Siah1a has been implicated in numerous signaling pathways because of its ability to induce ubiquitin-mediated degradation of many protein substrates. Siah1a knockout mice are growth-retarded, exhibit early lethality, and display spermatogenic defects. In this study we identified a striking low bone phenotype in these mice (trabecular bone volume was halved compared with wild type mice), linking Siah1a to bone metabolism for the first time. Markers of bone formation, including osteoblast numbers and osteoid volume, were decreased by up to 40%, whereas the number of osteoclasts was more than doubled in Siah1a mutant mice. However, ex vivo osteoclast formation occurs normally and hematopoietic osteoclast progenitor cell types were present in normal numbers in Siah1a mutant mice. Moreover, adoptive transfer of Siah1a mutant bone marrow into wild type mice failed to reproduce the osteopenia or increased osteoclast numbers observed in mutant mice. Although ex vivo osteoclast colony formation was normal in Siah1a mutant mice, mineralization from these cells was elevated in cultures from Siah1a mutant mice, which may explain the reduction in osteoid volume seen in vivo. These findings suggest that although Siah1a is clearly essential for normal bone metabolism, the bone defect in Siah1a mutant mice is not due to cell-autonomous requirements for Siah1a in osteoblast or osteoclast formation. We propose that bone metabolism defects in Siah1a mutant mice are secondary to an alteration in an unidentified systemic, paracrine, or metabolic factor in these mice.

Ubiquitination is increasingly recognized as an important regulatory mechanism in diverse cellular processes. Tagging proteins with chains of Lys 48-linked ubiquitin induces their rapid degradation via the proteasome. It is therefore essential that the process of selecting substrates for ubiquitination is tightly controlled. One family of proteins that regulates this selection are Siah proteins, which are members of the RING domain-containing family of E3 ubiquitin ligase enzymes. Mice mutant for these proteins display spermatogenic defects. In this study we identified a significant skeletal defect in Siah1a mutant mice. These mice exhibit high numbers of osteoclasts with low numbers of osteoblasts, resulting in severe osteopenia (low bone volume). We present evidence that this phenotype is not due to cell autonomous requirements for Siah1a in osteoclast or osteoblast differentiation. Furthermore, analysis of the hematopoietic system also supports the conclusion that growth defects in Siah1a mutant mice are not due to a cell autonomous requirement for Siah1a in cellular proliferation.

EXPERIMENTAL PROCEDURES

Antibodies—Unless otherwise stated, antibodies were from BD Pharmingen. The following anti-mouse antibodies were used in this study: anti-B220 (RA3-6B2), anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD28 (37.51), anti-CD40 (3/23), anti-CD45.2 (104), anti-F4/80 (Coltage), anti-Gr-1 (RB6–8C5), anti-IgM (Fab'), fragments (ICN), and anti-Mac-1 (M1/70).

Mice—Siah1a−/− mice were generated as described previously (5). Littermate controls were used for all experiments. All animal work was approved by an institutional ethics committee in compliance with National Health and Medical Research Council (Australia) guidelines.

