Pharmacological inhibition of SUMOylation recapitulates genetic hypoSUMOylation in the murine epididymal white adipose tissue

By

Damien Dufour [a,f], Xu Zhao [b,g], Magnar Bjørås [c,d,e], Antoine Martinez [a,*] and Pierre Chymkowitch [b,*]

[a] Institut Génétique, Reproduction & Développement (iGReD), CNRS, INSERM, Université Clermont Auvergne, Clermont-Ferrand, F-63000, France.

[b] Department of Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, 0316, Oslo, Norway

[c] Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, 7491, Trondheim, Norway.

[d] Department of Microbiology, Oslo University Hospital and University of Oslo, 0424 Oslo, Norway.

[e] Centre of Healthy Embryology (CRESCO), 0424, Oslo, Norway.

[f] Present address: Department of Molecular Medicine, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, 0317 Oslo, Norway.

[g] Present address: Movel As, Tordenskioldsgate 2, N-0160 Oslo.

[*] Corresponding authors

Running title: SUMOylation inhibition triggers lipoatrophy *in vivo*

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Abstract

SUMOylation plays a critical role in regulating cellular reprogramming and stem cell differentiation. Interestingly, while most studies suggest that SUMOylation constrains these processes, both *in vitro* and *in vivo* knockdown (KD) and knockout (KO) studies, as well as experiments employing SUMOylation inhibitors, have demonstrated that adipose tissue formation and adipocyte differentiation require an active SUMOylation pathway. This apparently positions adipose tissue as a bystander in the broader context of SUMOylation-regulated cell fate and identity.

To address this *in vivo*, we investigated the effects of SUMOylation inhibition in mice using TAK-981, a pharmacological SUMOylation inhibitor. Our results indicate that mice treated with TAK-981 develop lipoatrophy, characterized by reduced adipose tissue, due to impaired expression of adipogenic genes. These findings provide evidence that SUMOylation is essential for fat accumulation in vivo.

Furthermore, given that TAK-981 is currently under clinical evaluation for the treatment of solid tumors, our findings underscore the importance of considering potential unintended effects of SUMOylation inhibition on adipose tissue in patients.

Introduction

Post-translational modification by the small ubiquitin-like modifier SUMO is the reversible addition of the SUMO peptide to a protein. During the past decade, numerous studies investigated the function of the SUMOylation pathway during cellular differentiation using multiple cellular and *in vivo* models. Although the mechanisms regulated by SUMOylation and the target specificity of this modification remain largely unclear, it becomes evident that an active SUMOylation / deSUMOylation cycle is essential to the formation and function of many cellular types and organs (1-3).

Importantly, SUMOylation also safeguards the cellular identity of stem cells and differentiated cells by restricting cellular identity conversions (4-6). To reveal the SUMO-regulated mechanisms supporting cellular differentiation and fate, SUMO targets and SUMO-chromatin landscapes were characterized in mouse organs, ESCs, MEFs and adipocytes using mass spectrometry and ChIP-sequencing (2, 7, 8). These data revealed that SUMO targets are for the most part nuclear, and that chromatin-bound SUMO is linked to proteins involved in chromatin, epigenetic and transcription regulation. The use of specific SUMOylation inhibitors like ML-792 and TAK-981 showed that SUMOylation can both promote and repress transcription, depending on the cellular system (9, 10).

Interestingly, numerous *in vitro* and *in vivo* studies indicated that the formation of the adipose tissue was particularly dependent on an active SUMOylation / deSUMOylation pathway. Mice lacking *Sumo-1* or *Senp2* are resistant to high fat diet (HFD)-induced obesity (11, 12). Specific loss of *Ubc9* in white adipose tissue triggers lipoatrophy (13) and maturation of lipid droplets is impaired in mice lacking *Senp7* (14). Consistently, loss of *Sumo-1, Ubc9, Senp1* and *Senp2* in cellular models limits adipogenesis and is associated with altered expression of adipogenic genes, as well as genes involved in lipid and energy metabolism (15-17).

