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STAT3 Suppresses Wnt/ β -Catenin Signaling During the Induction Phase of Primary Myf5+ Brown Adipogenesis

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Abstract

Thermogenic fat is a promising target for new therapies in diabetes and obesity. Understanding how thermogenic fat develops is important to develop rational strategies to treat obesity. Previously, we have shown that Tyk2 and STAT3, part of the JAK-STAT pathway, are necessary for proper development of classical brown fat. Using primary preadipocytes isolated from newborn mice we demonstrate that STAT3 is required for differentiation and robust expression of Uncoupling Protein 1 (UCP1). We also confirm that STAT3 is necessary during the early induction stage of differentiation and is dispensable during the later terminal differentiation stage. The inability of STAT3^{-/-} preadipocytes to differentiate can be rescued using Wnt ligand secretion inhibitors when applied during the induction stage. Through chemical inhibition and RNAi, we show that it is the canonical β -catenin pathway that is responsible for the block in differentiation; inhibition or knockdown of β -catenin can fully rescue adipogenesis and UCP1 expression in the STAT3^{-/-} adipocytes. During the induction stage, Wnts 1, 3a, and 10b have increased expression in the STAT3^{-/-} adipocytes, potentially explaining the increased levels and activity of β -catenin.

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6. Competing Interests

The authors have no competing interests to declare.

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Our results for the first time point towards an interaction between the JAK/STAT pathway and the Wnt/ β -catenin pathway during the early stages of *in-vitro* adipogenesis.

Keywords

Adipogenesis; STAT3; Wnt; β -Catenin; BAT; UCP1

1. Introduction

Obesity is an epidemic in Western countries. In the United States one-third of adults are obese and another third are overweight [1]. Rates of obesity in the pediatric population have also increased [2]. Increases in affordable high-caloric foods coupled with increased sedentary life styles are major contributors to the epidemic. Excess adiposity is a risk factor for a host of chronic diseases such as diabetes, cardiovascular disease, and cancer [3].

One area of research to address the obesity epidemic is centered on a type of adipose tissue called brown adipose tissue (BAT). BAT metabolizes fat for heat production as opposed to white adipose tissue (WAT), which stores fat for use during periods of starvation. BAT derives its name from the brown color of the tissue; this color is due to the large amounts of mitochondria present in these fat cells. The mitochondria in BAT express a unique protein called Uncoupling Protein 1 (UCP1). UCP1 dissipates the electrochemical gradient of hydrogen ions between the intermembrane space and the matrix of the mitochondria generated by components of the electron transport chain. Instead of the hydrogen ions moving through ATP-synthase, which captures most of the energy in the electrochemical gradient into ATP, the hydrogen ions move through UCP1 and produce no useful chemical work, being instead lost as heat. BAT is therefore a thermoregulatory tissue and is used in mammals to maintain body temperature.

There are two distinct lineages of thermogenic adipose tissue [4]. The classical brown fat, found in the interscapular fat pad of mice, is derived from a myf5+ progenitor, a lineage closely related to skeletal muscle. A myf5-lineage is present in scattered depots in subcutaneous fat; these cells are termed beige or brite cells/fat. Both lineages share some common mechanisms in their development, namely through the action of a protein called PR Domain Containing 16 (PRDM16) [5].

It was thought that adult humans do not possess significant stores of BAT; however, it is now clear that humans possess beige fat, which is interspersed within WAT in the supraclavicular, paraspinal, and suprarenal depots [6, 7]. These findings have generated interest in identifying the differentiation mechanisms that lead to brown adipocyte development, as this tissue could potentially serve as a therapy for obesity due to its ability to increase the number of calories burned daily by an individual.

The JAK/STAT pathway is a highly conserved signaling pathway that couples cytokines to rapid activation of transcription factors [8]. There are seven members of the STAT family, and STAT1, STAT3 and STAT5 have been implicated in differentiation of white adipose tissue (WAT), both *in-vitro* and *in-vivo* [9, 10]. Through Gamma interferon (IFN γ),

activated STAT1 binds to the promoter of the Peroxisome Proliferator-Activated Receptor gamma (PPAR γ), is a critical regulator of adipogenesis, and represses its expression [11]. Overexpression of STAT5A in non-precursor fibroblast cells is sufficient to promote adipogenesis *in-vitro* [12].

Previous studies of STAT3 in regulation of differentiation of adipocytes have mainly used 3T3-L1 cells, a white fat immortalized cell line. Knockdown of STAT3 with shRNA or chemical inhibitors of the JAK/STAT pathway prevent differentiation of 3T3-L1 cells. The function of STAT3 during adipogenesis remains unclear. To our knowledge, there have been no reported studies of STAT3's function in adipogenesis of primary preadipocytes. STAT3 can bind to DNA in the promoter of CCAAT/enhancer binding protein beta (CEBP β) [13, 14]. Additional evidence that STAT3 is involved in adipogenesis comes from studies examining Protein Inhibitor of Activated STAT3 (PIAS3). PIAS3 inhibits STAT3 signaling by binding and sequestering tyrosine phosphorylated STAT3. Overexpression of PIAS3 leads to reduction in markers of mature adipocytes, providing further evidence that STAT3 is a positive regulator of adipocyte differentiation [15]. *In-vivo*, STAT3 was knocked out using an aP2 driven Cre, which resulted in hypertrophy of adipose cells and increased weight of the rodents [16]. However, aP2 Cre is not specific to adipocytes, and is induced late in the differentiation of adipose cells, so this animal model is unlikely to capture STAT3's role in regulating differentiation of adipocytes [9].

