Proportionate Dwarfism in Mice Lacking Heterochromatin Protein 1 Binding Protein 3 (HP1BP3) Is Associated With Alterations in the Endocrine IGF-1 Pathway

Benjamin P. Garfinkel, Shiri Arad, Phuong T. Le, Michael Bustin, Clifford J. Rosen, Yankel Gabet,* and Joseph Orly*

Department of Biological Chemistry (B.P.G., S.A., J.O.), The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel; Center for Clinical and Translational Research (P.T.L., C.J.R.), Maine Medical Center Research Institute, Scarborough, Maine 04074; Protein Section (M.B.), Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and Department of Anatomy and Anthropology (Y.G.), Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239, Israel

Heterochromatin protein 1 binding protein 3 (HP1BP3) is a recently described histone H1-related protein with roles in chromatin structure and transcriptional regulation. To explore the potential physiological role of HP1BP3, we have previously described an *Hp1bp3*−/− mouse model with reduced postnatal viability and growth. We now find that these mice are proportionate dwarfs, with reduction in body weight, body length, and organ weight. In addition to their small size, microcomputed tomography analysis showed that *Hp1bp3*−/− mice present a dramatic impairment of their bone development and structure. By 3 weeks of age, mice of both sexes have severely impaired cortical and trabecular bone, and these defects persist into adulthood and beyond. Primary cultures of both osteoblasts and osteoclasts from *Hp1bp3*−/− bone marrow and splenocytes, respectively, showed normal differentiation and function, strongly suggesting that the impaired bone accrual is due to noncell autonomous systemic cues in vivo. One major endocrine pathway regulating both body growth and bone acquisition is the IGF regulatory system, composed of IGF-1, the IGF receptors, and the IGF-binding proteins (IGFBPs). At 3 weeks of age, *Hp1bp3*−/− mice exhibited a 60% reduction in circulating IGF-1 and a 4-fold increase in the levels of IGFBP-1 and IGFBP-2. These alterations were reflected in similar changes in the hepatic transcripts of the *Igf1*, *Igfbp1*, and *Igfbp2* genes. Collectively, these results suggest that HP1BP3 plays a key role in normal growth and bone development by regulating transcription of endocrine IGF-1 components. (Endocrinology 156: 4558–4570, 2015)

We have recently described a mouse model with reduced postnatal viability and growth observed upon targeted deletion of the histone H1-like gene heterochromatin protein 1 binding protein 3 (*Hp1bp3*) (1). Growth in mammals generally begins in early embryogenesis and continues postnatally until a typical species-specific size is attained. During postnatal growth, there is a proportionate increase in the size and weight of organs, as well as linear and radial growth of bones. Although linear growth of bones determines stature, radial growth (ie, thickening) of bones is critical for their ability to function in giving the organism mechanical stability and strength.
(2). Childhood and adolescence is a particularly important time to maximize bone acquisition because the skeleton undergoes rapid changes owing to the processes of growth, modeling, and remodeling (3, 4). The peak bone mass achieved at this stage is a key factor in bone health later in life. In humans, by the fifth decade of life, a process of inexcorable bone loss begins, potentially leading to osteoporosis (5, 6). Therefore, information learned from studies on the mechanisms regulating bone acquisition during postnatal growth periods is of considerable importance in developing strategies to postpone or reduce the risk of osteoporotic fractures.

Studies of human populations have demonstrated a strong genetic component in the determination of peak bone mass, a phenomenon observed in mouse models as well (7–9). One such genetic regulatory pathway with key roles in postnatal growth and bone acquisition is the GH and IGF-1 axis (10, 11). GH secreted from the pituitary induces IGF-1 expression in the liver, and this is the major source of systemic IGF-1. However, the dependence of IGF-1 expression on GH in mice does not begin immediately after birth as suggested by studies with GH or GH receptor-deficient mice. In such animals, no growth retardation is observed before 2 weeks of age (12–14), despite the critical role of IGF-1 in early postnatal growth and bone development, which has been described in both mouse models (15–18) and in humans (19–21).

In addition to its regulation at the transcriptional level, much of the biological activity of IGF-1 is modulated by the IGF-binding protein (IGFBP) family (22–24). These proteins bind to IGF-1 with high affinity and play a complex role in its regulation. On the one hand, binding to IGFBPs can stabilize IGF-1 in the plasma, increase its half-life, and provide local binding sites in the extracellular matrix (25). On the other hand, IGF-1 bound to an IGFBP may be unavailable to receptors, and thus inactive. In addition to their role in regulating IGF-1 function, IGFBPs can also act independently, directly mediating cellular effects (25). Studies in humans and in mouse models have shown that the different IGFBPs have complex and crucial functions in growth and bone development (26, 27).

