

BRIEF REPORT



## Wnt inhibition enhances browning of mouse primary white adipocytes

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### ABSTRACT

The global epidemic in obesity and metabolic syndrome requires novel approaches to tackle. White adipose tissue, traditionally seen as a passive energy-storage organ, can be induced to take on certain characteristics of brown fat in a process called browning. The “browned” white adipose tissue, or beige fat, is a potential anti-obesity target. Various signaling pathways can enhance browning. Wnt is a key regulator of adipocyte biology, but its role in browning has not been explored. In this study, we found that in primary mouse adipocytes derived from the inguinal depot, Wnt inhibition by both chemical and genetic methods significantly enhanced browning. The effect of Wnt inhibition on browning most likely targets the beige precursor cells in selected adipose depots.

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### KEYWORDS

beige fat; brite fat; browning; C59; Wnt

### Introduction

Obesity has reached an epidemic level globally. In the simplest sense, obesity arises from an imbalance of energy intake and energy expenditure. Methods that could increase energy expenditure are potential anti-obesity strategies. Adipose tissue is the primary energy storage organ in the body. There are 2 main types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is responsible for storing excess energy as triglycerides for use in times of scarcity. Excess accumulation of WAT leads to obesity and increases the risk for metabolic disorders. On the other hand, BAT, which expresses UCP1 and other thermogenic proteins, has anti-obesity properties as it dissipates energy via non-shivering thermogenesis. This is a mechanism to maintain core body temperature and prevent hypothermia in mammals and human infants. BAT and WAT are different in their functions, anatomical locations, gene expression, and origins.<sup>1</sup>

A third type of fat, known as beige or brite (brown-in-white) fat, are brown fat-like cells found in certain depots of white fat.<sup>2</sup> Beige adipocytes have been traced to have a smooth muscle-like,<sup>3</sup> endothelial or perivascular cell origin.<sup>4</sup> In response to certain conditions such as cold exposure,<sup>5</sup> PPAR $\gamma$  agonists,<sup>6</sup> exercise,<sup>7</sup> or  $\beta$ -adrenergic stimulation,<sup>8</sup> beige adipocytes located within white fat are activated in a process known as

browning. Once activated, they express UCP1 and some other BAT genes, and they are also capable of thermogenesis.<sup>9</sup> Morphologically, they are characterized by multilocular lipid droplets and a high mitochondrial content. Certain depots of WAT, such as the inguinal and retroperitoneal depots, are more susceptible to browning.<sup>10</sup>

Different signaling pathways and regulators have been shown to have an effect on browning of white fat.<sup>11</sup> For example, inhibition of Notch<sup>12</sup> and JAK signaling<sup>13</sup> have been shown to promote browning in mouse and human white adipocytes, respectively. Recently, it has been shown that Lrp5, a Wnt coreceptor, is a regulator of adipose progenitor biology.<sup>14</sup> Adipose progenitors have been postulated to be a source of beige fat through a *de novo* adipogenesis process,<sup>15</sup> although it was also suggested that beige cells could arise from existing mature white adipocytes.<sup>16</sup>

Wnts are a family of 19 glycoproteins that act in an autocrine and paracrine manner to regulate tissue homeostasis and remodeling. These molecules signal through diverse non-canonical and canonical pathways, the latter of which converges on the transcriptional regulator  $\beta$ -catenin. Wnt signaling is a key regulator of mesenchymal stem cells (MSC) biology and adipogenesis.<sup>17</sup> Conditional deletion of  $\beta$ -catenin in mouse uterus leads to a switch from myogenesis to adipogenesis.<sup>18</sup> Wnt signaling also has an inhibitory

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role in both white and brown adipogenesis.<sup>19,20</sup> Interestingly, overexpression of Wnt10b under the control of Ucp1 promoter results in BAT gaining molecular and histological features of WAT.<sup>20</sup> It has not been explored if altering Wnt signaling in white fat would have any effect on browning. In this current study, we find that in primary adipocytes derived from mouse inguinal fat inhibition of Wnt signaling enhances browning. The effect of Wnt inhibition on browning appears to target adipocyte progenitors rather than mature adipocytes or the lineage-determined preadipocyte population, lending support to the theory that beige fat is derived from *de novo* differentiation of beige precursors. Furthermore, Wnt inhibition enhanced the thermogenic properties of primary brown adipocytes, but did not induce browning of primary white adipocytes derived from epididymal depots, suggesting that the effect of Wnt inhibition on browning is specific to certain fat depots.

