Title
Differential Roles of the TRAF3 Adapter Protein in Adipogenesis and Glucose Metabolism

Author
Loo, Lotus Kyi

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Differential Roles of the TRAF3 Adapter Protein in Adipogenesis and Glucose Metabolism

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics

by

Lotus Kyi Loo

2015

ABSTRACT OF THE DISSERTATION

Differential Roles of the TRAF3 Adapter Protein

in Adipogenesis and Glucose Metabolism

by

Lotus Kyi Loo
Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics
University of California, Los Angeles, 2015
Professor Genhong Cheng, Chair

The main goal of this project is to delineate the roles and mechanisms of constitutive type II nuclear factor-kappa B (NF-κB) activation on adipogenesis and glucose metabolism. Our laboratory has shown that the tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) is a critical negative modulator of type II NF-κB and TRAF3 knockout (TRAF3-/-) have constitutive activation of non canonical or type II NF-κB pathway. However, TRAF3-/- pups die within two weeks after birth and the function of TRAF3 remain largely elusive. TRAF3 null mice have greatly reduced serum glucose levels, elevated serum corticosterone and minute amount of fat. On the other hand, the development of Smac mimetic compounds (SM), inhibitors of cellular inhibitors of apoptosis (cIAPs), has been a major goal in advancing cancer

therapeutics, as cIAPs are believed to antagonize activated caspases and are often amplified in human cancers. Two reports using two different second generation Smac (SM) mimetic compounds, including LBW242 showed that inhibition of cIAPs can activate NF-κB noncanonically. TRAF3 recruits nuclear factor κB-inducing kinase (NIK) and TRAF2 recruits cIAP1/2 to TRAF3/NIK, and then promote NIK ubiquination and degradation. On the other hand, SM can prevent NIK degradation and initiate constitutive processing of type II NF-κB. Here, we have shown that TRAF3-/- murine embryonic fibroblasts (MEFs) and bone marrow mesenchymal stem cells (BMs) do not differentiate into adipocytes compared to WT, p100-/- and TRAF3-/-p100-/- counterparts. Treatment with SM can also prevent adipogenesis in WT MEFs and BMs as well as 3T3-L1 and 3T3-F422A preadipocytes. We have found that different generations of SM as well as agonistic lymphotoxin-β receptor (LTβR) antibody can inhibit PPARγ, C/EBPα and downstream adipocyte specific genes at both mRNA and protein levels. We have demonstrated that this inhibition by constitutive p52 is due to upregulation of GATA3, the inhibitor of adipogenesis, which directly inhibit PPARγ and C/EBPα. As TRAF3 null mice have low serum glucose level and compound loss of TRAF3 and p100 can rescue this phenotype, we investigated effect of TRAF3 null on glucose related metabolic pathways. Here, we demonstrated that TRAF3-/- MEFs and 3T3-L1 cells treated with SM have higher insulin sensitivity. In addition, liver isolated from TRAF3-/- mice have increased expression of genes involved in gluconeogenesis such as PEP carboxykinase (PEPCK) and pyruvate dehydrogenase kinase (PDK) as well as lactate dehydrogenase (LDH). Investigations presented in this thesis help elucidate novel mechanisms responsible for the crosstalk of non-canonical NF-kB with adipocyte differentiation and host metabolism.

The dissertation of Lotus Kyi Loo is approved.

Kelsey Martin

Carrie Miceli

Stephen Smale

Genhong Cheng, Committee Chair

University of California, Los Angeles

2015

University of California, Los Angeles

2015

Dedication

This thesis is dedicated to my parents and Genhong: the illness of my dad during first two years of medical school and that of my mom during PhD years heighten my interest in biomedical research and drug development as well as make me persevere to finish this dissertation. Genhong has not only been a mentor but also a father figure who taught me to appreciate my scientific discoveries. 15 years after TRAF3 null mice generation by Genhong, I have discovered new roles of TRAF3. Finally, I hope that this hard work will contribute to development of therapies for Obesity and diabetes.

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Genhong Cheng for the opportunity to work in his lab and his enthusiasm for science which has been driving force for my development as a researcher and instrumental for finishing my PhD thesis. Genhong has been very patient and understanding during the course of my PhD which consists of personal and family problems. I would also like to thank the member of my thesis committee, Dr. Kelsey Martin, Dr. Carrie Miceli and Dr. Stephen Smale for all of the time and help given to me over the years.