We now find that HP1BP3 deficiency in mice leads to severe proportionate dwarfism and low bone mass, which begin at birth and persist into adulthood and old age. We further show that the impaired growth is probably due to severe alterations in circulating levels IGF-1 and IGFBP-1-2-3 that correlate with altered hepatic transcription of these genes.
were calculated as predictors of the potential effect of changes in cortical parameters on biomechanical strength. pMOI and aMOI are proportional to the torsional and bending forces leading to bone fracture, respectively (28).

**Histomorphometric analysis**

After μCT image acquisition, the specimens were dehydrated in progressive concentrations of ethanol, cleared in xylene, and embedded in low-temperature polymethylmethacrylate embedding system (Technovit 9100; Heraeus Kulzer). Undeplasticized longitudinal 5-μm sections from the center of each bone were left unstained for dynamic measurements. To identify osteoclasts, additional sections were deplasticized and submitted to tartrate-resistant acid phosphatase (TRAP) histochemical procedure (Sigma). Histomorphometric analysis was performed using Image-Pro Discovery software (Media Cybernetics; Silver Spring). The measurements, terminology, and units used for the histomorphometric analysis were according to the convention of standardized nomenclature.

**Primary osteoblast culture**

Bone marrow cells were harvested from femurs and tibias of 4- to 8-week-old Hp1bp3−/− and Hp1bp3+/+ control mice and plated at 20 × 10⁶ cells per well on 6-well plates in α-MEM and 10% fetal calf serum. Media were changed on day 1 and day 3. Fibroblast colony-forming units (CFUs) were assessed by crystal violet staining at day 6. Differentiation media containing 10% fetal calf serum. Media were changed on day 1 and day 3. For the differentiation assay, adherent cell number was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. For the differentiation assay, adherent cells were stained using TRAP kit (Sigma), and the number of multinucleated TRAP-positive cells was counted.

**Primary osteoclast culture**

Splenocytes were extracted from 1-day-old Hp1bp3−/− and Hp1bp3+/+ control pups, and preosteoclasts were obtained after 3 days of culture in nontissue-culture treated 12 wells in “standard” medium (α-MEM, 10% fetal bovine serum, and 1% penicillin/streptomycin) supplemented with 100-ng/mL macrophages colony-stimulating factor (M-CSF). For the proliferation assay, preosteoclasts were grown for 3 days in this medium, and cell number was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. For the differentiation assay, adherent preosteoclasts were plated in 96-well plates with standard medium supplemented with 20-ng/mL M-CSF and 100-ng/mL receptor activator for nuclear factor κB ligand. On day 6, multinucleated osteoclasts were stained using TRAP kit (Sigma), and the number of multinucleated TRAP-positive cells was counted.

**Blood biochemistry analysis**

Blood collected by cardiac puncture was allowed to clot in blood collection tubes (BD), then centrifuged (6000g, 5 min at 4°C), and serum was separated. Serum concentrations of the amino-terminal propeptide of type I procollagen (P1NP), cross-linked C-telopeptide (CTX), and IGF-I were measured using commercially available kits from IDS according to the manufacturer’s instructions. The assay sensitivities were 0.7, 2, and 2.8 ng/mL for P1NP, CTX, and IGF-I, respectively. The intraassay variations were 6.3%, 6.9%, and 5.8%, and the interassay variations were 8.5%, 12%, and 7.8%, respectively. The serum concentration of IGFBPs was measured using a mouse IGFBP magnetic bead panel (Millipore) according to the manufacturer’s instructions. The assay sensitivities (ng/mL), intraassay variations (%), and interassay variations (%) for each of the binding proteins were: IGFBP1: 0.037, 1.7, 6.9; IGFBP2: 0.018, 1.6, 6.3; IGFBP3: 0.039, 1.4, 4.7; IGFBP5: 0.321, 1.8, 6.9; IGFBP6: 0.063, 1.2, 7.9; and IGFBP7: 0.025, 2.0, 15.6.

