Φ for 24 hours. This experimental setting was used to determine the effect of the restoration of p62 expression on NF- κ B activity and signaling pathway. As a positive control of optimal NF- κ B

translocation, we used stimulation with TNF- α on Ob (Fig. S6B). We confirmed that M Φ signaling was necessary for p62-dependent NF- κ B nuclear translocation, since exogenous expression of p62 did rescue and abrogate the nuclear localization of active NF- κ B p65 in p62-deficient Ob in the presence of M Φ (Fig. 5A), but not in their absence (Fig. S6B). Reintroduction of p62 decreased the cellular levels of NF- κ B p65 protein (Fig. 5B) due to both lysosomal degradation and a loss of nuclear translocation, as assessed by co-localization in lysosomes and diminished nuclear translocation (Fig. 5A- and 5C). Inhibition of NF- κ B p65 activation by p62 was secondary to restored catalytic activity of IKK activity, since the absolute cytosolic levels of p-I κ B α were found to inversely correlate with the expression of p62 (Fig. 5D). These changes were not associated with changes in the expression levels of the IKK catalytic subunits α , β , or γ (Fig. S6C), indicating that p62 controls IKK activity, but not its expression.

Similarly, exogenous expression of p62 in p62^{-/-} Ob in the presence of M Φ rescued the expression of Ob Runx2 (Fig. 5E), confirming that p62 expression is associated with Ob differentiation. Overexpression of p62 also rescued the expression and production of Ccl4 by Ob (Fig. 6A–C) indicating that the concentration of Ccl4 is directly related to p62 expression, and that it is inversely related to the activity of NF- κ B in Ob cultured with M Φ .

Protein degradation by p62-dependent autophagy mechanisms may be determined by the oligomerization-dependent autophagosome localization (Itakura and Mizushima, 2011). To identify the functional domain in p62 responsible for Ccl4 production, p62^{-/-} Ob were transduced with a retroviral vector expressing the p62⁶⁹⁻⁷³ mutant which fails to oligomerize, a process required for autophagosome formation (Duran et al., 2011; Duran et al., 2008; Moscat et al., 2006). The chemoattraction of HP towards Cxcl12 which was added to cultures of Wt M Φ and Wt Ob was significantly inhibited by the autophagy inhibitor, bafilomycin A1, to the same level seen in cultures of Wt M Φ and p62^{-/-} Ob (Fig. 6D, compare with Fig. 3K). This suggests that the effect of p62-deficiency is equivalent to that induced by bafilomycin A1 through autophagic inhibition. Ectopic expression of the p62⁶⁹⁻⁷³ mutant (Fig. S6D) which disrupts the p62 oligomerization required for autophagosome formation failed to rescue Ccl4 expression or production (Fig. 6A–C), suggesting that p62-dependent Ccl4 expression depends on oligomerization which is required for localization to the autophagosome formation site.

Together, these data indicate that p62 represses M Φ -dependent NF- κ B signaling, and that it is necessary for Ob differentiation and Ccl4 production through its role in autophagy.

Nbr1 antagonizes p62 and its deficiency rescues BM M Φ -dependent Ob Ccl4 expression and HP retention

Phox bemp1-(PB1)-domain-containing proteins p62 and Nbr1 share domain architecture and play overlapping roles in cell signaling through protein-protein interaction. A C-terminus deletion of Nbr1 results in osteoblast differentiation (Whitehouse et al., 2010). To identify whether Nbr1 has overlapping or distinct roles in osteogenesis and HP retention, we crossed CMV-Cre; Nbr1^{fl/fl} (Nbr1^{-/-}) mice (Yang et al., 2010), where Nbr1 is absent in germinal cells, with p62^{-/-} mice (p62^{-/-}; Nbr1^{fl/fl}). We analyzed the effect of HM deficiency of full-length Nbr1 and of double Nbr1/p62 on osteogenesis and HP mobilization in chimeric

animals. We identified that Ob isolated from $p62^{-/-}$ bones express increased levels of Nbr1 (Fig. 6E), suggesting a compensatory role. The deficiency of Nbr1 in vivo does not significantly impair osteogenesis, Ccl4 production, or levels of circulating HP (Fig. 6F–J). Surprisingly, the double deletion of Nbr1 in $p62^{-/-}$ HM mice ($p62^{-/-}; Nbr1^{-/-}$ HM) ameliorates bone architecture, Ccl4 production, and HP egress, which suggests distinct roles for $p62$ HM and Nbr1 in hematopoiesis, and an antagonistic effect of Nbr1 on $p62$ activity..

DISCUSSION

The circulation of HSC/P in PB is crucial as part of a system of immunosurveillance of the peripheral organs and to foster the local production of tissue-resident innate immune cells (Massberg et al., 2007). Niche-regulation of HP trafficking in vivo is incompletely understood. Cellular and molecular specificity, as well as crosstalk, are important aspects in order to understand how cell signaling within complex networks generates such precise cellular responses to a myriad of different stimuli. A key to this process are intracellular protein scaffolds, which are multidomain proteins that assemble specific signaling complexes in different cellular locations so as to assure a spatially and temporarily controlled signal.

The role of Ob in HSC/P traffic remains controversial, and the genetic characterization of the molecular signatures within the Ob microenvironment which have been implicated in contributing to HSC/P trafficking in vivo, is quite incomplete. Previous reports have indicated that Ob activity controls HSC/P retention within the BM (Calvi et al., 2003; Raaijmakers et al., 2010; Visnjic et al., 2004; Zhang et al., 2003), and MΦ that are in contact with Ob (called osteomacs) (Winkler et al., 2010) have been recently implicated as intermediate cells in G-CSF mobilization, Ob depletion and in HSC/P egress from the BM (Katayama et al., 2006; Winkler et al., 2012). However, modulation of Ob numbers does not necessarily alter HSC numbers (Kiel et al., 2007; Zhu et al., 2007), suggesting that functional changes in Ob activity may be responsible for their effect on BM HSC/P retention. Cxcl12-dependent progenitor retention (Greenbaum A et al, 2013) and B-lymphopoiesis (Zhu et al., 2007; Greenbaum et al., 2013; Ding et al., 2013) are functional roles that have been assigned to Ob lineage populations of the BM. However, the specific role of Ob in HSC/P mobilization remains controversial (Kiel et al., 2007; Ding and Morrison, 2013).

