We thank the editor and two reviewers for the helpful comments to improve our manuscript. As described in the detailed point-by-point rebuttal below, we have carefully addressed all the concerns and suggestions raised by the reviewers in the revised manuscript.

We wish that you and the reviewers would be satisfied with our revision. Should you need additional information, please let us know.

Thank you again for your kind consideration of our manuscript.

Sincerely,

Kyoung-Han Kim and Hoon-Ki Sung

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you. We carefully went through the manuscript for grammatical and spelling errors during this revision.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Thank you. We have formatted our revised manuscript based on the JoVE guidelines.

3. Please revise lines 223-230 and 237-241 to avoid overlap with previous publications.

Thank you for the comment and sorry the overlapping issue with our previous work. To address this, we have re-written the indicated lines to avoid the overlapping as follows:

PREVIOUS VERSION (lines 223-230 of the original manuscript): Body composition analysis revealed that IF specifically reduced fat mass without changes in lean mass in wild-type mice (**Figure 3C**). We speculated that the lower body weight of IF animals might be attributed to the slight decrease in accumulated energy intake over 16 weeks of the IF program. However, IF experiment with the pair-feeding regimen revealed that IF-mediated decrease in body weight is not attributed to an energy intake difference (**Figure 3D-E**). Unlike wild-type animals, *ob/ob* mice subjected to IF exhibited lower body weight than Ob-AL mice (**Figure 3G**). This is due to hyperphagic behaviors of *ob/ob* mice, leading to mild (21%) increase in total food intake in AL mice, compared to IF-treated animals (**Figure 3H**).

→ REVISED VERSION (lines 317-325 of the revised manuscript): Body composition analysis revealed that IF specifically reduced fat mass without changes in lean mass in wild-type mice (**Figure 3C**). It is possible that a slightly, albeit not significantly, lower accumulated energy intake over 16 weeks of the IF program could result in reduced body weight gain of IF animals. However, IF experiment with the pair-feeding regimen confirmed that the decreased body weight gain by IF was not due to altered energy intake (**Figure 3D-E**). Unlike wild-type animals, body weight of *ob/ob* mice subjected to IF (Ob-IF) was lower than that of Ob-AL mice (**Figure 3G**). This is due to hyperphagia (excessive eating) of *ob/ob* mice, leading to mildly higher (21%) food intake in AL mice, compared to IF-treated animals (**Figure 3H**).

PREVIOUS VERSION (lines 237-241 of the original manuscript): As shown in **Figure 4A-D**, HFD-IF mice showed improved glucose homeostasis with smaller glucose excursion in GTT, increased insulin sensitivity in ITT, compared to HFD-AL or HFD-PF mice.

→ REVISED VERSION (lines 331-335 of the revised manuscript): As shown in **Figure 4A-D**, HFD-IF mice exhibited a significant improvement in glucose homeostasis. GTT showed that blood glucose is more rapidly cleared in IF-treated mice, while ITT revealed higher insulin sensitivity in HFD-IF mice, compared to HFD-AL or HFD-PF mice.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Harlan, Research Diets, Echo-MRI

Thank you. We have removed commercial language, including Harlan and Research Diets and replaced them with generic terms.

Protocol:
1. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Thank you for the comment. We have revised our manuscript to address this comment.

Figures:
1. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, or .pdf file (4 in total). Please remove ‘Figure 1’ etc. from the figures themselves.

We have made individual PDF files for each figure. ‘Figure X ’ labelling of each figure has been removed.

2. Figure 1: Please define the error bars. Also, please use ‘day 1/2/3’ instead of ‘day1/2/3’ (include a space).

Thank you for the comments. The definition of the error bars for Figure 1 has been added and the X-axis title, Day 1/2/3, has been modified.

References:
1. Please do not abbreviate journal titles.