## Results

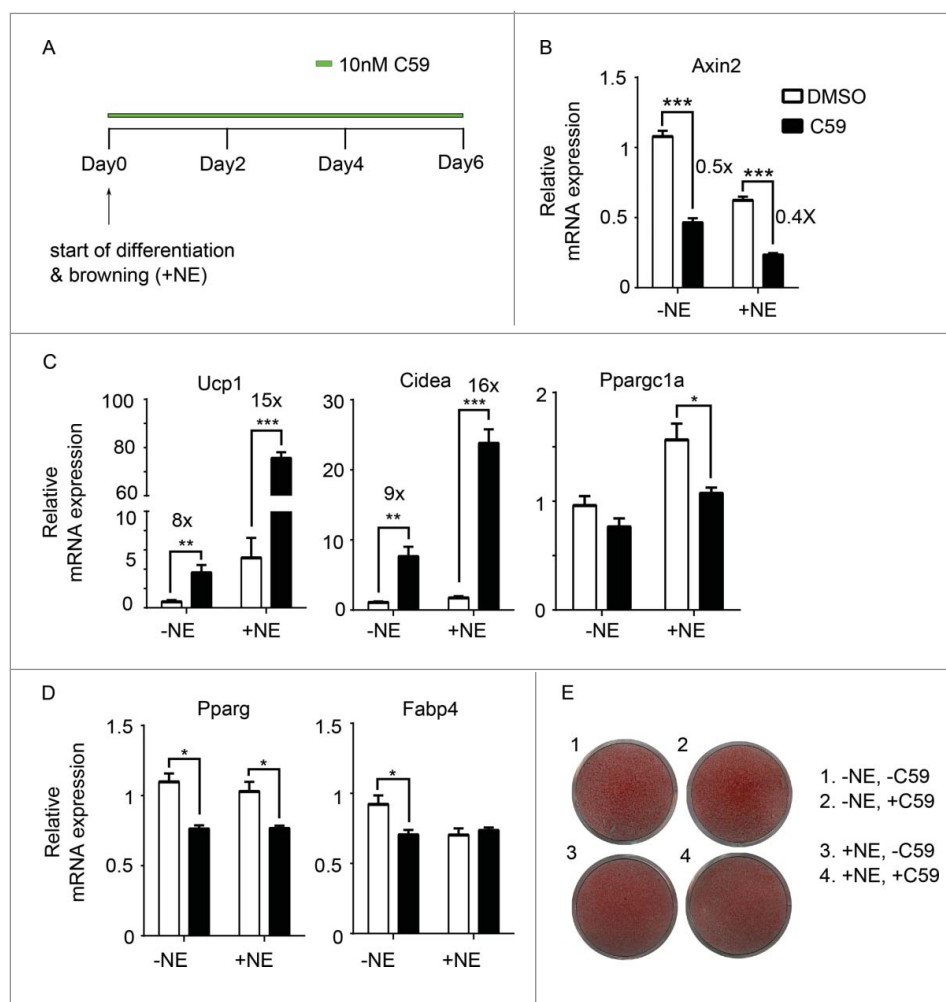
To test if Wnt inhibition would have any effect on browning, the stromal vascular fraction (SVF) from mouse inguinal WAT was isolated and differentiated into mature adipocytes using a standard cocktail (see Methods). Wnt inhibitor C59 or vehicle was added for 6 d from the start of differentiation (Fig. 1A). C59 blocks Wnt signaling by inhibiting porcupine (PORCN),<sup>21</sup> which is an acyltransferase responsible for palmitoylation of all Wnt proteins.<sup>22,23</sup> Inhibiting PORCN blocks Wnt secretion and down-stream Wnt signaling, which was confirmed by the down-regulation of *Axin2* (Fig. 1B) by around 50% both in the presence and absence of norepinephrine. Remarkably, C59 treatment led to increased expression of thermogenic markers (*Ucp1* and *Cidea*), the expression of these 2 genes was increased by more than 15-fold in the presence of norepinephrine (Fig. 1C), though the expression of *Pparg1a*, an important coregulator in brown fat markers, was not elevated. The induction of the expression of BAT genes occurred in a dose-dependent manner (Supplementary Fig. 1A) and could be observed as early as Day 4 (Supplementary Fig. 1B), though the induction of Ucp1 on the protein level is minimal on Day 6 (Supplementary Fig. 1C). Wnt inhibition has been shown to enhance adipogenesis.<sup>19,20</sup> To test if the induction of thermogenic genes is a consequence of enhanced adipogenesis, we checked the expression of *Pparg* and *Fabp4*, which did not show any up-regulation upon C59 treatment (Fig. 1D). Furthermore, oil red O staining shows similar differentiation efficiency in all groups (Fig. 1E).