I also would like to thank Dr. Laurent Vergnes for helping me with tissue isolation. I want to thank Bahram Razani, Brain Zarnegar, Erin Tricker, Eric Pietras and Shanker Iyer for teaching me molecular biology techniques. I could not finish this thesis without emotional support from Gayle, Anna, Maxime and Saba.

BIOGRAPHICAL SKETCH

8/98- 7/00	City College of San Francisco, San Francisco, CA. pre-med
9/00- 9/03	University of California, Los Angeles, Los Angeles, CA B.S. Neuroscience (with departmental honor), minor in Public Policy; Concentration in Social Welfare.
8/04- 06/06	Medical Student, David Geffen School of Medicine at UCLA
9/06-3/11	Graduate student Department of MIMG, UCLA School of Medicine
9/13- 12/14	Graduate student Department of MIMG, UCLA School of Medicine
7/09-6/11	National Institute of Health- NIDDK pre-doctoral fellowship award (F31DK084768)
7/05-6/11	Medical Scientist Training Program Fellow (T32 GM008042)

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2. Wu J, Ohlsson M, Warner E, **Loo KK**, Hoang T, Voskuhl RR, Havton L. Glial Reactions and Degeneration of Myelinated Processes in Spinal Cord Gray Matter in Chronic Experimental Autoimmune Encephalomyelitis. Neuroscience. 2008 Oct 15; 156(3):586-96.

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2. Liu, H.; **Loo, K**; Voskuhl, R. The distribution of estrogen receptors in immune tissues and Estrogen's possible mechanism in regulating immune system. (84th Annual Endocrine Society's meeting)

3. Liu, H.; **Loo, K**; Voskuhl, R. The distribution of estrogen receptors in the peripheral immune system of mice and a possible mechanism of estrogen's action. 2002 June, 103 (3): S89.

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CHAPTER 1

Introduction

The NF-κB pathways

The nuclear factor-κB (NF-κB)/ Rel family of transcription factors are critical regulators of apoptosis, proliferation, inflammatory and immune responses and have been linked to autoimmune diseases and tumorigenesis. The NF-κB signaling pathways have been categorized into two distinct pathways: the canonical pathway, which is initiated by complexes of Rel A, c-Rel, and NF-κB1 (p50), and noncanonical pathway which is initiated by complexes of NF-κB2 (p52) and Rel B (p68). In unstimulated cells, NF-κB dimers are sequestered by one of the family of inhibitory molecules, inhibitors of κB (IκBs). The canonical pathway requires activation of trimetric IκB kinase (IKK) complex (IKKα, IKKβ, and IKKγ) which releases p50: Rel A and p50: cRel dimers by phosphorylation and degradation of IκBα and IκBβ. The noncanonical pathway involves NF-κB-inducing kinase (NIK) which activates IKKα homodimers, leading to the phosphorylation and processing of p100 to p52 and the release of p52: Rel B dimers. In contrast to receptor-mediated activation of the canonical NF-κB pathway, which occurs within minutes, activation of the noncanonical NF-κB pathway takes several hours and requires new protein synthesis.

TRAF3 adapter protein

TRAF3 plays a role as negative regulator of both canonical (type I) and noncanonical (type II) NF-κB signaling (Zarnegar et al., 2008, He et al., 2007). Our lab has previously shown that TRAF3 recruits E3 ubiquitin ligases, cellular inhibitor of apoptosis (cIAP) 1 and 2, to NIK. The degradation of NIK occurs upon assembly of a regulatory complex through TRAF3 recruitment of NIK and TRAF2 recruitment of cIAP1 and cIAP2. In addition to TRAF3 constitutive

activation of p52, recent reports demonstrated spontaneous p100-to-p52 processing in mice with a conditional disruption of the gene for TRAF2 in B lymphocytes. Unlike TRAF2 and TRAF3, inhibition of both cIAPs was required for constitutive noncanonical NF-kappaB activation and increased survival and proliferation of primary B lymphocytes. Smac mimetic (cIAP inhibitors) can prevent NIK degradation and initiates constitutive processing of type II NF-κB. Thus, cIAP1 and cIAP2 seem to play redundant roles in the degradation of NIK (Zarnegar et al., 2008, Darding et al., 2011).

A recent characterization of primary multiple myelomas showing high NIK levels and constitutive p100-to-p52 processing identified several classes of chromosomal alterations such as TRAF2 or TRAF3 chromosomal deletions and NIK amplifications. These analyses also identified a subset of samples showing dual chromosomal loss of cIAP1 and cIAP2, suggesting that they are also involved in the negative regulation of NIK (Keats et al., 2007).