Since the mechanism by which SUMOylation supports adipogenesis remained poorly described, we recently used adipocyte differentiation as a model to investigate the regulation of differentiation and fat accumulation by the SUMOylation pathway *in vitro* (2). We found that the SUMOylation pathway is strongly activated upon adipogenic induction and that SUMOylation supports both the repression of pre-adipogenic genes and the activation of adipogenic genes (2). Our mass spectrometry data point towards complexes involved in SUMO dependent heterochromatin formation and DNA methylation leading to preadipocyte genes repression, which is consistent with a previous report (7). Mass spectrometry, ChIP-sequencing and chronic treatment with the SUMOylation inhibitor ML-792 indicated that SUMOylation enforces the specificity of PPARγ-RXR binding to their target DNA sequences and thus the proper expression of their target genes during adipogenesis, which is consistent with previously reported functions of SUMOylation (18). Our findings thus indicated that differentiating adipocytes require an active SUMOylation pathway to achieve full differentiation, which further points at an adipogenesis-specific function of the SUMOylation pathway when compared to other cellular systems (4, 5).

The epididymal white adipose tissue (eWAT) develop post-natal with the first adipocytes observed at post-natal day 7 (p7) (19). Then tissue undergoes further development with lipogenesis and adipogenesis taking place simultaneously. The post weaning period taking place after p21 is a period of adipose tissue remodeling particularly sensitive to environmental factors (20, 21). These make the study of hypoSUMOylation in the eWAT after p21 particularly interesting.

Given the lipoatrophic effect of pharmacological SUMOylation inhibition *in vitro*, we hypothesized in this study that SUMOylation inhibition using the small molecule SAE inhibitor TAK-981 leads to weight loss and lipoatrophy *in vivo*.

Results

SUMOylation inhibition leads to lipoathrophy in mice

To investigate *in vivo* the effect of pharmacological SUMOylation inhibition on the adipose tissue, we injected mice with the SUMOylation inhibitor TAK-981 (22, 23) using our previously described protocol (1). During this procedure, TAK-981 was injected twice a week during five weeks. We monitored body mass and found that mice treated with TAK-981 featured a significantly slower rate of weight gain compared to untreated mice with significance increasing with time (Fig. 1A) consistent with an observation from another cohort (1). This weight gain deficiency was associated with lipoatrophy illustrated by a decrease of gonadal WAT (eWAT) mass while the mass of the adrenal glands remained unaffected (Fig. 1B and 1C). To further investigate the origin of the decrease of eWAT mass, we assessed the size of lipid droplets which were smaller in TAK-981 treated mice (Fig. 1D, 1E and 1F).

This prompted us to assess WAT beiging. However, we were unable to detect UCP1-positive cells in the eWAT in both treatments (Fig. 2A) and we barely detected the expression of the browning marker *Ucp1* using qPCR (Fig. 2B). Furthermore, *Ucp1* expression was not affected by TAK-981, so was the expression of *Cidea*, another beiging marker (Fig. 2B). Together, our data indicate that SUMOylation inhibition leads to lipoatrophy in mice without triggering beiging.

Immune cells infiltration in the WAT

Further analysis of the tissue structure revealed accumulation of small cells with round nuclei located between adipocytes resembling immune cells (Fig. 3A). Accumulation of immune cells is a hallmark of lipoatrophy and has already been observed in adipocyte specific Ubc9 cKO (12). To test this hypothesis, we performed immunohistofluorescence for lymphocyte

marker CD45 and macrophage marker IBA1 in eWAT. Quantification revealed an increase in the proportion of both CD45+ cells and IBA1+ cells (Fig. 3B). Moreover, we observed a trend of increased presence of IBA1+ crown-like figures indicating active phagocytosis (Fig. 3C). These results show that SUMOylation is involved in maintaining immune microenvironment of the eWAT.

SUMOylation inhibition leads to downregulation of adipogenic genes.