**Quantitative real-time RT-PCR**

Livers, kidneys, and tibiae of 3- and 8-week-old mice were collected and snap frozen in liquid nitrogen. RNA was extracted using Tri-Reagent (Sigma). Bones were homogenized with a Mini-bead-beater (Biospec Products, Inc) in 2-mL screw-cap tubes prefilled with 3-mm zirconia beads (Benchmark Scientific). Livers and kidneys were homogenized in Dounce homogenizers. Samples containing 2 μg of RNA were converted to cDNA in a reverse transcription reaction using the RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific) and oligo-dT primers. Igfl1 was analyzed using the forward primer 5′-TACAAAGACGGACCCGCTCTA-3′ and the reverse primer 5′-TCTCCCTTG-CAGCTTGTTT-3′. For Igfbp1, the primers used were forward 5′-CCCTGCAACAGAACTCTA-3′ and reverse 5′-TCTC- CATCCAGGGATGTCTC-3′. Igfbp2 was amplified with the forward primer 5′-GCCCTGGAACATCCTCTAC-3′ and the reverse primer 5′-GGATTGGGTTCCACACCC-3′. For Igfbp3, the primers used were forward 5′-CTGCTTGTTGTGGG-TCAAGT-3′ and reverse 5′-CCAGCTGCTGATCTGTGTTG-3′. The mitochondrial protease Yme1 was used as a reference gene, and was amplified with the forward primer 5′-TCAATCAGGCAATT-GACAG-3′ and the reverse primer 5′-CAATGCTTCTGCAGGT-3′. The real-time PCR was carried out using the KAPA SYBR Fast kit (KAPA Biosystems) for 40 cycles.

**Antibodies**

The anti-HP1BP3 antibody was created by injecting guinea pigs with the peptide STRETPKSKLAEGEEEKPEPD-C corresponding to amino acids 47–68 of the murine protein as previously described (1). Peptide synthesis, keyhole limpet hemocyanin conjugation and immunization of guinea pigs were carried out by Peptide Specialty Laboratories GmbH. Antitubulin (T5168) was purchased from Sigma-Aldrich. Anti-Signal Transducer and Activator of Transcription 5 (STAT5) (sc-835) was from Santa Cruz Biotechnology, Inc, and the antiphospho-STAT5 antibody (ab32364) was from Abcam (see Table 1).

**Tissue lysis and SDS-PAGE**

Livers of 3-week-old mice were extracted, washed twice in PBS, minced, and 100 mg were homogenized in 0.5-mL radioimmunoprecipitation assay buffer (containing protease inhibitors and NaCl, NaF, and sodium pyrophosphate) in Dounce homogenizers. Extracts were then left on ice for 30 minutes, followed by 10 minutes of centrifugation at 10 000g. The supernatant was separated from pellet and fat and centrifuged again. This was repeated until the sup was cleared, at
which point it was resuspended in sample loading buffer. Total protein in each sample was quantified using the Bradford assay. Fifteen micrograms protein from each sample were separated by electrophoresis in 10% polyacrilamide gels and further prepared for Western blot analysis as described previously (1).

Statistical analysis
Mice were grouped according to age, sex, and genotype. Differences between genotypes, lines, ages, or genders at selected times were determined by unpaired Student’s t tests. Bivariate correlations were performed with Pearson’s product moment correlation. All results were expressed as mean ± SD.

Results
Severe dwarfism and low bone mass in 3-week-old Hp1bp3−/− mice
Both male and female Hp1bp3−/− mice that survive to weaning have a normal lifespan (1). However, we have shown that at birth they are somewhat smaller than their littermates and weigh 10% less (1). The growth retardation of the Hp1bp3−/− mice is notably exacerbated postnatally (Figure 1A) and continues during the first 3 weeks of life in both males and females. There was a more pronounced weight difference in females (Figure 1, B and C) and beyond 3 weeks of age, Hp1bp3−/− mice grow at a rate similar to that of control littermates but remain smaller than them (Figure 1, B and C). Because the most severe effect was seen in 3-week-old mice, we explored this age in more depth. At 3 weeks, females and males weigh 44% and 36% less, respectively, than their WT littermates (both \( P < .0001 \)) (Figure 1D). The weights of hearts, kidneys, and spleens were measured as representatives of internal organs and found to be proportionally reduced in the Hp1bp3−/−.
mice (Figure 1, E1–G2), other than the spleen that remained substantially smaller after normalizing to body weight in the HP1BP3-deficient mice (Figure 1G2). Because body weight does not necessarily reflect longitudinal skeletal growth, the lengths of the body, tail, and femur were determined. At 3 weeks of age, body length of Hp1bp3−/− female and male mice (nose to anus) was reduced by 19.2% and 12%, respectively, when compared with that of WT siblings (both \( P < .005 \)) (Figure 1H). Tail length was reduced by 20.2% (\( P < .05 \)) for females and 13.1% (\( P < .01 \)) for males (Figure 1I). Finally, the femoral length of Hp1bp3−/− mice was significantly decreased by 16.2% (\( P < .01 \)) and 9.3% (\( P < .01 \)) compared with WT mice.

