Oxidative Stress Antagonizes Wnt Signaling in Osteoblast Precursors by Diverting β-Catenin from T Cell Factor- to Forkhead Box O-mediated Transcription*

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We have elucidated that oxidative stress is a pivotal pathogenic factor of age-related bone loss and strength in mice, leading to, among other changes, a decrease in osteoblast number and bone formation. To gain insight into the molecular mechanism by which oxidative stress exerts such adverse effects, we have tested the hypothesis that induction of the Forkhead box O (FoxO) transcription factors by reactive oxygen species may antagonize Wnt signaling, an essential stimulus for osteoblastogenesis. In support of this hypothesis, we report herein that the expression of FoxO target genes increases, whereas the expression of Wnt target genes decreases, with increasing age in C57BL/6 mice. Moreover, we show that in osteoblastic cell models, oxidative stress (exemplified by H2O2) promotes the association of FoxOs with β-catenin, β-catenin is required for the stimulation of FoxO target genes by H2O2, and H2O2 promotes FoxO-mediated transcription at the expense of Wnt-/T-cell factor-mediated transcription and osteoblast differentiation. Furthermore, β-catenin overexpression is sufficient to prevent FoxO-mediated suppression of T-cell factor transcription. These results demonstrate that diversion of the limited pool of β-catenin from T-cell factor- to FoxO-mediated transcription in osteoblastic cells may account, at least in part, for the attenuation of osteoblastogenesis and bone formation by the age-dependent increase in oxidative stress.

Increased levels of reactive oxygen species (ROS) influence numerous cellular processes and have been linked to aging and the development of age-related diseases, but the molecular details of the pathogenetic effects of oxidative stress on tissue homeostasis are not well understood. Recent studies from our group and others provide compelling evidence that the age-associated increase in oxidative stress is a pivotal pathogenic mechanism for skeletal involution. Indeed, in the accompanying manuscript by Almeida et al. (52), we have determined that sex steroid sufficient female or male C56BL/6 mice lose bone strength and mass progressively between the ages of 4 and 31 months. These changes are temporally associated with increased osteoblast and osteocyte apoptosis, decreased osteoblast number and bone formation rate, increased levels of ROS, and a corresponding increase in the phosphorylation of p53 and p66shc, two key components of a signaling cascade that influences apoptosis and lifespan in invertebrates and mammals. In agreement with our findings, others have shown that H2O2 suppresses osteoblastic differentiation of bone marrow progenitors and promotes osteoblast apoptosis in an ERK-dependent manner in vitro and that an increase in ROS leads to osteopenia in other murine models and, perhaps, in humans (1–3). In addition, osteoporosis has been noted in mouse models of premature aging (4–6).

Cells attempt to counteract the adverse effects of ROS by several defense mechanisms, including the up-regulation of free radical scavenging enzymes such as manganese superoxide dismutase (Mn-SOD), Catalase, as well as DNA damage repair genes, such as Gadd45 (7–10). This response requires activation of a family of ubiquitous transcription factors, known as Forkhead box O (FoxO), which consists of four members: FoxO1 (or Fkhrl), FoxO3a (or Fkhrl1), FoxO4 (or Afx), and FoxO6 (11). FoxOs promote mammalian cell survival by inducing cell cycle arrest and quiescence in response to oxidative stress (7, 12–16), and also regulate longevity in model organisms (17).