The deficiency of $p62$ in the non-hematopoietic compartment of BM results in osteopenia as a result of loss of Ob differentiation, and induces egress of ST-HSC and myeloid progenitors. This effect is due to impaired Ob signaling since the deficiency of $p62$ in Ob, as identified by the expression of $Col1\alpha1$ driven by its 2.3Kb promoter/enhancer, phenocopies the mobilization of HSC/P of mice with $p62$ -deficient HM (Dacquin et al., 2002). However, the deficiency of $p62$ does not result in either significant mobilization of long- or medium-term repopulating HSC or changes in the BM content or mobilization of B lymphopoietic cells, suggesting that $p62$ regulates some, but not all, of the activities associated with Ob activity in the BM (Winkler et al., 2010; Chouo et al., 2011; Zhu et al., 2007; Greenbaum et al., 2013). This mobilization effect results in decreased homing of ST-HSC and LSK/LK progenitors in non-myeloablated recipients, but not in myeloablated recipients. The effect of

myeloablation is restored by transplantation, since $p62^{-/-}$ HM mice display increased numbers of circulating HSC/P after transplantation. We hypothesized that a Wt radiosensitive cell population with the ability to be regenerated by 6 weeks post-transplantation may be responsible for this differential effect, and may contribute to significant changes in the content or activity of the osteoblastic niche. Several reports have provided information about the functional defects and apoptosis of irradiated M Φ in vivo in C57Bl/6 mice as early as within the first 24 hours after irradiation (Coates et al., 2008; reviewed in Mukherjee et al., 2014). While the mechanisms of these changes induced by myeloablation remain unclear and are likely to be indirect, we hypothesized that a radiosensitive cell which could be regenerated by as early as 6 weeks post-transplantation may be responsible of this differential effect, being tissue macrophages major candidates in relation to the BM Ob niche. Other radiosensitive populations such as osteoclasts, which have also been reported to control HSC/P traffic in the BM (Kollet et al., 2006), were analyzed in the BM of Wt HM and $p62^{-/-}$ HM mice and found not to be regenerated by 6 weeks post-transplantation (data not shown).

A putative effect of p62 on BM HSC/P proliferation (Meenhuis et al., Blood 2011) as a downstream target of microRNAs 17/20/93/106 was explored. Our data could not confirm changes in HSC/P proliferation, as assessed by flow cytometry analysis of BrdU uptake in vivo, in either primary mice or HM chimeric BM HSC/P. Analysis of $p62^{-/-}$ and $p62^{-/-}$ HM mice for as long as 6 months after birth or transplantation did not show any significant sign of hematopoietic failure (data not shown). While it is possible that long-term aging of $p62^{-/-}$ or $p62^{-/-}$ HM mice results in significant defects in BM HSC/P content, our data focuses on the effect of p62 expression on the ST-HSC and myeloid progenitor populations.

This report describes and analyzes a novel mechanism of M Φ -Ob-dependent hematopoietic progenitor retention in the BM, in which M Φ regulate Ob differentiation. We explored the existence of functional crosstalk between M Φ and Ob lineage cells that would regulate HSC/P trafficking, and identified a novel three-cell (M Φ , Ob and HSC/P) interplay of interactions in vivo which could be recapitulated and mechanistically analyzed in cellular models. Our data provide evidence that a) cell contact between p62 in M Φ and Ob induces Ob NF- κ B activity and differentiation; b) the maintenance of low levels of Ob NF- κ B activity is crucial for Ob differentiation, Cxcl2-directed chemotaxis, BM retention of myeloid progenitors, and the upregulation of NF- κ B activity secondary to the loss of p62 results in egression of myeloid progenitors and ST-HSC to the PB; c) autophagy p62 is a negative regulator of NF- κ B activity controlling the levels of p-FAK, p-I κ B α , and NF- κ B translocation; and d) Nbr1, a PB1 binding partner of p62, antagonizes p62 activity as a negative regulator, and the loss of Nbr1 rescues the deficient osteogenesis of p62-deficient animals. This signaling pathway is summarized in Fig. S6E.

Circulating HP can foster the local production of tissue-resident innate immune cells in response to inflammatory signals (Baldrige et al., 2010; Essers et al., 2009; Massberg et al., 2007). The transcription factor NF- κ B has a key role in inflammation and immune responses and has been recently shown to play a role in myeloid progenitor response to stress hematopoiesis (Zhao et al., 2014). Inflammatory signals through NF- κ B probably underlie the stress circulation of HSC/P. M Φ are well known mediators of intrinsic activation of NF-

κ B activity in response to infection or other inflammatory cues. Here, we demonstrate that M Φ signaling is required for Ob differentiation and Ob NF- κ B activation, and is crucial for the expression and production of Ob Ccl4, a modulator of Cxcl12 activity which effects HP retention in the BM (Basu and Broxmeyer, 2009). Ob would act as signal amplifiers and coordinators of inflammatory signals initiated by infection responsive macrophages. Our data reveals for the first time Ccl4 as a chemokine expressed and secreted by differentiated Ob in contact with BM M Φ , and confirms in vivo previous data on the role of Ccl4 as a modulator of Cxcl12-dependent HSC/P traffic in the BM (Basu and Broxmeyer, 2009).

