nature portfolio

Peer Review File

Dysfunctional Adipocytes Promote Tumor Progression Through YAP/TAZ-dependent Cancer-Associated Adipocyte Transformation

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript reports a tumor suppressive role of BECN1 in control of cancer-associate adipocytes (CAAs) to inhibit cancer development in adipose-rich tissues. The authors show that the BECN1-deficient adipocytes promote breast and colon tumor growth, which is independent of BECN1-mediated autophagy but involved in the activation of Hippo pathway effectors YAP and TAZ. Upon BECN1 loss, activated YAP/TAZ drive the transcription of genes in inflammation, TNF-alpha signaling, and secreted adipokines to induce tumor progression, phenocopying the oncogenic events observed in CAAs. Targeting YAP/TAZ genetically and pharmacologically inhibits adipose-associated tumor growth.

Although the proposed model is potentially interesting, some of the results presented are too preliminary and require more robust data to support the authors' conclusions. Another weakness of this study is that the presented findings are mostly descriptive, lacking mechanistic insights into the observations.

The following improvements are suggested for the authors.

1. The xenograft tumor studies (Fig. 1a-d, 4e-g, 7) were performed subcutaneously, which lacks biological relevance for the related tumor microenvironment. Orthotopic xenograft should be performed. Additional cell lines should be included to address the potential cell line specificity issue.

2. It is unclear how YAP/TAZ were identified to act downstream of BECN1 in adipocytes. If this is the case, why was YAP/TAZ or Hippo signaling gene signature not identified as the top altered gene set in the BECN1-deficient cells (Fig. 2a, 3f)? How do YAP/TAZ regulate the transcription of inflammation genes as well as LCN2 (Fig. 7f-7i)? Are YAP/TAZ and TEAD able to directly bind the promoters/enhancers of these genes? ChIP assay should be performed to test this hypothesis. To strengthen this conclusion, the authors should also examine the adipocytes with Hippo deficiency (e.g., Lats1/2 KO) or YAP activation (e.g., YAP-5SA) to see whether they could similarly modulate the inflammation/LCN2 genes and tumor growth.

3. In Fig. 5a and 5c, YAP expression is significantly suppressed by BECN1. However, in Fig. 5h, loss of BECN1 did not affect YAP protein level. This inconsistency should be addressed. Would YAP transcription be affected in Fig. 5a, 5c, and 5h? This brings in another question – how does BECN1 regulate the Hippo pathway? Although the authors cited some literatures, they should at least examine additional Hippo pathway components like LATS1, MOB1, MST1 (both expression and phosphorylation) in the BECN1-deficient cells.

4. In Fig. 4, the authors show a dispensable role of autophagy in BECN1-mediated regulation of YAP/TAZ and tumor growth. To strengthen the conclusion, the authors should reconstitute the BECN1-deficient adipocytes with wild-type BECN1 and its mutants – the ones that cannot activate autophagy and the Hippo pathway, respectively, and use these cells to evaluate their roles in driving inflammation genes' transcription and tumor growth.

5. BECN1 protein expression in adipocytes were tightly controlled by cancer cells (Fig. 3c), adipocyte differentiation (Fig. 5a), and high-fat diet (Fig. 7e). Since these data provide the physiological/pathological regulation of BECN1 in adipocytes, the underlying mechanisms

should be elucidated.

6. The reviewer is curious about what if BECN1 is depleted in other types of cells like fibroblasts and epithelial cells. Would these BECN1-deficient non-adipocytes cells also show YAP/TAZ activation and enhanced inflammation, and drive tumor growth by modulating the microenvironment?

Minor points:

1. In Fig. 2j, the authors should knockdown LCN2 in adipocytes to repeat the co-culture experiment. In addition, treatment with LCN2 antibody in the co-culture media would help further strengthen the conclusion.

2. In Fig. 3c-d, were the q-PCR and WB studies performed in the mixed adipocyte-cancer cells or specifically in the isolated adipocytes from the co-culture system?

3. The provided Figure Legends are quite concise, making it hard to determine how the experiments were exactly performed.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors employed an adipose tissue-specific BECN1 knockout (BaKO) mouse model to investigate the effects of dysfunctional adipocytes (caused by loss-of-function of Beclin1) on the growth of certain types of tumors. They reported that BaKO adipocytes promote tumor growth both in vitro and in vivo using a combination of multiple mouse models. Furthermore, they demonstrated the involvement of the YAP/TAZ signaling pathway in tumor progression. As a demonstration, deletion or inhibition of YAP/TAZ in the BaKO adipocytes restored normal cell functions, suppressed lipodystrophy, and mitigated inflammatory phenotypes. Overall, the data are robust, and the findings are intriguing. The conclusions are well-supported by their results. However, there are significant concerns regarding unclear presentations and a lack of sufficient mechanistic studies, which somewhat diminish the enthusiasm for this otherwise interesting study. The following concerns, including both major and minor issues, are outlined below:
Major Concerns:
1). Several result titles start with "Dysfunctional adipocytes," which fails to provide precise information about the adipocytes in the respective paragraph. If these cells are BECN1 KO cells, it is recommended to use "BaKO adipocytes."; If the cells were isolated from diet-induced obese adipose tissue, then the term "diet-induced obese adipocytes" should be used. Employing clearer terminology would help readers better understand the nature of these cells.
2). It has been reported that loss-of-function of BECN1 promotes tumor growth in cancer cells. Similarly, here, the authors found that BaKO in adipocytes stimulates co-cultured tumor cells and activates downstream YAP/TAZ pathways, paralleling the role observed previously in cancer cells. The stimulation of the YAP/TAZ signaling pathway might explain the impact on adipogenesis reduction and inflammation enhancement. However, one significant effect of BaKO in adipocytes is enhanced lipolysis (Fig. 5g, Supplementary Fig. 5e and i). Considering that lipolysis can promote cancer growth, it's surprising that the authors didn't investigate the mechanism underlying how BaKO adipocytes increase

lipolysis.
3). The manuscript lacks crucial information about the recipient mice for co-injected WT or Becn1 KO cells with 4T1. Are these nude mice with compromised inflammatory reactions for the injected cancer cells? Additionally, the nature of the co-injected fat cells remains unclear, as there is mention of imSVCs (line 99) and differentiated adipocytes (line 100).
4). There's a need to reconcile the observations of BECN1-deficient adipocytes and HFD-feeding induced obese adipocytes. In Fig 6, the authors note that inhibition of YAP/TAZ activity in both BECN1-deficient and HFD-fed obese adipocytes yields the same tumor suppression effect. However, these two adipocyte types exhibit stark morphological differences: BECN1-deficient adipocytes have small lipid droplets and are prone to dedifferentiation to fibroblasts, while HFD-induced obese adipocytes possess unusually large lipid contents and do not transform into fibroblasts. Moreover, there's confusion in line 298-299, where they state that "the WATs of the HFD-fed mice had decreased levels of BECN1," while these fat cells do not share the lipid droplet phenotype of BECN1 KO adipocytes. Addressing these discrepancies is crucial.
5). The autophagy-independent effect of BECN1 in adipose tissue needs further experimentation to provide solid support. While the authors discuss the involvement of key factors such as LATS1 and MST1 (lines 303 to 312), more experiments are required to establish their role in regulation.

6). The discussion lacks depth and detail. The authors should provide more comprehensive mechanistic insights into the effects of BECN1 in adipocytes. Particularly, they should elucidate how BECN1 suppresses the YAP/TAZ signaling pathway specifically in adipocytes and compare their findings about BECN1/YAP/TAZ signaling in adipocytes with previous reports in other cell types.
Minor Concerns:
1). The RNA-seq results (Fig. 2a&b) were obtained from iWAT, which includes multiple cell populations. However, BECN1 deficiency is specific to adipocytes in the isolated fat tissue.
2). In line 165, clarify whether "Co-regulated" means "Co-upregulated" or "Co-downregulated."
3). In line 219, clarify what "restored phenotypes" refer to. Also, given that the KO effects are specific to adipocytes, it's important to clarify that the liver phenotypes aren't a direct result of YAP/TAZ KO.
4). Line 279: Clarify the specific KO mouse models used in the study.
5). In line 290, specify which phenotypes are meant in relation to the effects of BECN1 depletion.
6). In line 298, explain what is meant by the "quality of adipose tissue." Does "quality" refer to the capacity for lipolysis, appropriate adipokine secretion, or another aspect?

Reviewer #3 (Remarks to the Author):

Summary:
This paper demonstrates Becn1 conditional KO in adipocytes can promote tumor growth of breast cancer and colorectal cancer cells in vivo and in vitro. Adipokine array analysis of serum plasma from Becn1 mutant mice, and RNA sequencing of white adipose tissue of mutant and control mice revealed upregulation of LCN2 and TNFa signaling in mutants in concordance with the tumor growth phenotype. Further analysis suggests that BECN1 KO in adipocytes creates a pro-tumorigenic environment by eliciting gene transcription and

markers associated with CAA (cancer associated adipocyte) status. Upregulation of YAP/TAZ gene signatures was observed in both BECN1 KO and peritumoral adipocytes, suggesting a potential mechanism for the tumor growth phenotypes observed. Beclin KO mice also lacking YAP and TAZ reversed many lipodystrophy phenotypes observed, as well as the increase in TNFa levels. Since Atg7 conditional KO did not elicit similar YAP/TAZ or tumor growth phenotypes in vitro or in vivo that were observed in BECN1 KO, the authors speculate that perhaps BECN1 function via PI3K/MTOR may be responsible for some of the phenotypes. It is my opinion that this work is novel and important, and suitable for publication in this journal with some revisions to characterize the phenotypes and bolster molecular data.

Major Comments:
1. It would be good to look at LCN2 (TNFa?) in the context of ATG7/ Baf/HCQ in vitro.
2. Fig 1h, add p62 and/or lc3b to western blot, to help add context for the Atg7 data.
3. Along with changes in tumor cell proliferation, it would be good to evaluate changes in cell death, i.e. by CC3 staining (i.e. Fig 6), since autophagy can regulate cell death.
4. Please comment/discuss on YAP/TAZ inhibitor effect on other stromal cells? Could be relevant given this paper: The β-catenin/YAP signaling axis is a key regulator of melanoma-associated fibroblasts
5. Are there any changes in Wnt signaling given the beta catenin changes? Can you comment on IL6 given its previous role? Do you think IL6 changes could also drive phenotypes?
6. Is LCN2 or the receptor expressed in the tumor cells of interest? Consider providing some staining and/or citations. (same for TNFa/ receptor?) Is there any other previous data regarding LCN2 in breast/colorectal cancer specifically?
7. Please mention whether obesity is predictive of poor outcome in breast and colon cancer respectively. Overall, the discussion of cancer is very general, some more specifics for breast and colon (as well as cancers where there was no difference!) would be useful.
8. EMT: all the data for this are RNA levels/ gene signatures. Please add some protein data as well for a few markers. Please discuss, are EMT signatures associated with CAA status?
9. Any thoughts as to why the 2 other tumor cell types did not show the same phenotypes?
Minor comments:
1. Spell out LCN2 fully the first time it appears in the text.
2. Line 151 use “co-culture” instead of co cultivation
3. Should mention/discuss and cite Beige Adipocyte Maintenance Is Regulated by Autophagy-Induced Mitochondrial Clearance paper
4. Please spell out what are imSVCs the first time it’s mentioned since I’m not familiar with this acronym.
5. Supp fig 4a: please add quantification
6. Supp. Fig 5c could benefit from BOPIPDY stain for ease of interpretation.
7. Fig. 6g: is WT versus Beclin/YT CKO significantly different? Please add this to the figure.

Response to Reviewers

We highly appreciate the constructive comments provided by the reviewers. We acknowledge that these concerns are reasonable and should be addressed in our manuscript (especially the mechanistic insights into YAP/TAZ regulation and its potential effects on other stromal cells in the tumor microenvironment). In this regard, we have included our thorough responses here in blue and revised the text in the manuscript in red. By doing so, we could further solidify our work, leading to a substantial improvement in the quality of the manuscript. Once again, we express our great thanks to the reviewers for their invaluable time and effort.

REVIEWER COMMENTS

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This manuscript reports a tumor suppressive role of BECN1 in control of cancer-associate adipocytes (CAAs) to inhibit cancer development in adipose-rich tissues. The authors show that the BECN1-deficient adipocytes promote breast and colon tumor growth, which is independent of BECN1-mediated autophagy but involved in the activation of Hippo pathway effectors YAP and TAZ. Upon BECN1 loss, activated YAP/TAZ drive the transcription of genes in inflammation, TNF-alpha signaling, and secreted adipokines to induce tumor progression, phenocopying the oncogenic events observed in CAAs. Targeting YAP/TAZ genetically and pharmacologically inhibits adipose-associated tumor growth.

Although the proposed model is potentially interesting, some of the results presented are too preliminary and require more robust data to support the authors' conclusions. Another weakness of this study is that the presented findings are mostly descriptive, lacking mechanistic insights into the observations.

We appreciate the reviewer's valuable feedback, emphasizing the need for more robust data and mechanistic insights to support our conclusions. In response, we have dedicated efforts to enhance the clarity and depth of our manuscript, specifically delving into the sequential mechanism for BECN1 to YAP/TAZ and LCN2, addressing the reviewer's concerns and elevating the overall quality of our work.

The following improvements are suggested for the authors.

1. The xenograft tumor studies (Fig. 1a-d, 4e-g, 7) were performed subcutaneously, which lacks biological relevance for the related tumor microenvironment. Orthotopic xenograft should be performed. Additional cell lines should be included to address the potential cell line specificity issue.

We appreciate the reviewer's thoughtful suggestions and apologize for any confusion in presenting the methodology of our in-vivo studies (Main Fig. 1,4,7). Our cancer cell injection data comprise both breast and colon cancer models (EO771, 4T1, and MC38), along with the MMTV-PyMT mouse model. Here we provide a detailed explanation of how we implemented these models in our study.

