

## RESEARCH ARTICLE

# SIRT1 is a positive regulator of *in vivo* bone mass and a therapeutic target for osteoporosis

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## Abstract

Overexpression or pharmacological activation of SIRT1 has been shown to extend the lifespan of mice and protect against aging-related diseases. Here we show that pharmacological activation of SIRT1 protects in two models of osteoporosis. Ovariectomized female mice and aged male mice, models for post-menopausal and aging-related osteoporosis, respectively, show significant improvements in bone mass upon treatment with SIRT1 agonist, SIRT1720. Further, we find that calorie restriction (CR) results in a two-fold upregulation of *sirt1* mRNA expression in bone tissue that is associated with increased bone mass in CR mice. Reciprocally, SIRT1 whole-body knockout (KO) mice, as well as osteoblast and osteoclast specific KOs, show a low bone mass phenotype; though double knockout mice (containing SIRT1 deleted in both osteoblasts and osteoclasts) do not show a more severe phenotype. Altogether, these findings provide strong evidence that SIRT1 is a positive regulator of bone mass and a promising target for the development of novel therapeutics for osteoporosis.

## OPEN ACCESS

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## Introduction

The SIR2 family of proteins (named the Sirtuins) are NAD<sup>+</sup> dependent enzymes that are evolutionarily conserved from yeast to humans [1]. Yeast SIR2 utilizes NAD<sup>+</sup> as a co-substrate to catalyze deacetylation of histone proteins, thereby linking metabolism with gene silencing [2]. Importantly, increased dosage and/or pharmacological activation of SIR2 orthologues have been shown to extend the lifespan of model organisms, including mice [3–10]. The mammalian genome contains seven Sirtuins, of which SIRT1 shares the greatest sequence similarity with yeast SIR2. Activation of SIRT1 in mice is associated with a delay in the onset of many aging-related diseases, including osteoporosis [10–19].

Another regimen that extends lifespan in a wide variety of organisms is calorie restriction (CR). Mice fed a diet 30–40% below *ad libitum* (AL) levels live longer and are more resistant to the development of a number of aging-related diseases [20]. Evidence has emerged that SIRT1 may act as an important mediator of the beneficial effects of CR [21]. For instance, SIRT1

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knockout mice fail to display a number of phenotypes associated with CR, including increased activity, higher respiration, and lifespan extension [22–24]. Importantly, CR has been shown to stimulate the expression and activity of SIRT1 in various mammalian tissues, suggesting activation of SIRT1 may mimic the effects of CR [25–26]. Consistent with this, mice treated with SIRT1 agonists show a transcriptional profile that closely mirrors that of CR [16–17]. While CR has been shown to protect against a number of aging-related diseases, its role in osteoporosis has been far less conclusive [27–30].

Osteoporosis is a classic aging-related disease that occurs due to an imbalance in bone remodeling. This can arise either through hyperactivation of osteoclasts (the hematopoietic cells that break down bone) or hypoactivation of osteoblasts (the mesenchymal cells that form bone), resulting in post-menopausal (or Type 1) or aging-related (or Type 2) osteoporosis, respectively. There are currently no effective treatments for aging-related osteoporosis [31].

To better define the role of SIRT1 in bone remodeling, we have taken a systematic *in vivo* approach. Using both whole-body and tissue-specific knockouts, we show that deletion of SIRT1 invariably results in an osteoporotic phenotype. Reciprocally, short-term pharmacological activation of SIRT1 in two models of osteoporosis leads to significant increases in bone mass. Interestingly, calorie restriction also results in improved bone mass that is associated with increased expression of SIRT1 in bone tissue. Our data solidify SIRT1 as an important regulator of bone mass and promising target for the treatment of osteoporosis.

## Materials and methods

### Animal experimentation

All mice were cared for in accordance with the MIT Committee on Animal Care (MITCAC), which approved this study. Mice were housed under controlled temperature ( $25 \pm 1^\circ\text{C}$ ) and lighting conditions, and fed standard chow *ad libitum* unless otherwise stated.

Sirt1<sup>flox/flox</sup> mice and osteoclast Cre mice (Lysozyme M promoter) were obtained from Jackson Laboratory; osteoblast specific Cre mice (2.3kb Collagen type 1 promoter) were obtained from the Mutant Mouse Regional Resource Center (MMRRC). Whole-body knockouts were in a CD1 and 129/Sv mixed background (due to lethality in an inbred background), osteoblast knockouts were in a mixed C57BL/6  $\times$  BALB/c background, and osteoclast knockouts and Sirt1<sup>flox/flox</sup> were in a C57BL/6 background. Female mice were used for all experiments unless otherwise stated.

Calorie restricted (CR) C57BL/6J male mice that had been on a 60% *ad libitum* diet for 4 months starting at 4 months of age were obtained from the National Institutes of Aging (NIA). Once received, mice were kept on CR for an additional 2 weeks in order to re-equilibrate mice to the new surroundings.

For SRT1720 feeding experiments, 12 month old male C57BL/6J mice were fed 100mg/kg/day SRT1720 (SirTris) [32] or vehicle control in their chow for 5 months. 3 month old ovariectomized or sham treated C57BL/6J female mice were obtained from Jackson Laboratories and similarly fed for 1 month with SRT1720. Mice were euthanized with carbon dioxide asphyxiation as approved by MITCAC and bones fixed in 10% formalin overnight at  $4^\circ\text{C}$ , and then stored in 70% ethanol.

### Microcomputed tomography ( $\mu\text{CT}$ ) analysis

Dissected bones were analyzed by  $\mu\text{CT}$  using a Skyscan 1172 instrument (60kV, 150 $\mu\text{A}$ , using a 0.5-mm aluminum filter) at a resolution of 5 $\mu\text{m}$ . Measurements were taken from 200 slices immediately distal of the primary spongiosa and analyzed for alterations in trabecular bone parameters. Resulting images were reconstructed using the Skyscan NRecon program and

analyzed using Skyscan CTAN software. Analyses were performed typically for the femur or tibia, except for double knockout mice (DKO) for which analysis was only performed for the spine.

### Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells or tissues using Trizol (Invitrogen) and the RNeasy MinElute Cleanup kit (Qiagen). 1  $\mu$ g of RNA was used for cDNA synthesis using random hexamers with the SuperScript III reverse transcriptase kit (Invitrogen). cDNA was then subjected to quantitative PCR analysis in the presence of iQ-SYBR green (Bio-Rad). Relative mRNA abundance was obtained by normalization to ribosomal *rpl19* levels.