The mechanism of Adipogenesis

Adipose tissue plays an essential role in energy homeostasis. While adipogenesis is known to be controlled by a complex network of transcriptional factors, including transcriptional activators, coactivators, and repressors, much need to be studied to get a fuller understanding of the molecular processes governing adipogenesis. In 3T3-L1 preadipocytes, adipogenic stimulus triggers expression and activation of CCAAT-enhancer-binding proteins (C/EBP)β and C/EBPδ which then stimulate expression of peroxisome proliferator-activated receptorγ (PPARγ) and

C/EBPα. PPARγ is both necessary and sufficient for adipocyte differentiation and PPARγ2 is expressed specifically in adipocytes (Rosen et al, 2006). No factor which promotes adipogenesis in the absence of PPARγ has been discovered and forced expression of PPARγ is sufficient to induce adipocyte differentiation in fibroblasts. It is consistent with most pro-adipogenic factors seem to function at least in part by activating PPARγ expression or activity. PPAR is not only crucial for adipogenesis but is also required for maintenance of the differentiated state as introducing a dominant-negative PPARγ into mature 3T3-L1 adipocytes causes loss of lipid accumulation with decreased expression of adipocyte markers (Tamori et al., 2002).

C/EBPα induces many adipocyte genes directly and some studies indicate an important role for this factor in development of adipose tissue (Rosen et al., 2006). C/EBPα mice are devoid of white adipose tissue except within mammary gland and analyses of these mice are complicated by profound hypoglycemia and perinatal lethality (Linhart et al., 2001). C/EBPβ is crucial for adipogenesis in immortalized pre-adipocytes lines such as 3T3-L1 but its effect is less obvious in MEFs. It is possible that C/EBPδ can compensate for the loss of C/EBPβ. However, C/EBPβ and C/EBPδ promote adipogenesis at least in part by inducing C/EBPα and PPARγ. The amounts of C/EBPα and PPARγ mRNA are normal in the remaining adipocytes of C/EBPβ and C/EBPδ double-knockout mice in contrast to C/EBPβ- and C/EBPδ-deficient MEFs, which do not express C/EBPα and PPARγ. These findings indicate that there might be factors that allow some cells to escape the developmental requirement for C/EBPβ and C/EBPδ in vivo (Rosen et al., 2006).

The bone marrow cells are pleiotropic and are differentiated into fibroblasts, adipocytes, osteoblasts and chondrocytes (Takada et al., 2007). Thus, it is evident that there are regulators to govern the cell fate switching, and particularly in normal bone continuous osteoblastogenesis is maintained while adipogenesis appears suppressive. Since activated PPARγ is the prime regulator to stimulate adipogenesis from pleiotropic mesenchymal stem cells, it is inhibited in bone marrow of young animals. Osteoblastogenesis is governed by a number of regulators including cytokines and Wnt peptide ligands. Activated canonical Wnt signaling is shown to stimulate osteoblastic differentiation at several steps of cytodifferentiation. According to Takada et al., mesenchymal stem cells can be switched to osteoblasts from adipocytes by transrepression of PPARγ by two transcriptional pathways (1) activating NF-κB which blocks the DNA binding of PPARγ (2) by activating of non-canonical Wnt pathway through CaMKII-TAK1/TAB2-NLK (Takada et al., Nat Cell Biology, 2007).

TRAF3 null mice

Although our lab generated TRAF3 knockout mice (TRAF3-/-) over10 years ago, TRAF3 deficient mice die within two weeks after birth and the function of TRAF3 remains largely elusive (Xu Y, Cheng G et al., 1996). TRAF3 null mice are easily distinguishable as early as day 5 from their WT or heterozygous littermates with their smaller size and wasted phenotypes. Several abnormalities in TRAF3-/- such as drastically reduced spleen size and lymphocyte count, and elevated serum corticosterone have been reported. TRAF3-deficient cells show constitutive processing of p100 to p52 processing as a result of accumulated NIK. Using genetic approach, we have demonstrated that loss of TRAF3 results in constitutive type 2 NF-κB activities, and the

early post-natal lethality observed in TRAF3 deficient mice is rescued by compound loss of type 2 NF-κB *p100* gene. The early post-natal lethality of $TRAF3^{-/-}$ is also rescued in TRAF3-/-NIK-/- DKO mice (unpublished data). The abnormalities such as reduced spleen size, greatly reduced serum glucose level and elevated serum corticosterone level were also rescued in TRAF3-/-P100-/- mice (He et al., 2006).