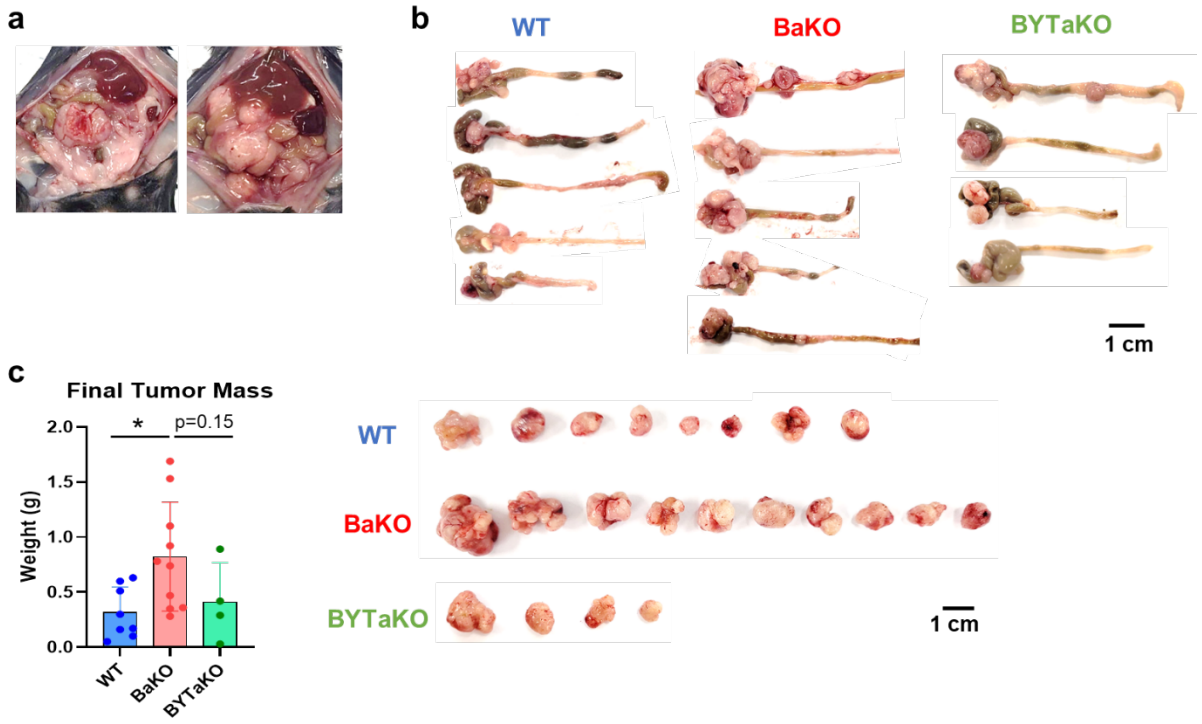
EO771 – Breast cancer cells were allografted by mammary fat pad injection.

4T1 – Breast cancer cells were co-injected subcutaneously with differentiated adipocytes. This approach aimed to minimize the influence of normal adipose tissue, focusing primarily on the impact of co-injected BECN1 WT or KO adipocytes.

MC38 – Colorectal cancer cells were allografted by subcutaneous injection.

MMTV-PyMT – Spontaneous breast cancer model mice were mated with BECN1 aKO mice.

It is important to note that, our breast cancer models were performed orthotopically, while the colon cancer model was not. To address the concerns raised, we performed orthotopic injection of MC38 into WT, BaKO, and BYTaKO to confirm the effect of dysfunctional adipocytes on tumor growth particularly in the context of tumor microenvironment (TME). MC38 cells were injected in the mouse cecum, and the tumors were resected after 4 weeks **(Revised Fig. 1a).** Consistently, MC38 grew much faster in BaKO colon, but not as much in that of BYTaKO **(Revised Fig. 1b, and c).** We hope this clarification addresses the reviewer's concerns regarding the biological relevance of our xenograft tumor studies.



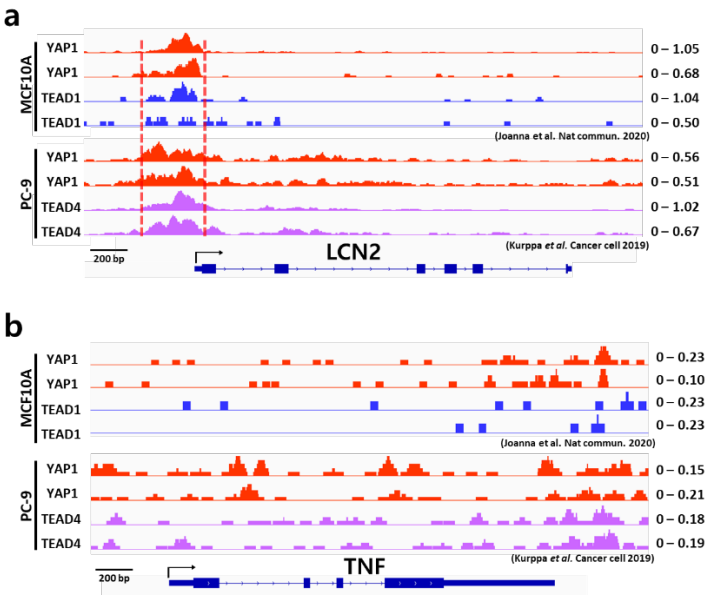
Revised Fig. 1a-c – (a) Colon orthotopic injection of MC38 into mouse cecum. Mice were sacrificed after 4 weeks post-injection. (b) Image of resected colons harboring tumors. (c) Final tumor mass measured (WT n = 8, BaKO n = 10, BYTaKO n = 4).

The results have been incorporated into our Supplementary Figure 9.

2. It is unclear how YAP/TAZ were identified to act downstream of BECN1 in adipocytes. If this is the case, why was YAP/TAZ or Hippo signaling gene signature not identified as the top altered gene set in the BECN1-deficient cells (Fig. 2a, 3f)? How do YAP/TAZ regulate the transcription of inflammation genes as well as LCN2 (Fig. 7f-7i)? Are YAP/TAZ and TEAD able to directly bind the promoters/enhancers of these genes? ChIP assay should be performed to test this hypothesis. To strengthen this conclusion, the authors should also examine the adipocytes with Hippo deficiency (e.g., Lats1/2 KO) or YAP activation (e.g., YAP-5SA) to see whether they could similarly modulate the inflammation/LCN2 genes and tumor growth.

The reason why YAP/TAZ or Hippo signaling did not appear in the altered gene set is that Main Fig. 2a and 3f are based on the Hallmark gene set enrichment analysis (HGSEA), which only consists of 50 general gene sets. To address this, we browsed the 'C6: oncogenic signature gene sets' in Molecular Signature Database (MSigDB). The result is shown in Supplementary Fig. 3c and d, indicating that YAP conserved signature gene set was at the 14th position among of the most significantly altered gene set.

We thank the reviewer to pinpoint the specific mechanism that remained vague in our initial conclusion. We relied on literatures to support our hypothesis that links YAP/TAZ to the upregulation of inflammatory gene expression. Furthermore, the reviewer's comment to check TEAD binding motif at the promoters/enhancers of associated genes (including LCN2) was very helpful [1]. We collected ChIP results of YAP and TEAD from PC-9 and MCF10A cell lines in ChIP database [2, 3]. The following figure illustrates the potential binding region of YAP and TEAD at the promoter regions of LCN2 and TNFα (**Revised Fig. 2a and b**).

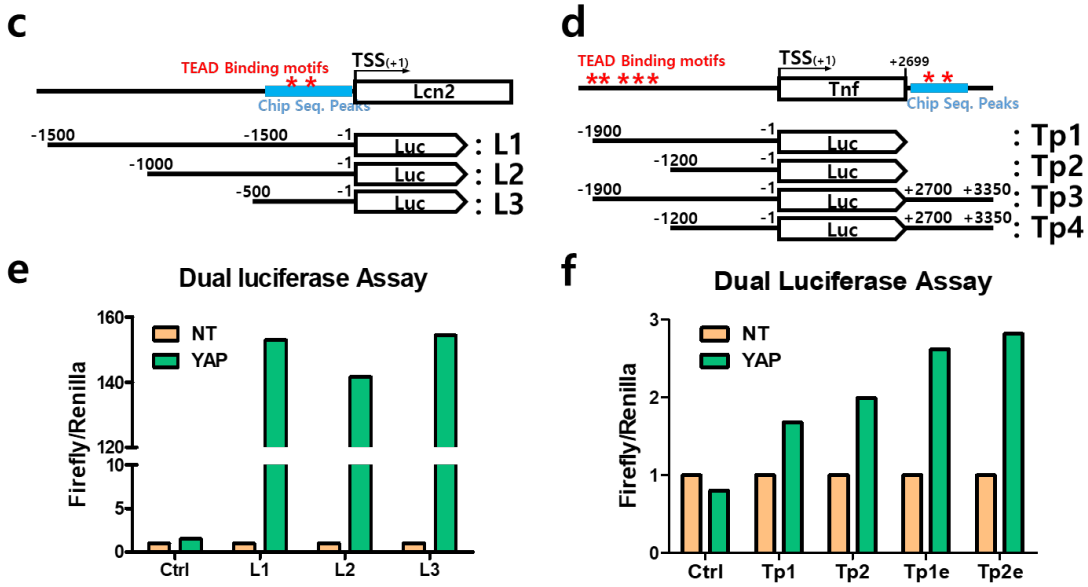


Revised Fig. 2a,b – (a,b) ChIP-seq result of YAP and TEAD in MCF10A and PC-9 cells at the genomic loci of LCN2 (a) and TNFα (b). ChIP-seq results were collected from 'chip-atlas.org'. Intensified peaks indicate potential binding regions of the proteins.

To further validate the regulatory role of YAP/TAZ-TEAD activity on TNFα and LCN2 transcription, we constructed luciferase vectors containing TNFα or LCN2 promoters/enhancers **(Revised Fig. 2c and d).** After transfection of each plasmid, we treated YAP recombinant protein to activate transcription factor efficacy. The result of our dual luciferase assay showed only a mild increase from the TNFα constructs **(Revised Fig. 2e).** Unlike TNFα, LCN2 constructs containing the binding motifs of YAP and TEAD exhibited enhanced luciferase activities by more than 140-fold compared to the control **(Revised Fig. 2f).** Taken together, we show that adipocyte-LCN2 could be enhanced by active YAP/TAZ-TEAD, which can be applicable to dysfunctional adipocytes that exhibit YAP/TAZ activation.

Initially, our summary figure illustrated that TNFα contributes to LCN2 amplification in dysfunctional adipocytes. This was supported by Supplementary Fig. 2c, where recombinant TNFα protein was sufficient to increase LCN2 level in adipocytes. However, we now show that Hippo pathway directly contributes to LCN2 production in adipocytes. Therefore, we have revised our summary figure to better represent the direct regulation of LCN2 via

YAP/TAZ, emphasizing the contribution of Hippo pathway to LCN2 production in adipocytes (Main Fig. 7h).



Revised Fig.2c-f – (c,d) Design of the luciferase reporter plasmids containing of TNFα (c) and LCN2 (d) promoters/enhancers. Red stars indicate the DNA recognition sequence for TEAD. Blue bar indicates the potential binding region according to ChIP-seq results in **Revised Fig. 2a,b**. (e,f) Dual luciferase assay of the luciferase reporter constructs for both LCN2 and TNFα. HEK293 cells were transfected with the indicated plasmids, then treated with recombinant YAP protein (1 μg, 48 hr).

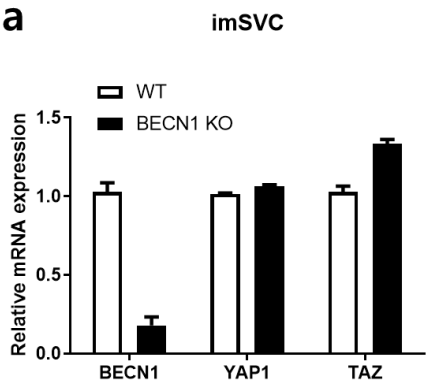
The results have been incorporated into our Main Figure 6 and Supplementary Figure 8.

3. In Fig. 5a and 5c, YAP expression is significantly suppressed by BECN1. However, in Fig. 5h, loss of BECN1 did not affect YAP protein level. This inconsistency should be addressed. Would YAP transcription be affected in Fig. 5a, 5c, and 5h? This brings in another question – how does BECN1 regulate the Hippo pathway? Although the authors cited some literatures, they should at least examine additional Hippo pathway components like LATS1, MOB1, MST1 (both expression and phosphorylation) in the BECN1-deficient cells.

We appreciate the reviewer's keen observation regarding the apparent inconsistency in our data. It is important to note that different cell lines were used in Fig. 5a, c and Fig.5h. Specifically, in Fig. 5a, c, we used 10T1/2 cell line, which derived from mouse embryonic fibroblasts and established as preadipocytes. However, in Fig. 5h, we used stromal vascular cells (SVCs) extracted from inguinal white adipose tissue (iWAT) of an adult mouse. Given the distinct origins at different developmental stages and distinct pluripotency of these two cell lines, variations in results were anticipated. The 10T1/2 results show more dynamic regulation of YAP protein, while the effect was more subtle in SVCs. These properties of 10T1/2 and SVCs were advantageous in identifying the mechanism of how BECN1 regulates YAP.

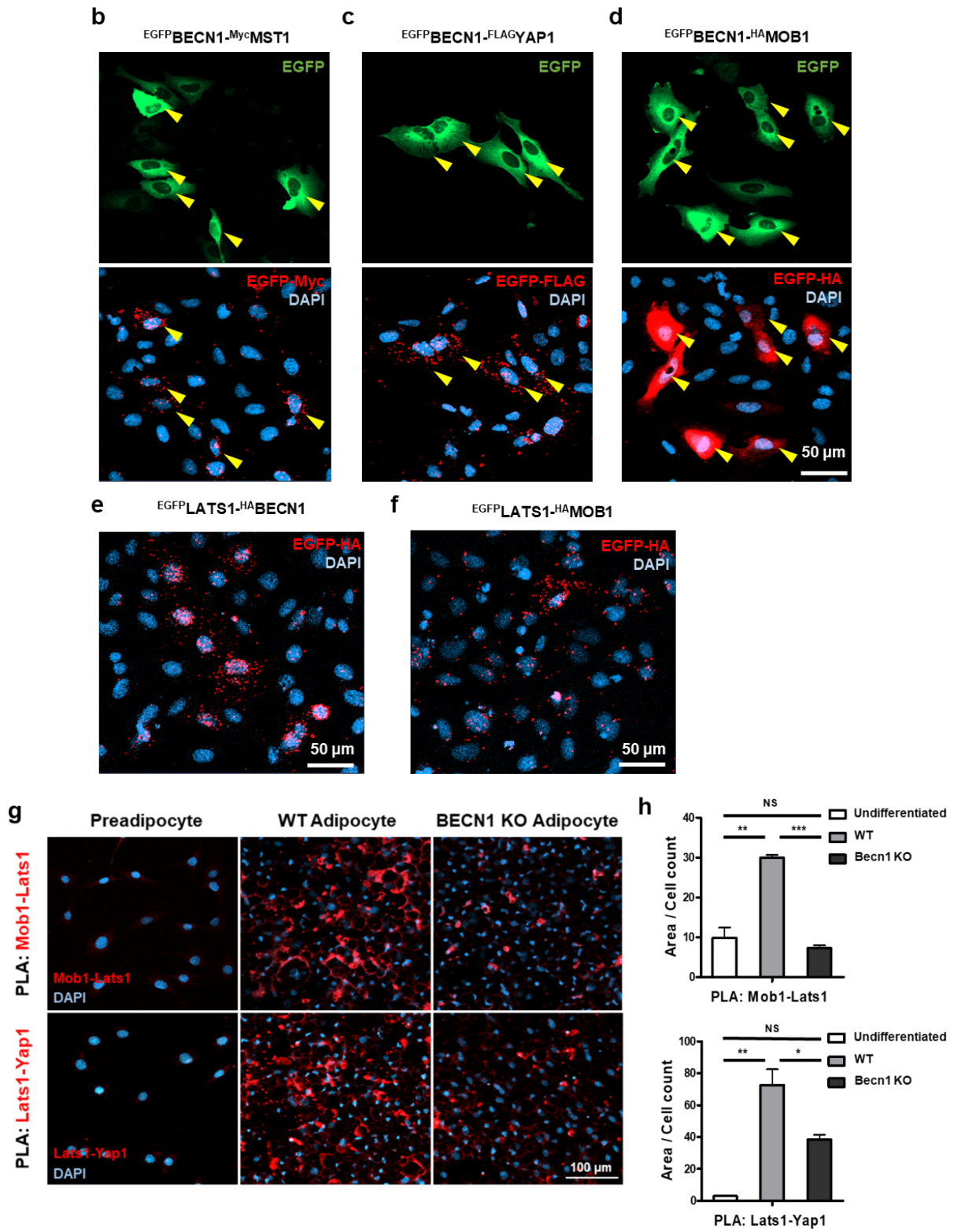
Here, we organized multiple experimental designs to uncover the regulation of YAP/TAZ.