For RNA extraction from bone tissue, calvaria (top of the skull) of mice was dissected out, washed with cold PBS to remove non-osseous tissue, and then flash-frozen in liquid nitrogen. Calvaria was then minced in Trizol, and homogenized using a Tissue Tearor homogenizer (VWR) in the fume hood. Lysates were then spun down at 15,000g for 10 minutes, with the resulting supernatant used for RNA isolation using the RNeasy MinElute Cleanup kit (Qiagen).

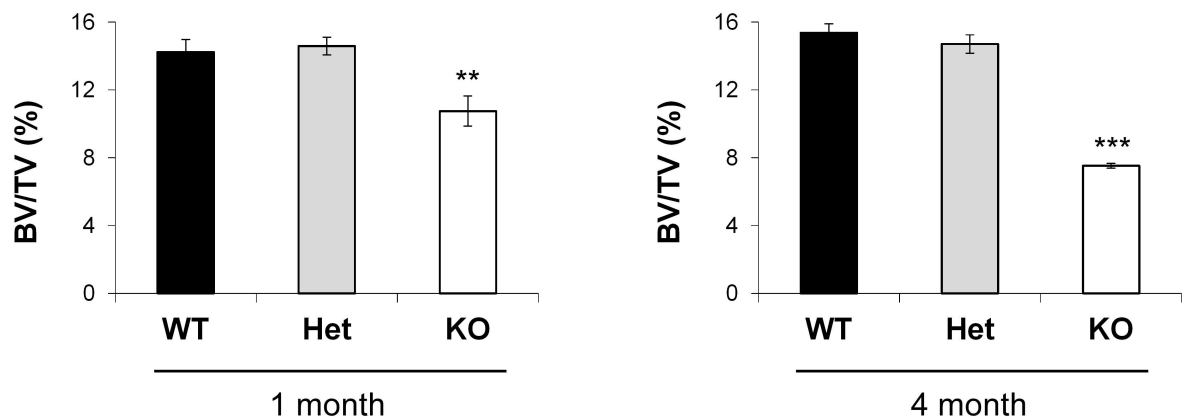
### Statistical analysis

Statistical analysis was performed using an unpaired Student's t test, with significant differences indicated by single asterisk (\*) when  $p < 0.05$ , double asterisk (\*\*) when  $p < 0.01$ , and triple asterisk (\*\*\*) when  $p < .005$ . All data is presented  $\pm$  standard error of the mean (SEM).

## Results

### SIRT1 knockout mice display a low bone mass phenotype

To comprehensively address the role of SIRT1 in bone remodeling, we first analyzed whole-body SIRT1 knockout mice using microcomputed tomography ( $\mu$ CT). We found that SIRT1 knockout mice show marked reductions in bone mass at both 1 month and 4 months of age, with the phenotype appearing to worsen with age (Fig 1). Heterozygous knockout mice did not show any differences in bone mass at either age (Fig 1).



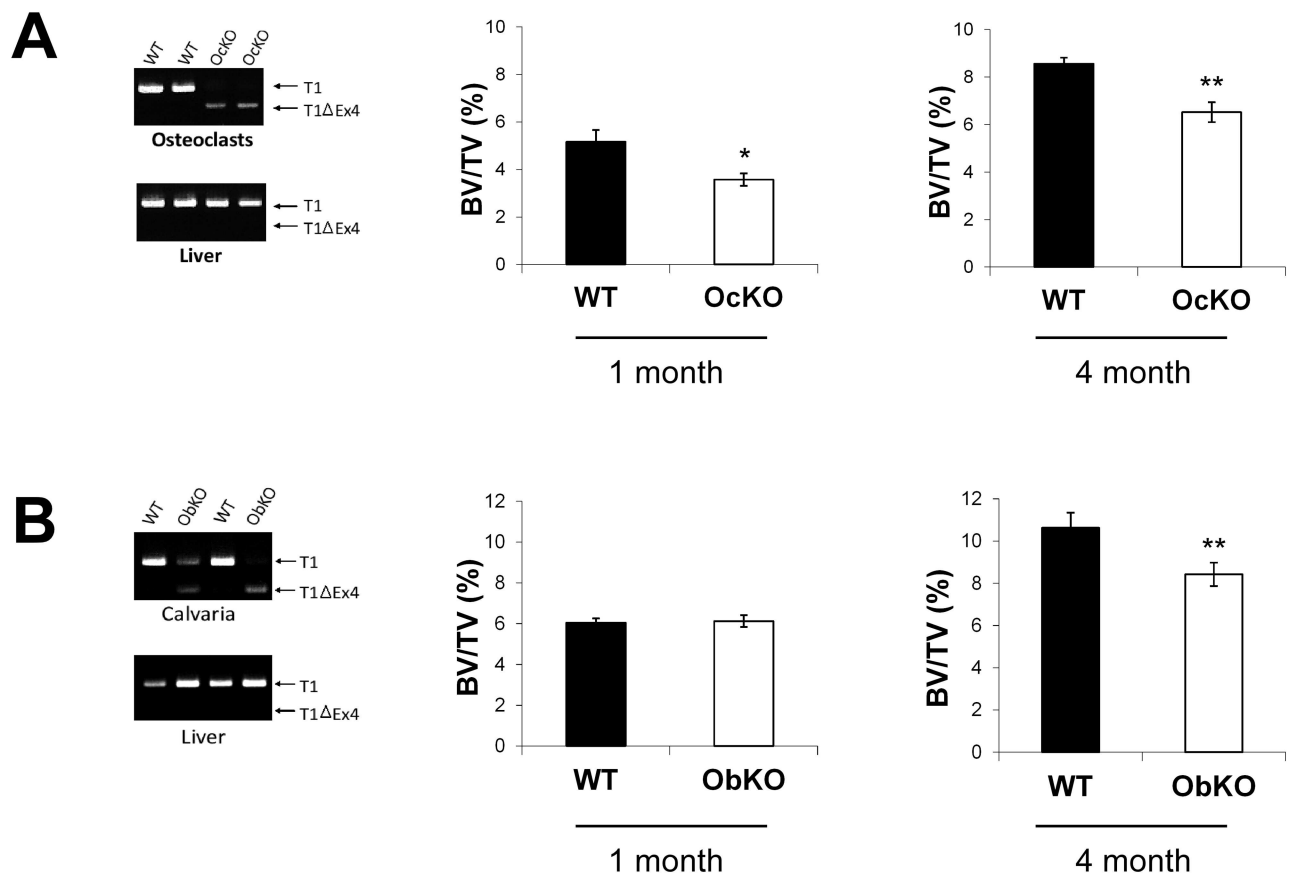
**Fig 1. Deletion of SIRT1 leads to a low bone mass phenotype.** SIRT1 whole-body knockout mice show decreased bone volume/total volume (BV/TV) at both 1 month and 4 months of age as assessed by microcomputed tomography ( $\mu$ CT). ( $n \geq 4$  for each group; \*\*  $p < .01$ ; \*\*\*  $p < .005$ ).