We have revised references with non-abbreviated journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Thank you. We have added all materials and equipment used in the protocol to the Tagble of Materials.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This manuscript provides a valuable perspective on the appropriate methodology to apply to intermittent fasting as a nutritional intervention, particularly to ensure that the fasting effects can be isolated from additionally inadvertently underfeeding. The authors provide evidence describing the importance of applying a 2-day feeding to 1-day fasting interval, relative to a 1-day feeding:1-day fasting, to ensure that the protocol provides sufficient energy in the refeeding period to compensate for the energy deficit accrued in the fasting day. The authors also provide evidence for the application of pair-feeding in particular animal models or experimental approaches expected to alter feeding behavior. The authors provide a comprehensive description of the 2-day feeding:1-day fasting isocaloric intermittent fasting feeding regimen, the application of a Pair-feeding control group within this context, a description of the body composition analysis and description of the glucose and insulin tolerance tests and provide data representing the application of these feeding approaches.

Major Concerns:
This manuscript nicely describes the application of the intermittent fasting feeding regimen on body composition and glucose homeostasis and ensuring that energy intake is carefully matched. However, energy balance and therefore body composition depends not only on energy intake but also energy expenditure. While the described protocol very nicely controls for the energy intake, it would be worth providing representative data on how such an intervention can alter energy expenditure to provide readers further perspective on the phenotype that might be manifested under this type of regimen and its influence on energy balance. For example, the HFD-IF show a reduced increase in body weight relative to their HFD-PF, clearly showing that the fasting itself is eliciting an effect on body weight. Since energy intake is matched, presumably this difference in body weight gain is due to an increase in whole-body energy expenditure in the HFD-IF. In contrast, the Ob-PF show no difference in body weight relative to the Ob-IF, yet oxygen consumption is lower only in the fasted state in the Ob-IF (2nd attached manuscript). These are very interesting findings resulting from the fasting itself.

We thank the reviewer for the positive comments and in particular, very constructive suggestions about energy expenditure data.

We cannot agree more that one of main mechanisms of IF-mediated metabolic benefits is adipose thermogenesis thereby increasing energy expenditure without affecting food intake. Particularly, we have previously shown that energy expenditure was increased during refeeding period after fasting and it is largely mediated by adipose thermogenesis, including browning of white adipose tissue and activation of brown adipose tissue [Kim et al, Cell Res, 2017]. On the other hand, in our follow-up study, we have demonstrated that this elevated energy expenditure with increased adipose browning was not evident in IF-treated *ob/ob* mice, suggesting that the effect of IF on energy expenditure can be changed in different types of obesity [Kim et al., Sci Rep, 2019]. As the reviewer commented, data above would presumably explain why wild-type mice subjected to IF exhibited the reduced body weight gain without a difference in food intake, compared to AL mice. However, we respectively disagree that the body weight reduction effect by IF is mediated by IF because we have shown that adipose-specific VEGF KO (Ad-VEGF-KO) mice, which exhibited impaired IF-induced adipose thermogenesis, still showed reduced body weight gain compared to AL-treated Ad-VEGF-KO mice. This suggests the absence of effect in body weight gain observed in IF-treated *ob/ob* mice is not simply due to inhibited thermogenesis, but likely caused by lack of leptin's metabolic functions. Nevertheless, we agree with the reviewer that the addition of energy expenditure data is beneficial to potential readers who would use this protocol.

To address the Reviewer's comment, thus, we have added new representative results of energy expenditure in IF-treated wild-type and *ob/ob* mice as Figure 5, and have demonstrated that as follows:

(lines 343-354 of the revised manuscript)
One of the metabolic effects of IF in wild-type mice is higher total O_2 consumption, used to estimate the energy expenditure (**Figure 5A-B**). This elevation in O_2 consumption was found only during feeding period in IF mice, but not fasting period, compared to AL mice. The increased energy expenditure was largely mediated by adipose thermogenesis, such as browning of white adipose tissues and activation of brown adipose tissue (data not shown)[8,16]. IF-mediated adipose thermogenesis would presumably explain how wild-type mice subjected to IF exhibited the reduced body weight gain with no difference in food intake, compared to AL mice. On the other hand, IF failed to increase O_2 consumption in *ob/ob* mice (**Figure 5C-D**), and even led to a reduction in energy expenditure during fasting period. Consistently, IF-induced adipose thermogenesis was completely abolished in *ob/ob* mice (data not shown). These data suggest a possible limitation of IF as it may work differently for individuals with different genetic and environmental backgrounds.