Our comprehensive investigation, incorporating both in-vitro and in-vivo results revealed that BECN1 does not regulate YAP/TAZ at the transcriptional level **(Revised Fig. 3a;** Main Fig. 6f**).**



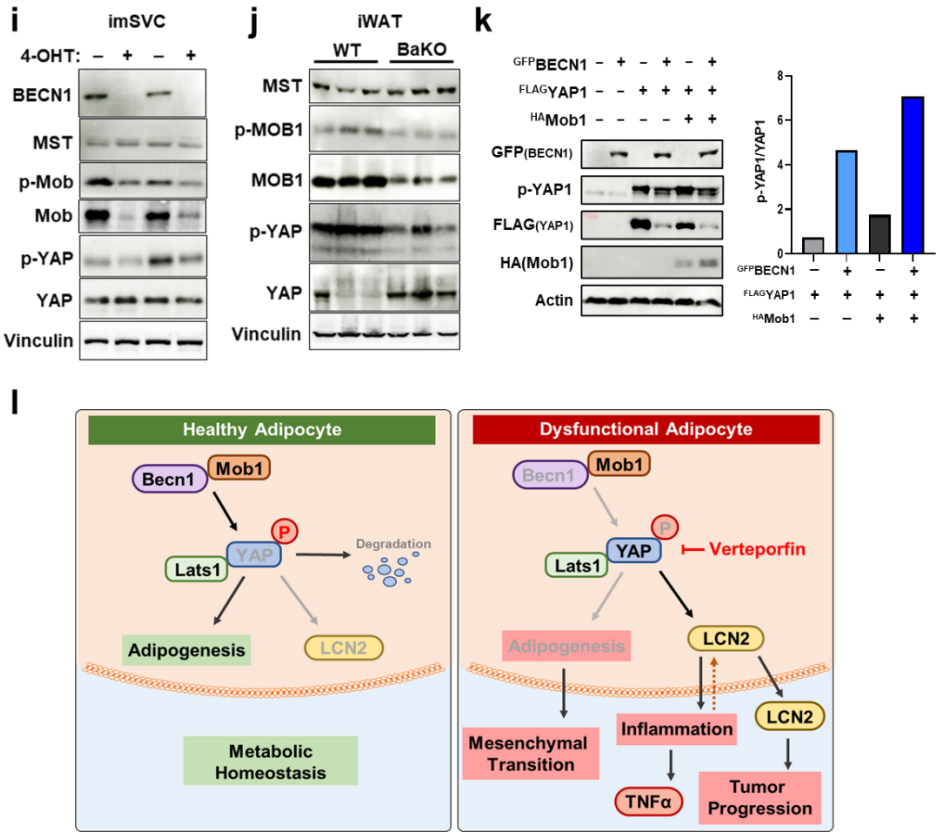
Revised Fig. 3a – Immortalized SVC (imSVC) was differentiated into mature adipocytes, then treated with 4-OHT to deplete BECN1 expression. Expression of BECN1, YAP1, and TAZ were evaluated using RT-qPCR.

As mentioned, we acknowledge the significance of comprehensively exploring the mechanical aspects of how BECN1 regulates YAP/TAZ through experimental investigations. Therefore, we conducted a proximity ligation assay (PLA) to check the interactions between BECN1 and Hippo-associated proteins. Since PLA reveals the proximity between two proteins, such interactions may impact their functional activity or protein stability [4]. We used the U2OS cell line to overexpress BECN1 along with Hippo-associated proteins: YAP1, LATS1, MST1, and MOB1. Intriguingly, we observed a remarkably strong interaction of BECN1 with MOB1 compared to that with other Hippo-associated proteins **(Revised Fig. 3b-e**), and the interaction was higher than that between LATS1 and MOB1 **(Revised Fig. 3f).** Since MOB1 accounts for LATS phosphorylation, which is necessary for its kinase activity, we thought the interaction between BECN1 and MOB1 would regulate the Hippo pathway in adipocytes [5]. Therefore, we tested whether loss of BECN1 regulates LATS activity through MOB1 in adipocytes. The results showed that the loss of BECN1 in mature adipocytes could drive the reduction of MOB1-LATS interaction **(Revised Fig. 3g)**, leading to inhibition of LATS-YAP interaction **(Revised Fig. 3h).**



Revised Fig. 3b-h – (b-e) Proximity ligation assay (PLA) performed in U2OS cells transfected with BECN1^{GFP} and other Hippo-associated proteins (MOB1, YAP1, MST1, and LATS1). Yellow arrows locate the interacting regions between the two proteins indicated. (f) LATS and MOB1 were transfected in U2OS as a positive control. (g-h) PLA was performed before and after the differentiation of 10T1/2 cells. Endogenous protein-protein interactions of MOB1-LATS1 and LATS1-YAP1 (h) were quantified.

To verify the interaction of BECN1-MOB1 causing Hippo pathway suppression, we tested the protein expression in both in-vitro and in-vivo system. imSVC result showed BECN1 deletion did not alter the MST1 level, but it regulated YAP phosphorylation through the downregulation of MOB1 **(Revised Fig. 3i).** Consistently, mouse iWAT of BaKO contained similar levels of MST1 but low levels of MOB1 that leads to inhibition in YAP phosphorylation **(Revised Fig. 3j).** Next, we confirmed the effect of BECN1 on Hippo pathway via BECN1 overexpression. We transfected BECN1, YAP1, and MOB1 as indicated and assessed the changes in YAP phosphorylation. Overexpression of BECN1 increased YAP phosphorylation level, which was more severely increased with the presence of MOB1 protein **(Revised Fig. 3k).** Taken together, these findings suggest that BECN1 interacts with MOB1 to enhance Hippo pathway activity. Accordingly, we have included supporting results and revised our summary figure with more mechanistic insight **(Revised Fig. 3l).**



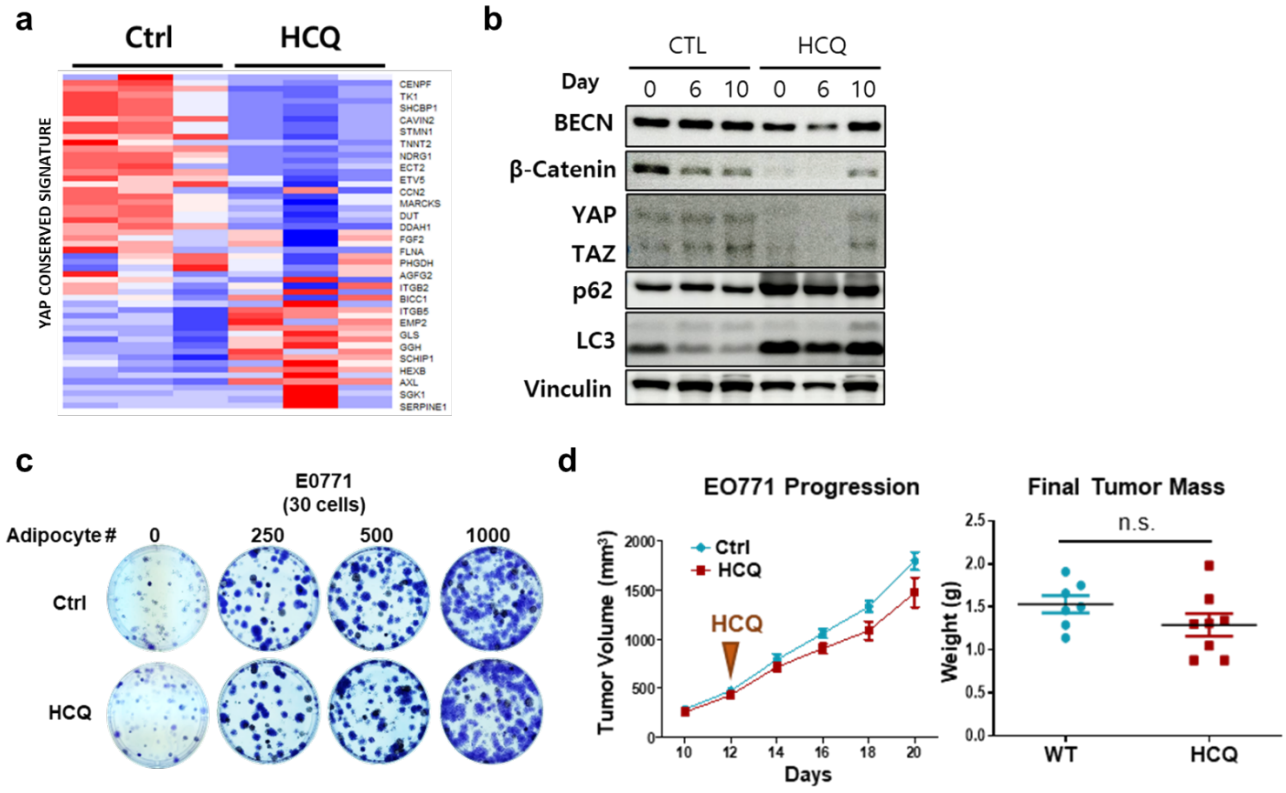
Revised Fig. 3i-l – (i) Western blot analysis of imSVCs differentiated into mature adipocytes. 4-OHT was treated to deplete BECN1 after differentiation (j) Western blot analysis of protein samples isolated from WT or BaKO mouse iWATs (8 weeks old, n = 3). (k) Western blot analysis of HEK293 cells transfected with BECN1, YAP1, and MOB1 as indicated. Protein lysates were prepared 2 days post-transfection. (l) Revised summary figure.

The results have been incorporated into our Main Figure 5 and Supplementary Figure 9.

4. In Fig. 4, the authors show a dispensable role of autophagy in BECN1-mediated regulation of YAP/TAZ and tumor growth. To strengthen the conclusion, the authors should reconstitute the BECN1-deficient adipocytes with wild-type BECN1 and its mutants – the ones that cannot activate autophagy and the Hippo pathway, respectively, and use these cells to evaluate their roles in driving inflammation genes' transcription and tumor growth.

We agree that Main Fig. 4 should be more deliberately presented to emphasize the dispensable role of autophagy in BECN1-mediated regulation of YAP/TAZ. Unfortunately, the non-autophagic function of BECN1 has not been profoundly studied. Additionally, no specific domain of BECN1 that ablates its autophagic function *per se* has been identified. While mutation in the BH3 domain, which dissociates BECN1 from BCL2, is known to inhibit autophagy flux, its impact is not limited to autophagy and could affect other potential roles of BECN1 [6, 7]. Hence, our study is confined to testing of autophagy inhibition across different stages of the autophagic process, which may lead to alteration in the Hippo pathway.

To validate the effect of autophagy inhibition in adipocytes, we conducted RNA-sequencing on hydroxychloroquine (HCQ) treated adipocytes. Its effect on the Hippo pathway was rather opposite compared to what we observed in BECN1 deficient adipocytes **(Revised Fig. 4a).** Additionally, HCQ treatment in general reduced YAP/TAZ as well as β-Catenin throughout adipocyte differentiation **(Revised Fig. 4b).** Next, we evaluated how HCQ-treated adipocytes contribute to cancer growth. We pretreated HCQ on differentiated adipocytes and then co-cultured them with cancer cells (30 cells of EO771). After 10 days of co-cultivation of different number of adipocytes and cancer cells, the growth of EO771 was measured through crystal violet staining. The results revealed that cancer cells tended to form bigger colonies with an increased number of adipocytes; however, no remarkable difference was observed between the Ctrl and HCQ treated adipocytes **(Revised Fig. 4c).** Further, we tested this phenomenon in-vivo by co-injecting adipocytes and cancer cells. For this, adipocytes were pretreated with HCQ for 2 days and mixed with cancer cells and the mixture was injected subcutaneously into the mice. While EO771 co-injected with BECN1-deficient adipocytes enhanced tumor progression (Main Fig. 1i), HCQ-treated adipocytes did not **(Revised Fig. 4d).** Our revised data demonstrate that mere autophagy inhibition in adipocytes is not sufficient to regulate YAP/TAZ or promote tumor progression. This suggests that the distinctive function of BECN1 is responsible for driving alterations in the Hippo pathway.



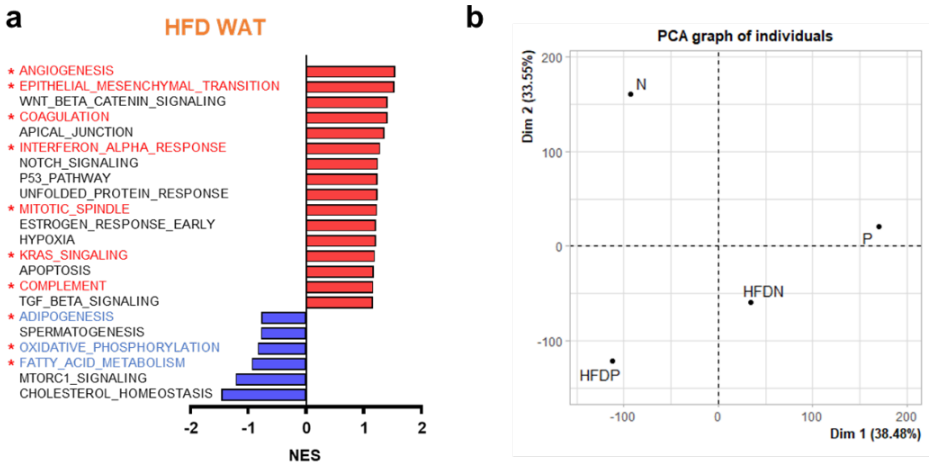
Revised Fig. 4a-d – (a) Heat map analysis of YAP-conserved signature gene set (GSEA) from RNA-seq results of Control versus HCQ-treated adipocytes. Fully differentiated adipocytes were treated with HCQ (20 μM, 48 hr). (b) Western blot analysis of Control and HCQ treated (50 μM, 48 hr) adipocytes at different stages of differentiation. Day 0, 6, and 10 represent different timepoints after differentiation stimulation. (c) Colony assay of EO771 co-cultured with adipocytes treated with or without HCQ. HCQ was pretreated (20 μM, 48 hr) to the number of adipocytes as indicated. (d) Tumor progression evaluated by the rate of tumor growth in volume and final mass. Mature adipocytes were treated with either PBS or HCQ for 48 hr and then mixed with an equal number of EO771 cancer cells. Mice were sacrificed 20 days after injection.

The results have been incorporated into our Main Figure 4 and Supplementary Figure 4.

5. BECN1 protein expression in adipocytes were tightly controlled by cancer cells (Fig. 3c), adipocyte differentiation (Fig. 5a), and high-fat diet (Fig. 7e). Since these data provide the physiological/pathological regulation of BECN1 in adipocytes, the underlying mechanisms should be elucidated.