We have shown in mouse 3T3-L1 cells that SUMOylation inhibition correlated with the downregulation of adipogenic genes (2). To investigate this in mice, we purified RNA from eWAT collected from mice treated or not with TAK-981 and assessed adipogenic gene expression by RT-qPCR. We found that WAT markers such as *Cebpa*, *Pparg*, *Cd36*, *Lipe*, *Adipoq*, *Fasn* and *Dgat2* were significantly downregulated (Figure 4). These data indicate that TAK-981inhibits adipogenic transcription *in vivo*. This suggests that adipocyte differentiation was inhibited by TAK-981, which is consistent with our results in 3T3-L1 cells, and the results presented in figures 1 (2).

Discussion

Recent studies using various cellular and *in vivo* systems have clearly indicated that SUMOylation limits differentiation and favors stemness and cellular identity over reprogramming (1, 4, 5). However, other works have shown that the formation and function of the adipose tissue depends on an active SUMOylation pathway. Indeed, SUMO1 KO mice and the conditional KO of *Ubc9* in WAT correlated with resistance to high-fat diet induced obesity and WAT atrophy, respectively (11, 13, 24). Furthermore, us and others have shown that maintaining low SUMOylation levels in differentiating mouse 3T3-L1 cells disrupts the dynamics of adipogenic gene expression, which leads to lipoathrophy (2, 15). These works

suggested that adipocyte differentiation is specifically and negatively affected by low SUMOylation levels. To further investigate this, we treated mice with TAK-981 to achieve global SUMOylation inhibition. Pharmaceutical SUMOylation inhibition resulted in eWAT atrophy caused by lipoatrophy.

The anti-differentiation effect of SUMOylation has mainly been shown at the level of stem cells, which fail to differentiate if maintained with high SUMOylation levels (1, 4, 5). The adipose tissue contains ~5% of mesenchymal adipose stem cells. Thus, even if eWAT is overall reduced, we cannot exclude that TAK-981 has had a pro-adipogenic effect on adipose stem cells, which would result in a higher number of adipocytes. This is highly likely as the eWAT undergoes both lipogenesis and adipogenesis between p7 and adulthood (25) and will be the focus of a follow up study. However, our data suggest that at the level of the whole tissue, pharmaceutical SUMOylation inhibition mainly affects mature adipocytes, which represent most cells in eWAT. So, we propose that TAK-981-dependent lipoathrophy is due to the failure of committed adipocytes to accumulate fat and that the effect on stem cells could exist but remains undetectable within the larger population of mature adipocytes. This is supported by the fact that adipogenic genes (e.g. Pparg, Fasn, etc) are downregulated, which indicated adipogenic failure due to alterations of the chromatin, transcription, likely epigenetics, and is consistent with our previous data (2). In this mechanism SUMOylation inhibition would destabilize chromatin-bound complexes and negatively affect transcriptional regulators as described before (2, 26-28).

Furthermore, previous study directly linked SUMOylation to the maintenance of the metabolic pathways involved in fatty acid synthesis (29). These studies indicated that in addition to regulate the expression of genes like Fasn, SUMOylation inhibition could directly affect the activity of enzymes that synthesize fatty acids. Our recent mass spectrometry

experiments indicate that these enzymes (e.g. Fasn) are SUMO targets in 3T3-L1 cells (2), which suggests that SUMOylation can modulate their activity.

Although this work focused on the adipose tissue, this organ is particularly sensitive to the energy state of the organism. As observed in the adipocyte specific *Ubc9* KO, it is very likely that energy balance is affected following TAK-981 treatment. However, the consistency with cellular models and the similarity of our model with the adipose tissue specific ablation of *Ubc9* (13) support the adipose origin of the phenotype. Similarly, even though we have not observed TAK-981 induced beiging, adipocytes from male eWAT do not seem to be able to transdifferentiate to a beige identity (30) and it cannot be excluded that thermogenesis took place in the iWAT or BAT after TAK-981 treatment. Moreover, the absence of beiging in TAK-981 treated mice is consistent with the observation that beiging of iWAT is dependent on active SUMOylation as evidenced by adipose tissue-specific *Senp2* KO (31).