Importantly, it has been recently shown that FoxO-mediated transcription requires binding of β-catenin (18), a scaffold protein that is also required for the transcriptional activity of the T-cell factor (Tcf) family of transcription factors, which are the downstream effectors of the Wnt/β-catenin pathway (19, 20). Oxidative stress and FoxO/β-catenin signaling have also been linked in worms (18). In sharp contrast to the adverse effects of oxidative stress on osteoblastogenesis and survival, the Wnt/β-catenin pathway provides an essential signal for osteoblastogenesis (21). This background and in particular the evidence for the utilization of β-catenin by both FoxO- and Tcf-mediated transcription led us to hypothesize that ROS may sup-
press osteoblast differentiation by antagonizing the Wnt/Tcf pathway.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins, Plasmids, and Oligonucleotides**—Wnt3a and Dkk1 recombinant proteins were purchased from R&D Systems (Minneapolis, MN). pcDNA, was purchased from Invitrogen. A reporter plasmid containing 3 Tcf binding sites upstream of a minimal c-fos promoter driving the firefly luciferase gene (Tcf-Luc) (22), and a construct expressing constitutively active β-catenin (S33Y) (23) were provided by B. Vogelstein, John Hopkins University Medical Institutions, Baltimore, MD. A plasmid expressing Axin (24) was provided by F. Costantini, Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York. Hemagglutinin-FoxO3aWT (25) was provided by M. Greenberg, Harvard Medical School, Boston (Addgene plasmid 1787). A reporter plasmid containing 6 copies of daf-16 family protein binding element (FoxO-luc) in the pGL3-basic firefly luciferase vector with a minimal TATA box (26) was provided by B. Burgering, University Medical Center, Utrecht, Netherlands.

**Cell Culture, Transfections, and Luciferase Activity**—OB-6 cells were cultured in α-minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), and 1% each of penicillin, streptomycin, and glutamine. C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and 1% each of penicillin, streptomycin, and glutamine and 1% sodium pyruvate. Luciferase reporter constructs were introduced into cells by transient transfection using Lipofectamine Plus (Invitrogen). C2C12 cells were plated in 48-well plates and transfected 16 h later with a total of 0.4 μg of DNA. Luciferase activity assays were performed as previously described (27).

**Immunoprecipitation and Western Blot Analyses**—C2C12 cells were treated with vehicle or H2O2 for 1 h. Cell lysates were immunoprecipitated with anti-IgG, anti-β-catenin, or anti-FoxO3a and Protein A/G Plus-agarose (Santa Cruz Biotechnology). Immunoprecipitates were analyzed by SDS-PAGE and Western blot analysis was performed using standard conditions and the following antibodies: mouse monoclonal anti-β-catenin (BD Biosciences), rabbit polyclonal anti-FoxO3a and normal mouse IgG (Santa Cruz Biotechnology). Quantification of the intensity of the bands in the autoradiograms was performed using a VersaDoc™ imaging system (Bio-Rad).

**Quantitative PCR**—Total RNA was extracted using Ultraspec RNA (Biotecx Laboratories, Houston, TX) and reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s instructions. TaqMan quantitative RT-PCR was performed as previously described (27). Primers and probes for alkaline phosphatase (AP), Axin2, osteoprotegerin (Opg), catalase, Mn-SOD, Gadd45, glyceralddehyde-3-phosphate dehydrogenase, and ribosomal protein S2 were manufactured as Assays-by-Demand™ (Applied Biosystems Inc.).

**Alkaline Phosphatase Activity**—C2C12 cells were seeded at a density of 2 × 10⁴/cm² in medium containing 10% fetal bovine serum. The following day, the medium was replaced with 5% serum-containing medium and the cells were either pre-treated with vehicle or H₂O₂ for 1 h. Vehicle and Wnt3a were added to the cells and cultures were continued for 24 h. Cells were lysed in 100 mM glycine, 1 mM MgCl₂, and 1% Triton X-100 at pH 10. AP activity in the cell lysate was determined using a buffer containing 2-amino-2-methylpropanol and p-nitrophenyl phosphate (Sigma).

**Statistical Analysis**—The data were analyzed by analysis of variance, using SigmaStat (SPSS Science, Chicago, IL). All experiments were repeated at least three times.

**RESULTS**

Organismal Aging is Associated with Decreased Expression of Wnt Target Genes and Increased Expression of FoxO Target Genes in Bone—To begin testing the hypothesis that the increased production of ROS with age may attenuate Wnt signaling in bone, we compared the expression of Wnt and FoxO target genes in C57BL/6 female mice between the ages of 4 and 31 months. In two different experiments transcripts encoding Axin2 and Opg, two known targets of the canonical Wnt signaling pathway (28–30), were significantly lower in calvaria of older animals (Fig. 1). Conversely, the transcripts for the FoxO target gene Gadd45 (10) was increased in the older as compared with the younger animals (Fig. 1).