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To determine whether this phenotype is due to a cell-autonomous effect, we next generated tissue specific SIRT1 knockout mice. To obtain osteoblast specific SirT1 knockouts (ObKO), we crossed  $Sirt1^{fllox/fllox}$  animals with mice expressing Cre under the 2.3kb Collagen type 1 promoter [33]. This well-characterized promoter is specific to osteoblasts that have exited the cell cycle and are actively undergoing differentiation [34]. To generate osteoclast specific knockouts (OcKO) we used the previously described Lysozyme-M Cre promoter which specifically targets the monocyte precursors that give rise to osteoclasts [35]. In each case, expression of Cre leads to excision of the catalytic domain of SIRT1 in osteoblasts or osteoclasts, respectively, but not in other tissue types such as the liver (Fig 2A and 2B).

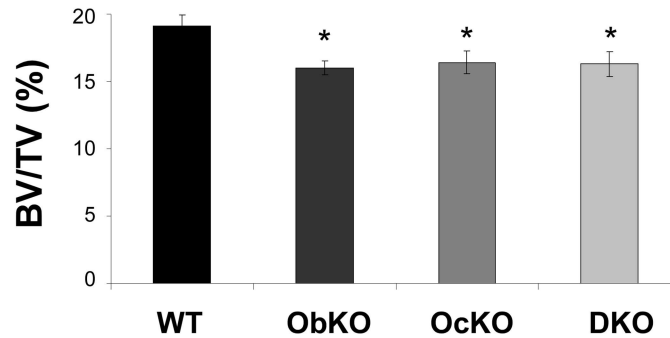
Unlike SIRT1 whole-body knockouts, ObKO and OcKO mice were obtained at expected Mendelian ratios and were indistinguishable from wildtype littermates, though we did note a modest but statistically significant increase in blood glucose levels for ObKOs (but not OcKOs) (S1 Fig).  $\mu$ CT analysis showed that OcKO mice displayed reduced bone mass at both 1 month and 4 months of age (Fig 2A). In contrast, ObKOs showed an osteoporotic phenotype only at 4 months of age (Fig 2B).

To determine whether deletion of SIRT1 in both cell types would result in an even more severe phenotype, we crossed ObKO and OcKO mice in order to obtain double knockouts



**Fig 2. Deletion of SIRT1 in osteoclasts or osteoblasts results in a low bone mass phenotype.** (A) Osteoclast tissue specific SIRT1 knockout mice (OcKO) show excision of SIRT1 in osteoclasts, but not liver, as indicated by a smaller PCR product obtained with primers flanking SIRT1 catalytic exon 4 (T1Δ4). OcKOs show decreased bone mass at both 1 month and 4 months of age. (B) Osteoblast tissue specific SIRT1 knockout mice (ObKO) mice show excision of SIRT1 in calvaria, but not liver. ObKOs show decreased bone mass only at 4 months of age. (n ≥ 5 for each group; \* p < .05; \*\* p < .01).

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**Fig 3. Deletion of SIRT1 in both osteoblasts and osteoclasts does not lead to a more severe phenotype.** Adult double knockout mice (DKO) containing SIRT1 deleted in both osteoblasts and osteoclasts show similar bone deficits as compared to individual osteoblast (ObKO) or osteoclasts (OcKO) knockout mice. ( $n \geq 6$  for each group; \*  $p < .05$ ).

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(DKO). Somewhat surprisingly, adult DKO mice did not show an even more pronounced phenotype as each individual knockout (Fig 3). Altogether, these data indicate that SIRT1 positively regulates bone mass, at least in part in a cell-autonomous manner, consistent with previous studies [36–39].

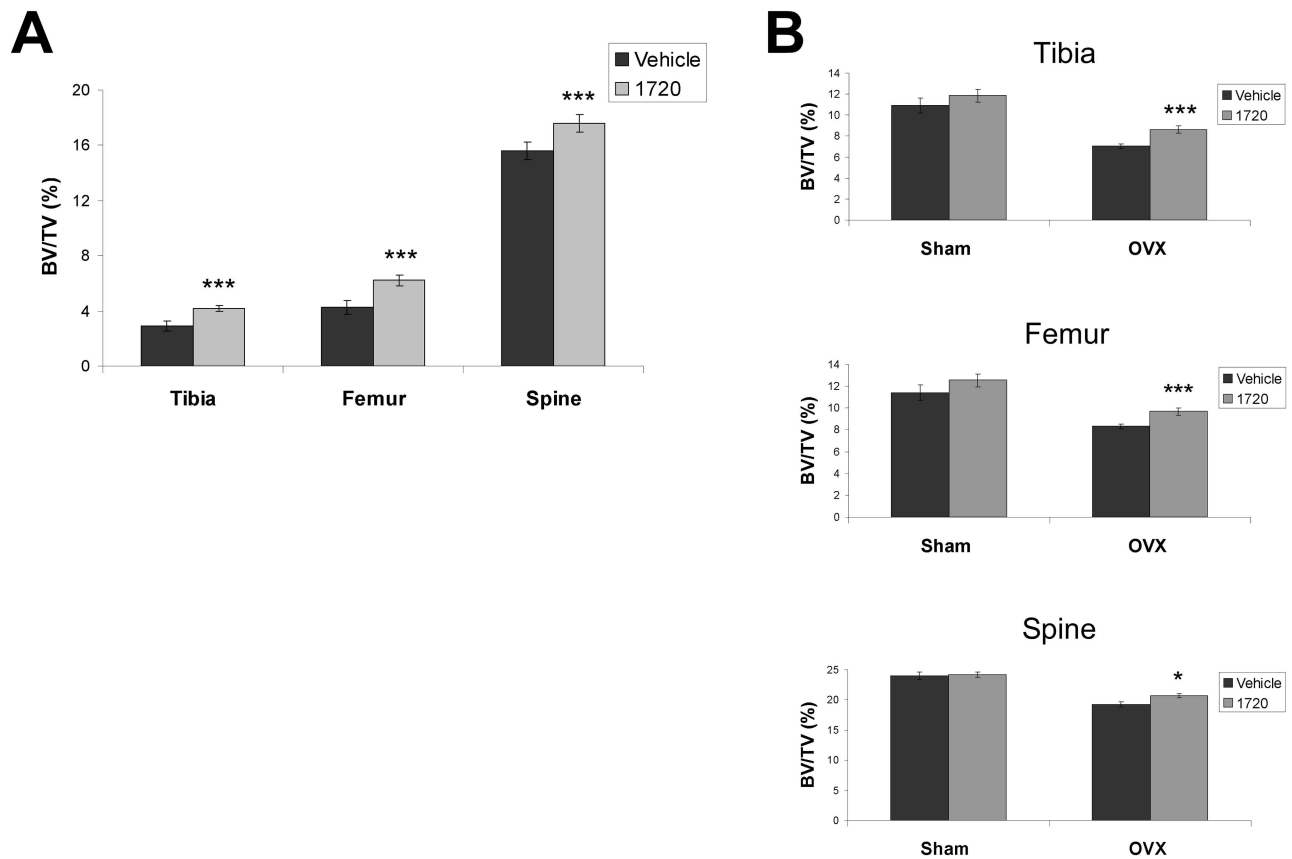
### Pharmacological activation of SIRT1 protects in two models of osteoporosis