These *in vivo* data suggest that the administration of TAK-981, which is currently in phase 1-2 clinical trial for solid tumors (22, 32), in individuals could result in weight loss and therefore constitute an inroad into the development of new anti-obese drugs. However, despite the apparent negative effect of TAK-981 on fat accumulation, such a strategy does not seem viable at this stage. First, lipoatrophy with constant energy intake is not to be sought to fight obesity since fatty acid will in return accumulate in other organs like the liver and lead to metabolic-associated fatty liver disease (33). Second, low SUMOylation levels in WAT correlated with infiltration of lymphocytes and activated macrophages, which suggests activation of the immune system as previously shown (13, 34). However, considering TAK-981 is currently being evaluated in clinical trials for solid tumors, our data provide valuable insights into potential unintended effects this compound may have on patients.

Overall, we show that pharmacological reduction of global SUMOylation phenocopies both conditional (12) and total (10, 20) genetic hypoSUMOylation models making it an interesting

tool to study generalized lipoatrophy syndroms. TAK-981 can allow easy manipulation the SUMOylation machinery during various stage of post-natal development and aging. However, authors wanting to use TAK-981 to study the effects of hypoSUMOylation should consider the effects that increased circulating free fatty acid resulting from lipoatrophy will have on metabolic organs (35).

Experimental procedures

Ethical approval declarations

Mouse experiments were conducted according to French and European directives for the use and care of animals for research purposes and were approved by the Comité d'Éthique pour l'Expérimentation Animale en Auvergne (project agreement #211522019061912052883), C2EA-02, at Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, Research Centre Clermont-Theix, France (C2E2A).

Animals

Mice bred in-house and maintained on a mixed sv129-C57Bl/6 genetic background were housed on a 12-h light/12-h dark cycle (lights on at 7:00 am). Mice were fed normal, commercial rodent chow and provided with water ad libitum. After weaning, mice were kept in siblings with a maximum of four animals per cage. At the end of experimental procedures mice were killed by decapitation around 8:30 am and trunk blood was collected in vacuum blood collection tubes (VF-053STK, Terumo). Mice aged 3 weeks were treated with vehicle control or TAK-981 (MedChemExpress, Sollentuna, Sweden) (7.5 mg/kg, intraperitoneally twice per week) for 5 weeks as previously described (1).

Histology

Tissues were fixed in 4% PFA for 24h and embedded in paraffin. 5 µm sections were deparaffinized and processed for hematoxylin/eosin staining. For immunofluorescence, deparaffinized slides were submerged in a citrate-based antigen retrieval buffer and microwaved for 8 min. After being rinsed with 1x PBS, slides were blocked for an hour with 2.5\% horse serum (Vector) and incubated overnight at 4C with rabbit anti-PLIN1 antibody (Cell signaling technology #9349, 1/500), anti-rat CD45 (550539 1/200) or anti rabbit IBA1 (ABIN2857032 1/500). After rinsing, they were incubated with ImmPRESS polymer for 30 min at room temperature. HRP activity was detected with Alexafluor (Thermo Fisher). Images were acquired with Zeiss Axioscan Z1 and ZEN 3.4 blue edition software and analyzed with QuPath 0.3.2 software (Bankhead et al, sci rep)

RNA purification, cDNA synthesis and RTqPCR

Total RNA purification from Formalin-Fixed Paraffin-Embedded eWAT was done using the RNeasy FFPE kit (73504, Qiagen) and cDNA synthesis was done using the QuantiTect Reverse Transcription Kit (205311, Qiagen) following manufacturer's instructions. RT-qPCR was performed using the HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne, 08-36-00001-5) on a LightCycler 96 instrument (Roche Diagnostics). Gene expression was normalized to the expression of *18S* and plots and data analyzed and visualized using GraphPad Prism 9. *P values* are indicated in figure legends. The following primer pairs were used for RT-qPCRs.