Ob have high autophagy activity during differentiation (Liu et al., 2013) and NF- κ B signaling prevents Ob differentiation (Chang et al., 2009). Oligomerization of p62 is required for autophagosome formation (Itakura and Izushima, 2011), where p62 aggregates ubiquitinated proteins (Bjorkoy et al., 2006) and has been shown to regulate the degradation of the RelA component of NF- κ B in Ob through selective autophagy (Chang et al., 2013). We have identified that p62 attenuates M Φ -dependent NF- κ B signaling in Ob through the downregulation of pFAK, NF- κ B and p-I κ B α , which results in impaired NF- κ B activation and Ob differentiation in vivo (Fig. S6E). Pharmacological inhibition of NF- κ B in M Φ /p62^{-/-} Ob cocultures restored Ob differentiation and Cxcl12-driven chemotaxis, confirming the mechanistic role of NF- κ B in the osteogenic defect of p62-deficient mice. Neutralization of Ccl4 and inhibition of autophagy also restored the deficient Cxcl12-directed chemotaxis seen in the same cocultures indicating that all are dependent on the same pathway and regulated by p62. Finally, restoration of p62 expression in the Ob represses NF- κ B activity to levels similar to Wt, and induces Ob differentiation, Ccl4 expression, and progenitor retention in vivo. Our data also suggest that during Ob-M Φ crosstalk, p62 relies on p62-PB1 oligomerization for Ccl4 expression.

BM M Φ are essential to understand Ob activity, osteogenesis and HP traffic. The interaction between differentiating cell- and niche-derived signals have also been shown to play an important role in *Drosophila* HP maintenance (Mondal et al., 2011). Clodronate-induced deficiency of M Φ results in progenitor mobilization (Winkler et al., 2010). We have confirmed that the deficiency of CD11b⁺/F4/80⁺/CD68⁺/CD115⁺ BM M Φ does result in 4-fold increase in myeloid progenitor mobilization, which is ~2-fold higher than the mobilization observed in p62-deficient animals. Interestingly, the Ob p62-deficiency does not add or synergize with the depletion of M Φ , indicating that the effect of Ob p62-deficiency on myeloid progenitor retention depends on the existence of BM M Φ in vivo. Subpopulations of M Φ may be specifically controlling myeloid progenitor retention. BM CD169⁺ M Φ have been shown to promote the retention of HSC/P in the mesenchymal stem cell niche (Chow et al., 2011). Our experiments did not specifically address the role of Ob activity in relation to CD169⁺ M Φ , since the cultured primary M Φ did not express CD169. It is possible that CD169⁺ BM M Φ share the activities of BM CD169⁻ M Φ in regards to the activation of Ob NF- κ B activity and downstream effects, however, this point is unproven and will require further dissection in vivo of the specific M Φ populations responsible for Ob NF- κ B activity.

In our system the interaction between Ob and M Φ has been exclusively contact-dependent as some of the phenotypes, such as upregulation of Ccl4 expression could not be reproduced

in non-contact transwell systems. We provide evidence that p62 regulates osteogenic signals in an Ob-M Φ coculture setup. This signal, probably mediated by integrins, relies, at least in part, on FAK or other redundant family proteins that are responsible for cell-to-cell anchoring. Integrins link the inside of a cell with its outside environment and in doing so regulate a wide variety of cell behaviors. Integrins play an important role in angiogenesis and cell migration, however their functions in bone formation are less clear. The majority of integrin signaling proceeds through FAK, an essential component of the focal adhesion complex. The loss of FAK does not perturb Ob differentiation in vitro or in vivo, owing to the compensatory increase in Pyk2 in Ob (Kim et al., 2007). FAK and Pyk2 are substrates of autophagy, and there is emerging evidence implicating autophagy as an important mediator of bone cell function in normal physiology (Hocking et al., 2012) and in pathology as documented by the role of p62 mutations in Paget disease of the bone (Rea et al., 2013).

Genetic truncation of Nbr1, a selective autophagic receptor for degradation of ubiquitinated substrates which can interact with p62 but not LC3, leads to increased Ob differentiation and activity in vivo (Whitehouse et al., 2010). As shown by our data, its deficiency restores in vivo BM Ccl4 production and HP mobilization, suggesting that Nbr1 plays an inhibitory role on the p62-dependent regulation of NF- κ B activity in Ob.

Our data define for first time a signaling network between BM M Φ and neighboring Obs with activity on the HP niche. We propose the existence of a regulatory signal from BM resident “osteomacrophages” where Ob NF- κ B signaling is connected with the immunosurveillance functions of circulatory HP. We also propose the existence of a homeostatic regulatory role of selective autophagy regulated by p62 on NF- κ B signaling pathway in Ob which is required for osteogenesis and BM progenitor retention. A connection between bone, innate immunity and progenitor traffic is proposed which has the ability to amplify or inhibit M Φ -dependent inflammatory signals. Our data support a role for p62 in the regulation of the intercellular signaling at the BM M Φ -Ob niche, and identifies the key molecular determinants of signaling regulating ST-HSC and myeloid progenitor trafficking.