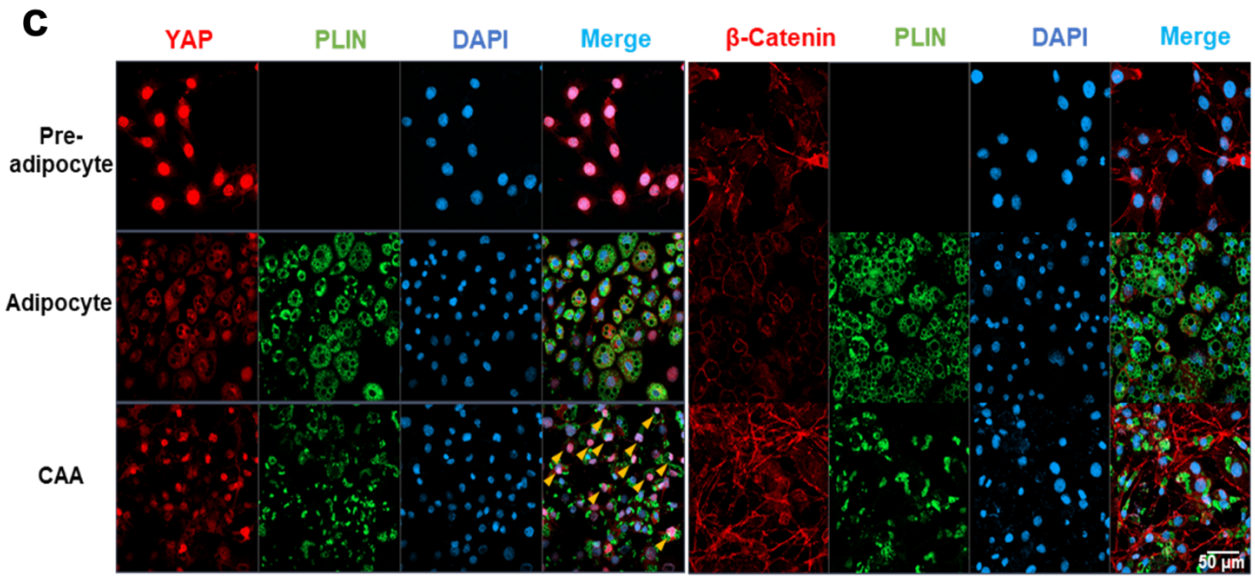
We apologize for providing only descriptive response to this issue. Our study primarily focuses on investigating the impact of dysfunctional adipocytes on tumor progression. We have identified YAP/TAZ activity as a critical determinant of adipocyte dedifferentiation, enhancing the formation of pro-tumorigenic environments. Here, BECN1-deficient adipocytes are utilized as a dysfunctional/dedifferentiated adipocyte model. Consequently, delving into what controls BECN1 expression may appear somewhat tangential to the primary focus of our study. We hope the reviewer understands this matter.

We have reported the two circumstances that result in suppression of BECN1 expression in adipocytes: adipocytes influenced by cancer cells and adipocytes from high-fat diet (HFD) fed mice. To extract common traits that could potentially regulate BECN1 levels, we compared the RNA-seq results of peritumoral WAT (Main Fig. 3f) and HFD-fed WAT. We newly conducted bulk-RNA sequencing in iWAT of HFD-fed mice and compared it to that of the normal chow diet (NCD)-fed mice. HGSEA was performed to classify the significantly upregulated and downregulated gene sets and identified 7 upregulated and 3 downregulated gene sets that were commonly observed in peritumoral WAT **(Revised Fig. 5a).** These results indicated the potential signaling pathways that can regulate BECN1 in two different circumstances. Furthermore, we conducted principal component analysis (PCA) between RNA-seq results of Control, peritumoral, HFD Control, and HFD peritumoral WATs. PCA depicts the relationships between those samples based on gene expression patterns, where the distance between samples reflects how similar or different these samples are from one another. The PCA result indicates that HFD-fed WAT and peritumoral WAT share somewhat similar traits **(Revised Fig.5b).** In other words, WATs under two different circumstances may acquire similar gene expression patterns.



Revised Fig. 5a,b – (a) HGSEA result of bulk RNA-seq conducted on NCD and HFD fed mouse iWATs. A positive NES score indicates that such gene sets were upregulated in HFD-fed iWAT. Red stars indicate that those gene sets were also altered in peritumoral WAT in a similar pattern. (b) PCA result of Naïve (N), Peritumoral (P), HFD Naïve (HFDN), and HFD peritumoral (HFDP) WAT RNA seq data. The distance between each dot reflects similarity in gene expression patter between samples.

Another aspect regarding BECN1 regulation is that its level is significantly altered depending on the adipocyte differentiation status (Main Fig. 5a, b). Furthermore, BECN1 depletion led to acquisition of dedifferentiation phenotypes through the expression of preadipocyte markers including YAP and β-Catenin (Main Fig. 5c-f). We tested whether similar phenomenon occurs in CAAs, which are well-known to undergo adipocyte dedifferentiation [8-11]. For this, we conducted immunocytochemistry (ICC) on YAP and β-Catenin to observe their dynamics upon adipocyte-cancer co-culture. Similar to BECN1-deficient adipocytes, co-cultured adipocytes showed increased nuclear YAP and cytoplasmic β-Catenin expression **(Revised Fig. 5c)**. These results reaffirm that the BECN1 level relies on the differentiation status. Though it is insufficient to pinpoint a specific molecule or signaling pathway, we can conclude that BECN1 is regulated by adipocyte plasticity and differentiation stimuli.



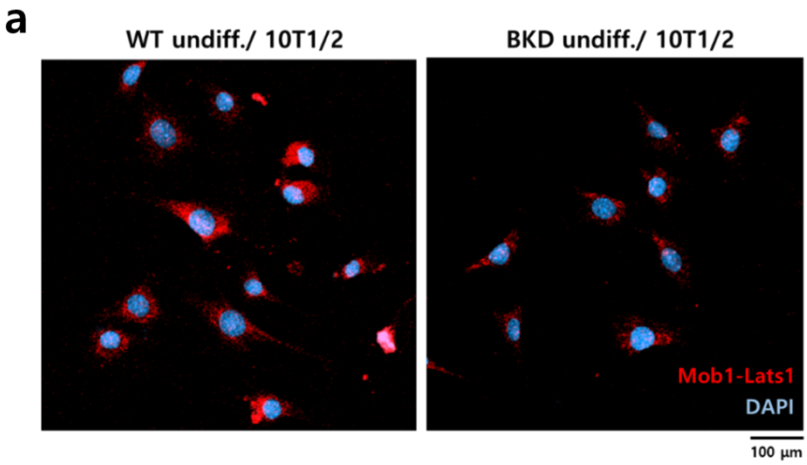
Revised Fig. 5c – Immunofluorescence staining of YAP, β-Catenin, PLIN, and DAPI in 10T1/2 cells. Cells were prepared as preadipocytes, adipocytes, and CAAs. Yellow arrows indicate the nuclear translocation of YAP.

The results have been incorporated into our Supplementary Figure 5

6. The reviewer is curious about what if BECN1 is depleted in other types of cells like fibroblasts and epithelial cells. Would these BECN1-deficient non-adipocytes cells also show YAP/TAZ activation and enhanced inflammation, and drive tumor growth by modulating the microenvironment?

This comment helped us to extend the applicability of our study to various components within the TME. Given the significance of cancer-associated fibroblast (CAF) as one of the key TME component, we conducted pertinent experiments using mouse embryonic fibroblasts (MEFs) and preadipocytes (10T1/2) [12]. By doing so, interesting results were obtained: (1) BECN-YAP/TAZ regulation was continually observable in fibroblasts and (2) BECN1-deficient fibroblasts were also sufficient to promote cancer growth.

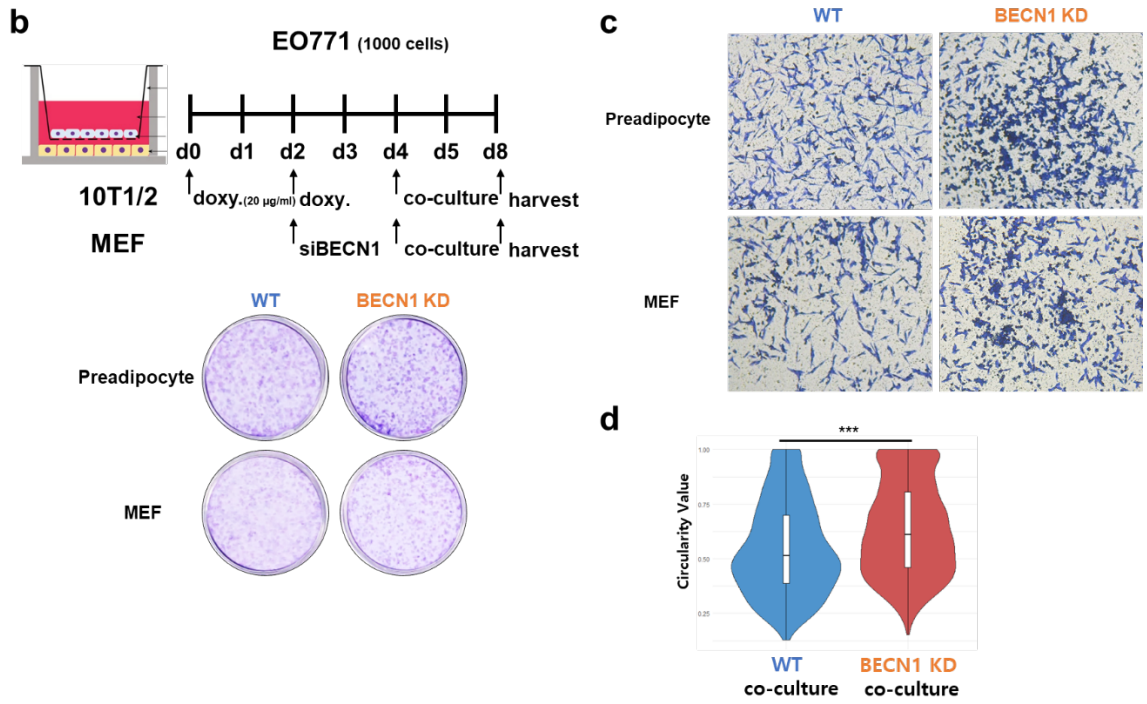
As previously mentioned, BECN1 depletion suppressed Hippo signaling **(Revised Fig. 3b-i)**. PLA assay was again conducted in preadipocytes to observe the interaction between Hippo-associated proteins. Similar to differentiated adipocytes, BECN1 depletion in preadipocytes also diminished MOB1-LATS1 interaction **(Revised Fig. 6a)**.

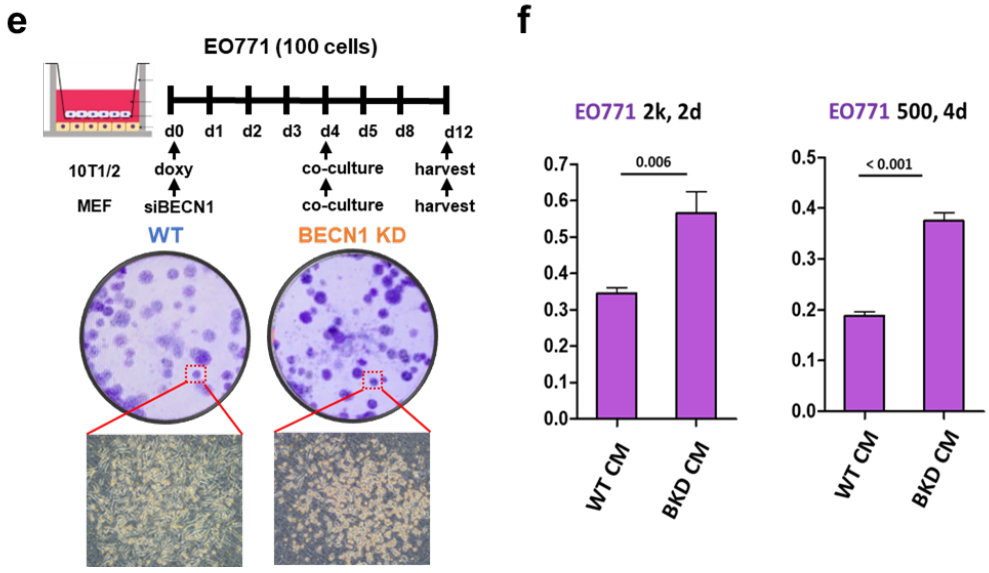


Revised Fig. 6a – PLA performed in WT and BECN1-deficient preadipocytes (10T1/2). The interaction between endogenous MOB1 and LATS1 was evaluated. Longer exposure was used compared to **Revised Fig. 3f**.

To assess the impact of BECN1-deficient fibroblasts on cancer growth, we co-cultured MEFs and preadipocytes with EO771 cells. Upon BECN1 depletion, MEFs and preadipocytes were co-cultured with 1000 cancer cells for 4 days, followed by crystal violet staining of the live cells. Although no notable difference between WT and BECN1 knockdown (KD) fibroblasts was observed initially **(Revised Fig. 6b)**, morphological difference was observed at a microscopic level **(Revised Fig. 6c)**. Furthermore, measurement of circularity of the cells was measured revealed that the cancer cells influenced by BECN1-deficient fibroblasts exhibited more round-shaped morphologies **(Revised Fig. 6d)**. To magnify the effect of fibroblast co-culture, we lowered the number of cancer cells and extended the exposure to 8 days instead of 4. In fact, changes in the cancer cell morphologies were more apparent **(Revised Fig. 6e)**. Furthermore, we verified the impact of conditioned media (CM) extracted

from WT and BECN1-deficient fibroblasts on cancer growth. Consistently, cancer cells grown in BECN1-deficient fibroblast CM exhibited an enhanced growth rate compared to WT fibroblast CM **(Revised Fig. 6f)**.