Cebpa: CAAAGCCAAGAAGTCGGTGGACAA – TCATTGTGACTGGTCAACTCCAGC

Pparg: CCGTAGAAGCCGTGCAAGAG – CCGTAGAAGCCGTGCAAGAG

Cd36: CGACTGCAGGTCAACATATT – TTAGCCACAGTATAGGTACA

Lipe: GCTCATCTCCTATGACCTACGG – TCCGTGGATGTGAACAACCAGG

AdipoQ: CTTGTGCAGGTTGGATGGCA – GGACCAAGAAGACCTGCATCT

Fasn: CACAGTGCTCAAAGGACATGCC – CACCAGGTGTAGTGCCTTCCTC

Dgat2: CTGTGCTCTACTTCACCTGGCT – CTGGATGGGAAAGTAGTCTCGG

Cidea: TGCTCTTCTGTATCGCCCAGT – GCCGTGTTAAGGAATCTGCTG

Ucp1: ACTGCCACACCTCCAGTCATT – CTTTGCCTCACTCAGGATTGG

18S: GTAACCCGTTGAACCCCATT – CCATCCAATCGGTAGTAGCG

Statistics

Results are presented as mean ± SD. Experiments were performed 5 times and statistical significance was calculated using unpaired, two-tailed Student's t tests using GraphPad Prism (v7). P values < 0.05 were considered as significant.

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Author contributions

D.D., A.M and. P.C. designed research; D.D. and X.Z. performed research; D.D. and X.Z. analyzed data; M.B., A.M. and P.C. resources, supervision; D.D., A.M. and P.C wrote the article.

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Conflict of interest

"The authors declare that they have no conflicts of interest with the contents of this article."

Figure legends

Figure 1. SUMOylation inhibition correlates with eWAT atrophy.

A, body mass follow-up of male mice treated with vehicle or TAK-981 between 3 and 8 weeks of age. *B-C,* absolute mass of eWAT *(B)* and adrenal glands *(C)* at 8 weeks of age. *D-F,* H&E staining (top) and PLIN1 immunofluorescence (bottom) *(D)* and quantification of adipocytes area *(E)* and lipid droplet *(F)* of eWAT of 8-week-old mice.

Figure 2. Absence of eWAT browning in TAK-981-treanted cells.

A, immunofluorescence staining of UCP1 in eWAT from vehicle or TAK-981 treated mice eWAT. *B,* qPCR analysis of genes associated with beige identity *Cidea* and *Ucp1*.

Figure 3. Immune cells infiltration in eWAT upon SUMOylation inhibition.

A, Coimmunofluorescent labelling of pan leukocyte marker CD45 (green) and macrophage marker IBA1 (purple) in 8-week-old eWAT from mice treated with vehicle or TAK-981. *B,* quantification of populations in *(A)*. *C,* quantification of crown-like structures observed in *(A*).

Figure 4. SUMOylation inhibition impairs adipogenic transcription.

RT-qPCR analyses of genes associated with white adipose tissue function from vehicle or TAK-981 treated 8-week-old-mice eWAT. *: P values ≤ 0.05, **: P values ≤ 0.01, ***: P values ≤ 0.001.