Given that loss of SIRT1 resulted in decreased bone mass, we next explored whether activation of SIRT1 could increase bone mass, particularly in models of osteoporosis. To determine the effects of SIRT1 activation on age-related osteoporosis, we treated 12 month old male C57BL/6 mice for 5 months with SIRT1 agonist SRT1720 (100mg/kg/day) [32]. We found that mice fed SRT1720 showed significant improvements in bone mass as compared to vehicle control animals (Fig 4A).

We next examined the effect of SIRT1 activation in a model of post-menopausal osteoporosis. This is a more acute model of osteoporosis as ovariectomized (OVX) mice lose 20–30% of their bone mass in just one month (Fig 4B). OVX mice were treated with 100mg/kg/day SRT1720 for one month immediately after their surgery. In contrast to animals treated with vehicle control, mice treated with SRT1720 showed a significant amelioration of the OVX-induced bone loss (Fig 4B). The effect was a partial rescue and a higher dose of SRT1720 (200mg/kg/day) did not improve this rescue any further.

### Calorie restriction leads to increased bone mass associated with upregulation of SIRT1 expression

Calorie restriction (CR) has been shown to forestall many diseases of aging, though its effects on osteoporosis have been more ambiguous [27–30]. In an attempt to resolve this, we obtained CR mice from the central colony maintained at the National Institutes of Aging (NIA). The mice (C57BL/6) used in this study were 8 months old and had been placed on a CR diet consisting of 60% *ad libitum* (AL) levels for 4 months (starting at 4 months of age). As CR has previously been shown to induce SIRT1 expression in certain tissues [25–26], we first analyzed the expression of SIRT1 in the calvaria (skullcap) of CR mice. We found a two-fold upregulation in SIRT1 mRNA expression in the calvaria of CR mice as compared to AL animals (Fig 5A and 5B). To determine the effects of CR on bone mass, we next performed  $\mu$ CT and found that CR mice showed a marked increase in bone mass for all bones examined (Fig 5C).



**Fig 4. SIRT1 agonist, SRT1720, increases bone mass in aged male mice and ovariectomized female mice.** (A) 12 month old male mice treated for 5 months with 100mg/kg/day of SIRT1 agonist, SRT1720, show increased bone mass compared to mice treated with vehicle control (final age 17 months). (B) Ovariectomized (OVX) female mice treated with SRT1720 for 1 month show a modest but significant increase in bone mass compared to vehicle treated controls (final age 4 months). (n ≥ 9 for each group; \* p < .05; \*\* p < .01; \*\*\* p < .005).

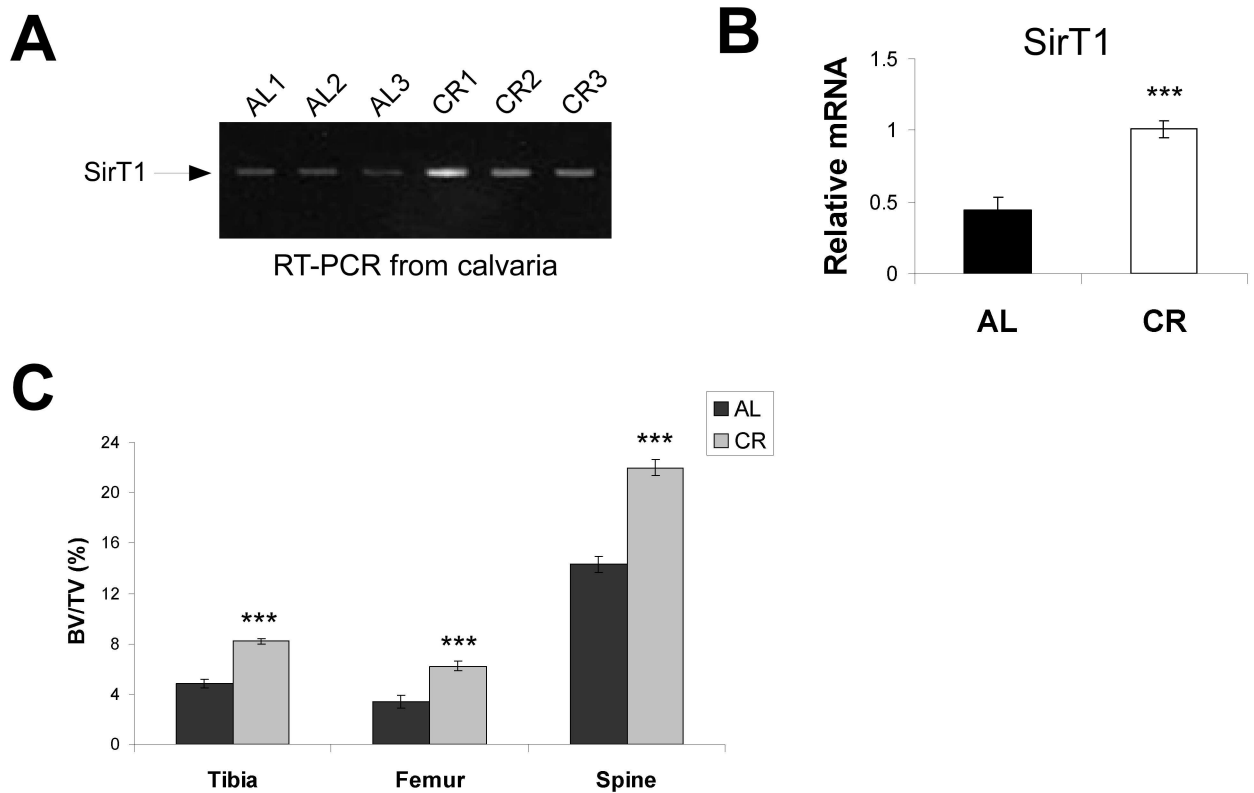
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### SIRT1 expression does not change with age in bone