In addition, we have observed that there is none to very little white adipose (WAT) and brown adipose (BAT) tissues as well as low serum glucose level in 7 day old TRAF3-/- mice compared to WT littermates. Thus, it raises the question that there could be metabolic abnormalities in addition to inflammatory abnormalities contributing to early post-natal lethality of TRAF3 null mice.

CHAPTER 2

Mechanism for regulation of constitutive non-canonical NF-κB signaling on adipogenesis

We have shown that the tumor necrosis factor receptor-associated factor 3 (TRAF3) is a critical negative modulator of type II NF-kappa B pathway and TRAF3-/- mice have constitutive activation of type II NF-κB. TRAF3-/- pups die within two weeks after birth and are easily distinguishable as early as day 5 from wild type littermates with their smaller size and runted phenotypes. Although our laboratory has already shown that TRAF3-/- mice have immunological defects due to TRAF3 null, we hypothesized that constitutive activation type II NF-κB also has effect on metabolic functions as TRAF3-/- pups have low glucose level (He JQ et al., 2006) and very lean.

No white and brown adipose tissue in TRAF3-/-

There is no white adipose tissue (WAT) (Fig 2.1, left) and little or no brown adipose tissue (BAT) (Fig 2.1, right) in dorsal interscapular region of 7 days old TRAF3-/- compared to WT littermates. There is no WAT in inguinal area of TRAF3-/- mice which can be seen in WT mice (pictures not shown). We then compared body weight (previously shown), BAT weight and body temperature of WT, TRAF3-/- and TRAF3-/-p100-/- mice. TRAF3-/-p100-/-mice are included as we previously reported that loss of both TRAF3 and p100 can rescue phenotypes of TRAF3-/- (He, JQ et al., 2006). We have found that BAT is significantly reduced in 7 days old TRAF3-/- mice compared to WT and TRAF3-/-p100-/- mice as well as body temperature (Fig 2.2).

In order to confirm reduction in adipose tissues and adipocytes, we have collected various tissues of 7 days old WT and TRAF3-/- littermates. The tissues were cryo-sectioned and stain with Oil Red-O stain according to protocol mentioned. As shown in Fig 2.3, there is no Oil Red-O

staining in different layers of dermis (panel A), adipose layer between skin and muscle (panel B) and reduced Oil Red-O staining in bone marrow (panel C).

TRAF3-/- MEFs do not differentiate into adipocytes

As defect in adipose tissue formation in TRAF3 null mice can be due to indirect effects of other metabolic abnormalities, we have tested whether adipogenesis is defected in TRAF3-/- cells. First, early passages of WT, TRAF3-/- and TRAF3-/-p100-/- MEFs, freshly obtained from E14 embryos were differentiated into adipocytes. As shown in Fig 2.4, WT MEFs differentiate into adipocytes while TRAF3 null MEFs do not. However, this defect is rescued in DKO MEFs due to compound loss of both TRAF3 and p100 genes.

Smac inhibits adipogenesis in WT MEFs

As previously shown by our lab (Zarnegar et al., 2008) SM can activate NF-kB noncanonically, we hypothesized that SM can also inhibit adipogenesis. Indeed, different second generations SMs inhibit adipogenesis in WT MEFs (Fig 2.5) but could not prevent adipogenesis in DKO MEFs (Fig 2.6). On the other hand, Tumor Necrosis Factor α (TNFα), activator of canonical NF-kB pathway, can inhibit adipogenesis in both WT and DKO MEFs (Fig 2.7). It shows that SM inhibits adipogenesis by constitutive activation of noncanonical NF-kB as well as defect in adipose tissue formation of TRAF3 null mice is attributed by this activation. In order to determine inhibition of adipogenesis by SM is not limited to MEFs, we have studied adipogenesis in BMs. We have found that SM can inhibit differentiation of BMs into adipocytes but could not prevent adipogenesis in both p100-/- and TRAF3-/-p100-/- BMs (Fig 2.8).