EXPERIMENTAL PROCEDURES

Mice

All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all protocols were approved by institutional care and use committees for animal research at the Cincinnati Children’s Hospital Research Foundation. p62^{-/-}, p62^{-/-}; Erk1^{-/-}, and CMV-Cre; Nbr1^{+/+} mice have been described previously (Lee et al., 2010; Rodriguez et al., 2006; Yang et al., 2010). p62^{-/-} mice were crossed with Nbr1^{+/+} mice to generate p62^{-/-}; Nbr1^{+/+} mice. Genetically modified Col1(I) (2.3 Kb promoter/enhancer)-Cre mice (Dacquin et al., 2002) were crossed with p62^{f/f} mice (Muller et al., 2013) for generation of osteolineage-specific deletion of p62. All primary mice were analyzed between 6 and 10 weeks of age. Chimeric (HM) mice were generated by non-competitive transplantation of Wt BM nucleated cells (BMNC) from B6.SJL^{Ptprca Pepcb/BoyJ} (CD45.1⁺) mice into lethally irradiated 6–8 week old CD45.2⁺ Wt or p62-deficient mice. All mice were maintained in C57Bl/6 background. C57Bl/6 (CD45.2⁺)

and B6.SJL^{Ptprca Pepcb/BoyJ}(CD45.1⁺) mice were obtained commercially (Jackson Laboratory, Bar Harbor, ME; Harlan Laboratories, Frederick, MD). Littermate mice from the same breeding were used in all experiments. Ubiquitin C-EGFP mice have been described previously. These mice had been backcrossed >10 generations into C57Bl/6 mice.

Quantification of HP egress

PB total and differential counts were analyzed using a Hemavet 950 (DREW Scientific). PB was isolated by retro-orbital bleeding. Hematopoietic progenitors isolated from BM, spleen or PB were grown on methylcellulose medium supplemented with cytokine cocktails (Stem Cell Technologies) and colony forming progenitors (CFU-C) were scored on day 9.

HSC repopulation

Adult recipient mice were lethally irradiated with a Cs¹³⁷ gamma irradiator as previously described (Sengupta et al., 2011). For competitive repopulation experiments CD45.2⁺ BMNC or PB (50–150 μ L) were mixed with 500,000–3 \times 10⁶ CD45.1⁺ BMNC and were transplanted into lethally-irradiated B6.SJL^{Ptprca Pepcb/BoyJ}(CD45.1⁺) recipient mice. HSC engraftment was measured by chimera assessment using flow cytometry at 6 weeks, 10 weeks and 16 weeks post-transplantation. Chimera level was normalized to Wt levels as 100% to allow inter-experiment comparison.

Homing assays

For homing assay to nonmyeloablated BM, 20–25 \times 10⁶ carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen) labeled BM cells from Wt mice were prepared and used for transplantation through the tail vein injection. Wt or p62^{-/-} animals were used as recipients and sacrificed after three hours post transplantation. The single cell suspensions of the BM were subjected to FACS analysis to measure homing of HSC (CFSE⁺ lineage⁻ Sca-1⁺ cKit⁺ CD135⁻), LSK, and LK cells. The percent of homing was calculated from the input and output cell numbers. To measure homing to myeloablated BM, 23 \times 10⁶ Wt BM cells were transplanted into lethally irradiated Wt or p62^{-/-} recipient mice. Sixteen hours after transplant, the recipient mice were sacrificed and the BM cells were harvested and cultured in triplicate for CFU-C assay as well as FACS analysis. BM homing was calculated as previously reported (Boggs, 1984).

Rest of experimental procedures is described in the Supplemental Experimental Procedures section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Heimlich Institute of Cincinnati, the Department of Defense (10580355), the National Institutes of Health (NIH; R01-HL087159 and HL087159S1) and funds from Hoxworth Blood Center and Cincinnati Children's Hospital Medical Center (to J.A.C.); R01-CA132847 and R01-CA170225 (to J.M.), R01-CA134530 (to M.T.D-M); and R01-AR041255, R01-AR055913 and R01-AR056678 (to R.C.). K.H.C is a National Blood Foundation research funding awardee. A.S. was an awardee of The International Lady Tata Memorial Trust,

London, and A.S. acknowledges funding from CSIR, DST-SERB (SB/SO/HS-053/2013) and Ramalingaswami Fellowship (BT/RLF/RE-ENTRY/06/2010), Department of Biotechnology, Govt. of India. We thank Cincinnati Children's Research Foundation Mouse, Flow Cytometry and Immunobiology Cores for services, supported by the Center of Excellence in Molecular Hematology (P30 DK090971). We also thank Yi Zheng and Hartmut Geiger for critical reading of the manuscript and Ms. Margaret O'Leary for editorial revision.

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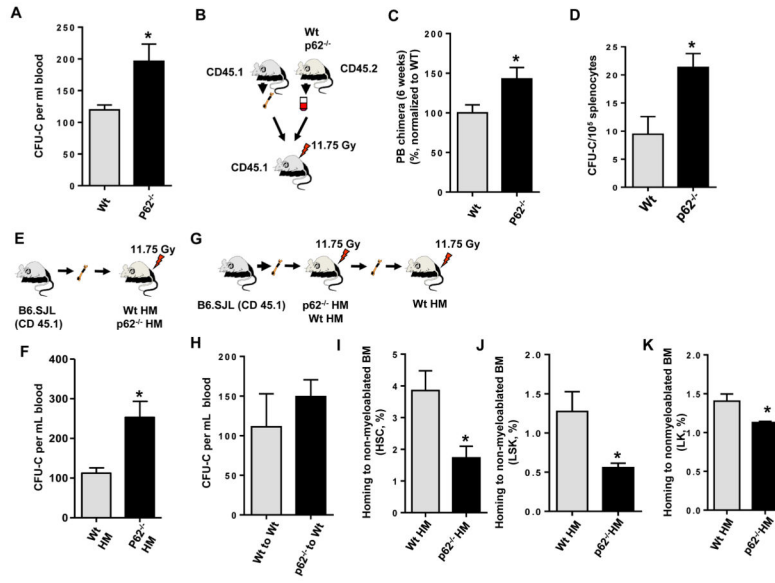