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Figure 1_Dufour

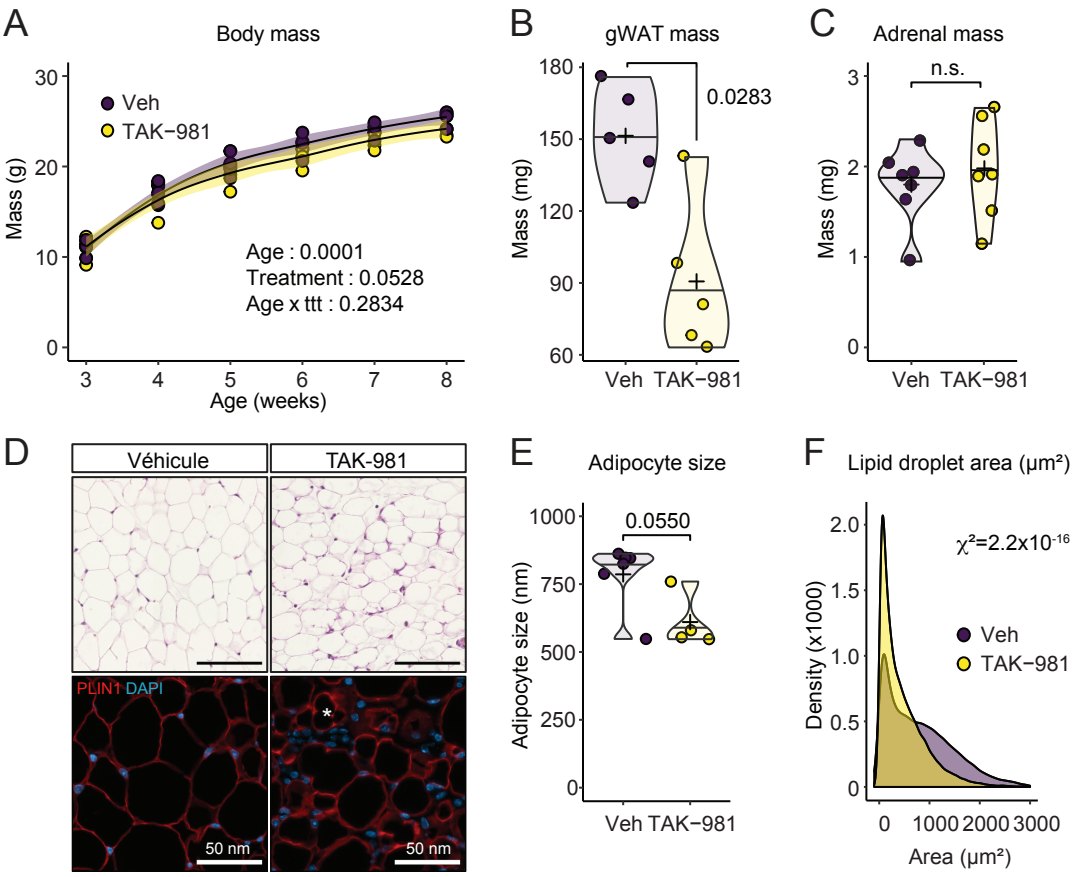


Figure 2_Dufour

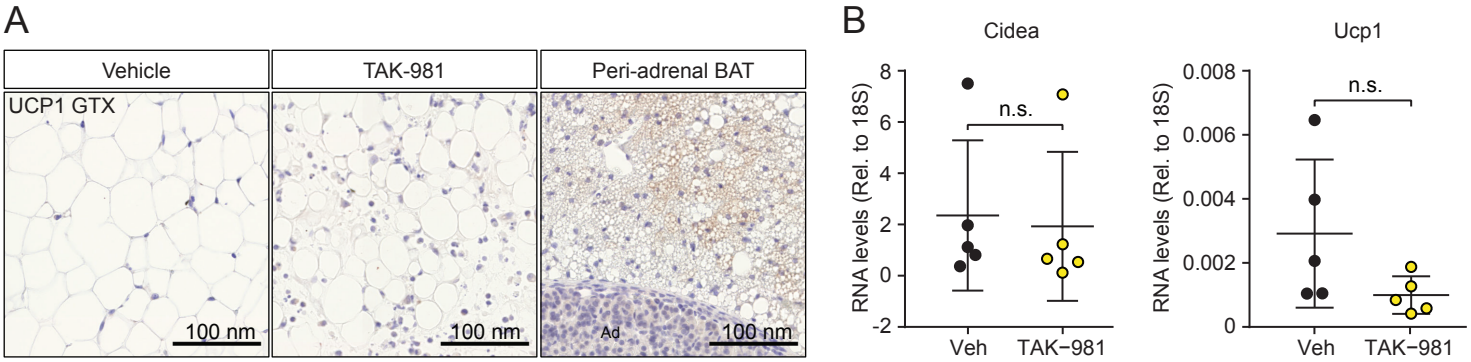
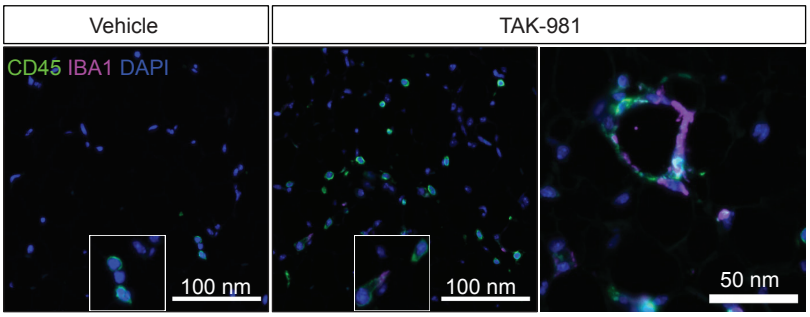