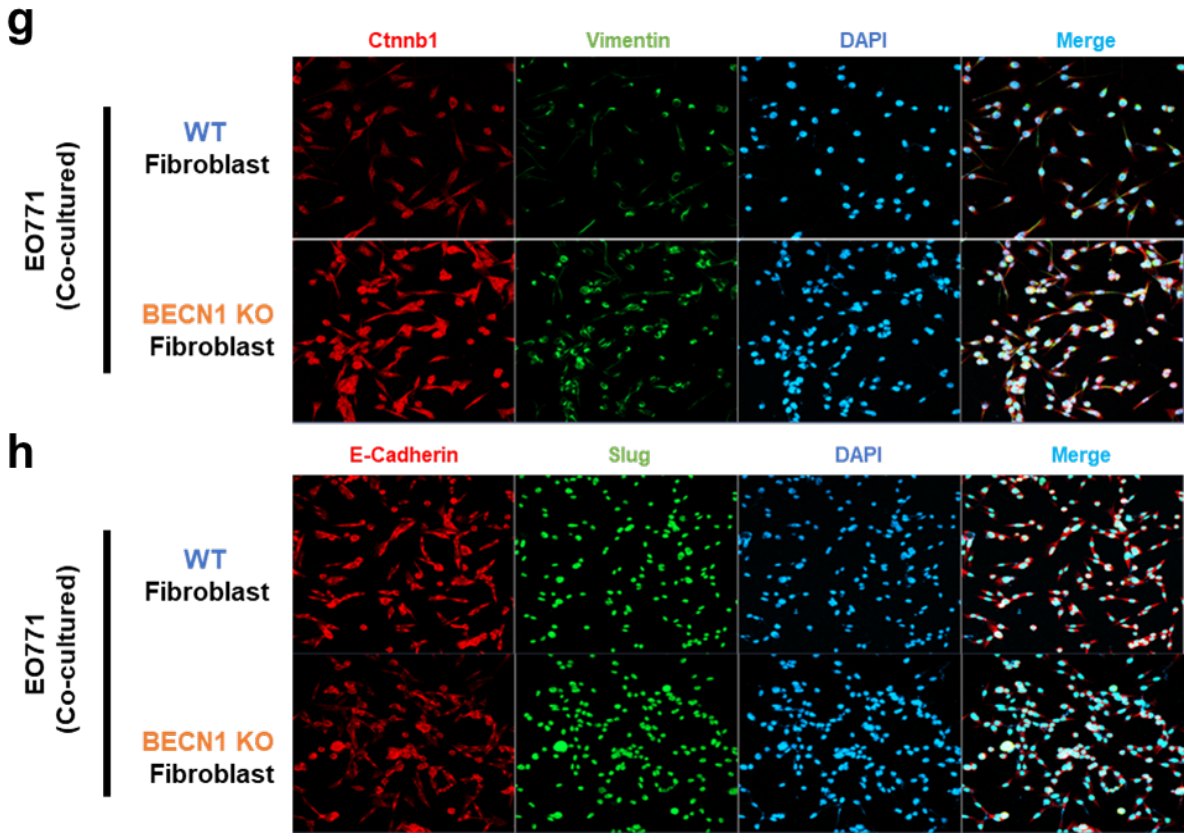




Revised Fig. 6b-f – (b) Schematic overview of fibroblast-cancer co-culture system. Cancer cells were stained with crystal violet after exposure to WT and BECN1-deficient fibroblasts. (c) Microscopic image of crystal violet stained cancer cells (d) Morphology of each cancer cell in (c) was evaluated for its circularity using *imageJ.* (e) Schematic overview of the co-culture system. The live image of a cancer cell colony displays distinct morphologies.

The morphological change in cancer cells signifies modifications in the cellular cytoskeleton, cell-cell adhesion molecules, and the acquisition of stemness characteristics. These alterations contribute to the transition of cancer cells, affecting their shape, migratory capacity, and invasive behavior, typically known as epithelial to mesenchymal transition

(EMT) [13, 14]. To evaluate the physiological implication driven by WT and BECN1-deficient preadipocytes, we observed changes in the EMT markers. In parallel to the enhanced circularity, cancer cells underwent EMT upon co-culture with BECN1-deficient preadipocytes **(Revised Fig. 6g,h).** While our results are not sufficient to conclusively establish the BECN1-YAP/TAZ-LCN2 axis as the key modulator for this phenomenon, we can assert that the level of BECN1 in fibroblasts is equally critical in shaping a malignant TME. In summary, BECN1-deficient fibroblasts demonstrate the capability to induce cancer malignancy through enhanced proliferation and EMT.



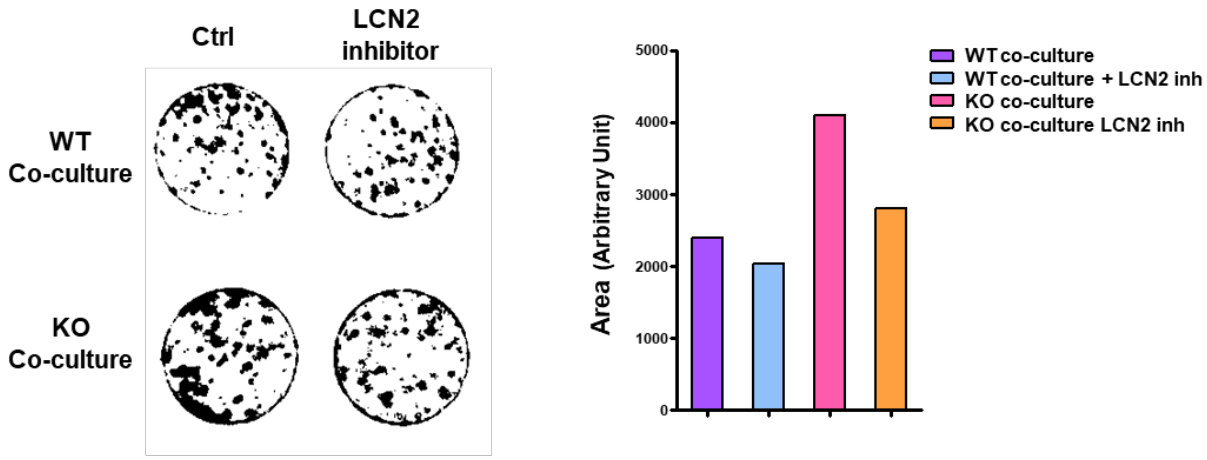
Revised Fig. 6g,h - Immunofluorescence staining of β-Catenin, Vimentin, E-Cadherin, Slug, and DAPI in co-cultured EO771 cells. Cancer cells were co-cultured with WT and BECN1-deficient preadipocytes for two days.

The results have been incorporated into our Supplementary Figure 11.

Minor points:

1. In Fig. 2j, the authors should knockdown LCN2 in adipocytes to repeat the co-culture experiment. In addition, treatment with LCN2 antibody in the co-culture media would help further strengthen the conclusion.

Thank you for highlighting the need to complement our adipocyte-cancer co-culture experiment. We have addressed this by treating LCN2 inhibitor (ZINC00640089). The result indicates that EO771 growth was mitigated by LCN2 inhibitor especially when they were co-cultured with BECN1-deficient adipocytes **(Revised Fig. 7a)**.



Revised Fig. 7a – EO771 co-cultured with WT or BECN1 KO adipocytes for 7 days. LCN2 inhibitor (ZINC00640089) at 5 μM was administered bi-daily to inhibit BECN1 KO adipocyte-induced cancer cell growth during co-culture. Cell viability was assessed with crystal violet staining and quantified using *ImageJ*.

2. In Fig. 3c-d, were the q-PCR and WB studies performed in the mixed adipocyte-cancer cells or specifically in the isolated adipocytes from the co-culture system?

Our co-culture system is performed on a trans-well culture plate, which consists of a permeable membrane separating two cell populations. This allows the exchange of soluble factors while isolating each population in distinct layers. As a result, our q-PCR and WB analyses could be exclusively performed on each cell population. For clarity, we have revised the method section of the manuscript (line 430-432).

3. The provided Figure Legends are quite concise, making it hard to determine how the experiments were exactly performed.

The Figure Legend section has been updated with additional details.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors employed an adipose tissue-specific BECN1 knockout (BaKO) mouse model to investigate the effects of dysfunctional adipocytes (caused by loss-of-function of Beclin1) on the growth of certain types of tumors. They reported that BaKO adipocytes promote tumor growth both in vitro and in vivo using a combination of multiple mouse models. Furthermore, they demonstrated the involvement of the YAP/TAZ signaling pathway in tumor progression. As a demonstration, deletion or inhibition of YAP/TAZ in the BaKO adipocytes restored normal cell functions, suppressed lipodystrophy, and mitigated inflammatory phenotypes. Overall, the data are robust, and the findings are intriguing. The conclusions are well-supported by their results. However, there are significant concerns regarding unclear presentations and a lack of sufficient mechanistic studies, which somewhat diminish the enthusiasm for this otherwise interesting study. The following concerns, including both major and minor issues, are outlined below:

We appreciate the positive feedback and interest expressed by the reviewer. Acknowledging the validity of the reviewer's concerns, we have made deliberate attempts to address them within the manuscript. Specifically, the molecular insights were clarified to resolve any obscurity in our text. We believe these efforts have enriched our discussion section and the overall contents of the manuscript.

Major Concerns:

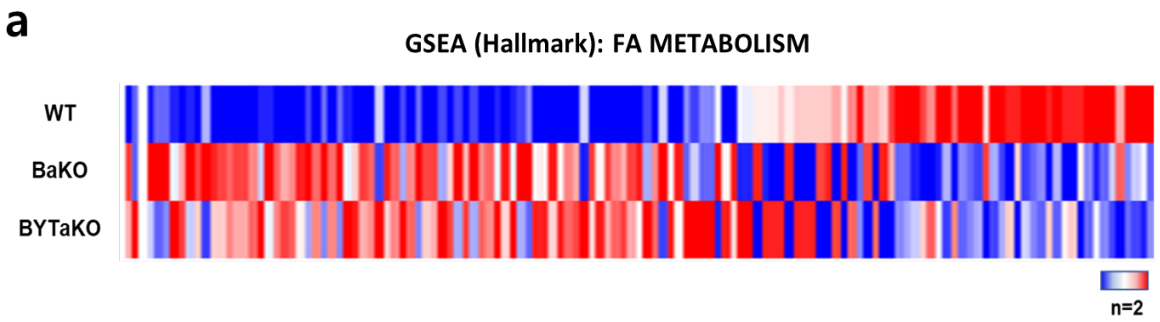
1). Several result titles start with "Dysfunctional adipocytes," which fails to provide precise information about the adipocytes in the respective paragraph. If these cells are BECN1 KO cells, it is recommended to use "BaKO adipocytes."; If the cells were isolated from diet-induced obese adipose tissue, then the term "diet-induced obese adipocytes" should be used. Employing clearer terminology would help readers better understand the nature of these cells.

We appreciate the reviewer's observation. Upon reviewing our manuscript, we acknowledge that the use of "Dysfunctional adipocytes" in several result titles may lead to ambiguity. To address this concern, we have revised the titles to provide more specific information.

2). It has been reported that loss-of-function of BECN1 promotes tumor growth in cancer cells. Similarly, here, the authors found that BaKO in adipocytes stimulates co-cultured tumor cells and activates downstream YAP/TAZ pathways, paralleling the role observed previously in cancer cells. The stimulation of the YAP/TAZ signaling pathway might explain the impact on adipogenesis reduction and inflammation enhancement. However, one significant effect of BaKO in adipocytes is enhanced lipolysis (Fig. 5g, Supplementary Fig. 5e and i). Considering that lipolysis can promote cancer growth, it's surprising that the authors didn't investigate the mechanism underlying how BaKO adipocytes increase lipolysis.

We acknowledge that it is crucial to understand the mechanisms underlying how BaKO adipocytes increase lipolysis, considering its potential role in promoting cancer growth. Our investigation revealed that BECN1-deficient adipocytes exhibit enhanced lipolysis, as evidenced by the phosphorylation of multiple sites on hormone-sensitive lipase (HSL). Notably, HSL phosphorylation is commonly mediated by PKA (at S563, S660) and AMPK (at S565)[15], indicating heightened PKA activity and suppressed AMPK in BECN1-depleted adipocytes.

The issue is to determine how much lipolysis contributes to tumor progression in our models. To investigate the impact of lipolysis in-vivo, we conducted RNA-seq analysis on tumor cells transplanted in WT, BaKO, and BYTaKO. If the tumor cells rigorously utilized the lipid contents secreted from active lipolysis, their genes associated with lipid metabolism would have increased. Heatmap analysis focusing on genes associated with fatty acid (FA) metabolism revealed that tumors grown in BaKO displayed heightened FA metabolism, confirming increased lipolysis and FA release (**Revised Fig. 8a**). However, this enhancement was not restored in BYTaKO tumors, suggesting that factors other than lipolysis contribute significantly to the observed tumor regression in BYTaKO.



Revised Fig. 8a – Heatmap analysis of genes associated with FA metabolism. Bulk RNA-seq was conducted on MC38 tumors grown in WT, BaKO, and BYTaKO mice for 2 weeks.

While we acknowledge that lipolysis may have played a role in tumor progression in BaKO, our data suggest it is not the sole major factor. Importantly, we identified Lipocalin 2 (LCN2) as a key molecule secreted from BECN1-deficient adipocytes, promoting tumor progression. Deletion of the LCN2 receptor in cancer cells prevented the rapid growth induced by BECN1-deficient adipocyte co-cultivation (Main Fig. 2j). This underscores the complexity of the interactions, where lipolysis, though contributory, is not the primary driver of tumor growth in our models.

3). The manuscript lacks crucial information about the recipient mice for co-injected WT or Becn1 KO cells with 4T1. Are these nude mice with compromised inflammatory reactions for the injected cancer cells? Additionally, the nature of the co-injected fat cells remains unclear, as there is mention of imSVCs (line 99) and differentiated adipocytes (line 100).

For the adipocyte-cancer co-injection system, we utilized immunodeficient mice (N2G). This information has been incorporated into both the Results and Methods sections of the manuscript. To provide further clarity, we have expanded on the schematic details of the co-injection system in the main text (result section, line 114-118; Method section, line 411-413). Regarding imSVC establishment, please refer to our previous research article [16].

line 114-118

we established immortalized stromal vascular cell (imSVC) lines with conditional *Becn1 KO* system (ROSA-Cre^{ERT2}). imSVCs were fully differentiated into adipocytes and then treated with 4-hydroxytamoxifen (4-OHT) to deplete BECN1. Next, we co-injected WT or *Becn1* KO imSVCs and breast cancer cells (4T1) into the mouse flanks

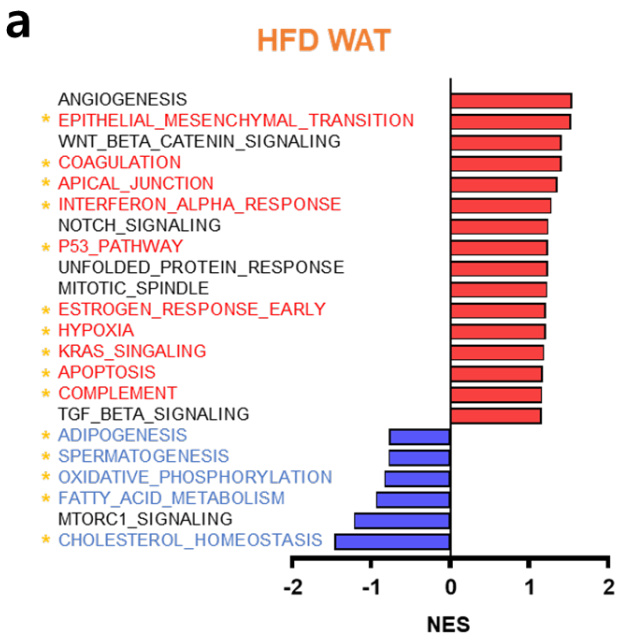
line 411-413

Immunocompromised N2G (NOD; Prkdc^{em1Gmcr}; Il2rg^{em1Gmcr}) mice were obtained from Gemcro Corp (Seoul, Republic of Korea). These mice were generated from NOD with deletion of entire exons of PRKDC and IL2RG.

4). There's a need to reconcile the observations of BECN1-deficient adipocytes and HFD-feeding induced obese adipocytes. In Fig 6, the authors note that inhibition of YAP/TAZ activity in both BECN1-deficient and HFD-fed obese adipocytes yields the same tumor suppression effect. However, these two adipocyte types exhibit stark morphological differences: BECN1-deficient adipocytes have small lipid droplets and are prone to dedifferentiation to fibroblasts, while HFD-induced obese adipocytes possess unusually large lipid contents and do not transform into fibroblasts. Moreover, there's confusion in line

298-299, where they state that "the WATs of the HFD-fed mice had decreased levels of BECN1," while these fat cells do not share the lipid droplet phenotype of BECN1 KO adipocytes. Addressing these discrepancies is crucial.