Figure 1. p62 regulates HP trafficking in a non-cell autonomous manner
 (A) CFU-C content in the PB (PB) of Wt or p62^{-/-} mice (n=8–10 mice per group). Values represent average of three independent experiments. (B) Experimental set up. PB mononuclear cells (PBNC) from CD45.2⁺ Wt or p62^{-/-} mice were mixed with CD45.1⁺ B6.SJL^{Ptprca Pep3b/BoyJ}Wt bone marrow (BM) cells and competitively transplanted into lethally irradiated CD45.1⁺ B6.SJL^{Ptprca Pep3b/BoyJ} mice. (C) CD45.2⁺ chimera in the PB of recipient mice (5–8 mice per group) after 6 weeks of competitive transplantation. (D) Frequency of hematopoietic progenitors in spleen of Wt or p62^{-/-} mice (n=3 mice per group). (E) Experimental set up. BM cells from CD45.1⁺ B6.SJL^{Ptprca Pep3b/BoyJ} mice were non-competitively transplanted into lethally irradiated CD45.2⁺ Wt or p62^{-/-} mice to generate chimeric Wt HM or HM p62^{-/-} mice. (F) Absolute numbers of CFU-C present in the PB of Wt HM or HM p62^{-/-} mice (n=4–7 mice per group). (G) Experimental set up. BM cells from CD45.1⁺ B6.SJL^{Ptprca Pep3b/BoyJ} mice were non-competitively transplanted into lethally irradiated CD45.2⁺ Wt or p62^{-/-} mice to generate chimeric Wt HM or HM p62^{-/-} mice. BM cells isolated from primary Wt HM or HM p62^{-/-} mice were transplanted into lethally irradiated CD45.2⁺ to generate secondary Wt recipients. (H) Absolute numbers of CFU-C present in the PB of secondary Wt recipient mice (n=4–6 mice per group). Values represent mean ± SEM. p=ns. (I–K) Homing (%) of Wt ST-HSC (I), LSK (J) and LK (K) BM cells to non-myeloablated BM from Wt or p62^{-/-} mice. A minimum of 5 mice were analyzed per group. Values represent mean ± SEM. * p<0.05.

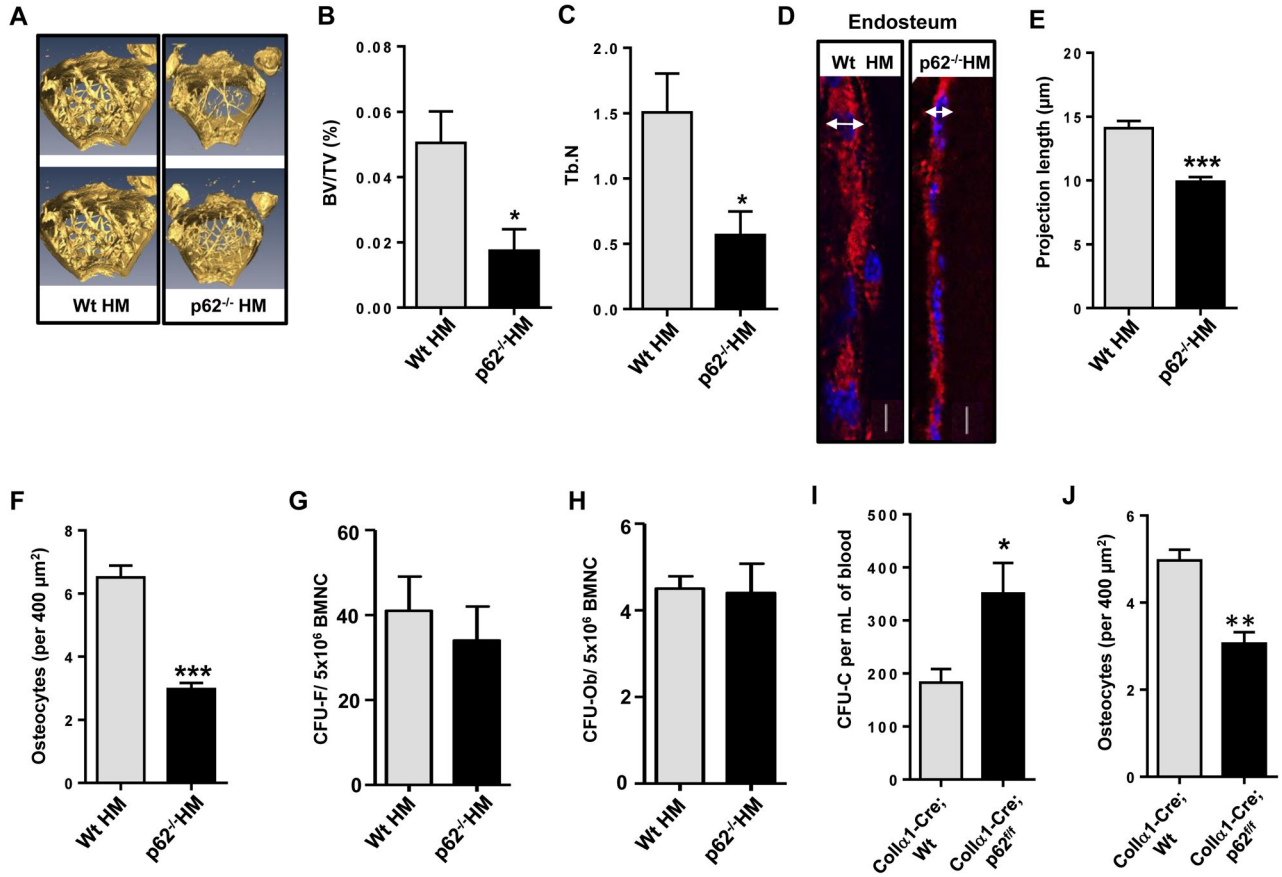


Figure 2. Ob p62-deficiency impairs Ob differentiation and osteogenesis in vivo resulting in HP egress