Bone Histology and Histomorphometry—Tibiae and femora were collected for histology and histomorphometric analyses at 7–8 weeks of age. After fixation overnight in 4% paraformaldehyde, legs were x-rayed (Faxitron, Wheeling, IL) for measurements of bone length and width using ImageJ 1.23 analysis software as described previously (6). Tibiae were processed into methylmethacrylate for histomorphometric analysis of undecalcified bone by standard protocols in the secondary spongiosa of the proximal tibial metaphysis using the Osteomeasure image analysis system (Osteometrics, Decatur, GA), with the size of the region corrected for bone size as described previously (6).
sions were prepared from spleen or lymph nodes (mesenteric, inguinal, and cervical) by organ separation through a wire mesh and passing through a 40 μm cell strainer (BD Biosciences). Red blood cells were removed from spleen suspensions by incubation for 5 min at room temperature in red cell lysis buffer (0.15 M NaCl, 1 M KCl, 0.1 mM EDTA, pH 7.2), followed by two washes in phosphate-buffered saline, 2% fetal bovine serum. Viable resting splenic B cells were purified by fluorescence-activated cell sorter (FACStar, BD Biosciences) on the basis of low forward and side scatter, exclusion of propidium iodide, and failure to stain for CD4 and CD8. Lymph node T cells were purified using nylon wool columns. Nylon wool (Robbins Scientific Corp.) was soaked overnight in 0.2 M HCl and washed with water until neutral pH was obtained. Approximately 1–2 g (dry weight) of wool was packed into the barrel of a 10-ml syringe and autoclaved. Prior to use, freshly washed with 20 ml each of 0.9% saline, Dulbecco’s modified Eagle’s medium, and growth medium. Lymph node cell suspensions were loaded onto the column in a volume of 4 ml, allowed to flow through, and washed with 1 ml of medium. The column was closed, and a further 2 ml of medium was added prior to incubation at 37°C for 45 min. Non-adherent cells (T cells) were eluted from the column in a volume of 13 ml. B cells and T cells were typically enriched by these procedures to greater than 95% purity based on B220 and CD4/CD8 staining of purified cells. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 250 μM 1-asperagine, 13 μM folic acid, penicillin (50 I.U/ml), streptomycin (500 μg/ml), and 50 μM β-mercaptoethanol. Cultures were initiated in 96-well plates (100 μl/well) at starting cell densities of 5 × 10^4 cells/ml and mitogenically stimulated by the addition of lipopolysaccharide (Escherichia coli 0111: B4, Sigma), anti-IgM or anti-CD40 antibodies, or concanavalin A (Sigma) or by plating in wells that had been coated for several hours with anti-CD5 plus or minus anti-CD28 antibodies. After 2 days, 0.5 μCi of [3H]thymidine (30 Ci/mmol, Amersham Biosciences) was added for the final 16 h of culture before harvesting of the cells onto glass filter paper with several washes of water (Filtermate 196 Harvester, Packard). Incorporated radioactivity was determined by scintillation counting using ReadySafe scintillant (Beckman-Coulter) and a Tri-car 2100TR liquid scintillation analyzer (Packard).

**Adoptive Transfer Experiments**—For competitive reconstitution experiments, 5 × 10^5 whole bone marrow cells from femurs of wild type or Siah1a−/− mice (CD45.2 allotype) were injected intravenously along with 5 × 10^5 whole bone marrow cells from a B6.SJL-Ptprc−/- mouse (CD45.1 allotype) into recipient B6.SJL-Ptprc−/- mouse (Animal Resources Centre, Canning Vale, Australia) that had been γ-irradiated (5 Gy followed 3 h later by 4.5 Gy, 137 Cs source, 0.75 Gy/min). Flow cytometry was conducted 15 weeks after injection. For analysis of bones after adoptive transfer, 2 × 10^6 whole bone marrow cells from femurs of wild type or Siah1a−/− mice were injected into recipient B6.SJL-Ptprc−/- mice as described above. Bones were harvested 8 weeks after injection, and flow cytometry analysis of bone marrow using anti-Mac-1 and anti-CD45.2 antibodies demonstrated that more than 99% of myeloid cells were derived from injected marrow.

**RESULTS**

**Osteopenia in Siah1a Mutant Mice**—While flushing bone marrow samples from femurs, we noticed that bones from Siah1a mutant mice appeared weaker than those from wild type mice. This prompted an investigation of the skeletal system of Siah1a−/− mice.

Femurs from Siah1a−/− mice were significantly shorter and narrower than those from wild type littermate controls, consistent with overall growth retardation of these mutants (Fig. 1, A and B) (5). However, although growth plate width, an indicator of chondrocyte proliferation, was slightly elevated in Siah1a−/− mice, suggesting a delay in growth plate maturation, this did not reach statistical significance (mean growth plate width (μm) ± S.E. in 7–8-week-old mice: wild type, 107 ± 7; Siah1a−/−, 142 ± 22, p = 0.25; n = 6/group).

Histological sectioning of tibiae revealed a striking low trabecular and cortical bone volume (osteopenic) phenotype in Siah1a mutant mice (Fig. 1C). Histomorphometric quantitation of undecalciﬁed tibial sections demonstrated thinner cortical bone (Fig. 1D), fewer and more dispersed trabeculae (Fig. 1E and F), and decreased trabecular thickness and volume (Fig. 1, G and H) in Siah1a−/− compared with wild type tibiae.