The Wnt pathway is important for many developmental processes including maintenance of stem cells and differentiation [17]. There are both canonical and non-canonical Wnt signaling pathways. The canonical pathway activates the transcription factor β -catenin, which is continually degraded unless Wnt ligands are present and bind to their cognate receptor complex Frizzled and LRP5/6. The canonical Wnt/ β -catenin pathway has been shown in multiple studies to be a negative regulator of adipogenesis and brown adipogenesis, in part through suppressing transcription factors like PPAR γ , CEBP α , and PGC1 α [18, 19].

Previously, we reported that a JAK family member, Tyk2, is necessary for differentiation of brown adipocytes, and that the kinase activity of Tyk2 is dispensable for adipogenesis [20, 21]. We also showed that STAT3 is necessary for PRDM16 expression; PRDM16 is considered a major regulator of BAT development [22]. In this report, we describe a link between STAT3 and Wnt signaling in classical, myf5+ brown adipose development. Using an *in-vitro* Tamoxifen-inducible Cre system, we show in primary brown preadipocytes that STAT3 is necessary during the induction period of differentiation and that loss of STAT3 can be rescued through inhibition of the canonical Wnt signaling pathway or knockdown of β -catenin. Deletion of STAT3 leads to increased expression of the Wnt ligands 1, 3a, and 10b during the induction period, potentially explaining the increased levels of β -catenin seen in the STAT3^{-/-} adipocytes. These observations delineate a previously not described cross talk between the Wnt/ β -catenin and JAK/STAT pathways in the development of classical brown adipose tissue.

2. Materials and Methods

2.1 Reagents and Antibodies

All chemicals and reagents were purchased from Sigma-Aldrich (St, Louis, MO, USA), unless indicated otherwise. See Supplementary Table 1 for the antibodies used in this manuscript.

2.2 Generation of Mouse Line

C57Bl6 STAT3^{flx/flx} mice strain #016923 (Jackson Laboratory, Bar Harbor, ME, USA) were crossed with a C57BL6 strain containing Tamoxifen-Inducible Cre in the ROSA locus, strain #008463 (Jackson Laboratory, Bar Harbor, ME, USA). The mice were bred and genotyped to be homozygous for both the floxed STAT3 allele and the Cre allele. All mice were bred and maintained in the Virginia Commonwealth University animal facility according to Institutional Animal Care and Use Committee Regulations.

2.3 Cell Culture and Differentiation

The interscapular fat pad from newborn pups (Day 1-Day3) was isolated and grown to confluence as previously described [21]. The cells were either plated into growth media containing 1 μ M 4-OH tamoxifen, or the ethanol vehicle for 48 hours. The cells were washed and replaced with growth media (20% FBS in 4.5 g/L Glucose DMEM-GIBCO, Waltham, MA, USA), until they reached 100% confluence (6–7 days after isolation). The cells were induced to differentiate using 100 μ M IBMX, 250 μ M Indomethacin, 2 μ g/mL Dexamethasone, and 1 μ M Rosiglitazone in basal differentiation media containing 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA), 20 nM insulin and 1 nM T3 in 4.5 g/L glucose DMEM for 2 days. The induction media was replaced with the basal media after the induction period and replaced every 2 days until the end of the experiment (7 days after start of induction). For the inhibitor experiments, the inhibitors were added one day before induction and remained to the end of the experiment unless otherwise stated. The concentrations used: 1 μ M IWP2 (Calbiochem, Burlington, MA, USA) 5 μ M DAPT (Tocris, Minneapolis, MN, USA), 100 nM SAG (Calbiochem), 5 μ M IWR-1-endo (Selleckchem, Houston, TX, USA), 10 ng/mL FK506 (Cayman Chemicals, Ann Arbor, MI, USA), 1 μ M Wnt-C59 (APEX-BIO, Houston, TX, USA), 1 μ M XAV939 (Cayman Chemical), 10 μ M CHIR99021 (Cayman Chemical), 10 μ M CL 316,243 (Sigma Aldrich).

2.4 Cell Proliferation

For analysis of proliferation prior to confluence, cells were labeled on the day of isolation using the Tag-IT VioletTM Cell Proliferation Dye (Biolegend, San Diego, CA, USA). Briefly, the primary cell pellet was resuspended in PBS containing 5 μ M of the dye and incubated for 20 minutes at 37°C with shaking. The cells were plated except for a sample that was retained for initial fluorescent measurement. Cells were fixed using 4% Formaldehyde for 10 minutes at room temperature and analyzed on the BD LSRFortessa-X20 system using the following laser and filter configuration: 405nm with BP 450/50. The data was analyzed using FCSEXPRESS software. For proliferation during and after induction, confluent cells were washed with PBS and were incubated with 5 μ M of Tag-IT in HBSS with Calcium and

Magnesium for 20 minutes at 37°C. The HBSS was replaced with media and control WT and KO cells were harvested and fixed as above to establish Day 0 fluorescence, while the rest of the cells were induced to differentiate.