(A) Representative micro-computer tomography (micro-CT) analyses of femoral trabecular bone of Wt HM or HM p62^{-/-} mice after 16 weeks of transplantation. (B and C) Percent ratio of trabecular bone volume versus tissue volume (BV/TV) and trabecular number (Tb.N) of Wt HM or p62^{-/-} HM mice (4–7 mice per group) at 16 weeks after transplantation. (D) Representative confocal microscopic images of collagen type1α1 (red) and nuclear counterstaining (DAPI, blue) in femur sections from Wt HM or p62^{-/-} HM. (E) Measurement of Ob length projections of bone lining Obs in longitudinal femoral sections from Wt HM (Ob n=24) or p62^{-/-} HM (Ob n>17 per group). Analysis was performed as measurement of the transversal diameter at the widest point of the Ob expressing Coll1α1 (space between arrow ends). (F) Counts of osteocytes in femoral cortical bone from Wt HM (n=47 fields) or p62^{-/-} HM (n=42 fields). Values represent mean ± SEM. ***p<0.001. (G and H) CFU-F and CFU-Ob from BMNC of chimeric Wt HM or HM p62^{-/-} mice. (I) Coll1a1-Cre;p62 p62^{f/f} mice phenocopy the hematopoietic egress of primary p62^{-/-} mice and p62^{-/-} HM mice. Values represent mean ± SEM. *p<0.05. (J) Osteocyte counts in femoral cortical bone from Coll1a1-Cre; Wt or Coll1a1-Cre; p62^{f/f}. N=33 fields were analyzed for each group. ***p<0.001.

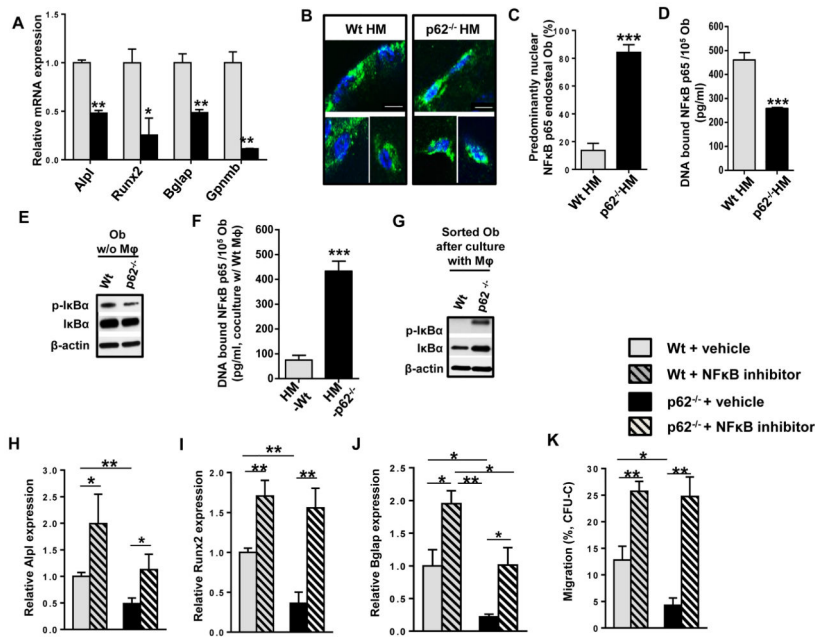


Figure 3. MΦs are in close proximity to endosteal Obs in vivo and p62 regulates NF-κB-dependent Ob differentiation through MΦ-mediated FAK signaling

(A) Relative gene expressions of *Alpl*, *Runx2*, *Bglap*, and *Gpnmb* in Wt (grey bars) or p62-deficient (black bars) Ob after 24 hours of culture with Wt MΦ. (B) Representative images of femoral bones from chimeric Wt HM (n=4) or p62^{-/-} BM (n=3) stained with anti-NF-κB p65 (green) and nuclear counterstaining (DAPI, blue). (C) Percent of Ob with predominant nuclear localization of NF-κB p65 from confocal microscopy images of a minimum of 25 Ob per group. (D and F) DNA-bound NF-κB p65 was measured by ELISA using isolated nuclear fractions of sorted Wt or p62-deficient Ob after culture alone (D) or with EGFP⁺ Wt MΦ for 24 hours (F). (E and G) Representative immunoblots of phosphorylated IκBα and IκBα expression in sorted Wt or p62-deficient Ob after culture alone (E), or with EGFP⁺ Wt MΦ for 24 hours (G). β-actin was used as a loading control. (H–K) Migration of HP towards Ob and Wt MΦ supplemented with 100 ng/mL Cxcl12, and either vehicle control or BAY 11-7085. (H). Relative gene expressions of *Alpl* (I), *Runx2* (J), and *Bglap* (K) in Wt or p62-deficient sorted Ob after 24 hours of coculture with EGFP⁺ Wt MΦ in the presence of 1 μM BAY 11-7085 (hatched bars) or vehicle control (DMSO, solid bars). Values represent mean ± SEM. **p<0.05, ***p<0.005.