SM Inhibits adipogenesis in adipocyte cell lines

In order to determine SM's adipogenesis inhibitory effect can be reciprocated in cell lines as well as to determine the mechanism of inhibition, we have used both NIH 3T3-L1 and 3T3-F422A cell lines. 3T3-F442A is generally regarded as a model with a more advanced commitment in the adipose differentiation process than 3T3-L1. We have demonstrated that various Smac mimetics (SM, LBW 242, and AEG) as well as LTβR antibody can inhibit adipogenesis in both cell lines (Fig 2.9, 2.10). Quantitative amount of triglycerides in each well is obtained by eluting Oil Red O in each well with 100% isopropanol and measuring absorbance at wavelength 500nm.

Time course of SM inhibition in 3T3-L1 adipocytes

We have found that inhibition of adipogenesis occur in first two days of adipocyte differentiation as adipogenesis can be rescued partially starting from day 3 of adipocyte differentiation (2.11). In order to study the mechanism of SM inhibition of adipogenesis, we have studied expression of genes that are crucial in adipocyte formation in 3T3-L1 cells. We have found that expression of PPARγ (master regulator of adipogenesis) and C/EBPα are inhibited in both mRNA (Fig 2.12A, 2.12C) and protein level (Fig 2.13) at day 6 of differentiation (144 hrs.). Expression of aP-2, downstream gene of adipocyte formation is also inhibited by SM (Fig 2.12D). However, expression of C/EBPβ (Fig 2.12B) and C/EBPδ are not affected by SM (data not shown).

GATA3-Inhibitor of Adipogenesis is upregulated with SM treatment

For the possibility of indirect effect of SM and LTβR antibody on PPARγ and C/EBPα, we have studied expression levels of inhibitors such as KLF-2, GATA2/3, SMAD, Wnt10b, Wnt3b and beta-catenin and activators of adipogenesis pathway such as Pref-1, KLF-5 and SREBP1c. We have shown that expressions of adipocyte differentiation inhibitor GATA3 (Fig 2.14) increased 3-5 folds in SM and LTβR antibody treated undifferentiated and differentiated 3T3-L1 cells in earlier time points (up to 24 hrs.) while there is no correlation with upregulation of other inhibitors such as KLF-2, SMAD, Wnt10b, beta-catenin as well as downregulation of activators of adipogenesis such as Pref-1 (2.14), and KLF-5 (data not shown).

PGL3-GATA3 Leuciferase Promoter Activated by noncanonical NF-κB subunits

Renilla Dual-Leuciferase Reporter Assay is conducted by transfecting 293T cells with PGL3-GATA3 promoter and noncanonical NF-κB subunits p52 or Rel B plasmids, and by measuring Leuciferase activity. Stat6 plasmid is used as positive control for PGL3-GATA3 promoter and empty pCDNA plasmid is used as negative control. Either p52 or Rel B subunit can activate GATA3 promoter, even stronger than stat6 (2.15). We have also done promoter analysis by searching NF-κB binding site on GATA3 promoter. We have found 2 NF-κB binding site on GATA3 promoter, one at -11 and one at -1000 (data not shown).

Inhibition of Adipogenesis by Constitutive Activation of non-canonical NF-κB depends on GATA3 upregulation

In order to determine involvement of GATA3 in SM inhibition of adipogenesis, we overexpressed 3T3-L1 with empty pBabe, full-length GATA3 and dominant negative GATA2/3 (KRR) plasmids. Overexpression of GATA3 is determined by both QPCR and western blot analysis. We have found that SM can no longer inhibit adipogenesis in 3T3-L1 overexpressed with dominant negative pBabe-KRR (Fig 2.16).

Summary of Results

In this report, we have shown that MEFs from TRAF3-/- mice do not differentiate into adipocytes. In addition, SM (cIAP inhibitors) and LTβR antibody can inhibit adipogenesis in WT MEFs and BMCs as well as 3T3-L1 and 3T3-F422A cell lines. In addition, we have found that constitutive activation of noncanonical NF-κB by SM inhibits adipogenesis by decreasing expression of C/EBPα and PPARγ at both mRNA and protein levels. Then, we have demonstrated that this inhibition by constitutive p52 is due to upregulation of GATA3, the inhibitors of adipogenesis, which directly inhibit PPARγ and C/EBPα. Thus, we hypothesize that constitutive type II NF-κB (p52) inhibits adipogenesis by upregulating GATA3 causing metabolic abnormality in TRAF3 mice (Fig 2.17).