Thank you for highlighting this critical aspect. We acknowledge the noticeable morphological differences between BECN1-deficient adipocytes and HFD-induced obese adipocytes. Despite these distinctions, we were able to identify certain shared phenotypes between the two samples. BECN1-deficient adipocytes exhibit reduced expression of adipogenic genes and elevated levels of inflammatory signals (Main Fig. 2a-c, 5f). These features are known to be analogous to the HFD-induced obese adipocytes [17, 18]. To further characterize these adipocytes, we conducted RNA-seq from HFD-induced obese adipose tissue. Next, we compared the HGSEA results between HFD-fed and BaKO WATs. The significantly upregulated / downregulated gene sets were arranged regarding their normalized enrichment score (NES), and gene sets that commonly appear in BaKO WAT were marked with yellow stars **(Revised Fig. 9a)**. Both BaKO WAT and HFD WAT shared inflammatory phenotypes followed by downregulation of genes associated with adipogenesis, FA metabolism, and cholesterol homeostasis [18]. Additionally, having EMT signaling as the second most significantly upregulated gene set aligns well with what we observed not only in BaKO WAT but also in Peritumoral WAT (Supplementary Fig. 3b). Presumably, HFD-fed adipocytes acquire partially dedifferentiated phenotypes, as observed in BECN1-deficient adipocytes and CAAs. Thus, though BECN1-deficient adipocytes and HFD-induced obese adipocytes evidently differ in their morphologies, they share some common features in terms of adipocyte quality.



Revised Fig. 9a – HGSEA result of RNA-seq conducted on mice fed with HFD (60% fat kcal) for 12 weeks. Significantly altered gene sets were arranged according to NES. Yellow stars indicate the commonly altered gene sets in the HGSEA result of BaKO WAT.

The effectiveness of the YAP/TAZ inhibitor (verteporfin) on both BaKO and HFD-fed mice is explained by the high expression of YAP/TAZ in their adipocytes. We have shown that BECN1 depletion in adipocytes induce YAP/TAZ expression (Main Fig. 5). This phenomenon is crucial when determining the differentiation state, leading to disrupted adipocyte homeostasis and causing formation of malignant TME to promote tumor (Main Fig. 1) [19, 20]. Similarly, WATs of HFD-fed mice contained high levels of YAP/TAZ (Main Fig. 7e) [21]. Hence, we hypothesized that YAP/TAZ^{high} adipocytes in the TME contribute to tumor

progression and that verteporfin treatment could induce tumor regression both in BaKO and HFD-fed mice.

We added the following paragraph to address the discrepancy about the morphological difference between BECN1-deficient adipocytes and HFD-induced obese adipocytes in our discussion section (line 333-338).

line 333-338

Of note, BaKO WAT displays lipodystrophy phenotypes, such as shrinking adipocytes and lipid contents, which significantly differ from obese WAT. However, both conditions were suitable for rapid tumor progression and were sensitive to VP treatment. We identified multiple features of BaKO WAT that are analogous to those of obese WAT (Supplementary Fig. 9e). This highlights that instead of fat mass, the quality of adipose tissue, pertaining to inflammation, vascularity, adipokine secretion, and extracellular matrix integrity, is more crucial when evaluating the TME status.

5). The autophagy-independent effect of BECN1 in adipose tissue needs further experimentation to provide solid support. While the authors discuss the involvement of key factors such as LATS1 and MST1 (lines 303 to 312), more experiments are required to establish their role in regulation.

We appreciate the reviewer's insightful comment and acknowledge the need for additional experimentation to solidify the autophagy-independent effect of BECN1 in adipose tissue. In response to Reviewer #1's queries regarding the dispensability of autophagy for BECN1-YAP/TAZ regulation, we conducted a series of experiments to provide more robust evidence. Specifically, we demonstrated that inhibiting autophagy alone (using HCQ treatment) does not suffice to enhance YAP/TAZ levels, resulting in accelerated tumor growth **(Revised Fig. 4a-d).**

As the reviewer mentioned, we have only roughly discussed the relationship between BECN1 and other Hippo-associated proteins such as LATS1 and MST1. To further explore the interaction between BECN1 and other Hippo-associated proteins, we conducted PLA which indicates the protein-protein interactions determined by their proximity [4]. Consistently, we found that BECN1 makes moderate interactions with LATS1 and MST1 **(Revised Fig. 3d, e)**. However, when we checked the interaction between BECN1 and MOB1, its intensity exceeded all other interactions between BECN1 and Hippo-associated proteins **(Revised Fig. 3b)**.

Next, we investigated the physiological outcome of BECN1-MOB1 interaction in adipocytes. As we depleted BECN1 from adipocytes, there was suppression of Hippo pathway demonstrated by diminished interactions between MOB1-LATS1 and LATS1-YAP1 **(Revised Fig. 3g, h)**. These results prompted us to hypothesize whether BECN1 regulates MOB1 phosphorylation. However, BECN1 rather altered total MOB1 level instead of its phosphorylation rate **(Revised Fig. 3i),** and this phenomenon was also observed in BaKO mouse iWAT **(Revised Fig. 3j).** Finally, overexpression of *BECN1, YAP1*, and *MOB1* in HEK293 cells revealed that BECN1 regulates YAP phosphorylation through MOB1 **(Revised Fig. 3k).** Taken together, we could uncover the mechanistic insight into how BECN1 contributes to Hippo pathway. Accordingly, we revised our summary figure of the manuscript **(Revised Fig. 3l).** We hope these comprehensive findings address the reviewer's concerns and enhance the clarity of our study.

The results have been incorporated into our Main Figure 5 and Supplementary Figure 9.

6). The discussion lacks depth and detail. The authors should provide more comprehensive mechanistic insights into the effects of BECN1 in adipocytes. Particularly, they should

elucidate how BECN1 suppresses the YAP/TAZ signaling pathway specifically in adipocytes and compare their findings about BECN1/YAP/TAZ signaling in adipocytes with previous reports in other cell types.

We appreciate the reviewer's concern and admit that our discussion section needs more improvement. Recognizing this, we have expanded the discussion section to encompass the following key points: (1) Exploring the contribution of CAAs to cancer cachexia and adipose tissue wasting, (2) Contrasting the morphologies of BaKO and HFD adipocytes, noting shared features and their relevance to verteporfin response, (3) Unveiling mechanistic insights into the BECN1-MOB1-YAP/TAZ-LCN2 axis in adipocytes, and (4) Investigating the potential impact of BECN1 loss in other stromal cells, such as fibroblasts in the tumor microenvironment (TME), and its role in promoting tumor progression (line 321-396)

Interestingly, the shared interest among all three reviewers in the potential impact of the BECN1-YAP/TAZ axis in various cell types prompted us to delve deeper into the effect of BECN1-deficient fibroblasts and their influence on cancer growth. Our findings indicate that BECN1-deficient fibroblasts can enhance cancer proliferation rates and induce epithelial-mesenchymal transition (EMT) **(Revised Fig. 6b-h).** While we cannot definitively assert that the BECN-YAP/TAZ axis is the sole modulator for these effects on cancer cells, we emphasize the critical role of BECN1 levels in fibroblasts in shaping a malignant TME.

The results have been incorporated into Supplementary Figure 11.

Minor Concerns:
1). The RNA-seq results (Fig. 2a&b) were obtained from iWAT, which includes multiple cell populations. However, BECN1 deficiency is specific to adipocytes in the isolated fat tissue.

The reviewer's concern is valid, noting that iWAT consists of multiple cell populations, including preadipocytes, immune cells, fibroblasts, and endothelial cells [22]. Therefore, performing bulk RNA-seq could lead to misleading interpretations. To address this, we verified the RNA-seq results in-vitro. Indeed, Main Fig. 2b was performed in-vitro to confirm that the significantly upregulated gene sets, TNFα signaling and inflammatory response, were due to the alterations in adipocytes (not due to the interference of immune cells). Similarly, the regulations of YAP downstream signaling and adipogenic genes (Main Fig. 3h-j) were verified in-vitro (Main Fig. 5f-i).

2). In line 165, clarify whether "Co-regulated" means "Co-upregulated" or "Co-downregulated."

This has been fixed in the main text. (line184-186)

In concordance, the genes associated with adipogenesis and fatty acid (FA) metabolism were consistently downregulated, supporting the concept of mesenchymal transition of adipocytes

3). In line 219, clarify what "restored phenotypes" refer to. Also, given that the KO effects are specific to adipocytes, it's important to clarify that the liver phenotypes aren't a direct result of YAP/TAZ KO.

This has been fixed in the main text. (line 258-261)

The results showed impaired autophagy (Supplementary Fig. 7b); however, lipodystrophy phenotypes observed in BaKO were restored in BYTaKO (Fig. 6b-g). This process involved the restoration of adipose tissue mass, potentially leading to the alleviation of liver steatosis through successful lipid storage in the adipose tissue

4). Line 279: Clarify the specific KO mouse models used in the study.

This has been fixed in the main text. (line 303)

To complement our genetic mouse model (BYTaKO), we employed VP to inhibit YAP/TAZ activity in adipose tissue

5). In line 290, specify which phenotypes are meant in relation to the effects of BECN1 depletion.

This has been fixed in the main text. (line 324-327)

While only Wnt and Notch signaling have been identified as the mediators of adipocyte dedifferentiation for CAA development, we show that similar phenotypes, such as suppression of adipogenic markers and acquisition of fibroblast-like features, are obtained from BECN1 depletion

6). In line 298, explain what is meant by the "quality of adipose tissue." Does "quality" refer to the capacity for lipolysis, appropriate adipokine secretion, or another aspect?

We also acknowledge the usage of word ‘quality’ is very vague in the context. However, instead of removing the term entirely, we have provided additional details of the crucial features that may contribute to adipose tissue homeostasis. This way, we can suggest important ‘qualities’ of adipose tissue to consider when evaluating TME.

This has been fixed in the main text. (line 336-338)

This highlights that instead of fat mass, the quality of adipose tissue, pertaining to inflammation, vascularity, adipokine secretion, and extracellular matrix integrity, is more crucial when evaluating the TME status

Reviewer #3 (Remarks to the Author):

Summary:

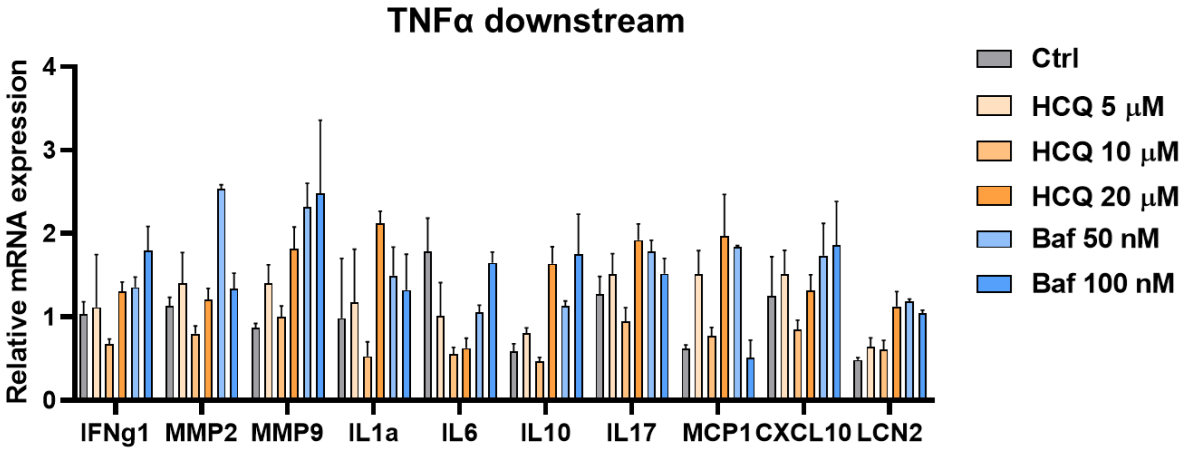
This paper demonstrates Becn1 conditional KO in adipocytes can promote tumor growth of breast cancer and colorectal cancer cells in vivo and in vitro. Adipokine array analysis of serum plasma from Becn1 mutant mice, and RNA sequencing of white adipose tissue of mutant and control mice revealed upregulation of LCN2 and TNFa signaling in mutants in concordance with the tumor growth phenotype. Further analysis suggests that BECN1 KO in adipocytes creates a pro-tumorigenic environment by eliciting gene transcription and markers associated with CAA (cancer associated adipocyte) status. Upregulation of YAP/TAZ gene signatures was observed in both BECN1 KO and peritumoral adipocytes, suggesting a potential mechanism for the tumor growth phenotypes observed. Beclin KO mice also lacking YAP and TAZ reversed many lipodystrophy phenotypes observed, as well as the increase in TNFa levels. Since Atg7 conditional KO did not elicit similar YAP/TAZ or tumor growth phenotypes in vitro or in vivo that were observed in BECN1 KO, the authors speculate that perhaps BECN1 function via PI3K/MTOR may be responsible for some of the phenotypes. It is my opinion that this work is novel and important, and suitable for publication in this journal with some revisions to characterize the phenotypes and bolster molecular data.

We appreciate the reviewer's thorough understanding of our manuscript and thank them for their positive perspective and constructive critiques. The acknowledgment of the novelty and importance of our work is encouraging. We are committed to addressing the suggested revisions to further characterize the phenotypes and enhance the molecular data. Your feedback is invaluable, and we will diligently incorporate the necessary improvements to ensure the manuscript meets the standards of this journal.

Major Comments:

1. It would be good to look at LCN2 (TNFa?) in the context of ATG7/ Baf/HCQ in vitro.

To enhance clarity, we have incorporated TNFα downstream and LCN2 expression data following Baf/HCQ treatment **(Revised Fig. 10a)**. Similar to YAP downstream result (Supplementary Fig. 4c), we could not observe a significant difference in TNFα downstream in adipocytes treated with Bafilomycin or HCQ. Although there was a moderate increase in LCN2 expression, it was not as extreme as observed in BECN1-deficient adipocytes (Main Fig. 2b and g).

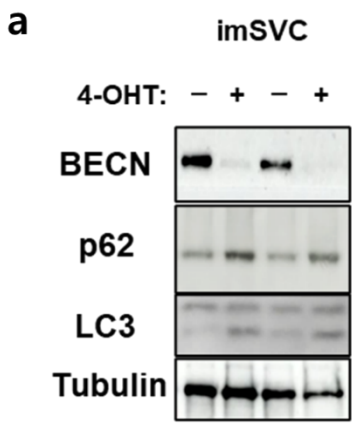


Revised Fig. 10a – Relative qPCR result of TNFα downstream and LCN2 in adipocytes treated with HCQ or Bafilomycin. Autophagy inhibitors were treated (48 hr) once adipocytes were fully differentiated.

The result has been incorporated into our Supplementary Figure 4.