In light of the observed reduction in linear skeletal growth, the effects of Hp1bp3 knockout on cortical and trabecular bone were evaluated by subjecting femora to \( \mu \)CT (Figure 2A). Both female and male Hp1bp3−/− mice presented a substantial reduction in mid-diaphyseal diameter measuring 22% and 14.3% less than WT, respectively (\( P < .01 \)) (Figure 2B, C and D). The Cort.Th was also reduced by 44% and 28% in females and males, respectively (\( P < .05 \)) (Figure 2C, D and E). The effect of the changes in bone diameter and thickness on the bone strength was assessed by calculating the pMOI and aMOI, 2 parameters correlated with the biomechanical properties of cortical bone (28). The pMOI showed a striking 72.4% decrease in Hp1bp3−/− females relative to femurs from control WT mice (\( P < .01 \)) (Figure 2D). Male femurs were also severely compromised, with a 52% decrease in pMOI (Figure 2D). The aMOI was similarly reduced by 67.3% and 46.1% in males and females, respectively (\( P < .01 \)) (data not shown).

Finally, trabecular bone in HP1BP3-deficient mice was also severely impacted, and female Hp1bp3−/− mice had 62.7% (\( P < .05 \)) lower BV/TV (Figure 2, F and G) associated with lower connectivity density (data not shown). Males had reduced trabecular bone volume as well, although it did not reach statistical significance.

Dwarfism and low bone mass of Hp1bp3−/− mice persist throughout life

In view of the observation that after 3 weeks of age the growth rates of Hp1bp3−/− mice increased to levels similar to those of control littermates (Figure 1, B and C), we aimed to examine the growth and bone characteristics of 8-week-old mice. At this age, both Hp1bp3−/− female and male mice remained small, weighing 21.4% (\( P < .05 \)) and 20.5% (\( P < .01 \)) less than control littermates (Figure 3A). Body length of Hp1bp3−/− female and male mice (nose to anus) was also reduced by 9.7% (\( P < .001 \)) and 8.5% (\( P < .05 \)), respectively (Figure 3B). Tail length was proportionately reduced by 8.5% for females and 10.9% for males (\( P < .05 \)) (Figure 3C). Finally, the femoral length of Hp1bp3−/− mice was also decreased by 8.4% (\( P < .05 \)) and 7.9% (\( P < .001 \)) (Figure 3D). The difference between the genotypes in all of these parameters is smaller than that observed at 3 weeks, but still highly significant and HP1BP3-deficient mice were still easily detectable.

A close examination of femurs from 8-week-old Hp1bp3−/− females and males revealed smaller bones when compared with those of WT mice (Figure 3E). Both female and male Hp1bp3−/− mice presented reduced mid-diaphyseal diameter by 10.5% (\( P < .005 \)) and 15%, respectively (Figure 3F and I). The Cort.Th was 20% lower for Hp1bp3−/− mice of both sexes (both \( P < .01 \)) (Figure 3G). The reduced diameter and thickness lead to a striking 40% (\( P < .05 \)) lower pMOI and a 33% (\( P < .05 \)) reduction.
in aMOI of both sexes relative to femurs from WT mice (Figure 3H).

Although the effects on cortical bone were identical for males and females, the trabecular bone volume fraction measured in the distal femur was sexually dimorphic. At the age of 8 weeks, male Hp1bp3−/− femurs presented a dramatic 40.7% reduction in BV/TV (P < .001) (Figure 3, J and K), and this was attributable to thinning of trabeculae, as well as a decrease in their number and connectivity (Supplemental Figure 1, A–C). In contrast, only a nonsignificant trend for reduced BV/TV was observed in female femora (Figure 3, J and K). It should be noted that most of the difference observed between sexes was in the dense distal-most 1.5 mm of the trabecular bone (Figure 3K and Supplemental Figure 1D). When the sparser region located more proximally is analyzed separately, both males and females show similar reductions in BV/TV of 54.1% and 46.4%, respectively (both P < .005) (Supplemental Figure 1E).