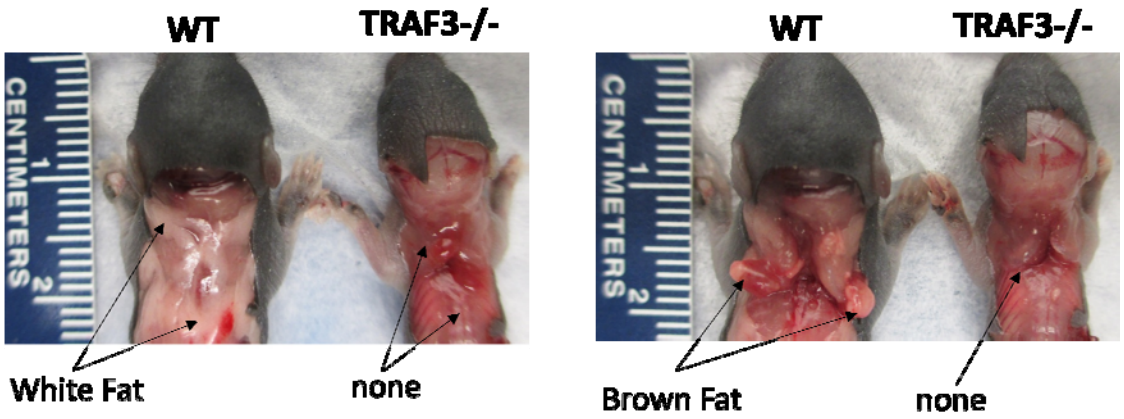

Figure 2.1. Dorsal view of 7 days old WT and TRAF3-/- mice indicating WAT (left) and BAT (right) in interscapular region.

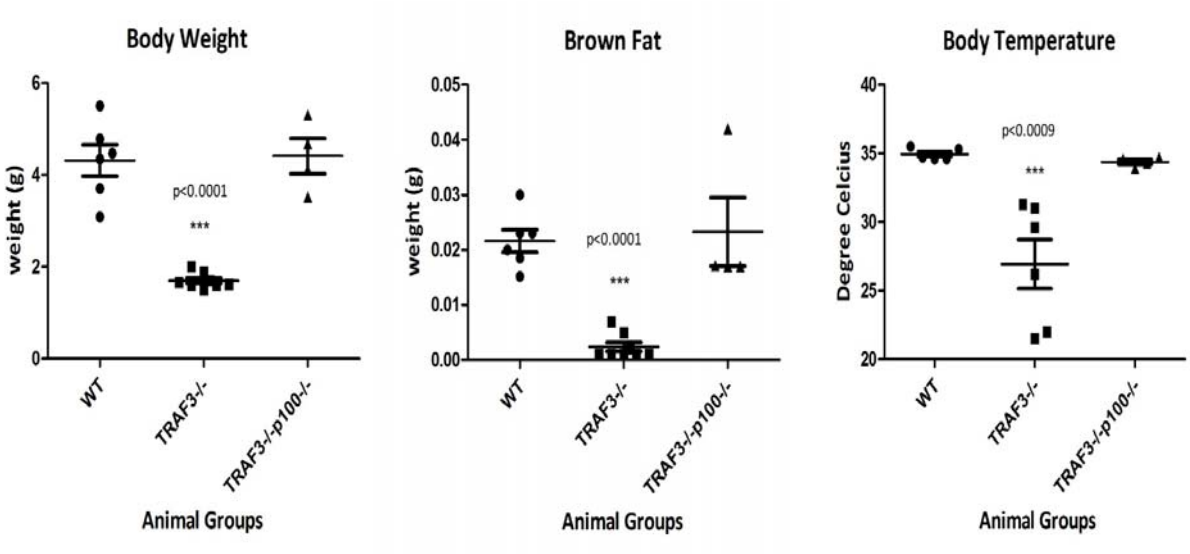

Figure 2.2. TRAF3-/- mice have only minute amount of BAT and lower body temperature. Body weight, BAT weight and Body Temperature of WT (n=6), TRAF3-/- (n=8) and TRAF3-/-p100-/- (n=5).