2. Fig 1h, add p62 and/or lc3b to western blot, to help add context for the Atg7 data.

We thank the reviewer for this comment, as BECN1-mediated autophagy has not been extensively addressed in the manuscript. Thus, we provide the data that autophagy was blocked in our imSVC cell line **(Revised Fig. 11a).**



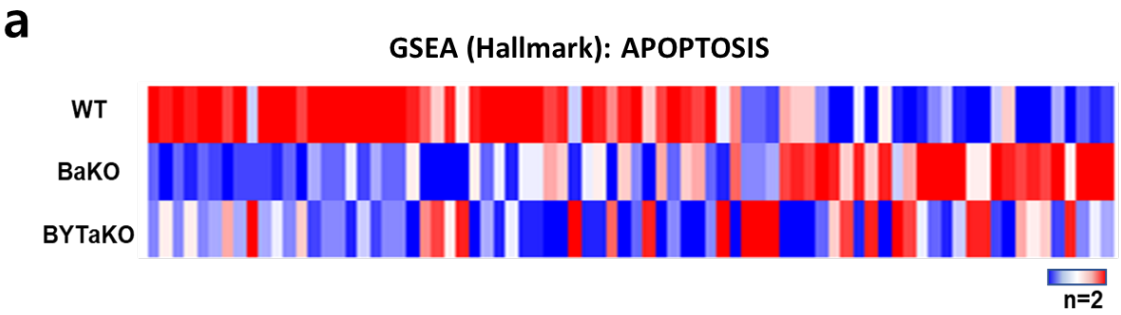
Revised Fig. 11a – Ablation of BECN1 leads to autophagy inhibition in differentiated imSVCs. Fully differentiated imSVCs were treated with 4-OHT to deplete BECN1.

The result has been incorporated into our Main Figure 1.

3. Along with changes in tumor cell proliferation, it would be good to evaluate changes in cell death, i.e. by CC3 staining (i.e. Fig 6), since autophagy can regulate cell death.

We apologize for any confusion regarding the experimental design we implemented in Fig.6. As the reviewer mentioned, autophagy ablation does play a crucial role to regulate cell survivability [23]. However, our manuscript mainly covers BECN1 depletion in adipocytes not in cancer cells [24]. It's worth noting that we have assessed adipocyte death in our previous report. We have shown that BECN1-deficient adipocytes experienced persistent accumulation of endoplasmic reticulum (ER) stress [24]. Furthermore, we have used TUDCA to relieve ER stress-mediated adipocyte death [24].

Nevertheless, we assume the reviewer was curious how cell death signal is altered in cancer cells when affected by adipocytes. It is reasonable to examine alterations in apoptosis, as Main Fig. 6j illustrates that BYT-deficient adipocytes could inflict apoptosis in cancer cells. Therefore, we conducted RNA-seq on the tumor cells transplanted in WT, BaKO, and BYTaKO. Here, we provide heatmap analysis for the expression pattern of genes associated with apoptosis. Surprisingly, tumors grown in BaKO exhibited downregulation of apoptosis; however, this effect was not significantly restored in cancer cells grown in BYTaKO **(Revised Fig. 12a).** Taken together, apoptosis induction is not considered the major factor to modulate tumor regression in BYTaKO. Instead, factors relating to cell proliferation such as adipokine secretion or environmental cues could drive the outcome. We hope these clarifications provide a better understanding of our experimental approach and results.



Revised Fig. 12a - Heatmap analysis of genes associated with apoptosis. Bulk RNA-seq was conducted on MC38 tumors grown in WT, BaKO, and BYTaKO mice for 2 weeks.

4. Please comment/discuss on YAP/TAZ inhibitor effect on other stromal cells? Could be relevant given this paper: The β-catenin/YAP signaling axis is a key regulator of melanoma-associated fibroblasts

First, we would like to express gratitude to the reviewer about the constructive comment. We admit that BECN1-YAP/TAZ axis is of great importance, especially in other stromal cells that are populated in TME. Before getting into this matter, we would like to share the mechanistic insight that was revealed throughout this study.

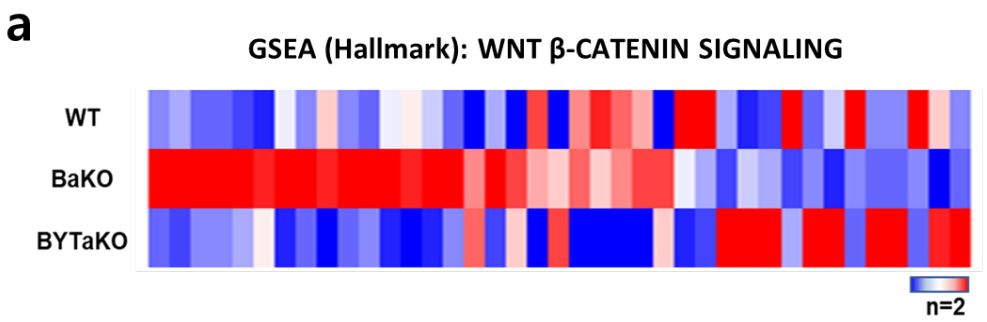
The Hippo pathway involves a cascade of kinase activities of different molecules such as MST1, LATS, and MOB1 [25]. Here, YAP/TAZ are tightly controlled by their phosphorylation status modulated by Hippo-associated proteins. Therefore, alteration of cellular YAP/TAZ level frequently involves alterations in kinase activities of Hippo-associated proteins. To identify the direct impact of BECN1 on Hippo-associated proteins, we conducted PLA which indicates the proximity between two proteins of interest [4]. PLA result between BECN1 and Hippo-associated proteins has shown modest interactions between BECN1-LATS1 and BECN1-MST1 interactions **(Revised Fig.3d, e).** However, when we checked the interaction between BECN1 and MOB1, its intensity exceeded all other interactions between BECN1 and Hippo-associated proteins **(Revised Fig. 3b).** Consistently, BECN1-deficient adipocytes exhibited downregulation of MOB1, indicating that loss of BECN1 leads to suppression of Hippo signaling through MOB1 **(Revised Fig. 3i).** This was confirmed in BaKO mouse iWAT **(Revised Fig. 3j).**

Implication of BECN1-MOB1-YAP/TAZ axis was also tested in preadipocytes, which are expected to have fibroblast-like features. BECN1-deficient preadipocytes also exhibited suppression of Hippo signaling demonstrated by the reduction in MOB1-LATS1 interaction **(Revised Fig. 6a**). Furthermore, we have shown that BECN1-deficient fibroblasts are sufficient to induce cancer malignancy through enhanced proliferation and EMT **(Revised Fig. 6b-h).** Therefore, usage of YAP/TAZ inhibitor could be effective on both adipocytes and fibroblasts in TME, which suggests somewhat broadened effects of verteporfin on the TME shown in Main Fig. 7d-g.

As the reviewer provided the paper, Wnt/β-catenin activity can possibly modulate YAP/TAZ in adipocytes [25, 26]. Our Main Fig. 5a-e also supports that there is a similar expression pattern between β-catenin and YAP/TAZ. We believe this discussion is highly relevant to our study and should be continued in the next reviewer's comment (Q5). Once again, we thank the reviewer for providing a thoughtful insight with a relevant literature.

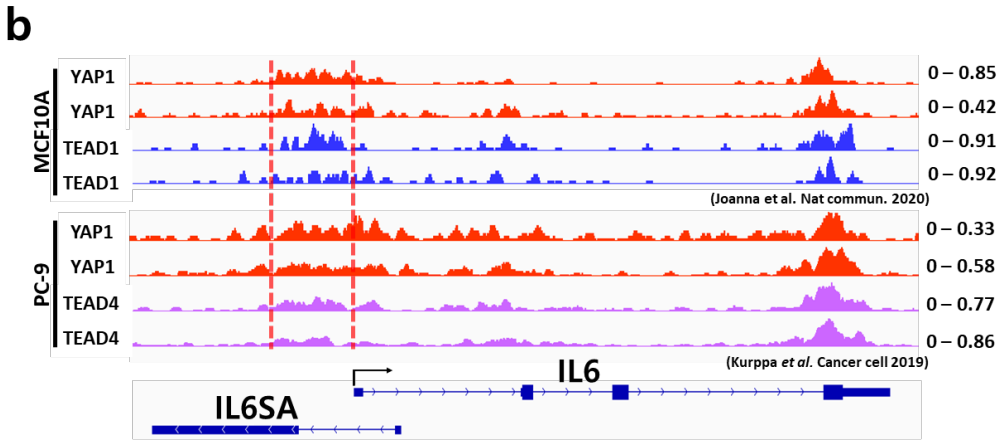
5. Are there any changes in Wnt signaling given the beta catenin changes? Can you comment on IL6 given its previous role? Do you think IL6 changes could also drive phenotypes?

As previously mentioned, Wnt/β-catenin signaling can be a strong candidate to control YAP/TAZ [25, 26]. Here, we provide heatmap analysis of Wnt/β-catenin signaling in WT, BaKO, and BYTaKO iWATs. The result was very striking, as it was highly activated in BaKO while the restoration in BYTaKO was evident **(Revised Fig. 13a)**. Such trend implies that Wnt/β-catenin signaling can be regulated by YAP/TAZ activity. In fact, there were multiple reports demonstrating the crosstalk between Hippo and Wnt signaling. Certainly, a few investigated how Hippo signaling triggered Wnt/β-catenin pathway [27-30].



Revised Fig. 13a – Heatmap analysis of genes associated with Wnt/β-catenin signaling. Bulk RNA-seq was conducted on WT, BaKO BYTaKO iWATs.

IL-6 has been reported to induce Wnt/β-catenin activity through JAK2 and FOXO3 in colorectal cancer (CRC) and kidney, respectively [31, 32]. Our RNA-seq result on BaKO WAT also illustrated that IL6-JAK-STAT3 signaling is the 5th most significantly upregulated gene set (Main Fig. 2a). Additionally, IL-6 itself was upregulated in BaKO WAT while restored in BYTaKO WAT (Main Fig. 6f). To resolve whether YAP/TAZ activity is capable of regulating IL6 expression, we checked the CHIP-seq result on IL6 loci. Interestingly, YAP-TEAD are found to be bound at the promoter region of IL6. Therefore, we can conclude that YAP-TEAD-IL6-JAK-Wnt/β-catenin axis is a plausible underlying mechanism in our study. Subsequent investigation should be performed to validate this pathway, and we have incorporated this aspect into the text; the following paragraph has been inserted in line 358-371 in the discussion section.



Revised Fig. 13b - ChIP-seq result of YAP and TEAD in MCF10A and PC-9 cells at the genomic loci of IL6. ChIP-seq results were collected from 'chip-atlas.org'. Intensified peaks indicate potential binding region of the indicated proteins at those chromatin regions.

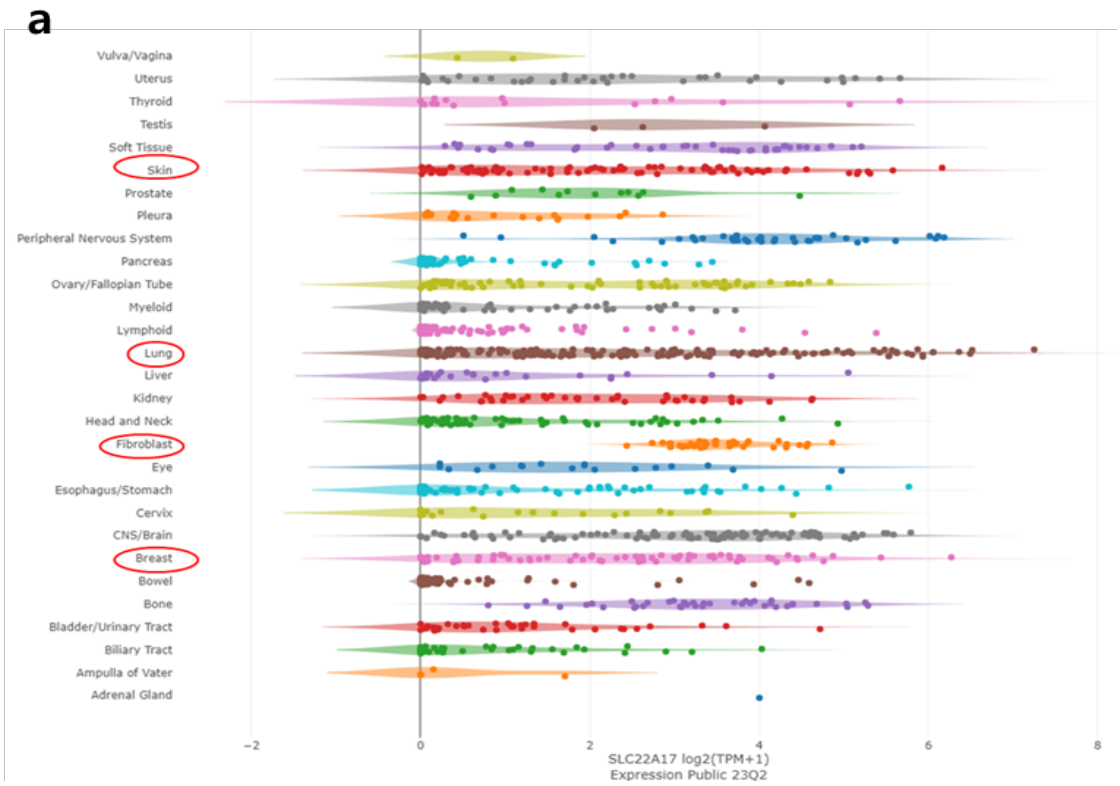
line 358-371

Our transcriptomic analyses on BaKO and peritumoral WATs well-characterized the dysfunctional adipocytes and identified IL-6-JAK-STAT3 signaling as the 5th and 9th most significantly upregulated gene sets (Fig. 2a, 3f) in BaKO and peritumoral WATs, respectively. Previous studies have shown that, IL-6 expression is not only relevant to the pro-inflammatory signals released from impaired adipose tissue, but also relevant to the induction of Wnt/β-catenin activity through JAK2 and FOXO3. In this study, we showed the upregulation of IL-6 in BaKO WAT while it was restored in BYTaKO WAT (Fig. 6f). Additionally, the CHIP-seq results of YAP and TEAD indicate potential binding regions at the IL-6 promoter, suggesting that the activation of YAP directly regulates IL-6 expression (Supplementary Fig.

10a). Heatmap analysis on Wnt/β-catenin signaling in WT, BaKO, and BYTaKO WATs revealed a significant activation in BaKO but restoration in BYTaKO (Supplementary Fig. 10b). This observed trend aligns well with our study, wherein lipodystrophy phenotypes and tumor progression were restored in BYTaKO (Fig. 6, 7). These findings suggest the IL6-JAK-Wnt/β-catenin axis as a plausible underlying mechanism for adipocyte transformation; however, further investigations are needed to validate this finding.

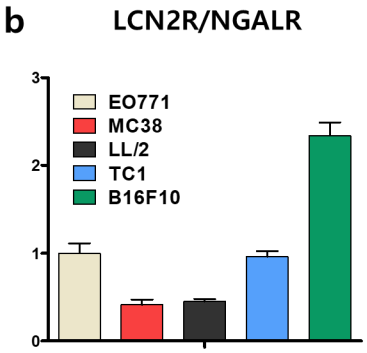
6. Is LCN2 or the receptor expressed in the tumor cells of interest? Consider providing some staining and/or citations. (same for TNFa/ receptor?) Is there any other previous data regarding LCN2 in breast/colorectal cancer specifically?