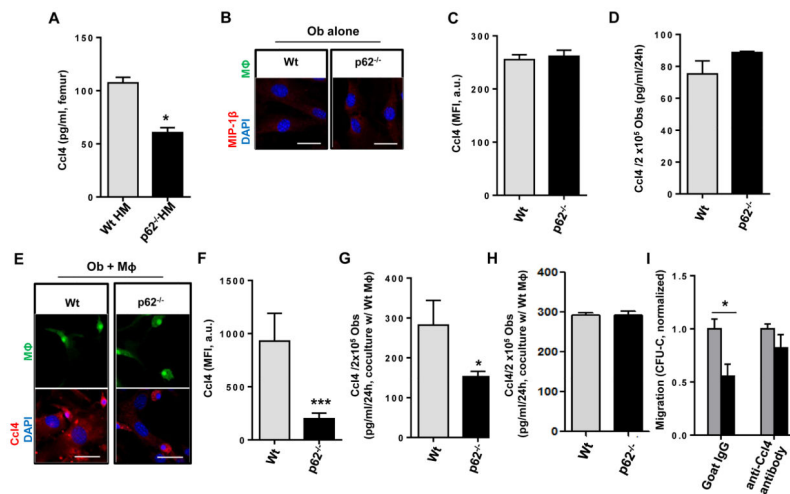


Figure 4. p62 regulates Ccl4-dependent HP retention

(A) BM extracellular levels of Ccl4. Extracellular fluid from Wt HM or p62^{-/-} HM femora (at 16 weeks after transplantation) were processed for ELISA. (B–D) In vitro analysis of Ccl4 in explanted Ob from Wt and p62^{-/-} mice cultured for 24 hours. (B) Representative confocal microscopic images of Ccl4 (red) and nuclear counterstaining (DAPI, blue) in Wt or p62^{-/-} Ob. Data are representative of two independent experiments with similar results. (C) Mean fluorescent intensity (MFI) of Ccl4 expression measured in (B). (D) Concentration of Ccl4 secreted from 10⁵ Ob after 24 hours culture without contact with Wt MΦ. Values are derived from two independent experiments. (E–G) In vitro analysis of Ccl4 in explanted Ob from Wt and p62^{-/-} mice cultured for 24 hours with EGFP⁺ Wt MΦ at a 1:1 ratio. (E) Representative confocal microscopic images of Ccl4 (red) and nuclear counterstaining (DAPI, blue). Data are representative of three independent experiments with similar results. (F) MFI of Ccl4 expression measured in (E). A minimum of 25 Ob were measured. (G) Concentration of Ccl4 secreted from 10⁵ Ob after 24 hours culture in contact with EGFP-expressing Wt MΦ. Values are derived from three independent experiments. (H) Effect of MΦ-Ob contact on Ccl4 production. Ccl4 production was not upregulated in Ob (lower chamber) from Wt and p62-deficient animals cultured in non-contact (transwell) systems after 24 hours of culture with MΦ (upper chamber). (I) Chemotaxis of HP towards Ob and of Wt MΦ towards a Cxcl12 gradient, in the presence or absence of an anti Ccl4 antibody. For all panels, values represent mean ± SEM. *p<0.05. ***p<0.001.

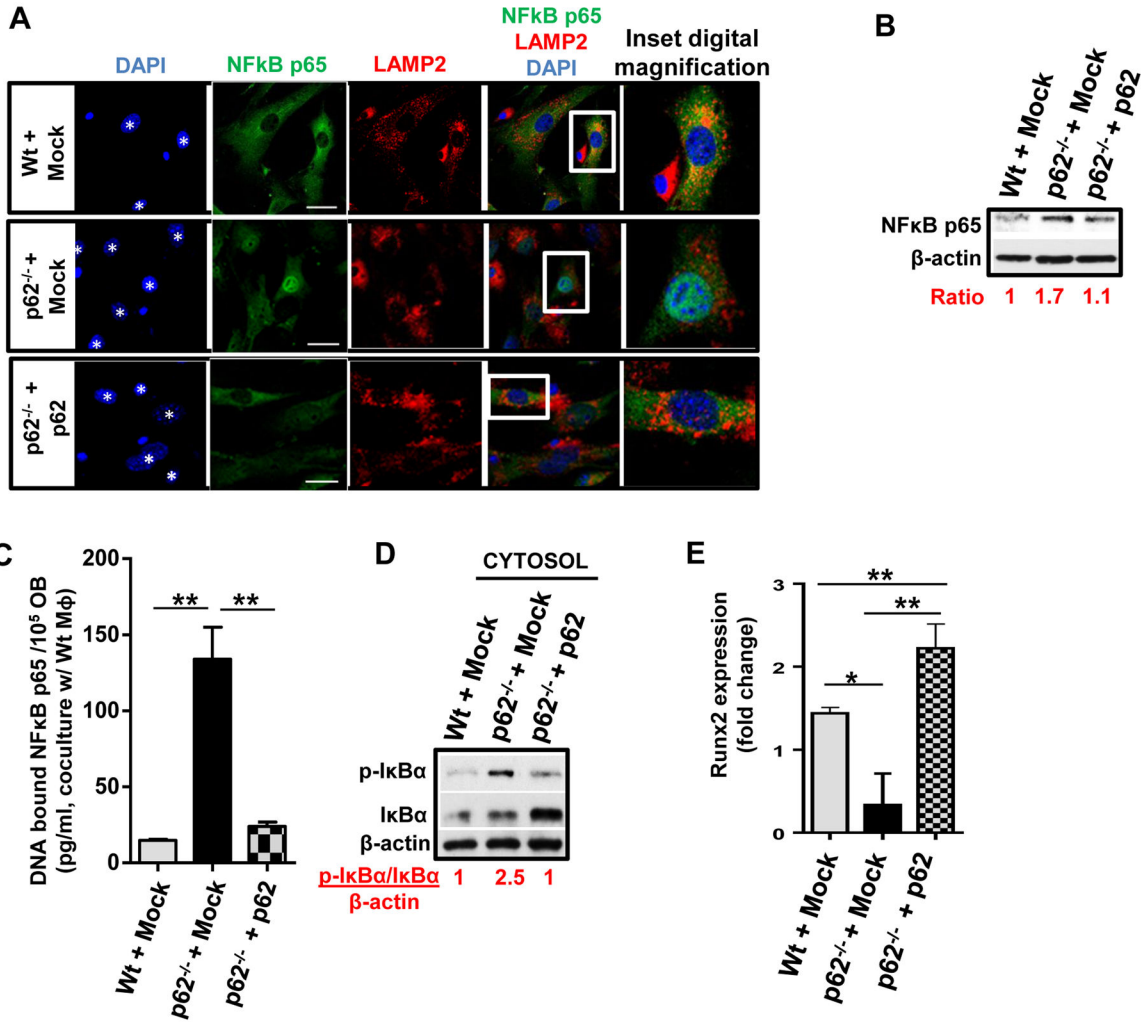


Figure 5. Ectopic expression of p62 abrogates nuclear translocation of NF-κB p65 and rescues differentiation and Ccl4 expression of p62-deficient Ob