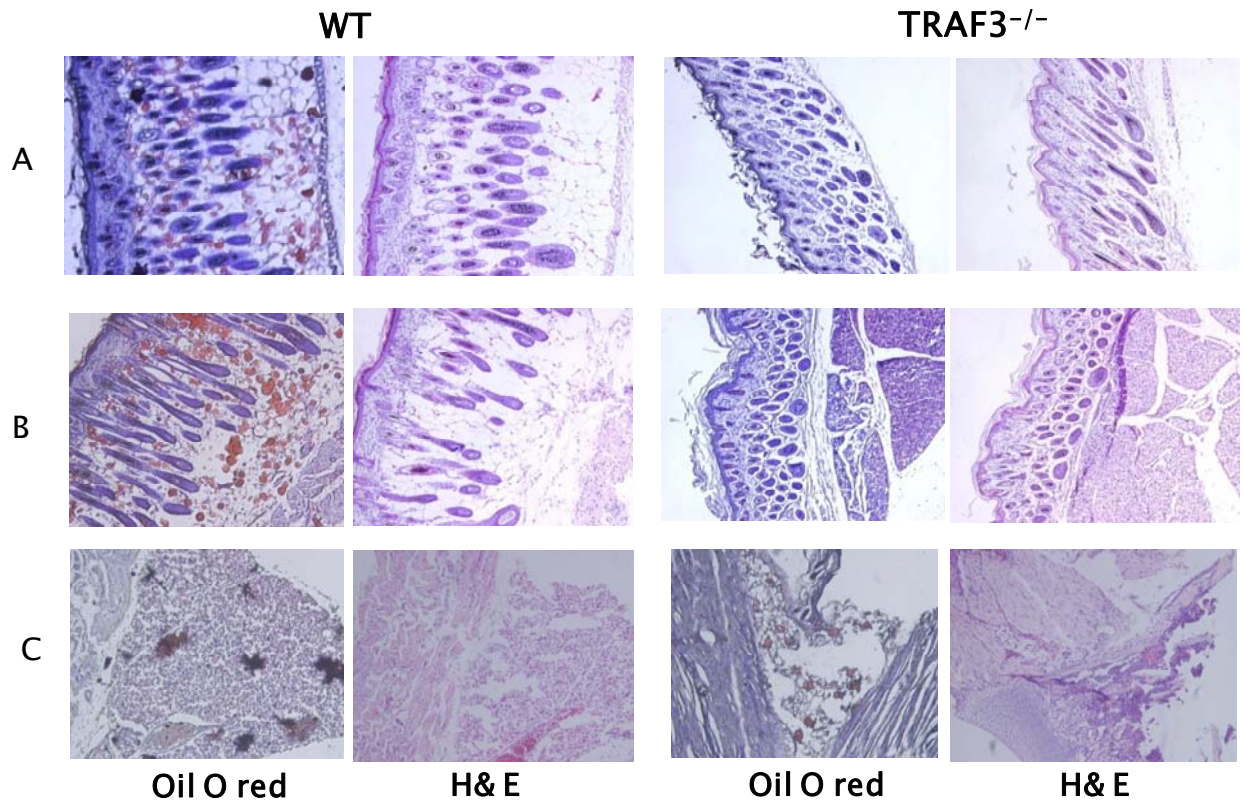

Figure 2.3. No Oil O-Red staining of adipocytes in TRAF3-/- tissues. Oil O-Red and H&E staining of frozen tissue sections from WT and TRAF3-/- mice panel A, skin; panel B, skin and muscle containing adipose layer; panel C, bone (C).