2.5 Oil Red O Staining and Imaging

Fully differentiated Brown Adipocytes (Day 7 after induction) were fixed in 10% formaldehyde for 1 hour at room temperature. The fixed cells were washed with PBS and then incubated with 60% isopropanol for 5 minutes. Stock Oil Red O stain (3mg/mL, 100% isopropanol) was diluted using 3 parts of the stock stain to 2 parts dH₂O and filtered through a Whatman paper filter. The filtered stain was applied to the cells for 1 hour at room temperature. The stain was removed and the plate was washed several times with water. The plates were imaged using an AxioObserver A1 microscope with a 10×/0.12 Ph1 lens and AxioCam MRc5 camera (Zeiss). Plates were quantitated for the area of the plate occupied by the stain by the threshold/analyze particles function in Image J (version 1.49, NIH). At least three random fields were imaged per sample.

2.6 Western Blot Analysis

Western blots were developed using the ECL system (GE Healthcare Life Sciences, Pittsburgh, PA, USA) or ECL2 system (Pierce, Rockford, IL, USA).

2.7 ChIP

Cells were fixed for 10 minutes at room temperature with 1% formaldehyde, then 5 minutes of 150 mM glycine, and washed with PBS. The cells were incubated in Farnham lysis buffer for 15 minutes on ice and spun down at 500g for 10 minutes to collect nuclei. The nuclei were lysed in sonication buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) and sonicated using a bioruptor pico sonicator for 5 cycles of 30sec ON/30sec OFF. The sonicated lysate was diluted 1:10 in dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1.0% Triton X-100) and manufacturer recommended amounts of antibodies were added and incubated overnight at 4 degrees. Non-specific matched species IgG was used as a control. 1% BSA blocked magnetic Protein A/G beads (Thermo-scientific, Waltham, MA, USA) were added for 2 hours at 4 degrees then washed twice with dilution buffer and once with high salt wash (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1.0% Triton X-100). The DNA was eluted using 1% SDS/0.1M Sodium Bicarbonate Buffer and de-crosslinked overnight at 65 degrees. RNase A (ThermoScientific,) and Proteinase K (New England Biolabs, Ipswich, MA, USA) were added, respectfully, for 1 hour each and the lysate was isolated using Qiaquick PCR Purification Kit (Qiagen, Germantown, MD, USA). The real-time quantitative polymerase chain reaction (qPCR) reaction was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using the manufacturer recommendations from the SensiMix SYBR and Fluorescein Kit (Bioline, Taunton, MA, USA). 5% of the input was saved for the calculations. The sequences of the primers can be found in Supplementary Table 2.

2.8 siRNA Transfection

siGenome SMARTpool control #1 and mouse specific β -catenin were purchased from Dharmacon and resuspended in siRNA buffer to 5 μ M (Dharmacon Inc, Lafayette, CO, USA). Primary cells were grown in 6 well plates. 48 hours before induction, the cells were washed three times with PBS and replaced with 1.6mL of antibiotic free primary media. The transfection cocktail was created as follows: for every well transfected, solution A contained 10 μ L of the siRNA and 190 μ L serum free media and solution B contained 4 μ L of Dharmafect Solution #1 and 196 μ L serum free media. After incubation for 5 minutes, solutions A and B were combined and incubated an additional 20 minutes at room temperature. 400 μ L of the transfection cocktail was added to each well and was left on the cells for 48 hours. After the transfection, the cells were washed with PBS and then were directly induced to differentiate or harvested in Trizol for mRNA analysis of the knockdown efficiency.

2.9 RT-qPCR

RNA was obtained using the Trizol method (Invitrogen). The RNA pellet was dissolved in DEPC-treated water (Sigma-Aldrich) and quantitated using absorbance at 260nm on a Nanodrop 200 (Thermo Fisher). 2 μ g of RNA was used in the first strand synthesis reaction. The High Capacity RNA-to-cDNA Kit (Applied Biosystems) was used according to manufacturer recommendations. The 20 μ L reaction volume was diluted with 380 μ L of dH₂O. 5 μ L of the cDNAs were combined with SYBR Green (SensiMix SYBR & Fluorescein Kit, Bioline), and with 250nM each of forward and reverse primers. The real-time PCRs were run on a CFX96 Real-Time PCR Detection System (Bio-Rad) using the following thermocycling conditions: 1) 95°C 10 minutes, 1 $\times$ 2) 95°C 15 seconds, 57°C 15 seconds, 72°C 15 seconds, 40 $\times$. A melt curve analysis was included at the end of each run. No-transcript controls (NTC) were used to assess for genomic DNA contamination. The data was analyzed using the C_t method. Primer sequences can be found in Supplementary Table 2.

2.10 Apoptosis Assay

Cells were incubated with AlexaFluor 488 annexin V/Propidium Iodide Kit (ThermoFisher) according to manufacturer protocols. The BD FACSCanto II analyzer was used for data acquisition. The following lasers and filter configurations were used: 488nm with BP 530/30 and BP 610/20. The data was analyzed using FCSExpress software. Cells incubated with 500 μ M H₂O₂ for 5 hours at 37°C was used as a positive control.

2.11 Statistical Analysis

Data is expressed as mean $\pm$ SEM, except for RT-qPCR data which is expressed as mean $\pm$ SD. Data was analyzed using Prism (Version 7; GraphPad Software, La Jolla, CA, USA). Comparisons were made using Student's t-test, one-way Analysis of Variance (ANOVA) or two-way ANOVA, where appropriate. Data are presented from at least 3 independent biological replicates (as indicated in the figure legend). A P value < 0.05 was considered significant and is indicated with (*). The exact comparisons made are noted in the figure legend.