H₂O₂ Reproduces the Reciprocal Changes in FoxO- and Wnt-mediated Transcription in Osteoblastic Cells—Encouraged by the in vivo findings, we proceeded to examine whether increased activation of FoxO transcription factors by ROS attenuates Wnt/Tcf-mediated transcription, using two different cell models: an uncommitted mesenchymal cell line, C2C12, capable of differentiating toward the osteoblastic lineage (31), and an osteoblastic cell line derived from the bone marrow, OB-6 (32). H₂O₂ increased the expression of transcripts for Gadd45 and Catalase in C2C12 cells, as measured by quantitative RT-PCR; whereas treatment with Wnt3a had no effect on the expression of these two genes (Fig. 2A). Likewise, H₂O₂ increased the expression of transcripts for Mn-SOD in OB-6 cells and Wnt3a promoted this effect (supplemental materials Fig. S1A). On the other hand, H₂O₂ decreased the basal levels, as well as Wnt3a-stimulated levels, of Axin2, AP, and...
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As a more direct measure of FoxO- and Tcf-mediated transcription, we next determined whether H2O2 altered the activity of either a FoxO- or a Tcf-transcriptional reporter construct, respectively. H2O2 dose-dependently attenuated Wnt3a-stimulated Tcf-luc activity in C2C12 cells (Fig. 3A). Conversely, H2O2 dose-dependently stimulated the activity of the FoxO-luc construct in the same cell line (Fig. 3B) and in OB-6 cells (supplemental materials Fig. S1B). The H2O2-induced suppression of Tcf transcription could be mediated by inhibition of the pathway upstream from β-catenin activation or by direct inhibition of β-catenin action. To distinguish between these possibilities, we activated Tcf transcription by co-expressing a mutant of β-catenin (S33Y) that cannot be targeted for degradation. The β-catenin mutant dramatically stimulated Tcf-luc activity. Moreover, similar to the effect of Wnt3a, H2O2 dose-dependently attenuated the transcriptional effect of the β-catenin mutant (Fig. 3C). These results indicate that H2O2 simultaneously activates FoxO-mediated transcription and suppresses Tcf-mediated transcription, and that the effect of H2O2 on canonical Wnt signaling is downstream of β-catenin.

Wnt/β-Catenin Signaling Is Required for and Potentiates FoxO-mediated Transcription—Next, we sought to determine whether β-catenin and FoxO physically interact in osteoblastic cells. As shown in Table 1, both C2C12 and OB-6 cells express FoxO1, -3a, and -4, as measured by quantitative RT-PCR. Immunoprecipitation of protein extracts from C2C12 (Fig. 4A) or OB-6 cells (data not shown) demonstrated that β-catenin was present in precipitates obtained using anti-FoxO3a antibody; and that this FoxO family member was present in precipitates obtained with anti-β-catenin antibody. These findings indicate that FoxO and β-catenin exist in a complex in these two cell lines. Moreover, treatment of cells with H2O2 enhanced the association of β-catenin and FoxO3a (Fig. 4A).

To determine whether β-catenin influences FoxO-mediated transcription in osteoblastic cells, we modulated β-catenin levels and determined the effect on FoxO-luc activity. H2O2-stimulated FoxO-luc activity was attenuated by blockade of Wnt signaling, and thereby reduction of β-catenin levels, via either treatment of C2C12 with the soluble LRP5/LRP6 inhibitor protein Dkk1, or by overexpression of Axin, an intracellular inhibitor of the canonical Wnt signaling pathway (Fig. 4B). Consistent with this, Dkk1 also blocked the increase in FoxO-mediated transcription promoted by co-transfection of a FoxO3a plasmid in C2C12 (Fig. 4C) or OB-6 cells (supplemental materials Fig. S2A).