SIRT1 expression has been reported to decline in certain tissues with aging [39–41]. To determine if a similar mechanism was at play in the skeleton, we next analyzed the expression of SIRT1 in the calvaria of young (3 month), old (12 month), and very old (24 month) C57BL/6 mice obtained from the NIA. In contrast to a previous report [39], we were unable to find any differences in SIRT1 mRNA expression in the bones of young versus old mice (Fig 6).

### Discussion

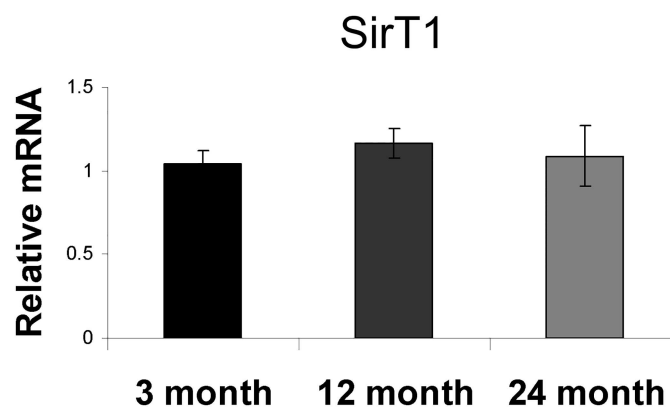
Activation of SIRT1 has previously been shown to increase the healthspan and delay the onset of many aging-related diseases in mice [7–19]. Here we examined the role of SIRT1 in osteoporosis, a classic aging-related disease, by taking a comprehensive *in vivo* approach. One shortcoming of this approach, however, is that we were unable to perform histology and thus bone histomorphometry data such as *in vivo* osteoblast and osteoclast counts is lacking. Nonetheless, we did find that deletion of SIRT1 invariably resulted in a low bone mass phenotype, consistent with previous studies [36–39]. Since deletion of SIRT1 in osteoblasts and osteoclasts also resulted in low bone mass, this indicates that SIRT1 regulates bone remodeling at least in part in a cell-autonomous fashion. While whole-body knockout mice did display a more severe



**Fig 5. Calorie restriction (CR) leads to upregulation of SIRT1 expression and increased bone mass.** (A) Calorie restricted (CR) mice show increased expression of SIRT1 in whole calvaria as assessed by reverse transcriptase polymerase chain reaction (RT-PCR). (B) Quantitative RT-PCR (qRT-PCR) confirms a two-fold increase in expression of SIRT1 (after normalization to RPL19) in the calvaria of CR mice. (C) 8 month old CR mice (placed on a CR diet for 4 months at 4 months of age) show marked increases in bone mass in all bones examined as compared to *ad libitum* (AL) fed controls. ( $n \geq 4$  for each group; \*\*\*  $p < .005$ ).

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phenotype than any of the tissue specific knockouts, direct comparisons between the two are difficult owing to the different backgrounds of the mice (deletion of SIRT1 in an inbred background is lethal). It should, however, be noted that SIRT1 has previously been shown to affect non-cell autonomous pathways such as the somatotrophic and endocrine axis [42–45] which



**Fig 6. SIRT1 expression in bone tissue does not change with age.** Expression of SIRT1 mRNA in the calvaria of mice remains constant with age. ( $n = 4$  for each group).

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would be expected to impinge on bone mass. In fact, SIRT1 overexpression in the brain was recently shown to be sufficient to extend the lifespan of mice, highlighting the powerful non-cell autonomous role SIRT1 plays in organismal biology [9]. It will therefore be interesting to determine the precise cell autonomous and non-autonomous role SIRT1 plays in bone remodeling, and whether any cross-talk (particularly in response to nutritional state) exists between the two. The mouse models developed in this study should be helpful in addressing these questions for future studies.

Unlike its widely accepted role in delaying the onset of aging-related diseases, the role of CR on bone mass has been somewhat murky. Some studies have shown a positive effect, while others have found either a negative or no effect at all [27–30]. This is likely due to the previous use of varying CR protocols, animal models, and strain backgrounds. Here, we have used mice from a central repository at the National Institutes of Aging (NIA) so as to use a standard model easily accessible by the scientific community. We show that mice in which CR was initiated during adulthood (at 4 months of age), and maintained for at least 4 months, show a marked increase in bone mass. A similar effect was observed for mice maintained for a longer 8 month period as well. This increased bone mass was associated with a two-fold induction of SIRT1 expression in the bone tissue of CR mice, similar to that observed for other tissues with CR [25–26]. At first glance, this appears as a rather peculiar finding—mice on a CR diet generally enter a catabolic state, resulting in loss of significant body weight. However, it is important to note that not all tissues shrink during CR: for example while the liver and white adipose tissue reduce in mass, the brain and heart do not [20]. These results, therefore, indicate that CR is more likely inducing a regulated response redirecting resources to different tissues and organs in order to meet the needs of the new low caloric diet. Behavior changes such as increased physical activity, presumably a foraging response, associated with CR are an example of this, and we believe the observed changes in bone mass are yet another example (and perhaps the two are related).

Most excitingly, pharmacological activation of SIRT1 resulted in significant increases in bone mass in two independent models of osteoporosis. Treating 12 month-old mice with SIRT1720 for 5 months resulted in an almost 30% increase in femoral bone mass; a site of frequent fractures in osteoporotic humans. While the differences in bone mass in ovariectomized (OVX) mice were not as dramatic, it should be noted that these mice were much younger (3 months) and were treated for only 1 month. Nonetheless, even with this short treatment, OVX mice showed a highly significant increase in bone mass as compared to vehicle treated animals. As the bone loss from ovariectomy is almost entirely due to increased osteoclast activity, it is reasonable to infer that the partial rescue is due to inhibition of osteoclast activity by activation of SIRT1. Consistent with this model, sham treated mice did not show any improvements in bone mass, indicating that SIRT1 is specifically acting to protect from OVX-induced bone loss. These findings are in agreement with previous studies showing SIRT1 acts as a negative regulator of both osteoclast activity and osteoclastogenesis [39, 46–47]. Encouragingly, a recent study using a more potent SIRT1 agonist resulted in an even more dramatic rescue in a similar OVX model of osteoporosis [48].