3. Results

3.1 STAT3 is required for robust differentiation of primary brown fat preadipocytes

Since our previous results indicated that STAT3 was required for differentiation of immortalized brown preadipocytes, we examined whether similar results were observed in primary preadipocytes. We created a mouse line containing floxed STAT3 and a Tamoxifen-Inducible Cre Recombinase. Stromal vascular fraction (SVF) from the interscapular region of new-born mice were plated into media containing either tamoxifen or vehicle. The cells were grown to confluence and then induced to differentiate for 48 hours using the standard cocktail. The cells were then allowed to fully differentiate in maintenance media until 7 days after the addition of the induction cocktail. Supplementary Figure 1A is a schematic of the *in-vitro* differentiation time course and naming conventions we use in this report. The tamoxifen inducible Cre system gives reproducible total deletion of STAT3 by the time the cells are confluent and ready to differentiate (Figure 1A, lane 2). STAT3^{+/+} (WT) cells differentiate and express UCP1 after 7 days (Figure 1A lane 3), but the STAT3^{-/-} (KO) cells do not express significant levels of UCP1 protein (Figure 1A lane 4). Additionally, levels of PPAR γ and CEBP α are reduced in the STAT3^{-/-} adipocytes, while aP2, a general fat marker, is relatively normal but trending down in the knockout. The expression of other markers of brown fat, such as PRDM16 and CIDEA are also significantly reduced five to twenty-fold in the STAT3 knockout adipocytes, while the general adipocyte markers are trending down (Figure 1B). Since UCP1 expression and protein levels are significantly decreased in the KO adipocytes (approximately 20-fold reduced), and it is the functional protein of BAT, we view it as the best marker of differentiation.

Visually, the STAT3 knockout adipocytes have very few cells that contain lipid droplets; in Figures 1C–E, Oil Red O staining is significantly reduced. While the STAT3 knockout cells are still confluent by Day 7, the number of cells on the plate appeared less than compared to STAT3 WT cells. It has been reported that STAT3 is required for the mitotic clonal expansion (MCE) that occurs during the induction period and early terminal differentiation phase [23]. There is reduced proliferation by Day 4 in the STAT3 KO primary adipocytes when measured with the Tag-IT dye (Figure 1F). This reduced proliferation is specific to the induction period. When the cells are labeled with Tag-IT at isolation and followed to confluence (approximately 6–7 days after isolation), there is no visible difference in proliferation between the STAT3 WT and KO preadipocytes (Supplementary Figure 1B). We also wanted to determine if the observed difference in cell density might be the result of increased apoptosis in the STAT3 KO adipocytes. Using Annexin V/Propidium Iodide Staining as a marker for apoptosis/cell death, we analyzed different time points along the differentiation time course (Figure 1G–H). Interestingly, there is a significant increase in Annexin V staining in the STAT3 KO cells at Day 4, but by Day 7 the levels of Annexin V return to WT levels. The propidium iodide positive/annexin V negative quadrant increases in the WT from Day 4 on and in the KO at Day 7. We believe this is an artefact of preparation as the cells with lipid droplets are fragile and membrane integrity is affected by the trypsinization and resuspension. As a positive control, we treated Day 0 and Day 4 WT cells with 500 μ M H₂O₂ for 5 hours (Supplementary Figure 1C); treatment with hydrogen peroxide increased Annexin V levels equivalent to Day 4 STAT3 KO.

The cells are treated with tamoxifen for 48 hours after isolation and then are washed and grown another five days, so it is unlikely that tamoxifen was responsible for the reduction in differentiation, however, since only the STAT3 KO cells are exposed to tamoxifen, we wanted to rule out tamoxifen as contributing to reduced differentiation. Treatment with tamoxifen of the parental flox STAT3 cells that lack the Cre Recombinase had no effect on the ability of the cells to differentiate, express UCP1, and form lipid droplets (Supplementary Figure 1D–E). Therefore, the results are likely due to the deletion of STAT3 KO, and not the tamoxifen that was used to generate the STAT3 KO cells.

Since STAT5 is known to play a role in adipogenesis, we wanted to determine if deletion of STAT3 affects STAT5 levels. In Supplementary Figure 2A, STAT5 levels are unchanged between WT and KO STAT3 at either Day 0 before induction, or Day 7 in terminally differentiated brown adipocytes. We also wanted to determine if the defect in differentiation was specific only to the cocktail used to induce differentiation. Therefore, we induced the cells with the 10 μ M of the β 3 agonist CL 316,243 for the entire 7 days of differentiation in the absence of the cocktail containing IMBX, Indomethacin, and Dexamethasone. We kept the 1 μ M Rosiglitazone during the first two days of differentiation to maximize the stimulus for differentiation. In Supplementary Figure 2B, treatment for 7 days with CL 316,243 recapitulates the results seen with the classic induction cocktail.

3.2 STAT3 is required only during the induction period of differentiation

To begin to dissect the function of STAT3-mediated differentiation in brown adipogenesis, we determined when in the differentiation program STAT3 is required. Classic STAT3 signaling requires tyrosine phosphorylation on Y705 in order for STAT3 dimers to form and act as a competent transcription factor [8]. Additionally, serine 727 phosphorylation has been shown to increase the strength of STAT3-mediated gene expression [24]. We assessed total- and phospho-STAT3 levels during the differentiation time course. Interestingly, STAT3 is both tyrosine and serine phosphorylated for the first 24–48 hours of differentiation, corresponding to the induction period (Figure 2A). After the induction period, both total, phospho-tyrosine 705 and phospho-serine 727 STAT3 levels decrease. These results are consistent with what has been observed in white preadipocytes [14, 23, 25]. We compared the total levels of STAT3 from Day 7 cells to mature WT BAT and found that STAT3 levels in the cells are equivalent to mature tissue (Supplementary Figure 3A).