Minor Concerns:
The Discussion describes two topics that are not particularly discussed in the Introduction or addressed in the Methods (housing density and temperature). I wonder whether it is worth describing how housing temperature can influence the phenotype and/or the fasting:feeding ratio - is it worth having more refeeding days if exposed to a colder environment? Can you run such a protocol in a cold environment? If so, how cold? Some animals struggle to survive in the cold when fasted for a prolonged period. This might be worth discussing as many groups are interested in combining these two metabolic stimuli.

We appreciate the reviewer's suggestion on housing temperature. It is indeed one of the most influential factors that largely affects feeding behaviour. For example, cold exposure around 4-6°C significantly increases energy intake to maintain core body temperature, whereas thermoneutral condition (30°C), where heat gain is balanced by heat loss, results in marked

reductions in food consumption. To address the reviewer's suggestion and questions, we have added a new paragraph in the Discussion as shown below. In particular, we discussed about a current interest in combining two interventions, IF and cold exposure, to maximize the metabolic benefits. We are hoping this revision improves the quality of our manuscript. We deeply thank the reviewer again for the supports.

(lines 471-488 of the revised manuscript)
An important factor to be considered for IF studies is housing temperature, which affects various physiological and behavioral parameters in mice. Particularly, cold exposure (4-6°C) significantly increases energy intake to maintain core body temperature[28], whereas under thermoneutral condition (30°C), where heat gain is balanced by heat loss, reductions in food consumption is markedly reduced[8]. With respect to metabolic outcomes, cold exposure induces adipose thermogenesis, which is hampered by thermoneutral condition. Therefore, it is expected that housing temperature would influence the metabolic phenotype of IF and the appropriate feeding-fasting ratio to achieve isocaloric IF. Indeed, we have previously demonstrated that isocaloric 2:1 IF can be achieved in thermoneutral condition, leading to improved metabolic health against diet-induced obesity and metabolic dysfunction without a differences in food intake between IF and AL groups[8]. However, isocaloric IF might not be achievable with 2:1 ratio at cold temperature because mice under cold exposure would show a hyperphagic phenotype, which would lead to underfeeding in the IF group. Since cold exposure and IF display comparable metabolic outcomes and mechanisms, such as adipose thermogenesis and improved glucose homeostasis, against obesity, there are interests in combining these two interventions to maximize the metabolic impacts. To properly test this, therefore, performing the feeding analysis before running IF experiment and utilizing a pair-feeding control group under cold exposure are recommended.

Reviewer #2:

Manuscript Summary:
This metods article describes protocols for isocaloric 2:1 intermittent fasting (IF) and pair-feeding that would be useful for studies investigating the effects of IF on other disease conditions. Overall, it is well summarized methods article on the IF using a mouse model and I have a minor point:

Minor Concerns:
- Introduction (the 2nd paragraph): It is recommended that the explanations on Figures 1A and 1B need to be more specifically described. It is not clear that the sentence in lines 64-65 is related with Figure 1C or 1A/1B.

We thank the reviewer for the positive comments. We also apologize if you felt that the paragraph in the Introduction was confusing. Our purpose was providing a brief introduction about 2:1 intermittent fasting (IF), compared to alternate day fasting, rather than specific description, since we provide the detail descriptions on results shown in Figure 1A-C in the sections of the REPRESENTATIVE RESULTS and FIGURE LEGENDS. To minimize a possible confusion, we have removed the indication to Figure 1 and we believe that in that way readers can easily find the detailed description below. We hope this change makes the revised manuscript improved and we thank the reviewer again for the support and comments.