Unlike a previous report [36], we did not find SIRT1 expression to decrease with age in bone. We attribute this discrepancy to the following: 1) We used calvaria for our qRT-PCR analysis, whereas the aforementioned study used whole long bones where the majority of RNA comes from hematopoietic and mesenchymal precursor cells in the marrow; 2) We used ribosomal gene *rpl19* for normalization, whereas the Edwards *et al* normalized with the metabolically relevant *gapdh* gene, the expression of which has previously been shown to change with age, nutritional status, and mechanical strain on bone [49–51]. In contrast, expression of *rpl19* has been shown to be more constant under a variety of conditions [52–53], including during



normal aging [54]. Regardless, the effect of aging on SIRT1 protein levels and activity in bone tissue is still an open question.

Overall, the data presented here support the model that SIRT1 acts as a positive regulator of *in vivo* bone mass. These findings add to our previous work showing that SIRT1 promotes osteoblast commitment and differentiation by positively regulating  $\beta$ -catenin and RUNX2, respectively [36, 55]. In fact, RUNX2 targets have previously been shown to be upregulated in bone tissue upon SIRT1 activation, consistent with an anabolic role for SIRT1 [55]. The current demonstration of the *in vivo* efficacy of the SIRT1 agonist SRT1720 on bone mass adds weight to the idea that this Sirtuin is a strong pharmacological target for treating both age-related and post-menopausal osteoporosis. Encouragingly, a recent human trial showed that treatment with resveratrol, another SIRT1 activator, led to a statistically significant increase in bone mass in elderly obese men [56]. Therefore new SIRT1 agonists, one of which is already in clinical trials [57–59], may pave the way towards novel therapeutics for osteoporosis.

## Supporting information

**S1 Fig. Deletion of SIRT1 in osteoblasts leads to increased blood glucose levels.** Osteoblast specific SIRT1 knockouts (ObKO) show higher blood glucose levels as compared to wildtype littermate controls under fed, but not fasted, conditions. Osteoclast specific knockouts (OcKO) do not show a difference under either condition. ( $n \geq 9$  for each group; \*  $p < .05$ ). (TIF)

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**Funding acquisition:** Leonard Guarente.

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**Methodology:** Kayvan Zainabadi, Leonard Guarente.

**Project administration:** Kayvan Zainabadi, Leonard Guarente.

**Resources:** Kayvan Zainabadi, Leonard Guarente.

**Supervision:** Kayvan Zainabadi, Leonard Guarente.

**Validation:** Kayvan Zainabadi.

**Visualization:** Kayvan Zainabadi.

**Writing – original draft:** Kayvan Zainabadi.

**Writing – review & editing:** Kayvan Zainabadi, Cassie J. Liu, Alison L. M. Caldwell, Leonard Guarente.

## References

1. Haigis MC, Guarente LP. Mammalian sirtuins—emerging roles in physiology aging and calorie restriction. *Genes Dev.* 2006; 20:2913–21. <https://doi.org/10.1101/gad.1467506> PMID: 17079682
2. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature.* 2000; 403:795–800. <https://doi.org/10.1038/35001622> PMID: 10693811
3. Kennedy BK, Austriaco NR Jr, Zhang J, Guarente L. Mutation in the silencing gene SIR4 can delay aging in *S. cerevisiae*. *Cell.* 1995; 80:485–96. PMID: 7859289
4. Kennedy BK, Gotta M, Sinclair DA, Mills K, McNabb DS, Murthy M, et al. Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*. *Cell.* 1997; 8:381–91.
5. Tissenbaum HA, Guarente L. Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature.* 2001; 410:227–30. <https://doi.org/10.1038/35065638> PMID: 11242085
6. Rogina B, Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A.* 2004; 101:15998–6003. <https://doi.org/10.1073/pnas.0404184101> PMID: 15520384
7. Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature.* 2004; 430:686–9. <https://doi.org/10.1038/nature02789> PMID: 15254550
8. Mitchell SJ, Martin-Montalvo A, Mercken EM, Palacios HH, Ward TM, Abulwerdi G, et al. The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet. *Cell Rep.* 2014; 6:836–43. <https://doi.org/10.1016/j.celrep.2014.01.031> PMID: 24582957
9. Satoh A, Brace CS, Rensing N, Cliften P, Wozniak DF, Herzog ED, et al. Sirt1 extends life span and delays aging in mice through the regulation of Nk2 homeobox 1 in the DMH and LH. *Cell Metab.* 2013; 18:416–30. <https://doi.org/10.1016/j.cmet.2013.07.013> PMID: 24011076
10. Mercken EM, Mitchell SJ, Martin-Montalvo A, Minor RK, Almeida M, Gomes AP, et al. SRT2104 extends survival of male mice on a standard diet and preserves bone and muscle mass. *Aging Cell.* 2014; 13:787–96. <https://doi.org/10.1111/acef.12220> PMID: 24931715
11. Herranz D, Muñoz-Martin M, Cañamero M, Mulero F, Martínez-Pastor B, Fernández-Capetillo O, et al. Sirt1 improves healthy ageing and protects from metabolic syndrome-associated cancer. *Nat Commun.* 2010; 1:3. <https://doi.org/10.1038/ncomms1001> PMID: 20975665
12. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, et al. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 $\alpha$ . *Cell.* 2006; 127:1109–22. <https://doi.org/10.1016/j.cell.2006.11.013> PMID: 17112576
13. Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, et al. SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. *EMBO J.* 2007; 26:3169–79. <https://doi.org/10.1038/sj.emboj.7601758> PMID: 17581637
14. Banks AS, Kon N, Knight C, Matsumoto M, Gutiérrez-Juárez R, Rossetti L, et al. SirT1 gain of function increases energy efficiency and prevents diabetes in mice. *Cell Metab.* 2008; 8:333–41. <https://doi.org/10.1016/j.cmet.2008.08.014> PMID: 18840364
15. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, et al. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature.* 2006; 444:337–42. <https://doi.org/10.1038/nature05354> PMID: 17086191
16. Barger JL, Kayo T, Vann JM, Arias EB, Wang J, Hacker TA, et al. A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. *PLoS One.* 2008; 3:e2264. <https://doi.org/10.1371/journal.pone.0002264> PMID: 18523577
17. Pearson KJ, Baur JA, Lewis KN, Peshkin L, Price NL, Labinskyy N, et al. Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. *Cell Metab.* 2008; 8:157–68. <https://doi.org/10.1016/j.cmet.2008.06.011> PMID: 18599363
18. Gräff J, Kahn M, Samiei A, Gao J, Ota KT, Rei D, et al. A dietary regimen of caloric restriction or pharmacological activation of SIRT1 to delay the onset of neurodegeneration. *J. Neurosci.* 2013; 33:8951–60. <https://doi.org/10.1523/JNEUROSCI.5657-12.2013> PMID: 23699506
19. Bordone L, Cohen D, Robinson A, Motta MC, van Veen E, Czopik A, et al. SIRT1 transgenic mice show phenotypes resembling caloric restriction. *Aging Cell.* 2007; 6:759–67. <https://doi.org/10.1111/j.1474-9726.2007.00335.x> PMID: 17877786
20. Koubova J, Guarente L. How does calorie restriction work? *Genes Dev.* 2003; 17:313–21. <https://doi.org/10.1101/gad.1052903> PMID: 12569120