Using the Tamoxifen-Inducible Cre system, we are able to delete STAT3 at different times during differentiation to test the hypothesis that STAT3 is required only during the induction period. Deletion of STAT3 prior to induction leads to loss of UCP1 expression (Figure 2B, Lane 1). However, once the induction phase is completed by Day 2, deletion of STAT3 does not affect the protein levels of UCP1 (Figure 2B, Lane 2).

Since STAT3 levels decrease after induction, and STAT3 is only required during the induction, we wanted to determine if JAK/STAT signaling is pro-thermogenic during the induction phase but becomes anti-thermogenic after the induction phase is complete. In Supplementary Figure 3B, we treated WT and KO cells with 2 μ M Ruxolitinib, a pan-JAK inhibitor, either during the induction phase (Day 0–Day 2) or after induction (Day 2–Day 7) and assessed UCP1 levels in Day 7 adipocytes. Treatment with Ruxolitinib during the

induction period inhibited UCP1 levels in the WT cells, but treatment post-induction actually increased UCP1 levels above vehicle. These results are consistent with the STAT3 knockout time course in Figure 2B.

3.3 Inhibition of Wnt ligand secretion during the induction phase rescues the adipogenic and thermogenic program in STAT3 KO preadipocytes

After establishing that STAT3 is necessary during the induction period and is dispensable afterwards, we wanted to determine the function of STAT3 during the induction period. We decided to conduct a screen to see if STAT3 regulates other pathways that are known to affect adipogenesis. We initially selected the Wnt pathway, the Notch Pathway, and the Hedgehog pathway based on a literature search for pathways that either positively or negatively regulated adipogenesis and have some evidence of cross-talk with the JAK/STAT pathway in other contexts [18, 19, 26–30]. We incubated preadipocytes with inhibitors or activators of these pathways, starting one day before induction and continuing throughout the full differentiation period (i.e. 8 days of treatment). We found that inhibition of the Notch pathway or activation of the Hedgehog pathway did not rescue differentiation of STAT3^{-/-} cells; however, treatment with IWP2, an inhibitor to Porcupine (PORCN), an enzyme critical in the Wnt signaling pathway, fully restored UCP1 expression in the STAT3^{-/-} cells (Figure 3A, lane 4). PORCN is an endoplasmic reticulum intramembrane O-serine palmitoyltransferase whose function is to add an acyl group to a conserved serine in all Wnt ligands [31]. This acylation is necessary for proper secretion and signaling of Wnt ligands [32]. We also tested a second PORCN inhibitor, Wnt-C59, to confirm that the results are due to inhibition of PORCN and not due to off-target effects. As expected, treatment with Wnt-C59 also restored UCP1 levels in the KO adipocytes (Supplementary Figure 4A–C).

To determine if STAT3's function is to regulate the Wnt pathway, we performed a time course with IWP2 where the inhibitor was added either during the induction period, or after the induction period. As Figure 3B shows, Wnt inhibition must occur during the induction period in order to rescue STAT3 KO adipocytes. Addition of IWP2 after the induction phase prevents rescue of UCP1 levels. These results establish a temporal correlation between when STAT3 is required and when inhibiting the Wnt pathway can restore differentiation in the KO.

Epigenetic changes occur during adipogenesis that regulate access to cell-specific genes involved in the mature functioning brown adipocyte [33]. We wanted to assess if the 20-fold reduction in UCP1 observed in the knockout may be due to defects in chromatin remodeling at the UCP1 locus, and if treatment with IWP2 could restore this defect. We performed ChIP assays at the promoter and enhancer regions of UCP1 with an antibody directed against Acetylated Histone H3 K27, which is recognized as a marker of open chromatin [34]. We selected Day 5 for analysis as this is the earliest we see UCP1 in a western blot time course (Figure 2A), and after the increased Annexin V staining we see in the KO (Figure 1G–H). As expected, the KO cells have reduced histone acetylation at both the promoter and enhancer regions of UCP1, and IWP2 treatment restores the acetylation (Figure 3C). We used the β -actin promoter and IGX1A, a region that is approximately 1 Mb from a known

transcription start site, as positive and negative controls (Supplementary Figure 4D). We also looked at the promoter region of PRDM16, as mRNA expression is 5-fold reduced in the KO. While it failed to reach statistical significance, there is reduced acetylation in the KO that can be rescued with IWP2 treatment.

3.4 The canonical β -Catenin Pathway is responsible for suppression of differentiation in STAT3 KO brown preadipocytes

Previous studies have shown that the canonical β -catenin pathway and the non-canonical Calcium-Calmodulin/Calcineurin pathway suppress adipogenesis in 3T3-L1 cells [35]. Since inhibition of Porcupine inhibits all Wnt pathways, we wanted to determine which pathway or pathways were responsible for suppression of UCP1 in KO adipocytes. We selected IWR1-endo to target the canonical β -catenin pathway and FK506, a calcineurin inhibitor, to target the non-canonical Calcium-Calmodulin/Calcineurin pathway. IWR1-endo increases levels of Axin2, an important scaffold of the β -catenin destruction complex [36]. We treated the cells with the inhibitors from the start of induction to day 7 and then analyzed their ability to rescue STAT3 KO adipocytes.