We found that the available literature on LCN2R (SLC22A17) is not sufficient to provide information on its expression across various mouse cancer cell lines. However, we could obtain LCN2R expression data for human cancer cell lines from the Cancer Cell Line Encyclopedia (https://sites.broadinstitute.org/ccle/). The expression of LCN2R varied across different tissues, with fibroblasts notably displaying relatively high levels of LCN2R. Human breast and bowel-related cancer cell lines exhibited a wide range of LCN2R expression distribution **(Revised Fig. 14a).**



Revised Fig. 14a – Distribution of LCN2R expression across multiple human cancer cell lines with various tissue origin.

LCN2 is known to promote breast and colon cancer progressions in both human and mouse [33-35]. When detecting the basal LCN2R expression across multiple cell lines, we observed considerable variation in its expression **(Revised Fig. 14b).** Notably, it was not necessarily upregulated in breast and colon cancers. Our thoughts on this phenomenon will be further discussed in Q7 and Q9.

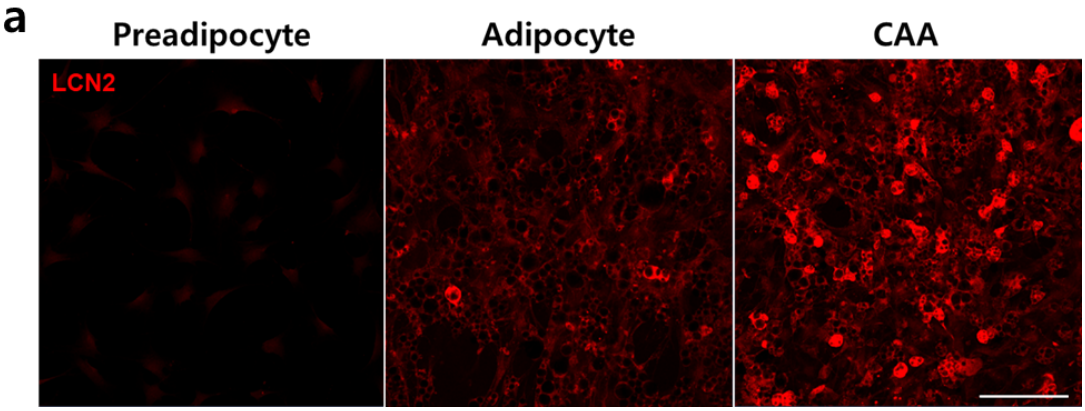


Revised Fig. 14b – qPCR result of LCN2R on multiple cancer cell lines that were used in our in-vivo study.

7. Please mention whether obesity is predictive of poor outcome in breast and colon cancer respectively. Overall, the discussion of cancer is very general, some more specifics for breast and colon (as well as cancers where there was no difference!) would be useful.

To date, obesity has been related to 13 different kinds of cancers, including breast and colon cancers [36]. Additionally, recent epidemiology on BMI and cancer risk identified that longer duration, greater degree, and younger age of onset are obese-related factors that correlate with cancer incidence [37]. Especially, longitudinal exposure to high BMI associated with 12 cancers: corpus uteri, kidney, gallbladder, myeloma, leukemia, breast, colorectal, liver, thyroid, brain, and head/neck cancers [36]. Of these cancers, tissue origins such as the kidney, bone marrow, breast, colorectal, and liver have a substantial presence of adipocytes within their environment. This suggests that some cancers, due to their proximity to large population of adipocytes, 'adipose health' becomes pivotal in determining the TME malignancy. Hence, we believe that the CAA literature will provide a profound understanding to obesity-related cancers.

We thank the reviewer for pointing out the obscurity regarding cancer specificity in our manuscript. We have noted that both breast and colon cancers are surrounded by substantial amount of adipose tissue, thereby being influenced by adipose milieu as a component of the TME. Considering the increased likelihood of interaction between cancer cells and adipocytes, we hypothesized a heightened crosstalk between these two cell types. This interaction is expected to lead to a more robust transformation of adipocytes, potentially increasing their tumorigenicity. Given our emphasis on LCN2 as a key factor derived from adipocytes that promotes breast and colon cancers, we assessed LCN2 levels in adipocytes cocultured with cancer cells. Notably, cocultured adipocytes exhibited elevated levels of LCN2 **(Revised Fig. 15a).** This finding supports the notion that with a greater presence of adipocytes in the TME, these cancers may become more susceptible to metabolites and cytokines derived from adipocytes.

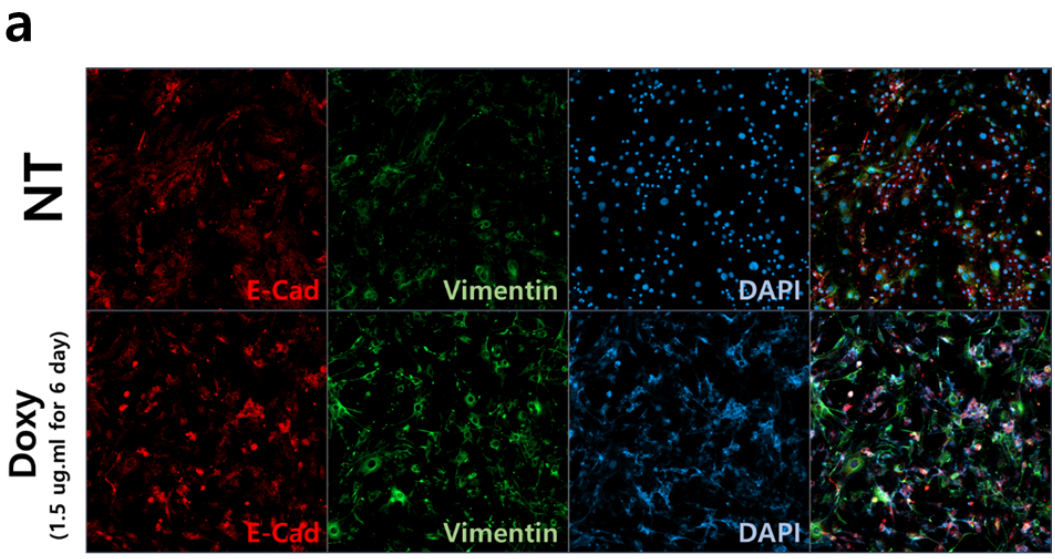


Revised Fig. 15a – Immunocytochemistry staining LCN2 level in preadipocytes, mature adipocytes, and cancer-co-cultured adipocytes. Adipocytes were co-cultured with EO771 cells for 2 days.

The results have been incorporated into our Supplementary Figure 3.

8. EMT: all the data for this are RNA levels/ gene signatures. Please add some protein data as well for a few markers. Please discuss, are EMT signatures associated with CAA status?

We admit that EMT markers should be confirmed through additional techniques. Accordingly, we stained Vimentin and E-cadherin in BECN1-deficient adipocytes. In line with RNA-seq results, BECN1-deficient adipocytes exhibit mesenchymal transition **(Revised Fig. 16a).**



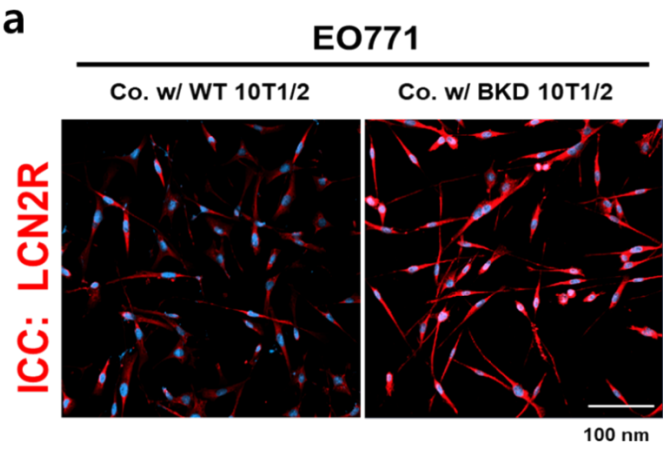
Revised Fig. 16a – Immunocytochemistry staining E-Cadherin (red) and Vimentin (green) in differentiated adipocytes treated with or without doxycycline.

CAAs are widely recognized for their propensity to undergo dedifferentiation, involving morphological alterations resulting in fibroblast-like and mesenchymal features [38]. Yiwei Li et al. referred to this transformation of adipocytes as adipocyte derived fibroblasts (ADF) [10]. In another recent study on CAAs, the term ‘adipocyte mesenchymal transition (AMT)’

was introduced [39]. Consequently, the acquisition of a mesenchymal signature well-characterizes CAA status.

9. Any thoughts as to why the 2 other tumor cell types did not show the same phenotypes?

Building on our response on Q6 and Q7, the basal LCN2R expression between different cancer cell lines **(Revised Fig. 14b)** does not explain their sensitivity to BECN1-deficient adipocytes in-vivo (Main Fig. 1). Therefore, we hypothesized that LCN2R expression could be altered situationally. To investigate the LCN2R level in our cell line of interest (EO771), we detected change in its expression upon adipocyte co-culture. While EO771 co-cultured with WT adipocytes show modest amount of LCN2R, EO771 co-cultured with BECN1-deficient adipocytes exhibited remarkably amplified levels of LCN2R **(Revised Fig. 17a).** This result suggests that LCN2R expression could be responsive to LCN2 accessibility. Additionally, whether other cancer cell types are capable of modifying adipocyte-LCN2, as observed in Revised Fig. 15a, remains unanswered. Therefore, the reason why the 2 other tumor cell types were not responsive to BECN1-deficient adipocytes could be attributed to the dynamic crosstalk between adipocytes and cancer cells, regulating LCN2 and LCN2R.



Revised Fig. 17a – LCN2R expression in EO771 co-cultured with WT or BECN1-deficient adipocytes. Cancer cells were co-cultured with adipocytes for 2 days.

This figure has been added into our Supplementary Figure 2

Minor comments:

1. Spell out LCN2 fully the first time it appears in the text.

This has been fixed in the main text.

2. Line 151 use “co-culture” instead of co cultivation

This has been fixed in the main text.

3. Should mention/discuss and cite Beige Adipocyte Maintenance Is Regulated by Autophagy-Induced Mitochondrial Clearance paper

This content has been added in line 200-202.

4. Please spell out what are imSVCs the first time it’s mentioned since I’m not familiar with this acronym.

This has been fixed in the main text. imSVC establishment procedure is further elaborated in the method section.

5. Supp fig 4a: please add quantification

Due to redundancy (with Main Fig. 4a), Supplementary Fig. 4a has been removed.

6. Supp. Fig 5c could benefit from BODIPY stain for ease of interpretation.

We appreciate the reviewer for positing a critical point. However, we would like to highlight our earlier publication has already demonstrated the development of a liver steatosis phenotype in BaKO attributed to lipodystrophy. Consequently, Supp. Fig 5c adequately illustrates the lipid accumulation in liver. Reference was added in line 111.

7. Fig. 6g: is WT versus Beclin/YT CKO significantly different? Please add this to the figure.

This has been fixed in the main figure.

Reference

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The revised study has been significantly improved. The reviewer appreciates the efforts of the authors to address the questions raised before.

One suggestion for the newly included luciferase reporter data (Fig. 6j-k):
- The predicted TEAD binding sites in L3 should be further mutated to strengthen the conclusion.

Reviewer #2 (Remarks to the Author):

The authors have carefully addressed all my questions by both new experiments and new discussions. I do not have more concerns, and suggest to consider the manuscript for publication in Nature Communications.

Kai Sun

Reviewer #3 (Remarks to the Author):

I recommend this paper for publication.

The authors have made a considerable effort in order to address the concerns of all 3 reviewers. The addition of further in vivo data using the colon cancer model is helpful, as are some of the mechanistic experiments i.e. ChipSeq investigating TEAD box binding, and investigating autophagy versus BECLIN specific functions.

Note: figures need to be edited to comply with the journal's requirements to show scatterplots (rather than bar graphs) for data where the n <10, including in the new data added. Additionally some of these new graphs need bars with *s (or ns) to indicate significance easily as well.
Fig 3b-e should have an insert panel at higher magnification because it is challenging to determine colocalization at the current magnification.

REVIEWERS' COMMENTS

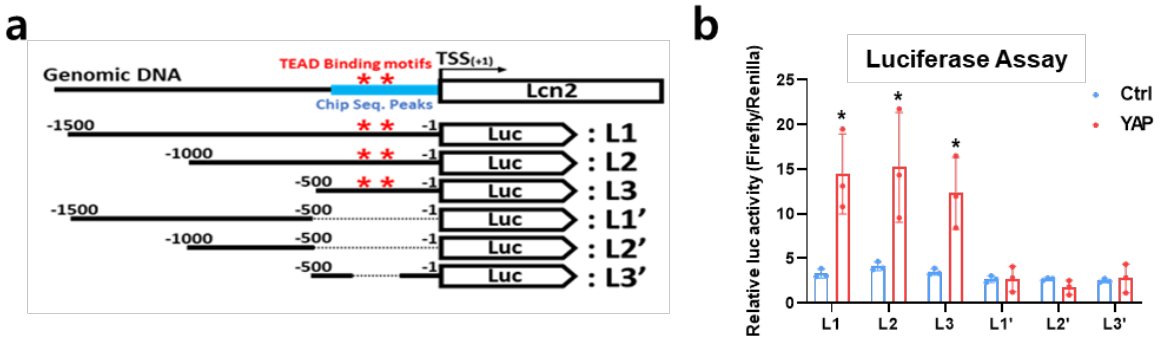
Reviewer #1 (Remarks to the Author):

The revised study has been significantly improved. The reviewer appreciates the efforts of the authors to address the questions raised before.

We express great appreciation to the reviewer for the positive evaluation.

One suggestion for the newly included luciferase reporter data (Fig. 6j-k):
- The predicted TEAD binding sites in L3 should be further mutated to strengthen the conclusion.

We thank the reviewer for the additional suggestion to strengthen our work. In response, we deleted the TEAD binding sites from our original luciferase constructs and detected luciferase activity (Revised Fig. 1a). The result revealed a marked reduction in LCN2 promoter activity in the absence of TEAD binding motifs, indicating that TEAD-YAP directly amplifies LCN2 expression (Revised Fig. 1b).



Revised Fig. 1

The main Figures 6j-k have been replaced

Reviewer #2 (Remarks to the Author):

The authors have carefully addressed all my questions by both new experiments and new discussions. I do not have more concerns, and suggest to consider the manuscript for publication in Nature Communications.

Kai Sun

We are grateful for the opportunity to have our manuscript evaluated by the reviewer.

Reviewer #3 (Remarks to the Author):

I recommend this paper for publication.

We are grateful to have our manuscript evaluated by the reviewer.

The authors have made a considerable effort in order to address the concerns of all 3 reviewers. The addition of further in vivo data using the colon cancer model is helpful, as are some of the mechanistic experiments i.e. ChipSeq investigating TEAD box binding, and investigating autophagy versus BECLIN specific functions.

Note: figures need to be edited to comply with the journal's requirements to show scatterplots (rather than bar graphs) for data where the n <10, including in the new data added. Additionally some of these new graphs need bars with *s (or ns) to indicate significance easily as well.
Fig 3b-e should have an insert panel at higher magnification because it is challenging to determine colocalization at the current magnification.

We sincerely appreciate the editor's careful consideration to our work.

We have revised the figures to align with the journal's requirements.