Having confirmed the requirement of the canonical Wnt signaling for FoxO-mediated transcription, we examined whether activation of canonical Wnt signaling and the downstream increase in β-catenin levels enhanced FoxO-mediated transcription. Co-transfection of C2C12 or OB-6 cells with the
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A, C2C12 cells were treated with vehicle or H₂O₂ (100 μM) for 1 h. Cell lysates were prepared and immunoprecipitated with Protein A-Sepharose in combination with the following primary antibodies: anti-IgG, anti-β-catenin, or anti-FoxO3a. Immunoprecipitates were analyzed by Western blot using anti-β-catenin or anti-FoxO3a antibodies. Graphs below the blots indicate the relative amounts of β-catenin in FoxO3a immunoprecipitates (left panel), or FoxO3a in β-catenin immunoprecipitates (right panel). Bars represent mean ± S.D. of triplicate determinations. B, C2C12 cells were co-transfected with the FoxO-luc reporter plasmid and either a vector control (pcDNA), or a plasmid expressing axin. Cells transfected with pcDNA were pre-treated for 1 h with vehicle or Dkk1 (500 ng/ml), and then with vehicle or H₂O₂ (100 μM) for 24 h. Cells transfected with the axin plasmid were maintained in parallel cultures with vehicle or H₂O₂ for 24 h. C, C2C12 cells were co-transfected with FoxO-luc and either pcDNA or FoxO3a, and then maintained with vehicle or Dkk1 (500 ng/ml) for 24 h. D, C2C12 cells were transfected with the FoxO-luc reporter plasmid and either pcDNA or a plasmid expressing FoxO3a. In both cases cells were either co-transfected with a constitutively active β-catenin (S33Y) expression construct or incubated with Wnt3a (50 ng/ml) for 24 h. Bars represent mean ± S.D. of triplicate determinations of relative luciferase units (RLU) normalized for Renilla activity.* p < 0.05 versus vehicle.

β-catenin S33Y mutant or treatment with Wnt3a significantly increased FoxO-luc activity (Fig. 4D and supplemental materials Fig. S2B). The enhancing effect of the S33Y mutant or Wnt3a on FoxO-mediated transcription was reproduced in cells overexpressing FoxO3a (Fig. 4D and supplemental materials Fig. S2B).

**β-Catenin Overexpression Prevents the Suppressive Effect of H₂O₂ on Tcf-mediated Transcription**—The results presented thus far suggest that H₂O₂ suppresses Tcf-mediated transcription in a manner that depends on the activation of FoxO-mediated transcription. Therefore, both events may utilize a limited pool of available β-catenin. Such a model would predict that overexpression of β-catenin would restore Tcf-mediated transcription suppressed by H₂O₂. To determine whether this was the case, increasing amounts of the β-catenin mutant S33Y were co-expressed with Tcf-luc in cells exposed to H₂O₂. As shown in Fig. 5A, β-catenin dose dependently increased Tcf-mediated transcription in the presence of H₂O₂. A similar result was obtained when FoxO transcriptional activity was stimulated by overexpression of FoxO3a (Fig. 5B).

**ROS and FoxO Suppress Osteoblast Differentiation**—Finally, we sought to determine whether increased levels of ROS were capable of suppressing osteoblastic differentiation of uncommitted precursors, as suggested by the decrease in osteoblast number with age in the C57BL/6 mice. To pursue this, we determined the effect of H₂O₂ on Wnt3a-induced osteoblast differentiation, as measured by AP activity. Wnt3a stimulated AP activity in C2C12 cells and this was suppressed by H₂O₂ (Fig. 6A). Moreover, overexpression of FoxO3α suppressed Wnt3a-induced AP activity (Fig. 6B).