As an alternative approach to inhibiting *Porcn* and hence Wnt secretion, we isolated SVF from inguinal WAT of *Rosa26-CreER<sup>T2</sup>/Porcn fl/fl* mice<sup>24-26</sup> and

differentiated it into mature adipocytes. The *Porcn* gene was then excised *in vitro* by addition of 4-hydroxytamoxifen (4-OHT) (Fig. 2A). We verified that Wnt signaling was inhibited as shown by 30% downregulation of *Axin2* (Fig. 2B). Wnt inhibition as a result of *Porcn* deletion led to an increased expression of *Ucp1*, *Cidea* and *Pparg1a* (Fig. 2C), though there was also a small but significant induction of *Pparg* and *Fabp4* (Fig. 2D). These data suggest that inhibiting Wnt signaling via both pharmacological and genetic approaches enhance expression of key thermogenic genes in primary inguinal adipocytes.

To further dissect the signaling pathway involved, a second Wnt inhibitor was used. XAV939 is a tankyrase inhibitor that specifically targets the canonical Wnt pathway by increasing Axin protein abundance, thereby stimulating  $\beta$ -catenin degradation.<sup>27</sup> Similar to C59, XAV939 induced the expression of *Ucp1* by 5-fold and that of *Cidea* by 1.4-fold but not *Pparg* and *Cebpa* (Fig. 3A), suggesting that the effect of Wnt inhibition on browning works through the canonical Wnt/ $\beta$ -catenin pathway. To further examine what cells are affected as a result of Wnt inhibition, XAV939 was administered for only 2 d starting at 3 different time points during the course of differentiation (Fig. 3B). Expression of *Ucp1* and *Cidea* increased by 3-fold and 1.3-fold respectively when XAV939 was administered from Day 0 to Day 2, indicating that the effect of Wnt inhibition on browning is strongest when inhibition takes place early during differentiation (Fig. 3C). Wnt inhibition was largely ineffective in inducing BAT genes when it occurred late in differentiation. Similar results were obtained using C59 (data not shown). This suggests that the positive effect of Wnt inhibition on browning most likely targets beige precursor cells rather than mature white adipocytes. We repeated the same experiment in 3T3-L1, a cell line which consists of clonal pre-determined preadipocytes prior to differentiation. There was no induction of thermogenic gene expression in 3T3-L1 upon XAV939 treatment (Fig. 3D). This suggests that the beige fat precursors responsive to Wnt inhibition are present in the SVF of primary cells but not in the lineage-predetermined 3T3-L1 cells.

Next, we explored if inhibiting Wnt signaling in primary brown adipocytes would enhance its thermogenic properties. C59 induced *Ucp1* and *Cidea* expression in a dose-responsive manner, while it had little effect on *Fabp4* and *Pparg* expression (Fig. 4A). The induction of Ucp1 was also observed at the protein level (Fig. 4B). Furthermore, the induction of BAT genes was observed as early as day 4 (*Ucp1*, 1.8-fold) or even day 2 (*Cidea*, 2.3-fold) (Fig. 4C), suggesting that Wnt inhibition targets precursors or differentiating cells rather than differentiated cells. Downregulation of Wnt signaling by C59