Although the activities of osteoblasts and osteoclasts are usually coupled in vivo such that bone formation and bone resorption are co-regulated (11) and trabecular bone volume is maintained at an appropriate level for mechanical strain, in the Siah1a mice, these activities seem to be dissociated. Histomorphometry revealed a low level of bone formation and low osteoblast numbers with a high level of osteoclastogenesis, leading to the osteopenia described above. Tibiae from Siah1a−/− mice have ~40% fewer osteoblasts per unit of bone perimeter and bone surface than wild type mice (Fig. 2, A and B). Osteoblasts form new bone by first secreting an extracellular matrix, termed osteoid, and then participating in the mineralization of this matrix. The thickness and volume of osteoid is a measure of the rate of osteoblastic bone formation. Consistent with low osteoblast numbers, Siah1a−/− mice also display low osteoid volume and thickness (Fig. 2, C and D), but fluorochrome-derived markers of bone formation were not sig-

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1 The abbreviations used are: RANKL, receptor activator of nuclear factor-κB ligand; BMP, bone morphogenetic protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1, interleukin; SCF, stem cell factor; TRAP, tartrate-resistant acid phosphatase; TGF-β, transforming growth factor-β; Gy, gray.
2.8 mg/dl; sorption showing number of osteoclasts per mm of bone perimeter

Vivo Osteoclast Formation in Siah1a Mutant Mice
—
oclasts are derived from hematopoietic precursors, we inves-
tigate whether altered hematopoiesis might account for in-
creased osteoclast numbers in Sia1a mutant mice. Siah1a−/−
mice are postnatally growth-retarded, and most organs display
a decrease in size and weight that is proportional to total body
weight (5). Consistent with reduced size, we found that spleen,
thyroid, lymph nodes, and bone marrow from Siah1a knockout
mice contained only 30–50% of the number of white blood cells
found in organs from wild type littermates (Table I). However,
a reduction in cellularity of hematopoietic organs does not
result from a change in a particular cellular lineage because

significantly altered (data not shown), possibly because of the high
level of bone resorption, which may have masked any effect on
these markers incorporated into the bone matrix during bone
formation. In contrast to the low level of bone formation, how-
ever, Siah1a−/− tibiae displayed more than double the number
of osteoclasts per unit of bone perimeter and bone surface than
wild type tibiae (Fig. 2, E and F). To investigate whether de-
fects in osteoblast and osteoclast number was related to altered
calcium homeostasis at the systemic level, serum calcium was
measured, but it was not altered in Siah1a−/− (10.7 ± 1.1 mg/dl; n = 6) compared with wild type mice (11.9 ±
2.8 mg/dl; n = 5).

Normal Osteoclast Precursor Populations and Normal ex
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a reduction in cellularity of hematopoietic organs does not
result from a change in a particular cellular lineage because

the percentages of cells in each tissue expressing T cell markers
(CD4, CD8), B cell markers (B220, IgM, IgD), and granulocyte
and monocyte markers (Gr-1, Mac-1, F4/80) were unchanged in
Siah1a−/− mice (Table I). Lymphocyte proliferation in vitro
in response to a range of mitogens is unaffected by loss of Siah1a
(Fig. 3, A and B), and wild type and Siah1a−/− bone marrow
cells competed equally well with a reference bone marrow in
the generation of T cell, B cell, and granulocyte lineages in a competitive hematopoietic adoptive transfer assay (Fig. 3C). These findings demonstrate that reduced overall hematopoietic cellularity of Siah1a−/− mice is unlikely to result from a cell-autonomous proliferative requirement for Siah1a.

Osteoclasts differentiate from bone marrow macrophages that are characterized by expression of Mac-1 and an intermediate level of expression of F4/80 (Mac-1+/− F4/80int) (12). Precursors of these cells, termed pre-osteoclasts, are enriched in a myeloid population of the bone marrow that does not express Mac-1 and also exhibits intermediate F4/80 staining (Mac-1− F4/80int) (12). Flow cytometry analysis showed that wild type and Siah1a−/− bone marrow contain similar percentages of cells that are Mac-1− F4/80int and Mac-1+ F4/80int (Table 1), suggesting that the steady state distributions of the most immediate osteoclast precursors are not altered in Siah1a−/− mice. More primitive osteoclast progenitor cells are present in hematopoietic colonies elicited by M-CSF and GM-CSF (13). In contrast to the expanded numbers of myeloid precursors observed in Siah2−/− mice (4), bone marrow from Siah1a−/− mice yielded the same number of colonies as bone marrow from wild type mice in response to all cytokine stimulations (Fig. 4A). This indicates that the increased number of osteoclasts in Siah1a−/− mice does not result from increased numbers of osteoclast progenitors.