**DISCUSSION**

ROS-activated FoxOs utilize β-catenin, a scaffolding protein that plays a critical role in skeletal homeostasis when bound to an alternative transcription factor, Tcf (19, 20). Prompted by this evidence, we have tested herein the hypothesis that ROS suppress osteoblast differentiation by antagonizing Wnt/Tcf-mediated transcription, and that this mechanism may be an important pathogenetic factor for the skeletal involution that is associated with old age. We report that advancing age in C57BL/6 mice is associated with increased expression of Gadd45, a target gene of FoxOs and decreased expression of the Wnt target genes Axin2 and Opg. Consistent with the in vivo findings, we found that β-catenin and FoxO3a physically associate in osteoblastic cells models and that H₂O₂ enhances this association. Exposure of osteoblastic cells to H₂O₂ in vitro stimulated the activity of a FoxO-luc reporter construct and the expression of Gadd45, Catalase, or Mn-SOD. On the other hand, H₂O₂ attenuated the activity of a construct driven by Tcf, as well as the expression of Axin2, Opg, and alkaline phosphatase. Moreover, stimulation of FoxO transcription by H₂O₂ in osteoblastic cells was abrogated by the Wnt antagonist Dkk1 as well as Axin, which accelerates β-catenin degradation. Conversely, β-catenin overexpression abrogated the suppressive effect of H₂O₂ on Tcf-mediated transcription. Last, Wnt3a-induced osteoblastic differentiation was abrogated by H₂O₂ or overexpression of FoxO. Taken together, these results demonstrate that ROS suppresses osteoblast differentiation by diverting a limited pool of β-catenin from Tcf to FoxO complexes.

It is widely accepted that decreased wall width, the histologic index of the amount of bone made by a team of osteoblasts, is the hallmark of age-associated osteoporosis and decreased bone formation in animals and humans, and results from decreased osteoblastogenesis (33). This is caused, at least in part, from a switch in the fate of mesenchymal stem cell progenitors that can give rise to either adipocytes or osteoblasts, to the former phenotype at the expense of the latter (34). This
reciprocal change may account for the well established phenomenon of increased bone marrow adiposity with age.

Wnt binding to the Frizzled-LRP5/6 receptor complex halts the proteolytic destruction of β-catenin, which then moves into the nucleus where it binds to and activates the Tcf family of transcription factors (Fig. 7). Importantly, among its many other biologic roles in development, β-catenin is a sine qua non factor for the commitment of mesenchymal stem cell progenitors to the osteoblast lineage and differentiation (21, 29, 35–41). This process continues throughout adult life and is essential for the regeneration of the skeleton. We have also shown earlier that Wnt-signaling prevents apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by β-catenin-dependent (as well as independent) signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/Akt (27). Collectively, all these actions as well as the ability of Wnt/β-catenin signaling to suppress osteoclastogenesis lead to a positive balance between bone formation and resorption and increased bone mass. On the other hand, Wnt/β-catenin suppresses adipocyte differentiation (42). Therefore, it is quite possible that the antagonism of Wnt signaling in osteoblast precursors by the diversion of β-catenin from Tcf- to FoxO-mediated transcription may be responsible not only for the decreased osteoblastogenesis and bone formation, but also the increased marrow adiposity associated with old age (Fig. 7). In
full support of the conclusions of the present report, high bone mass mutants of the LRP5 receptor, or Wnt10b overexpression in the marrow, prevents osteoblast apoptosis and the age-associated bone loss and strength in mice (35, 43).

Besides antagonizing mesenchymal progenitor commitment toward the osteoblastic lineage, oxidative stress operating through the molecular mechanism elucidated in the present paper may affect other aspects of osteoblastogenesis. Indeed, Tothova et al. (44) have recently provided compelling evidence from studies of hematopoiesis in mice with conditional deletion of FoxO1, FoxO3, and FoxO4 that FoxO deficiency depletes hematopoietic stem cells by inducing their apoptosis and by driving them into the cell cycle and terminal differentiation. More important, these workers were able to reverse completely the changes of the FoxO-deficient hematopoietic stem cell phenotype by the administration of the anti-oxidant n-acetyl cysteine, demonstrating that oxidative stress is a critical determinant of the long term regenerative potential of stem cells. In view of this evidence, it is tempting to speculate that increased levels of ROS with advancing age may also have deleterious effects on mesenchymal stem cell (MSC) self-renewal by increasing apoptosis and transiently enhancing MSC entry into the cell cycle and terminal differentiation.