Our results indicate that IWR1-endo fully restores UCP1 levels, similar to IWP2, while FK506 had no effect. Treatment with IWR1-endo also restores lipid droplet formation (Figure 4C–E). To confirm that the inhibitors are functioning as expected, we analyzed Axin2 expression at Day 2. Axin2 is directly regulated by β -catenin/TCF. As expected, treatment with either IWP2 or IWR1-endo, but not FK506, reduces Axin2 expression in KO cells (Figure 4F). Additionally, both IWP2 and IWR1-endo restore expression of other markers of adipogenesis, such as PRDM16, CIDEA, PPAR α , and aP2 in Day 7 adipocytes (Figure 4G).

The inhibitors were unable to rescue the proliferation defect seen at Day 4 in the KO cells (Figure 4H), indicating that the proliferation defect is not due to the Wnt pathway. However, the increased Annexin V staining seen in the KOs on Day 4 can be reduced with IWP2 and IWR1-endo treatment (Figure 4I–J), indicating that the apoptosis that occurs in the early terminal differentiation phase is due to Wnt signaling.

A second inhibitor of β -catenin, XAV939, also rescued the KO adipocytes (Supplementary Figure 5A–B). Additionally, activating the β -catenin pathway through inhibition of GSK3 β using CHIR99021 during the induction period only was sufficient to inhibit differentiation in the STAT3 WT adipocytes (Supplementary Figure 5C–D). Taken together, these results point to increased canonical Wnt/ β -catenin signaling in STAT3 KO adipocytes as the cause for reduced differentiation.

3.5 Knockdown of β -catenin restores differentiation

While the results with the chemical inhibitors point towards β -catenin being involved in the suppression of differentiation in the KO cells, we wanted to confirm that the results are not due to off target effects of the inhibitors. We transfected the preadipocytes 48 hours before induction with siRNA against β -catenin, or with non-targeting control siRNA. We assessed our ability to knockdown β -catenin by looking at mRNA levels of β -catenin at Day 0, which is just before the induction cocktail is added. In Figure 5A, transfection with β -catenin leads

to an 18–20 fold reduction in mRNA levels of β -catenin, indicating the knockdown is highly efficient. The cells were induced to differentiate and Day 7 adipocytes were analyzed for UCP1 protein levels. In Figure 5B, knockdown of β -catenin fully restores UCP1 protein levels in the KO adipocytes, which recapitulates the Wnt inhibitor results. The effect of siRNA is long lasting, as at Day 7 there is no detectable β -catenin present in the KO, while there is considerable β -catenin in the control siRNA KO group. Interestingly, there is no detectable β -catenin in either the control or β -catenin siRNA WT samples. We confirmed that by Day 7 there are higher levels of β -catenin protein in the KO compared to the WT (Figure 5C). This difference in protein levels is not due to differences in expression of β -catenin mRNA (Figure 5D), suggesting that the regulation of β -catenin is post-transcriptional.

3.6 Wnt Ligands 1, 3a, and 10b have Elevated Expression During the Induction Period

Since β -catenin is still present in the KO by Day 7 and this difference is regulated post-transcriptionally, this suggests there is an increase in Wnt ligands in the KO. The canonical mechanism of β -catenin involves continual turnover of β -catenin when Wnt ligands are not present. We analyzed Wnt signaling in the induction period since this is the critical period to rescue STAT3 KO differentiation. We began by comparing β -catenin levels during the induction period between WT and KO cells. If there is increased Wnt signaling, we would expect to see higher levels of β -catenin in the KO as the turnover of β -catenin would be reduced in the presence of Wnt ligands. Indeed, we saw a trend towards increased β -catenin during the induction period in the KO cells (Figure 6A). In fact, levels of β -catenin are down regulated in the WT by the end of the induction period, but significant levels of β -catenin still remain in the KO. We used mRNA expression of Axin2 as readout of β -catenin signaling. In Figure 6B, Axin2 levels are 3-fold elevated in the Day 1 KO adipocytes, confirming that there is increased Wnt/ β -catenin signaling in the KO compared to the WT. We further profiled expression of Wnt ligands that have previously been shown to be anti-adipogenic [35, 37]. In Figure 6C, there is increased expression of the Wnt ligands 1, 3a, and 10b, with Wnt3a being up regulated 50-fold in the KO compared to the WT. Dkk1, an antagonist of the Wnt pathway by its ability to prevent LRP6 from binding with Frizzled and a β -catenin regulated gene, is also significantly elevated in the KO (Figure 6C) [38].

4.1 Discussion

Using tamoxifen inducible Cre recombinase of floxed STAT3 primary adipocytes we demonstrate that STAT3 is required for differentiation during the induction period. It is interesting that STAT3^{-/-} cells respond to the induction cocktail in that general fat markers like aP2 and CEBP α are induced in the WT and KO cells, while the brown fat specific and selective markers are suppressed. This indicates that the cells are attempting to differentiate but are unable to properly express the mature brown adipocyte phenotype. One explanation for the 5–20 fold suppression of brown fat genes is a defect in chromatin remodeling, which would prevent access to regulatory transcription factors. At the UCP1 locus, STAT3^{-/-} adipocytes fail to acetylate Histone H3 at the same level as WT (Figure 3), and a similar trend is seen at the PRDM16 promoter (Supplementary Figure 4D). Therefore, it appears that the cells are pausing in early terminal differentiation before they execute the

thermogenic program, suggesting the presence of a checkpoint that is not being passed in the absence of STAT3.