To directly assay osteoclast formation from osteoclast precursors, osteoclast differentiation from bone marrow cells was induced in vitro by M-CSF and soluble RANKL-glutathione S-transferase fusion protein (7). No significant differences in osteoclast numbers were noted between wild type and Siah1a−/− cultured bone marrow (Fig. 4B). This finding is consistent with the lack of increased osteoclast progenitors noted above, and furthermore it suggests that Siah1a does not function as a cell-autonomous inhibitor of osteoclast differentiation.
gerin, vitamin D, M-CSF, Wnt, insulin-like growth factor, fibroblast growth factor-2, estrogen, androgens, TGF-β, BMP-2, activin A, tumor necrosis factor-α, IL-1, IL-12, interferon-β and -γ, osteonectin, and cadherin-11) regulate osteoblast and osteoclast differentiation and function (reviewed in Ref. 14). The possibility cannot be excluded that Siah1a is required for interpretation of a specific signal that we have not reproduced in our ex vivo assays.

To investigate whether the increase in osteoclasts in Siah1a−/− mice results from inappropriate behavior of Siah1a mutant cells in response to normal osteoclast differentiation signals, wild type or Siah1a mutant bone marrow cells were transplanted into irradiated wild type recipient mice in adoptive transfer experiments. Osteoclasts are derived from hematopoietic precursors and are thus transplantable by injection of whole bone marrow cells, whereas osteoblasts derive from mesenchymal stem cells and are not transplantable under these conditions. Eight weeks after transplantation, more than 99% of myeloid cells in the bone marrows of all recipients were derived from donor mice (data not shown), confirming successful adoptive transfer.

No significant alteration in the markers of bone mass (Fig. 5, A and B) or osteoblast (Fig. 5C) or osteoclast numbers (Fig. 5D) were detected following histomorphometric analysis of tibiae from mice that received Siah1a−/− bone marrow, indicating that the factor determining altered bone formation and resorption in Siah1a−/− mice is not derived from bone marrow cells. In contrast, systemic factors or mesenchymal cells may be responsible for this unusual phenotype of increased osteoclastogenesis in the presence of reduced bone formation.

**DISCUSSION**

This study demonstrates that in addition to growth, survival, and spermatogenic defects (5), Siah1a mutant mice exhibit skeletal abnormalities. Although the dynamics at the growth plate of long bones appear normal, bone remodeling is strikingly altered. Siah1a−/− mice exhibit osteopenia characterized by thinner cortical bone and fewer and smaller trabeculae. The cellular basis of this phenotype appears clear. Markers of bone formation, including osteoblast number and osteoid thickness and volume, are decreased by up to 40%, whereas the number of osteoclasts is more than doubled in Siah1a mutant mice. It is unlikely that the altered bone turnover described in these mice is merely a result of the reduced bone size, because the anatomical region used to measure trabecular bone mass is corrected for bone size, and other mice with similar or more dramatic dwarfism do not demonstrate osteopenia as a matter of course (6). To our knowledge, the only other published knock-out with reduced bone formation and increased bone resorption is the MT1-MMP knockout mouse (15). However, although grossly similar, the detailed phenotype of the MT1-MMP knockout mouse is quite different, with notable periosteal fibrosis, reduced mineral apposition, significant arthritis, and reduced growth plate width. None of those markers are detected in the Siah1a mice, so it appears unlikely that these phenotypes are related.