Because at the age of 8 weeks the bones in mice are approaching the end of their mass accrual, and the peak bone mass attained at a young age is a significant determinant of risk for osteoporosis later in life (29), we were next interested in assessing the effect of HP1BP3 deficiency on bone mass in aging mice. To this end, we analyzed femora of 7-month-old male and female mice by μCT. Both male and female Hp1bp3−/− mice presented substantial reductions in mid-diaphyseal diameter (Figure 4A) and Cort. Th (Figure 4B), similar to those observed at 8 weeks of age. As would be expected, these deficiencies lead to a dramatically decreased pMOI (Figure 4C), suggesting that they could be of functional importance. Finally, trabecular BV/TV was also affected in a manner similar to that observed at 8 weeks. In addition to a dramatic sex-dependent difference of the bone volume in WT animals, a significant 40% reduction of the BV/TV values were noted in the Hp1bp3−/− males, whereas a smaller nonsignificant decrease of the bone volume was measured in the Hp1bp3−/− females (Figure 4D).

Changes in bone mass are the result of an imbalance between the relative activity of osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells. First, we analyzed whether the loss of HP1BP3 has a direct influence on osteoblast function. To this end, differentiation of primary osteoblasts isolated from the bone marrow of Hp1bp3−/− mice or WT control animals was induced after confluence by adding osteogenic media containing ascorbic acid and β-phospho-glycerate. Staining of these cultures after 6 days with CV showed twice as many colonies in the Hp1bp3−/− culture, suggesting a significantly higher number of progenitor cells in the bone marrow of mice lacking HP1BP3 (Figure 5, A, D, and G). No significant alterations were detected in osteoblast differentia-
tion as visualized by determining the number of ALP-positive colonies relative to the CV-stained colonies (Figure 5, B, E, and H). Mineralization of osteoblast cultures was assessed after 18 days using Alizarin red solution, which stains calcium-containing hydroxyapatite deposits. Both cultures displayed clearly visible Alizarin red-stained mineral deposits (Figure 5, C, F, and I), but the staining intensities were proportional to the number of colonies observed with the CV staining, indicating normal mineralization by Hp1bp3−/− osteoblasts.

Because ex vivo culture conditions do not necessarily mimic the physiological circumstances in vivo, levels of the bone formation marker P1NP were examined in the serum of 8-week-old Hp1bp3−/− mice and littermate WT mice. No changes were observed in circulating P1NP, indicating normal rates of global bone formation at this age (Figure 5J). In order to assess bone formation directly in femoral samples, we performed dynamic histomorphometry measurements on femurs from 8-week-old Hp1bp3−/− and WT males. Males were chosen due to the more severe trabecular phenotype observed at this age. No differences were seen in the mineralizing perimeter (Figure 5K), mineral apposition rate (Figure 5L) or bone formation rate (Figure 5M), further supporting the observation that bone formation in 8-week-old mice is not impaired.

Low bone mass can also be the result of increased resorption by osteoclasts. To examine this possibility, primary splenocytes isolated from neonatal Hp1bp3−/− mice or WT control animals were induced to undergo osteoclast differentiation by incubating in culture medium containing M-CSF and receptor activator for nuclear factor κB ligand. Staining the 6-day colonies with the osteoclast-specific TRAP showed that Hp1bp3−/− preosteoclasts do not have a higher propensity to differentiate, and there was no increase in the number of mature osteoclasts (Figure 5, N and O). Hp1bp3−/− preosteoclasts also proliferate at a rate similar to that of control cells, as assayed by MTT (Figure 5P). To assess bone resorption in vivo, we measured circulating levels of the bone resorption marker Ctx in the serum of 8-week-old mice, and found no difference between Hp1bp3−/− mice and littermate WT mice of both sexes (Figure 5Q), indicating normal global bone resorption. Furthermore, no significant difference was observed in the number of osteoclasts per bone surface in femurs of 8-week-old male Hp1bp3−/− mice relative to WT controls, as determined by TRAP staining (Figure 5R). Collectively, these results suggest that both osteoblasts and osteoclasts from HP1BP3-deficient mice have the intrinsic capacity to function normally, and the low bone mass observed in Hp1bp3−/− mice is probably not due to cell autonomous effects in these cells. The fact that at 8 weeks of age Hp1bp3−/− bones remain affected despite apparently normal bone homeostasis further suggests that they probably suffered from severe growth impairment at an earlier stage. Thus, even when they attain normal rates of bone remodeling at adulthood, the bone mass being maintained is markedly reduced.