In studies by Almeida et al. (52) reported in the accompanying manuscript, we have found that loss of sex steroids acutely increases oxidative stress in mice. In addition, we have obtained evidence that both sex steroid deficiency and aging exert their adverse effects on bone by oxidative stress and that, in fact, sex steroid deficiency accelerates the effect of organismal aging on osteoclastogenesis and osteoblast and osteoclast survival.
Nonetheless, whereas acute estrogen or androgen deficiency is a state of high bone turnover and increased bone formation (albeit, unbalanced by the increased resorption), aging is a state of low turnover and decreased bone formation. We and others had previously established that acute loss of estrogens increases the rate of remodeling by up-regulating osteoblastogenesis and osteoclastogenesis. The effect of the loss of estrogens on the rate of remodeling and the up-regulation of osteoblastogenesis and bone formation in animals and humans, however, wanes with time (5–10 years in women and 6–8 weeks in mice) raising the possibility that aging might be overriding the acute effects of estrogen deficiency. Considering the evidence that unopposed oxidative stress promotes premature hematopoietic stem cell differentiation, it is possible that following acute loss of estrogens, the increased ROS levels in MSCs may promote their exit from quiescence, replication, and development of transit amplifying osteoblast progenitors, the latter already confirmed by our earlier work (45). These mechanisms, lead to up-regulation of osteoblast differentiation and thereby increased bone formation. However, this effect would be reversed with time because the gradual buildup of ROS production with aging (accelerated by estrogen deficiency) antagonizes Wnt signaling and also leads to a decrease in the size of the MSC compartment by increasing MSC apoptosis. In support of this scenario, we have developed methods of expanding the self-renewal of MSC progenitors of osteoblasts, whereas preserving stemness (46), and using this technology have determined that their replication capacity is significantly lower in old versus young mice.1 Hence, we propose that an acute increase in ROS may transiently stimulate osteoblast differentiation, whereas a long term increase in ROS may lead to depletion of the pool of osteoblast progenitors, accounting for the self-limiting effect of estrogen deficiency on osteoblastogenesis. Diver- sion of β-catenin from Tcf to FoxO may also represent an important molecular mechanism of the age-dependent increase in osteoblast and osteocyte apoptosis, which is well documented in aging mice and in humans (47, 48, 52).

Recently, during the writing of our paper, Mani and co-workers (49) reported a missense mutation in LRP6, a co-receptor for the Wnt-signaling pathway, in a family with autosomal dominant early coronary artery disease, features of the metabolic syndrome (hyperlipidemia, hypertension, and diabetes), as well as osteoporosis. Similar to the effects of H2O2 on Wnt transcription in the osteoblastic cells described in our paper, the LRP6 mutation in the report by Mani et al. (49) impaired Wnt-induced transcription in NIH 3T3 cells. It is, therefore, tempting to speculate that the antagonism of Wnt signaling by oxidative stress with increasing age may play a role not only in the development of involutional osteoporosis, but other common age-dependent diseases like coronary artery disease, Type II diabetes, and metabolic syndrome (50).

In contrast to the results of the present report, it has recently been shown that H2O2 can also activate Wnt/β-catenin signaling, via a mechanism involving binding of disheveled (Dvl) to nucleoredoxin. Specifically, Funato et al. (51) show that nucleoredoxin is a strong inhibitor of Wnt/β-catenin signaling and that the binding of nucleoredoxin to Dvl is inhibited by treatment with H2O2. One explanation for the apparent discrepancy between this study and the present work is the time frame of the events: Funato et al. (51) found that H2O2 induces a rapid increase in Wnt signaling that peaks ~20 min after stimulation, but the strength of this response is reduced when cells are analyzed several hours later. In contrast our studies were performed 5–24 h after H2O2, and Wnt3a treatment. Additional studies will, of course, be needed to fully elucidate the impact of acute versus chronic oxidative stress on Wnt signaling.