21. Guarente L, Picard F. Calorie restriction—the SIR2 connection. *Cell*. 2005; 120:473–82. <https://doi.org/10.1016/j.cell.2005.01.029> PMID: 15734680
22. Chen D, Steele AD, Lindquist S, Guarente L. Increase in activity during calorie restriction requires Sirt1. *Science*. 2005; 310:1641. <https://doi.org/10.1126/science.1118357> PMID: 16339438
23. Boily G, Seifert EL, Bevilacqua L, He XH, Sabourin G, Estey C, et al. Sirt1 regulates energy metabolism and response to caloric restriction in mice. *PLoS One*. 2008; 3:e1759. <https://doi.org/10.1371/journal.pone.0001759> PMID: 18335035
24. Mercken EM, Hu J, Krzysik-Walker S, Wei M, Li Y, McBurney MW, et al. SIRT1 but not its increased expression is essential for lifespan extension in caloric-restricted mice. *Aging Cell*. 2014; 13:193–6. <https://doi.org/10.1111/acer.12151> PMID: 23941528
25. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science*. 2004; 305:390–2. <https://doi.org/10.1126/science.1099196> PMID: 15205477
26. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, et al. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science*. 2005; 310:314–7. <https://doi.org/10.1126/science.1117728> PMID: 16224023
27. Kalu DN, Hardin RR, Cockerham R, Yu BP, Norling BK, Egan JW. Lifelong food restriction prevents senile osteopenia and hyperparathyroidism in F344 rats. *Mech Ageing Dev*. 1984; 26:103–12. PMID: 6748753
28. Tatsumi S, Ito M, Asaba Y, Tsutsumi K, Ikeda K. Life-long caloric restriction reveals biphasic and dimorphic effects on bone metabolism in rodents. *Endocrinology*. 2008; 149:634–41. <https://doi.org/10.1210/en.2007-1089> PMID: 17991723
29. Sanderson JP, Binkley N, Roecker EB, Champ JE, Pugh TD, Aspnes L, et al. Influence of fat intake and caloric restriction on bone in aging male rats. *J Gerontol A Biol Sci Med Sci*. 1997; 52:B20–5. PMID: 9008654
30. Redman LM, Rood J, Anton SD, Champagne C, Smith SR, Ravussin E, et al. Calorie restriction and bone health in young, overweight individuals. *Arch Intern Med*. 2008 22; 168:1859–66. <https://doi.org/10.1001/archinte.168.17.1859> PMID: 18809812
31. Rodan GA and Martin TJ. Therapeutic approaches to bone diseases. *Science*. 2000; 289:1508–14. PMID: 10968781
32. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, et al. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature*. 2007;29; 450:712–6. <https://doi.org/10.1038/nature06261> PMID: 18046409
33. Dacquin R, Starbuck M, Schinke T, Karsenty G. Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. *Dev Dyn*. 2002; 224:245–51. <https://doi.org/10.1002/dvdy.10100> PMID: 12112477
34. Liu F, Woitge HW, Braut A, Kronenberg MS, Lichtler AC, Mina M, et al. Expression and activity of osteoblast-targeted Cre recombinase transgenes in murine skeletal tissues. *Int J Dev Biol*. 2004; 48:645–53. <https://doi.org/10.1387/ijdb.041816fl> PMID: 15470637
35. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res*. 1999; 8:265–77. PMID: 10621974
36. Simic P, Zainabadi K, Bell E, Sykes DB, Saez B, Lotinun S, et al. SIRT1 regulates differentiation of mesenchymal stem cells by deacetylating  $\beta$ -catenin. *EMBO Mol Med*. 2013; 5:430–40. <https://doi.org/10.1002/emmm.201201606> PMID: 23364955
37. Iyer S, Han L, Bartell SM, Kim HN, Gubrij I, de Cabo R, et al. Sirtuin1 (Sirt1) promotes cortical bone formation by preventing  $\beta$ -catenin sequestration by FoxO transcription factors in osteoblast progenitors. *J Biol Chem*. 2014; 289:24069–78. <https://doi.org/10.1074/jbc.M114.561803> PMID: 25002589
38. Cohen-Kfir E, Artsi H, Levin A, Abramowitz E, Bajayo A, Gurt I, et al. Sirt1 is a regulator of bone mass and a repressor of Sost encoding for sclerostin, a bone formation inhibitor. *Endocrinology*. 2011; 152:4514–24. <https://doi.org/10.1210/en.2011-1128> PMID: 21952235
39. Edwards JR, Perrien DS, Fleming N, Nyman JS, Ono K, Connelly L, et al. Silent information regulator (Sir)T1 inhibits NF- $\kappa$ B signaling to maintain normal skeletal remodeling. *J Bone Miner Res*. 2013; 28:960–9. <https://doi.org/10.1002/jbmr.1824> PMID: 23172686
40. Chang HC, Guarente L. SIRT1 mediates central circadian control in the SCN by a mechanism that decays with aging. *Cell*. 2013; 153:1448–60. <https://doi.org/10.1016/j.cell.2013.05.027> PMID: 23791176
41. Gong H, Pang J, Han Y, Dai Y, Dai D, Cai J, et al. Age-dependent tissue expression patterns of Sirt1 in senescence-accelerated mice. *Mol Med Rep*. 2014; 10:3296–302. PMID: 25323555