**Bone and growth defects may be due to altered systemic IGF-1, IGFBP-1, IGFBP-2, and IGFBP-3**

The apparently normal function of HP1BP3-deficient osteoblasts and osteoclasts, together with the observation that Hp1bp3−/− mice are proportionate dwarfs, led us to suspect a systemic cause for the growth impairment and low bone mass. In this regard, IGF-1 and its binding proteins participate in a major growth promoting pathway with key roles in bone development (18). Indeed, we observed a dramatic reduction of 61% and 50% in systemic IGF-1 in 3-week-old Hp1bp3−/− females and males, respectively (Figure 6A). Notably, only some reduction in cortical bone and no observable growth retardation were documented in mice with liver-specific knockout of Igf1, despite similar reductions in systemic IGF-1 (18). Therefore, we assayed the levels of circulating IGFBPs as well. Three-week-old Hp1bp3−/− mice of both sexes presented
striking alterations in their IGFBP profile, with a 3- to 5-fold increase in the levels of IGFBP-1 and IGFBP-2, and decreased IGFBP-3 (Figure 6, B–D). No differences were observed in the circulating levels of IGFBP-5, IGFBP-6, or IGFBP-7 (Supplemental Figure 2, A–C).

The liver is the predominant source of systemic IGF-1 (18). We therefore assessed the expression of the hepatic Igf1 transcript using semiquantitative real-time-PCR that revealed a 3-fold reduction in Igf1 hepatic mRNA in the 3-weeks-old Hp1bp3+/− mice of both sexes (Figure 6E).

Such findings were consistent with the data observed for the systemic IGF-1 levels (Figure 6A). Serum IGFBPs are also known to be of hepatic origin, and indeed we observed a 10-fold increase in hepatic levels of Igfbp1 and Igfbp2 mRNA (P < .05) in the Hp1bp3+/− females (Figure 6, F and G). Male Hp1bp3+/− mice also showed a trend for higher levels of Igfbp1/2 transcripts, proportional to the changes in serum protein levels. No difference in hepatic Igfbp3 mRNA was observed in either of the sexes (Figure 6H). This suggests that the cause for the reduced serum
levels of IGFBP-3 in HP1BP3-deficient mice was protein degradation and not reduced hepatic mRNA levels; a similar pattern was previously described in liver-specific Igf1 knockout mice (18). In light of the marked variations between individual knockout mice in the levels of IGF-1 and IGFBPs, we also calculated the amounts of the proteins per body weight of the individual mice under study. The data revealed a very high correlation between the serum IGF-1 level and the mouse weight, whereas an expected inverse correlation was observed for IGFBP-1/2 and body weight (Figure 7, A–C). In light of the clear alterations in the levels of hepatic Igf1 transcript, we next assessed GH function in the livers of 3-week-old female mice. GH is a major regulator of hepatic Igf1 expression, and its effect is mediated downstream through phosphorylation of the transcription factor STAT5 (30), which binds to the Igf1 promoter and activates its transcription. Phosphorylation of STAT5 in liver extracts from 3-week-old females was therefore assessed by Western blot analysis (Figure 8A). We observed no difference between Hp1bp3−/− and control females, indicating that the altered regulation of Igf1 is probably GH independent.

In addition to the circulating complement of IGF-1 and IGFBPs, these factors are also produced locally within many tissues, where they act in autocrine/paracrine mechanisms to affect growth (31). We therefore compared the transcript levels of Igf1, Igfbp1, and Igfbp2 in kidneys and bones from 3-week-old Hp1bp3−/− and control females. In striking contrast to the liver, no changes were observed in the levels of Igf1 and Igfbp2 mRNA in tibiae from HP1BP3-deficient mice (Figure 8B), further supporting a systemic cause for the impaired bone growth. Igfbp1 levels in the tibiae were too low for reliable detection in both genotypes. Similarly, there was no difference in the levels of Igf1, Igfb1, or Igfbp2 in the kidney (Figure 8C), another organ known to express these factors (32).

Finally, the observed persistence of growth retardation throughout the entire lifespan of the Hp1bp3−/− mice was further supported by the similar changes of IGF-1 and IGFBP-1/2/3 in 8-week-old mice that still remained significantly smaller than littermate controls. In such mice, the circulating level of IGF-1 was still 30% reduced (Figure 9A) and IGFBP-1 and IGFBP-2 were moderately increased, whereas IGFBP-3 was slightly decreased (Figure 9, B–D). IGFBP-5, IGFBP6, and IGFBP-7 remained unchanged (Supplemental Figure 2, D–F). Taken together, these results strongly suggest that altered hepatic transcriptional regulation of Igf1, Igfbp1, and Igfbp2 is the underlying cause for the observed growth retardation.