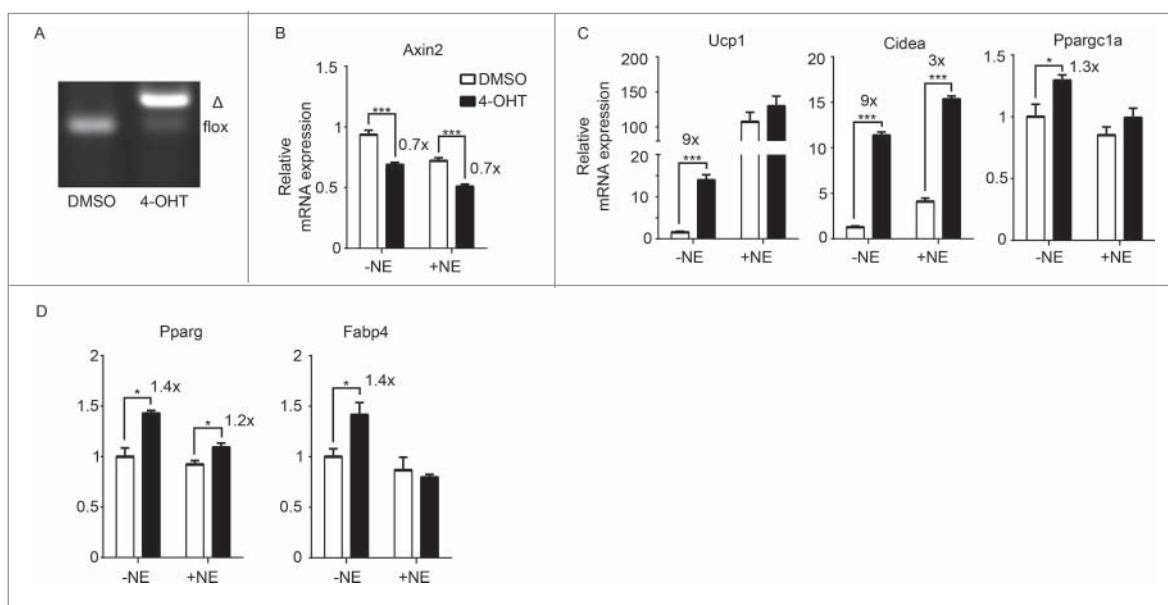
**Figure 1.** Inhibition of Wnt signaling pharmacologically enhances browning (A) Experiment schematics of *in vitro* browning experiment. Primary mouse adipocytes differentiated from the stromal vascular fraction (SVF) of inguinal fat pads were treated with 10 nM of C59 for 6 d from the start of differentiation. Browning was induced by adding 1  $\mu$ M norepinephrine. (B–D) qPCR analysis of Wnt signaling gene (*Axin2*) (B), BAT genes (*Ucp1*, *Cidea*, *Ppargc1a*) (C), and pan-adipocyte genes (*Pparg*, *Fabp4*) (D) on RNA extracted at Day 6 according to scheme 1(A). Shown is the relative mRNA expression values normalized with the housekeeping gene *Rpl23*. All data are presented as mean  $\pm$  SEM by Student's *t*-test. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  comparing with control. The fold of induction or inhibition is indicated when the change is significant and relevant. (E) Oil red O staining of primary mouse adipocytes at Day 6.

occurred as early as day 2 in both mRNA (*Axin2*, 60% inhibition) and protein level of  $\beta$ -catenin (Fig. 4D). However, Wnt inhibition in primary white adipocytes derived from the SVF of epididymal origin had no effect on browning (Fig. 4E).