42. Cohen DE, Supinski AM, Bonkowski MS, Donmez G, Guarente LP. Neuronal SIRT1 regulates endocrine and behavioral responses to calorie restriction. *Genes Dev.* 2009; 23:2812–7. <https://doi.org/10.1101/gad.1839209> PMID: 20008932
43. Toorie AM, Cyr NE, Steger JS, Beckman R, Farah G, Nilni EA. The Nutrient and Energy Sensor Sirt1 Regulates the Hypothalamic-Pituitary-Adrenal (HPA) Axis by Altering the Production of the Prohormone Convertase 2 (PC2) Essential in the Maturation of Corticotropin-releasing Hormone (CRH) from Its Prohormone in Male Rats. *J Biol Chem.* 2016; 291:5844–59. <https://doi.org/10.1074/jbc.M115.675264> PMID: 26755731
44. Kolthur-Seetharam U, Teerds K, de Rooij DG, Wendling O, McBurney M, Sassone-Corsi P, et al. The histone deacetylase SIRT1 controls male fertility in mice through regulation of hypothalamic-pituitary gonadotropin signaling. *Biol Reprod.* 2009; 80:384–91. <https://doi.org/10.1095/biolreprod.108.070193> PMID: 18987333
45. Lemieux ME, Yang X, Jardine K, He X, Jacobsen KX, Staines WA, et al. The Sirt1 deacetylase modulates the insulin-like growth factor signaling pathway in mammals. *Mech Ageing Dev.* 2005; 126:1097–105. <https://doi.org/10.1016/j.mad.2005.04.006> PMID: 15964060
46. Gurt I, Artsi H, Cohen-Kfir E, Hamdani G, Ben-Shalom G, Feinstein B, et al. The Sirt1 Activators SRT2183 and SRT3025 Inhibit RANKL-Induced Osteoclastogenesis in Bone Marrow-Derived Macrophages and Down-Regulate Sirt3 in Sirt1 Null Cells. *PLoS One.* 2015 Jul 30; 10:e0134391. <https://doi.org/10.1371/journal.pone.0134391> PMID: 26226624
47. Kim HN, Han L, Iyer S, de Cabo R, Zhao H, O'Brien CA, et al. Sirtuin1 Suppresses Osteoclastogenesis by Deacetylating FoxOs. *Mol. Endocrinol.* 2015; 29:1498–509. <https://doi.org/10.1210/me.2015-1133> PMID: 26287518
48. Artsi H, Cohen-Kfir E, Gurt I, Shahar R, Bajayo A, Kalish N, et al. The Sirtuin1 activator SRT3025 down-regulates sclerostin and rescues ovariectomy-induced bone loss and biomechanical deterioration in female mice. *Endocrinology.* 2014; 155:3508–15. <https://doi.org/10.1210/en.2014-1334> PMID: 24949665
49. Mozdziak PE, Dibner JJ, McCoy DW. Glyceraldehyde-3-phosphate dehydrogenase expression varies with age and nutrition status. *Nutrition.* 2003; 19:438–40. PMID: 12714097
50. Lowe DA, Degens H, Chen KD, Alway SE. Glyceraldehyde-3-phosphate dehydrogenase varies with age in glycolytic muscles of rats. *J Gerontol A Biol Sci Med Sci.* 2000; 55:B160–4. PMID: 10795720
51. Maran A, Hefferan TE, Zhang M, Turner RT. Unanticipated changes in steady-state mRNA levels for glyceraldehyde-3-phosphate dehydrogenase in rat tibiae. *Calcif Tissue Int.* 2004; 74:204–7. <https://doi.org/10.1007/s00223-002-1098-2> PMID: 14583840
52. Zhou L, Lim QE, Wan G, Too HP. Normalization with genes encoding ribosomal proteins but not GAPDH provides an accurate quantification of gene expressions in neuronal differentiation of PC12 cells. *BMC Genomics.* 2010; 11:75. <https://doi.org/10.1186/1471-2164-11-75> PMID: 20113474
53. Mohelnikova-Duchonova B, Oliverius M, Honsova E, Soucek P. Evaluation of reference genes and normalization strategy for quantitative real-time PCR in human pancreatic carcinoma. *Dis Markers.* 2012; 32:203–10. <https://doi.org/10.3233/DMA-2011-0875> PMID: 22377737
54. Warren LA, Rossi DJ, Schiebinger GR, Weissman IL, Kim SK, Quake SR. Transcriptional instability is not a universal attribute of aging. *Aging Cell.* 2007; 6:775–82. <https://doi.org/10.1111/j.1474-9726.2007.00337.x> PMID: 17925006
55. Zainabadi K, Liu CJ, Guarente L. SIRT1 is a positive regulator of the master osteoblast transcription factor, RUNX2. *PLoS One.* 2017; 12:e0178520. <https://doi.org/10.1371/journal.pone.0178520> PMID: 28542607
56. Ornstrup MJ, Harsløf T, Kjær TN, Langdahl BL, Pedersen SB. Resveratrol increases bone mineral density and bone alkaline phosphatase in obese men: a randomized placebo-controlled trial. *J. Clin. Endocrinol. Metab.* 2014; 99:4720–9. <https://doi.org/10.1210/jc.2014-2799> PMID: 25322274
57. Sands BE, Joshi S, Haddad J, Freudenberg JM, Oommen DE, Hoffmann E, et al. Assessing Colonic Exposure, Safety, and Clinical Activity of SRT2104, a Novel Oral SIRT1 Activator, in Patients with Mild to Moderate Ulcerative Colitis. *Inflamm Bowel Dis.* 2016; 22:607–14. <https://doi.org/10.1097/MIB.0000000000000597> PMID: 26595549
58. Krueger JG, Suárez-Fariñas M, Cueto I, Khacherian A, Matheson R, Parish LC, et al. A Randomized, Placebo-Controlled Study of SRT2104, a SIRT1 Activator, in Patients with Moderate to Severe Psoriasis. *PLoS One.* 2015; 10:e0142081. <https://doi.org/10.1371/journal.pone.0142081> PMID: 26556603
59. Baksi A, Kraydashenko O, Zalevkaya A, Stets R, Elliott P, Haddad J, et al. A phase II, randomized, placebo-controlled, double-blind, multi-dose study of SRT2104, a SIRT1 activator, in subjects with type 2 diabetes. *Br J Clin Pharmacol.* 2014; 78:69–77. <https://doi.org/10.1111/bcp.12327> PMID: 24446723