Since the finding that STAT3 was required for the MCE in 3T3-L1 cells, it has been hypothesized that STAT3 only functions during the induction period. Until now, this hypothesis has not been directly tested. With the tamoxifen inducible system, we were able to confirm that STAT3 regulates differentiation during the induction period (Figure 2). After the induction period, STAT3 is not required for full expression of UCP1. This is further suggestive of a checkpoint that requires STAT3.

The Wnt/ β -catenin pathway is well established as a suppressor of adipogenesis and thermogenesis both *in-vitro* and *in-vivo* [37, 39]. We have provided evidence that STAT3 functions during the induction period of brown adipogenesis to suppress Wnt signaling. This appears to be a critical function of STAT3, as chemical inhibition or knockdown of β -catenin can fully restore brown fat adipogenesis in KO adipocytes. The only process that could not be rescued by Wnt inhibitors was the reduction in proliferation after the STAT3^{-/-} cells have been induced to differentiate. The increased Wnt signaling appears to be the result of up regulation of anti-adipogenic Wnts, especially Wnt3a (Figure 6). It has been previously reported that Wnt3a can activate and drive STAT3 nuclear localization as a mechanism to protect the cells from exposure to oxidative stress in a retinal pigment epithelium cell line [28]. Additionally, Wnts 1 and 3a can drive increased STAT3 mRNA expression and protein levels in embryonic stem cells [40].

Wnt ligand secretion is reduced by the actions of the histone methyltransferase EZH2 [41]. EZH2 methylates and silences the promoter regions of Wnt ligands, leading to significantly reduced expression of these ligands as differentiation proceeds. IWP2, an inhibitor of Wnt ligand secretion, is only able to rescue STAT3^{-/-} cells when applied during the induction period. If there is a “restriction point” that the cells must pass during the induction period, then STAT3’s role may be to suppress Wnt signaling in the short term while EZH2 silences the Wnt ligand promoters for lasting suppression of the Wnt pathway. How STAT3 accomplishes this suppression of Wnt signaling is unknown.

Our studies focused on *in-vitro* differentiation of classical Myf5⁺ brown adipocytes. Whether this regulation of Wnt/ β -catenin signaling by STAT3 is unique to brown adipocytes from the Myf5⁺ lineage or is generally applicable to all types of adipocytes is not clear. We have begun to analyze primary beige cells to determine if this mechanism is more broadly applicable. *In-vivo*, there is some evidence that this interaction between STAT3 and Wnt is relevant. Deletion of PORCN in the mouse, the enzyme that acylates Wnt ligands and is inhibited by IWP2, can increase UCP1 expression in the inguinal fat pad [42]. Forced expression of Wnt10b using an aP2 driven Cre results in a 50% decrease in adipose tissue mass and complete inhibition of BAT development [37]. Knockout of STAT3 using an aP2 driven Cre also resulted in perturbations in adipose tissue development, but since aP2 is a late marker in adipogenesis, and our results indicate that STAT3 acts relatively early (Figure 2), it is unlikely that the aP2 driven Cre mouse is a good model of STAT3 in differentiation. Sorting through the signaling networks which contribute to BAT differentiation and function will require both *in-vitro* systems, and mouse models where the expression of transcription

factors such as STAT3 or β -catenin can be manipulated to tease out their physiological significance.

4.2 Conclusion

Primary BAT explants require STAT3 during the induction period (D0–D2). Loss of STAT3 leads to upregulation of canonical Wnt ligands, which signal through β -catenin. Inhibition of the Wnt/ β -catenin pathway or knockdown of β -catenin restores differentiation.

Acknowledgments

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Abbreviations

STAT	Signal Transducer and Activated Transcription
JAK	Janus Kinase
Tyk2	Tyrosine Kinase 2
UCP1	Uncoupling Protein 1
BAT	Brown Adipose Tissue
WAT	White Adipose Tissue
ChIP	Chromatin Immunoprecipitation
SVF	Stromal Vascular Fraction
MCE	Mitotic Clonal Expansion
PORCN	Porcupine
PRDM16	PR Domain Containing Protein 16
PPARγ	Peroxisome Proliferator Activated Receptor gamma
FABP4/aP2	Fatty Acid Binding Protein 4
TBP	TATA Binding Protein
CEBPα	CCAAT/enhancer binding protein alpha
CEBPβ	CCAAT/enhancer binding protein beta

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Highlights

- STAT3 is required during the induction phase for differentiation of primary brown pre-adipocytes.
- Deletion of STAT3 after the induction period does not affect UCP1 protein levels and differentiation.
- STAT3 KO cells can be rescued through inhibition of the canonical Wnt/ β -Catenin pathway or by knock down of β -catenin.
- STAT3 KO cells upregulate Wnt ligands during the induction phase.