## Discussion

The realization that white fat can take on a brown fat phenotype and function as genuine thermogenic cells opens up the possibility of harnessing this browned white fat to increase energy expenditure and reduce adiposity. Many different treatments, food components, drug substances, transgenes and gene knockouts have been shown to induce white fat browning.<sup>2,28</sup> In this study, we investigated if inhibiting Wnt signaling would enhance browning. Using

two different small molecule inhibitors of Wnt signaling – one specifically targeting the canonical Wnt pathway – we show in mouse primary inguinal adipocytes that Wnt inhibition led to upregulation of thermogenic genes. Furthermore, the induction and enhancement of browning is most prominent when Wnt signaling is inhibited at the start of differentiation, whereas late inhibition produces little or no effect. This suggests that the effect of Wnt on browning probably targets the beige precursor cells rather than mature white adipocytes. Moreover, the fact that Wnt inhibition has no effect on browning in 3T3-L1 cells suggests that lineage-predetermined white preadipocytes cannot be induced into beige fat upon Wnt inhibition. Interestingly, even primary white cells derived from epididymal fat pad (in contrast to those from inguinal origin) cannot be “browned” upon Wnt inhibition, suggesting



**Figure 2.** Inhibition of Wnt signaling genetically enhances browning (A) *Porcn* deletion in *Rosa26-CreER<sup>T2</sup>/Porcn<sup>fl/fl</sup>* mice upon addition of 4-hydroxytamoxifen (4-OHT).  $\Delta$ : PCR product after *Porcn* is deleted. (B-D) SVF was isolated from inguinal fat pads of *Rosa26-CreER<sup>T2</sup>/Porcn<sup>fl/fl</sup>* mice and differentiated to adipocytes in the presence or absence of 4-OHT to delete *Porcn*. RNA was extracted at Day 6 of differentiation. qPCR analysis of Wnt signaling gene (*Axin2*) (B), BAT genes (*Ucp1*, *Cidea*, *Pparg1a*) (C), and pan-adipocyte genes (*Pparg*, *Fabp4*) (D) on RNA extracted at Day 6 according to scheme 1(A). Shown is the relative mRNA expression values normalized with the housekeeping gene *Rpl23*. The fold of induction or % of inhibition is indicated when the change is significant and relevant. All data are presented as mean  $\pm$  SEM by Student's t-test. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  comparing with the control model.

that beige precursors likely reside in the heterogeneous SVF of certain fat depots, one example of which is the inguinal depot.

Pericytes have been suggested to be a source of beige precursors.<sup>4</sup> Interestingly, it has been shown that Wnt signaling inhibits adipogenic differentiation of pericytes and favors the osteogenic lineage.<sup>29</sup> We do not know if the effect of Wnt is cell-autonomous as we could not ascertain the cell type(s) that secrete and receive Wnt. Nevertheless, it is plausible that the immune milieu surrounding inguinal WAT, which has recently been shown to play a major role in browning,<sup>30-32</sup> could be a source of Wnt. Wnt could be inhibiting a group of bipotential precursor cells from developing into beige fat. The suppression of Wnt signaling thus abolishes this inhibition, leading to induction of thermogenic genes. Furthermore, Wnt inhibition also has a modest but significant effect on the thermogenic property of primary brown adipocytes derived from the interscapular depot.

In conclusion, our study shows that inhibition of Wnt secretion and downstream Wnt/ $\beta$ -catenin signaling enhances browning in primary inguinal mouse adipocytes. Future directions include identifying the source, identity and target of the Wnts, and the role of Wnt signaling in cell fate determination of beige precursor cells.