Discussion

The present study describes unique modulatory effects on gene expression assigned to HP1BP3, a structural protein that regulates chromatin architecture. We show that global deficiency of HP1BP3 in mice impinges on body growth and bone structure in 3 discrete phases of their lifespan. First, Hp1bp3−/− mice suffered mild intrauterine growth restriction (IUGR) that was amplified dramatically during the first 3 weeks of life resulting in 40% reduction of body weight, as well as functionally weakened femurs. Then, after 3 weeks of age, the growth rate of HP1BP3-deficient mice attained normal values, but the proportionate dwarfism and low bone mass persisted into adulthood and old age. This could be of relevance to osteoporosis in humans, a disorder characterized by enhanced skeletal fragility resulting from age-related bone loss and/or failure to achieve optimal peak bone mass in the growing years (6, 29, 33, 34). The impact of HP1BP3 revealed in its absence in the mouse model is also consistent with the notion that genetic factors and probably epigenetic
influences play a substantial part in determining the peak bone mass in humans (8, 9).

Although attempting to explain the severe impairment in bone accrual in Hp1bp3<sup>−/−</sup> mice, we examined the functions of osteoblasts and osteoclasts ex vivo and in vivo. However, primary osteoblasts displayed normal proliferation, differentiation, and ability to deposit a mineralized bone matrix in culture and in vivo. Similarly, osteoclast progenitor cells proliferated normally and underwent proper differentiation to functionally mature osteoclasts. These observations suggest that loss of HP1BP3 expression in the osteoblast and osteoclast is neither reflected in primary defects of growth and function of these cell types, nor is it a likely cause for the low bone mass in HP1BP3-deficient mice. Because no cell autonomous effect of the HP1BP3 loss could be directly assigned to cellular constituents of the bone structure, we turned to explore systemic alterations of potential relevance at 3 weeks of age when growth retardation was most pronounced. At this age, the circulating levels of IGF-1 were 60% lower in knockout mice, whereas the levels of serum IGFBP-1 and IGFBP-2 were increased over 4-fold. In this regard, various mouse models have indicated that a threshold level of circulating IGF-1 is required for normal body size and bone mass. For example, in a double knockout of liver-specific IGF-1 and its stabilizing protein ALS, circulating IGF-1 was reduced to about 10% of normal levels, leading to growth retardation and reduced bone mass (18). By contrast, IGF-1 amounting to 25% of normal levels was found to be sufficient to permit normal body size, as was demonstrated by the liver IGF-1-defi-

Figure 7. Circulating levels of IGF-1 and IGFBP-1 and IGFBP-2 relative to the individual mouse weight. The serum levels of IGF-1 and its binding proteins were plotted per weight of each Hp1bp3<sup>−/−</sup> mouse, both males (n = 5) and females (n = 5) (A–C). Correlation coefficient (R) and P value (P) are shown on each plot.

Figure 8. Altered levels of circulating IGF-1 and its binding proteins are growth hormone independent and liver specific. A, Western blot analysis of STAT5 activation. Liver extracts from 3-week-old Hp1bp3<sup>+/+</sup> + and Hp1bp3<sup>−/−</sup> female mice (n = 4 for each group) were separated by SDS-PAGE and analyzed by HP1BP3, STAT5, phospho-STAT5 (pSTAT5), and tubulin (loading control) antibodies. A representative blot is shown. B and C, Semiquantitative real-time PCR assay of Igf1, Igfbp1, and Igfbp2 in bone (tibiae) (B) and kidney (C) of 3-week-old Hp1bp3<sup>+/+</sup> (black bars) and Hp1bp3<sup>−/−</sup> (gray bars) female and male mice (n = 5 of each group). Data are mean ± SD.
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and its binding proteins reflect a severe liver-specific tran-

sient mouse model (35, 36). When compared with the
above models, HP1BP3-deficient mice display circulating
IGF-1 levels that are approximately 40% of normal, high
above the liver IGF-1-deficient mouse threshold. It is therefore likely that potentiation of growth restriction re-

sulted from the very high levels of circulating IGFBP-1 and
IGFBP-2, both of which may reduce IGF-1 bio-availability,
potentially pushing the levels of active IGF-1 far below
the threshold necessary for normal growth. Furthermore,
transgenic mice overexpressing IGFBP-1 exhibit IUGR and
delayed bone mineralization followed by postnatal
growth retardation (37–40). Also consistent with these con-

siderations is the IGFBP-2 transgenic mouse model ex-
hibiting reduced postnatal body weight gain (41). It is of
interest to note in this regard that overexpression of
IGFBP-2 in a transgenic mouse model affected spleen
weight more than any other organ (41, 42), consistent
with our finding of disproportionately small spleens in
Hp1bp3<sup>−−</sup> mice. Finally, circulating levels of IGFBP-1
have also been associated with IUGR in humans (43, 44),
and high IGFBP-2 was correlated with bone loss in aging men (45, 46). Collectively, HP1BP3 deficiency seems to
affect growth by dual synergizing effects on IGF-1 and its
binding proteins.