Figure 1. Deletion of STAT3 Significantly Reduces Differentiation

A. SVF were placed in media with or without tamoxifen for 48 hours and grown to confluence, then placed. Cells were harvested and lysates were blotted for STAT3, PPAR γ , CEBP α , FBP4, UCP1 and TATA binding protein (TBP) as a loading control. N=3 biological replicates. **B.** RT-qPCR of Day 7 WT and KO Adipocytes. N=4 biological replicates. TBP is used as loading control. * = $p < 0.05$. Student's t-test. Data is expressed as mean $\pm$ SD. **C.** Representative image of Oil Red O. N=5 biological replicates. **D.** Representative Micrograph of Day 7 WT and KO Oil Red O. 10 $\times$ magnification. Scale bar = 200 μ m. **E.** Quantitation of micrographs in D. Student's t-test. N=3 biological replicates. Data is presented as mean $\pm$ SEM. **F.** Tag-IT Proliferation Assay on Day 2 and Day 4 post-induction. N=3 for each time point. Two-way ANOVA. * = $p < 0.05$. **G.** Annexin V/Propidium Iodide Flow Assay Time Course. Each plot is a concatenation of 3 biological replicates. **H.** Quantitation of G. N=3 for each time point. Two-way ANOVA. * = $p < 0.05$. Data is presented as mean $\pm$ SEM.

A. **B.** **Figure 2. STAT3 is Required Only During the Induction Phase**

A. Representative immunoblot of WT STAT3 time course. pY705 = phosphorylated tyrosine 705; pS727 = phosphorylated Serine 727. N=3 biological replicates. **B.** Representative immunoblot of a knockout time course. KO D2 = tamoxifen added to WT STAT3 cells on Day 2 after induction (lane 2); KO D5 = tamoxifen added to WT STAT3 cells on Day 5 after induction (lane 3). All samples collected on Day 7.

Figure 3. A Wnt Ligand Secretion Inhibitor Rescues STAT3 KO Adipocytes

A. Representative immunoblot of Day 7 adipocytes treated for 7 days with either IWP2, DAPT, or SAG N=3 biological replicates. **B.** Representative immunoblot of Day 7 adipocytes subjected to an IWP2 time course. D0–D7 = IWP2 added for entire differentiation period; D2–D7 = IWP2 added after induction; D0–D2 = IWP2 added only during induction period. N=3 biological replicates. **C.** Day 5 ChIP of acetylated Histone H3 K27 (AcH3) at the UCP1 promoter and enhancer regions. IgG= non-specific matched species antibody. Data is presented as mean % input $\pm$ SEM. N=3 biological replicates. Two-way ANOVA. * = $p < 0.05$.

Figure 4. Inhibition of the Canonical β -Catenin Pathway Restores Differentiation in STAT3 KO Adipocytes

A. Representative immunoblot of Day 7 adipocytes treated for 7 days during differentiation with the indicated inhibitor. **B.** Densitometry of A. O.D.(A.U.) = Optical Density (Arbitrary Units). N=3–5 biological replicates. One-way ANOVA. * = p<0.05 compared to KO DMSO. Data is presented as mean ±SEM. **C.** Day 7 Whole Plate representative image of Oil Red O staining on cells treated with the indicated inhibitors for 7 days. N=3–5 biological replicates. **D.** Oil Red O micrographs of Day 7 adipocytes treated with the indicated inhibitors for 7 days. 10× magnification. Scale bar = 200 μm. **E.** Quantitation of Oil Red O micrographs in D. N=3–4 biological replicates. One-way ANOVA. * = p<0.05 compared to KO DMSO. Data is presented as mean ±SEM. **F.** RT-qPCR of Axin2 positive control from Day 2 STAT3 KO adipocytes treated with the indicated inhibitors for 2 days. N=4 biological replicates. One-way ANOVA. * = p<0.05 compared to KO DMSO. Data is presented as mean ±SD. **G.** RT-qPCR of Day 7 adipocytes for brown fat and general fat markers. N=4 biological replicates. One-way ANOVA. * = p<0.05 compared to KO DMSO. Data is presented as mean ±SD. **H.** Day 4 Tag-IT proliferation analysis of cells treated with the indicated inhibitors. One-way ANOVA. * = p<0.05 compared to KO DMSO. Data is presented as mean ±SEM. **I.** Day 4 Annexin V/Propidium Iodide Flow Assay. Each plot is a concatenation

of 3 biological replicates. **J.** Quantitation of I. N=3 biological replicates. One-way ANOVA.
* = $p < 0.05$ compared to KO DMSO. Data is presented as mean $\pm$ SEM.

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Figure 5. Knockdown of β -Catenin Rescues STAT3 KO Adipocytes

A. Day 0 RT-qPCR for β -catenin in cells transfected with control or β -catenin siRNA. N=3 biological replicates. Student's t-test. * = $p < 0.05$. Data is presented as mean $\pm$ SD. **B.** Representative immunoblot of Day 7 adipocytes transfected with control or β -catenin siRNA. N=3 biological replicates. **C.** Representative immunoblot of Day 7 adipocytes. Quantitation of A. O.D.(A.U.) = Optical Density (Arbitrary Units). N=5 biological replicates. Student's t-test. * = $p < 0.05$. Data is presented as mean $\pm$ SEM. **D.** RT-qPCR of β -catenin in Day 7 adipocytes. Student's t-test. N=5 biological replicates. N.S.= not significant. Data is presented as mean $\pm$ SD.

Figure 6. Wnt Signaling is Elevated in the STAT3 KO During Induction

A. Representative immunoblot of a time course during the induction period. N=3 biological replicates. **B.** RT-qPCR of Day 1 adipocytes for Axin 2. N=3 biological replicates. Student's t-test. * = $p < 0.05$. Data is presented as mean $\pm$ SD. **C.** RT-qPCR of Day 1 adipocytes for Wnt Signaling Components. N=3 biological replicates. Student's t-test. * = $p < 0.05$. Data is presented as mean $\pm$ SD.