Although Siah1a mutant mice exhibit an osteopenic phenotype, the molecular basis of the role of Siah1a in bone remodeling is less clear. Based on the results of other studies, it is possible to suggest several models by which Siah1a may participate in the regulation of osteoclast and osteoblast function. Firstly, receptors that mediate the differentiation and activation of osteoclasts, including RANK, tumor necrosis factor receptor, and IL-1 receptor, transduce their signals through TRAF family proteins (16). It is therefore noteworthy that Siah1a shares structural homology with TRAF proteins and that overexpression of Siah1a enhances TRAF2-mediated signaling induced by tumor necrosis factor-α (17). Secondly, overexpression of Siah1a induces the degradation of TIEG-1, a protein that is induced by signaling by TGF-β and bone morphogenetic proteins (BMPs) (18). BMPs control osteoblast proliferation and differentiation (19), and TGF-β has been implicated in the regulation of both osteoblast and osteoclast production (7, 20). Because TIEG-1 promotes signaling by TGF-β and BMPs by down-regulating negative feedback through Smad7 (21), Siah1a may provide an additional layer of regulation of signaling by these factors in osteoclasts and/or osteoblasts. Thirdly, signaling via the Wnt-β-catenin pathway has been implicated in the activation of osteoblast proliferation and bone matrix deposition (22). Siah1a may have an impact on such a pathway through its function as the rate-limiting component of a novel E3 ubiquitin ligase complex that targets β-catenin for degradation (23, 24), but such a role would predict increased osteoblast activity in Siah1a mutant mice.

These models propose that Siah1a functions cell-autonomously to regulate signaling pathways that control osteoblast and/or osteoclast differentiation and activities. However, our assays did not support such cell-autonomous models. Siah1a mutant mice exhibit a dramatic increase in osteoclast numbers in vivo, yet osteoclast generation in ex vivo bone marrow cultures from Siah1a mutant and wild type mice were equivalent. Thus, at least under in vitro conditions, Siah1a is dispensable for osteoclast differentiation. Although Siah1a mutant mice exhibit a decrease in osteoblast numbers in vivo, in vitro osteoblast colony formation and mineralization assays using bone marrow or calvariae from newborn mice as the source of osteoblast precursors showed elevated mineralization but no difference in osteoblast proliferation. Moreover, adoptive transfer experiments, in which hematopoietic cells (which include osteoclast progenitors) from Siah1a mutant mice were provided in the complex in vivo environment of a wild type mouse, failed to reproduce the enhanced osteoclast number and the low bone density phenotype observed in Siah1a mutant mice.

These findings argue that Siah1a is not required for interpretation of the normal autocrine or paracrine signals that regulate osteoclast or osteoblast differentiation because resident cells of bone were unable to recapitulate the in vivo phenotypes ex vivo. We suggest that the osteopenic phenotype in
Siah1a mutant mice reflects an alteration in an unidentified, possibly systemic factor that induces secondary effects on bone metabolism through the regulation of osteoclast and osteoblast activities. This factor may be a known systemic calcitropic hormone such as parathyroid hormone, calcitonin, or 1,25-dihydroxyvitamin D₃.

Bone mass and bone remodeling are also regulated at the local level by the coordinated actions of osteoblasts and osteoclasts within “basic multicellular units” in which each cell type regulates the differentiation and activity of the other. It is possible that this phenotype reflects a change in local communication between these cell types by altered release of, or altered sensitivity to, factors from the opposing cell type. This issue could be addressed by co-culture experiments, which unfortunately were precluded because of the low fertility and high perinatal mortality of the Siah1a mutant mice (5).

Consistent with a role for systemic or locally derived factors in the observed phenotype, we also present evidence that the growth retardation phenotype of Siah1a mutant mice is unlikely to result from a cell-autonomous requirement for Siah1a in somatic cell proliferation or survival. Despite Siah1a mutant mice having only 30–50% of the number of hematopoietic cells as wild type mice, Siah1a mutant lymphocytes proliferate normally in vitro, and Siah1a mutant bone marrow competes as effectively as wild type bone marrow in a competitive adoptive transfer setting.

The nature of the factor(s) that might induce such non-cell-autonomous phenotypes in Siah1a mutant mice is unknown. Circulating levels of bone growth hormone, testosterone, follicle-stimulating hormone, and luteinizing hormone are all normal in Siah1a mutant mice (5). We have also performed microarray comparisons of RNA prepared from spleen, thymus, kidney, and livers from wild type and Siah1a mutant mice, but to date we have found no convincing candidate gene product that may explain the phenotypic characteristic of the Siah1a bone cells (data not shown). The involvement of the central nervous system in this action also cannot be excluded, as contributions of the central nervous system in the regulation of bone mass have recently been proposed for leptin (25). Because Siah1a is an intracellular protein responsible for targeting proteins to the proteasome for degradation, we speculate that loss of Siah1a may result in aberrant secretion of an unknown factor from a central source (endocrine organ or central nervous system) that impacts on both osteoblasts and osteoclasts to alter normal bone homeostasis.

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