In closing, the evidence presented herein provides molecular details of a pathogenetic mechanism linking the increased oxidative stress that is associated with old age to the development of skeletal involution (Fig. 7). Ironically, in this particular instance, the diversion of β-catenin away from Tcf to the ROS-induced FoxO suggests that the adverse effects of aging on bone may be mediated by the same factor that was used during development and growth to build skeletal assets.

Acknowledgments—We thank R. L. Jilka and T. Bellido for helpful discussions, V. G. Lowe and A. D. Warren for technical assistance, and R. I. DeWall for assistance in the preparation of the manuscript.

REFERENCES

1. Bai, X. C., Lu, D., Bai, J., Zheng, H., Ke, Z. Y., Li, X. M., and Luo, S. Q. (2004) Biochim. Biophys. Res. Commun. 314, 197–207

2. Levasseur, R., Barrios, R., Elefteriou, F., Glass, D. A., Lieberman, M. W., and Karsenty, G. (2003) Endocrinology 144, 2761–2764

3. Basu, S., Michaelsson, K., Olofsson, H., Johansson, S., and Melhus, H. (2001) Biochem. Biophys. Res. Commun. 288, 275–279

4. Iljka, R. L., Weinstein, R. S., Takahashi, K., Parfitt, A. M., and Manolagas, S. C. (1996) J. Clin. Invest. 97, 1732–1740

5. Tyner, S. D., Venkataram, S., Choi, J. S., Jones, Y., Ghebranious, N., Igelmann, H., Lu, X., Soron, G., Cooper, B., Brayton, C., Hee, P. S., Thompson, T., Karsenty, G., Bradley, A., and Donehower, L. A. (2002) Nature 415, 45–53

6. De Boer, J., Andressoo, J. O., de Wit, J., Huijmans, J., Beems, R. B., van Steeg, H., Weeda, G., van der Horst, G. T., van Leeuwen, W., Themmen, A. P., Meradji, M., and Hoeijmakers, J. H. (2002) Science 296, 1276–1279

7. Kops, G. J., Dansen, T. B., Polderman, P. E., Saarloos, I., Wirtz, K. W., Coffer, P. J., Huang, T. T., Bos, J. L., Medema, R. H., and Burgering, B. M. (2002) Nature 419, 316–321

8. Nemoto, S., and Finkel, T. (2002) Science 295, 2450–2452

9. Ramaswamy, S., Nakamura, N., Sansal, I., Bergeron, L., and Sellers, W. R. (2002) Cancer Cell 2, 81–91

10. Tran, H., Brunet, A., Grenier, J. M., Datta, S. R., Fornace, A. J., Jr., DiStefano, P. S., Chiang, I. W., and Greenberg, M. E. (2002) Science 296, 530–534

11. Katoh, M., and Katoh, M. (2004) Int. J. Oncol. 25, 1495–1500

12. Medema, R. H., Kops, G. J., Bos, J. L., and Burgering, B. M. (2000) Nature 404, 782–787

13. Kops, G. J., Medema, R. H., Glassford, J., Essers, M. A., Dijkers, P. F., Coffer, P. J., Lam, E. W., and Burgering, B. M. (2002) Mol. Cell. Biol. 22, 2025–2036

14. Burgering, B. M., and Kops, G. J. (2002) Trends Biochem. Sci. 27, 352–360

15. Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., Tran, H., Ross, S. E., Mostoslavsky, R., Cohen, H. Y., Hu, L. S., Cheng, H. L., Jedrychowski, M. P., Gyg, S. P., Sinclair, D. A., Alt, F. W., and Greenberg, M. E. (2004) Science 303, 2011–2015

16. Essers, M. A., Weijzen, S., Vries-Smits, A. M., Saarloos, I., de Ruiter, N. D., Bos, J. L., and Burgering, B. M. (2004) EMBO J. 23, 4802–4812

17. Kenyon, C. (2005) Cell 120, 449–460

3 X-D. Chen, M. Almeida, L. Han, M. Martin-Millan, C. A. O’Brien, and S. C. Manolagas, unpublished observations.