This study reveals that the circulating levels of IGF-1 and
its binding proteins reflect a severe liver-specific tran-
scriptional misregulation of the Igf1, Igfbp1, and Igfbp2
genes. In adult mice, transcriptional regulation of hepatic
Igf1 responds to GH released by the pituitary gland. The
crucial importance of the GH/IGF-1 axis for bone miner-
alization has been demonstrated in several rodent models
and was implicated in the pathogenesis of osteopenia and
osteoporosis in humans (13, 47). Interestingly, GH itself is
not necessarily involved in the growth and skeletal defects
of the HP1BP3-deficient pups, because their growth is se-
verely compromised during the first 2–3 weeks of post-
natal life. During this period, the intrauterine and post-
natal regulation of growth by IGF-1 (13, 33) is rather GH
independent (48, 49), whereas the entire GH axis becomes
active only later than 2 weeks of age (49, 50). In fact, we
observed no impairment in the activation of the GH down-
stream effector STAT5 in the livers of HP1BP3-deficient
mice, indicating normal GH signaling, and further sup-
porting a GH-independent phenotype. Because very little
is known to date about the GH-independent mode of sys-
temic IGF-1 regulation (51), it is likely that the Hp1bp3
knockout mice may provide in future studies a valuable
model for understanding this mode of IGF-1 action.

When approaching adulthood at the age of 8 weeks,
HP1BP3-deficient mice have only a 30% reduction in cir-
culating IGF-1 levels. Also, the circulating levels of IGFBPs
were reduced, although IGFBP-1 was still 2-fold higher in
HP1BP3-deficient mice of both sexes. This partial nor-
malization of circulating levels may be due to the devel-
opmental shift from GH-independent to GH-dependent
regulation, which occurs around the age of 2–3 weeks (49,
50), and could explain the partial “catch up” growth ob-
served in the HP1BP3-deficient mice after the age of 3
weeks. During this time the mice partially closed the gap
in weight, length, cortical and trabecular bone mass rel-
ative to their control littermates. This effect is reminiscent
of a previously described model in which increasing the
serum IGF-1 level in a total IGF-1 knockout mouse model
compensated for the absence of locally produced IGF-1
after the age of 4 weeks (52). It is thus conceivable that the
partial restoration of the circulating IGF-1 network con-
stituents in the Hp1bp3<sup>−−</sup> mice allowed the correspond-
ing improvement in growth.

We observed a clear sexual dimorphism in growth and
bone mass during postnatal development of Hp1bp3<sup>−−</sup>
mice. At the age of 3 weeks, females were more severely
impacted in all measures, including body weight and
length, cortical bone diameter, and thickness and trabec-
ular bone volume. These effects were mirrored by similar
differences in IGF-1 and its binding proteins, with greater
changes observed in females. Conversely, by 8 weeks, fe-
nemales and males were equally affected in body weight and
length as well as in cortical bone diameter and thickness.
Interestingly, at this age, it was the males who had a greater reduction in trabecular bone volume, and this was also the case later in life. Our results are therefore consistent with sexual dimorphism observed in mice lacking IGFBP-2 (53), and its high levels in circulation may play a part in the observed differences between males and females. Furthermore, sexual dimorphism in bone growth appears under physiological conditions as well, due to the key roles played by estrogens and androgens in bone homeostasis (54–57). Of particular interest is the finding that estrogens can compensate for GH deficiency and trigger hepatic IGF-1 synthesis, thus rescuing skeletal phenotypes in GH-deficient mice (14). Therefore, it is tempting to speculate that estrogens are responsible for the greater growth compensation observed in female Hp1bp3<sup/^−</sup>−<sup/^−</sup> mice relative to males.

In conclusion, lack of HP1BP3 in mice led to severe misregulation of the endocrine IGF-1 pathway due to altered hepatic transcriptional regulation. Therefore, in light of the fact that HP1BP3 is a chromatin binding protein, further studies will aim to provide insight into the molecular mechanisms by which this novel H1-like protein controls the expression of IGF-1 and its binding proteins.

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Address all correspondence and requests for reprints to: Joseph Orly, PhD, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: orly@vms.huji.ac.il; or Benjamin P. Garfinkel, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: benny.garfinkel@mail.huji.ac.il.

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