(A) Representative confocal microscopy images of NF-κB p65 (green), lysosomes (LAMP2, red) and nuclear counterstaining (DAPI, blue) in cocultures of FACS-sorted, mock vector transduced Wt (Wt + Mock), p62-deficient (p62^{-/-} + Mock) or p62 transduced- p62-deficient (p62^{-/-} + p62) Ob (DAPI, asterisks) cultured with unlabeled MΦs (DAPI, small nuclei with condensed chromatin) for 24 hours. (B) Representative immunoblot of total NFκB p65 in post-culture isolated Ob cell lysates from (A). β-actin was used as a loading control. (C) DNA-bound NF-κB p65 in lysates of the nuclear fraction of Ob cells isolated post-culture from (B). (D) Immunoblot of phosphorylated IκBα and total IκBα expression in the cytosolic fraction of cells from (B). (E) Change (fold) in Runx2 mRNA expression of transduced Ob after 24 hours of coculture with Wt MΦ. Values are derived from three independent experiments. For all panels, values represent mean ± SEM. *p<0.05; **p<0.01.

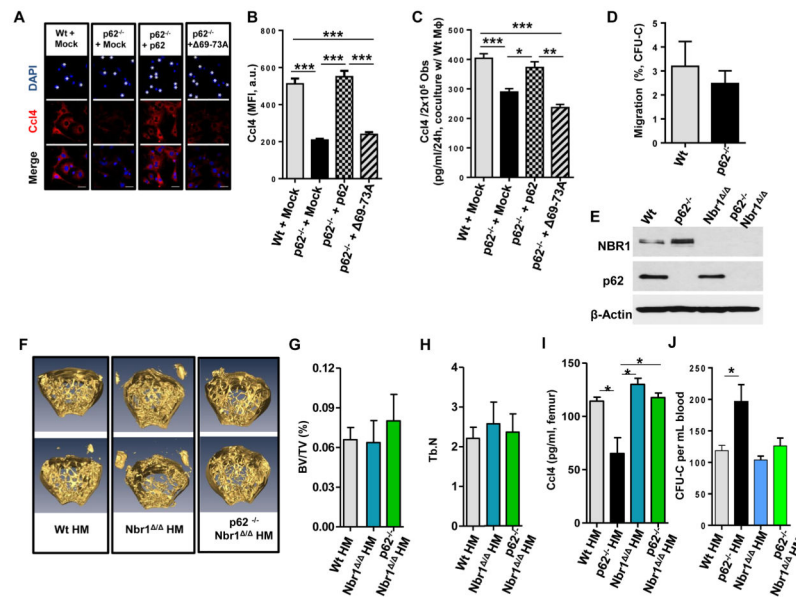


Figure 6. p62 oligomerization is required for Ccl4 expression and a double deficiency of p62 and Nbr1 rescues Ccl4 expression and HP mobilization in vivo

(A) Representative confocal microscopy images of Ccl4 (red) and nuclear counterstaining (DAPI, blue) in mock vector transduced Wt (Wt + Mock), p62-deficient (p62^{-/-} + Mock), p62 transduced- p62-deficient (p62^{-/-} + p62) or deletion of 69-73A in p62, disrupting PB1-mediated p62 oligomerization, transduced-p62-deficient (p62^{-/-} + 69-73A) Ob (DAPI, asterisks) after coculture for 24 hours with Wt MΦ (DAPI, small nuclei with condensed chromatin). (B) MFI of Ccl4 expression measured in (A). Values are derived from three independent experiments. (C) Secreted Ccl4 concentration from 2×10⁵ sorted, retrovirally transduced Obs after 24 hours culture in contact with Wt MΦ. Values represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001. (D) Chemotaxis of CFU-C (%) towards Ob and Wt MΦ supplemented with Cxcl12 and bafilomycin A1 or vehicle (control). (E) Representative immunoblot confirming the protein expressions of Nbr1 and p62 in Ob from p62^{-/-}, Nbr1^{Δ/Δ}, or p62^{-/-}Nbr1^{Δ/Δ} mice. (F) Representative micro-CT analyses of femoral trabecular bone of Wt HM, Nbr1^{Δ/Δ} HM or p62^{-/-} Nbr1^{Δ/Δ} HM mice at 16 weeks post-transplantation. (G and H) Percent ratio of trabecular bone volume versus tissue volume (BV/TV) and trabecular number (Tb.N) of the Wt HM, Nbr1^{Δ/Δ} HM or p62^{-/-} Nbr1^{Δ/Δ} HM mice analyzed in (F). (I) BM extracellular levels of Ccl4 from Wt, p62^{-/-}, Nbr1^{Δ/Δ}, or p62^{-/-} Nbr1^{Δ/Δ} mice (n=12 mice per group). (J) CFU-C contents in the PB of Wt HM, p62^{-/-}, Nbr1^{Δ/Δ} HM, or p62^{-/-} Nbr1^{Δ/Δ} HM mice (n=4–7 mice per group).