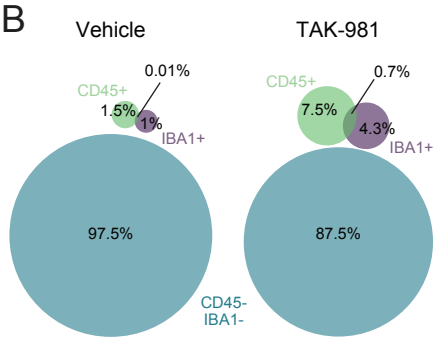


Figure 3_Dufour

A



B



C

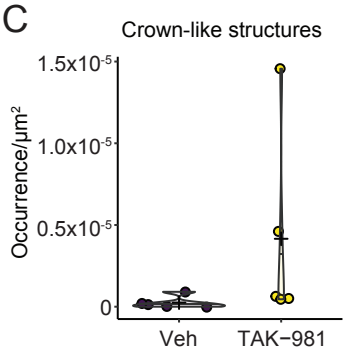
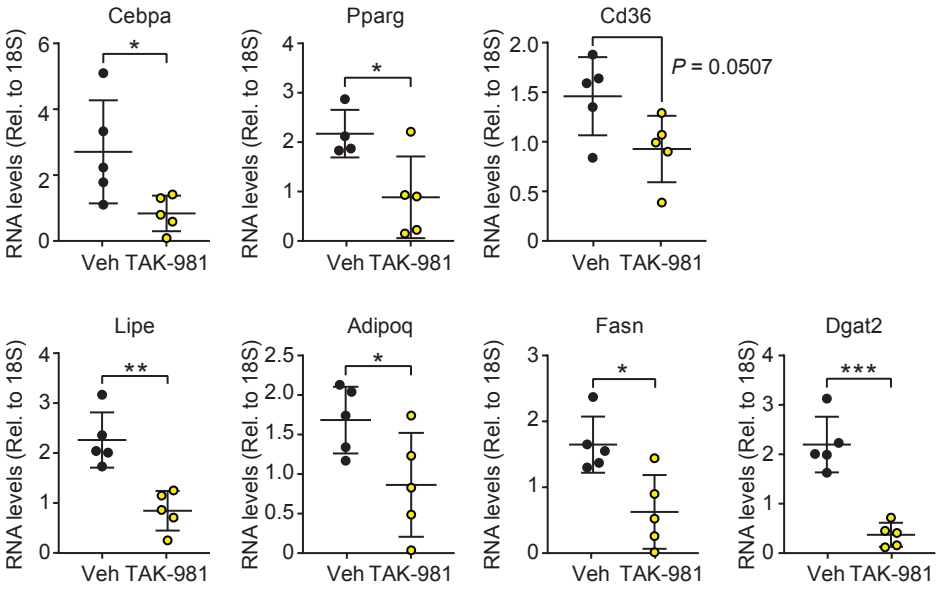



Figure 4_Dufour