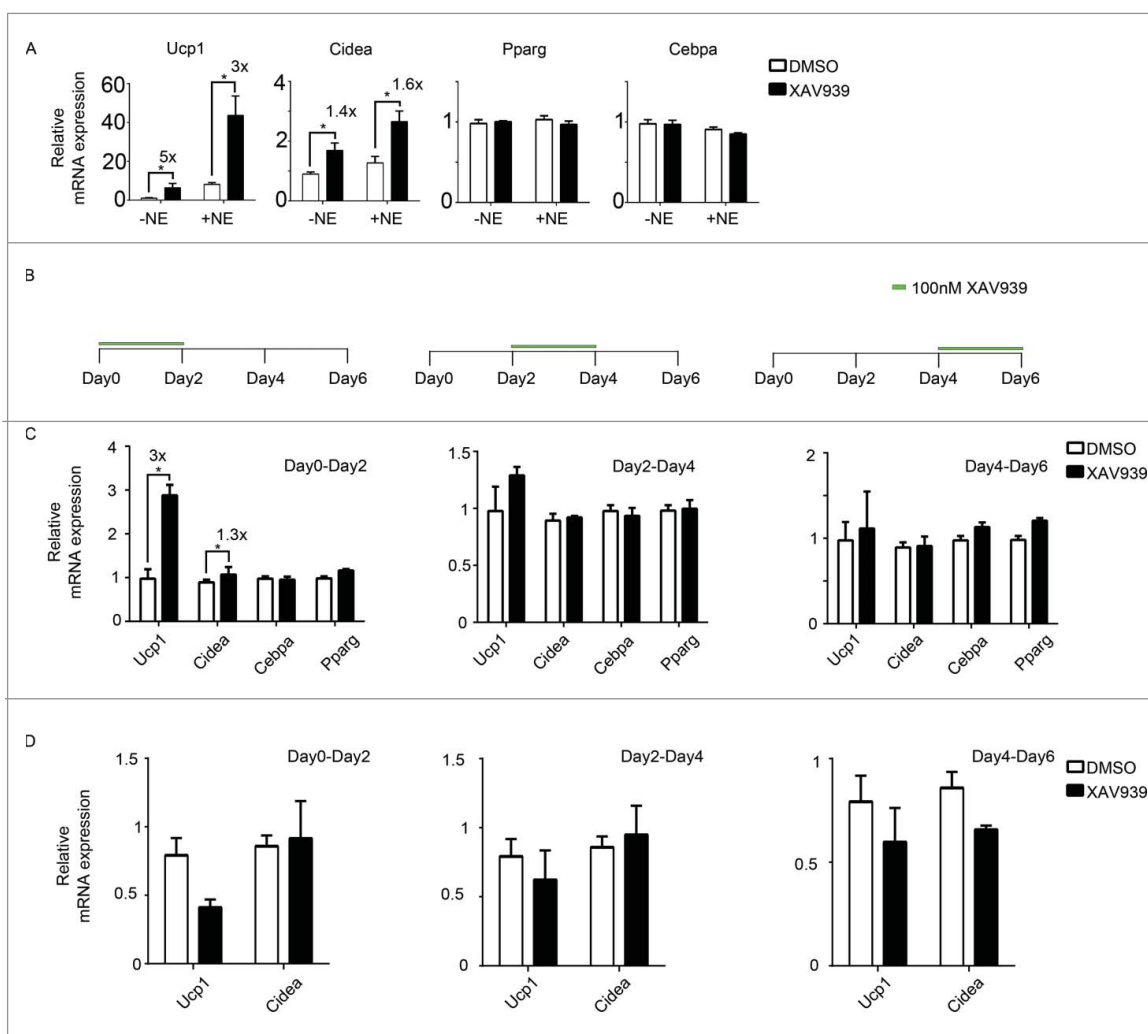
## Materials and methods

### RNA isolation and reverse transcription-PCR

Total RNA was extracted using Qiagen kit. 300ng of RNA was reverse-transcribed to cDNA and used for quantitative PCR using SYBR Green I master mix (BioLigne) in HT7900 Real Time PCR System (Applied Biosystems). At least 3 biological replicates were performed for each experiment.

### Primary adipocyte culture, differentiation and treatment

Inguinal fat pads from 3-week-old mice were excised, minced and digested in collagenase at 37 °C for 20min. The suspension was then spun at 2000 rpm, washed with PBS, and the pelleted stromal vascular fraction was resuspended in 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% new calf bovine serum (Invitrogen), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 10  $\mu$ g/ml of gentamicin (Invitrogen). Medium was changed the next day and then every 2 d. Cells were grown to confluence, and differentiation was initiated at Day0 with DMEM containing 10% fetal bovine serum (FBS), 0.5 $\mu$ M dexamethasone, 850nM insulin, 0.25mM 3-isobutyl-1-methylxanthine and 1 $\mu$ M



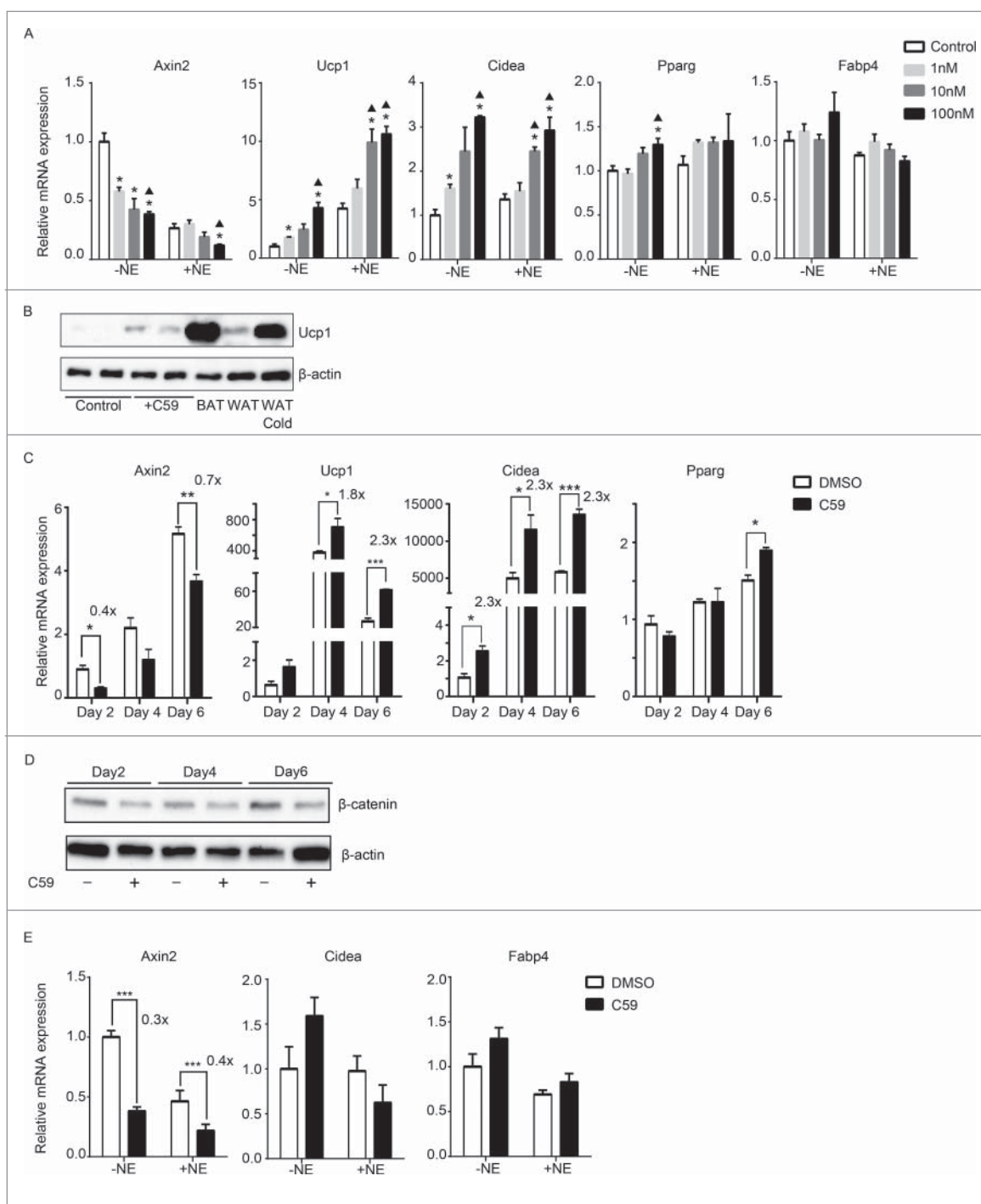
**Figure 3.** Wnt inhibition likely targets beige precursor cells to enhance browning (A) Primary adipocytes were treated with a second Wnt signaling inhibitor, XAV939. Shown is the qPCR analysis of marker genes after treatment following scheme 1(A). (B) Experiment schematics of *in vitro* browning experiment using primary adipocytes. Cells were treated with 100 nM XAV939 for only 2 d starting from Day 0, Day 2 or Day 4 during differentiation. RNA was harvested at Day 6 for qPCR analysis. (C) qPCR analysis of markers genes according to experiment plan 3 (B). (D) qPCR analysis of markers genes according to experiment plan 3(B) using 3T3-L1. Shown is the relative mRNA expression values normalized with housekeeping genes Rpl23. The fold of induction is indicated when the change is significant and relevant. All data are presented as mean  $\pm$  SEM by Student's t-test. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  comparing with control.

Rosiglitazone for 2 d. Cells were then incubated in DMEM containing 10% FBS and 850 nM insulin for 2 more days. After Day 4, cells were maintained for 2 more days in DMEM containing 10% FBS. For most experiments, RNA and protein were harvested from mature adipocytes at Day 6.

For primary adipocyte culture involving Rosa26-CreER<sup>T2</sup>/Porcn fl/fl mice, inguinal fat pad were isolated from 6-month-old mice and cultured as wild-type mice. Deletion of *Porcn* was initiated by adding 100nM of 4-hydroxytamoxifen (4-OHT) at the start of differentiation.

### Western blot

Primary white or brown adipocytes were treated with or without 10nM C59 during the course of differentiation. Cells were lysed in RIPA buffer with protease inhibitor. Proteins were extracted and quantified using Bradford reagent. 30ug of soluble protein was boiled with sample loading buffer for 10min and loaded onto 10% TGX handcast gel (Bio-Rad) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the SDS-PAGE gel were then immunoblotted onto PVDF membranes. Primary antibody was then used to



**Figure 4.** Wnt inhibition also enhances thermogenesis in primary cells derived from brown adipose tissue but not epididymal adipose tissue (A) Dose-response curve of primary brown adipocytes treated with increasing dose of C59. RNA was extracted at Day 6 for qPCR analysis. Shown is the relative mRNA expression values normalized with housekeeping genes Rpl23. All data are presented as mean  $\pm$  SEM by Student's t-test. \* $p < 0.05$  comparing with control;  $\blacktriangle p < 0.05$  comparing with 1nM. (B) Western Blot analysis on protein extracted at Day 6 from primary brown adipocytes with or without 10 nM C59 treatment. Also shown is protein extracted from brown adipose tissue (BAT), inguinal adipose tissue (WAT) and inguinal adipose tissue from mice underwent 7 d of 5°C cold exposure (WAT cold). (C) Primary brown adipocytes treated with 10 nM of C59 were harvested at 3 different time points (Day 2, Day 4 and Day 6) and subjected to qPCR analysis. (D) Western Blot analysis on protein extracted at Day2, Day4 and Day 6 from primary brown adipocytes during the course of differentiation treated with or without 10 nM C59 (E) Primary white adipocytes derived from epididymal fat pad were subjected to qPCR analysis. All data are presented as mean  $\pm$  SEM by Student's t-test. \*\*\*  $p < 0.001$  comparing with the control model. The fold of induction or inhibition is indicated when the change is significant and relevant.

probe for Ucp1 (abcam ab10983, 32kDa) and  $\beta$ -catenin (abcam 32572, 92kDa). Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody. Detection of signal was performed using Gel Doc XR system (Bio-rad). Membranes were then stripped for 10 min, re-blocked and incubated with  $\beta$ -actin antibody (Sigma A1978, 43kD) as loading control.

### Mouse studies

Mice on the C57BL/6 background were kept at Duke-NUS Medical School animal facilities. Mice were fed a normal chow diet during the course of the study. For cold exposure, 7-week-old male mice were housed at 5°C in a cold chamber (Geneva Scientific) for one week. All mice were sacrificed at 8 weeks of age and interscapular brown adipose tissue and inguinal white adipose tissues were harvested from control and cold-exposed mice and subjected to Western Blot analysis.

### Abbreviations

<i>Axin2</i>	axis inhibition protein 2
<i>BAT</i>	brown adipose tissue
<i>brite fat</i>	brown-in-white fat
<i>Cidea</i>	Cell death-inducing DFFA-like effector a
<i>Fabp4</i>	Fatty acid-binding protein 4
<i>MSC</i>	mesenchymal stem cells
<i>PORCN</i>	Porcupine; Pparg, Peroxisome proliferator-activated receptor gamma
<i>SVF</i>	stromal vascular fraction
<i>UCP1</i>	uncoupling protein 1
<i>WAT</i>	White adipose tissue

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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