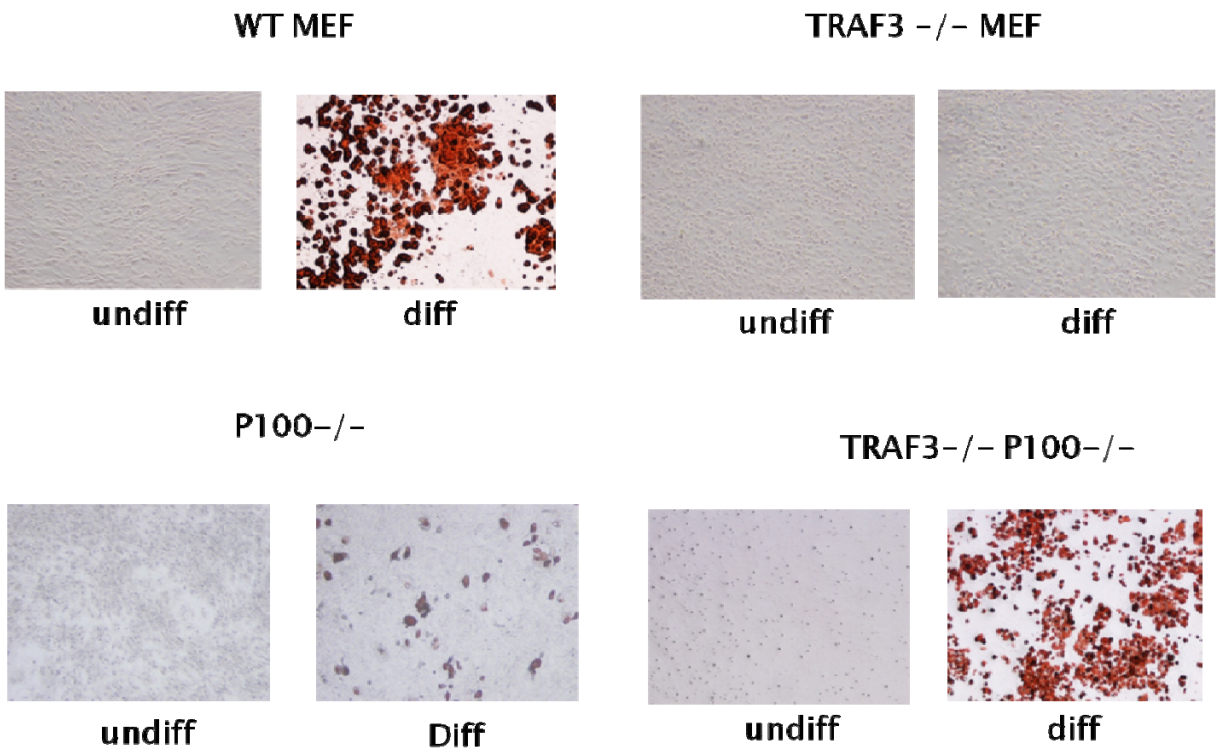

Figure 2.4. TRAF3-/- MEFs do not differentiate into adipocytes. Adipocyte differentiation of WT, TRAF3-/- and TRAF3-/-p100-/- MEFs. MEFs were differentiated in culture media (CM) with MIX and 1µM rosiglitazone (BRL) at day 0 and 2 days later (day 2), the medium was switched to CM containing 5ug/ml insulin and 1uM BRL and maintained in this media.

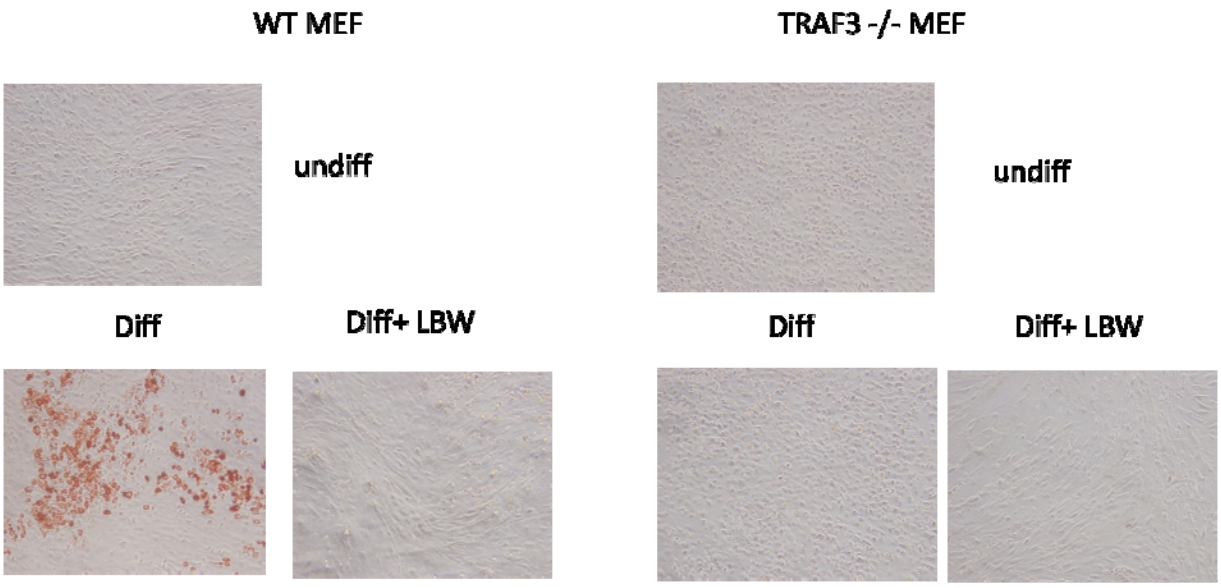

Figure 2.5. SM can inhibit adipogenesis in WT MEFs. Adipocyte differentiation of WT and TRAF3-/- MEFs without SM and with SM. Here, LBW242 (200nM) is shown as representative for SM.

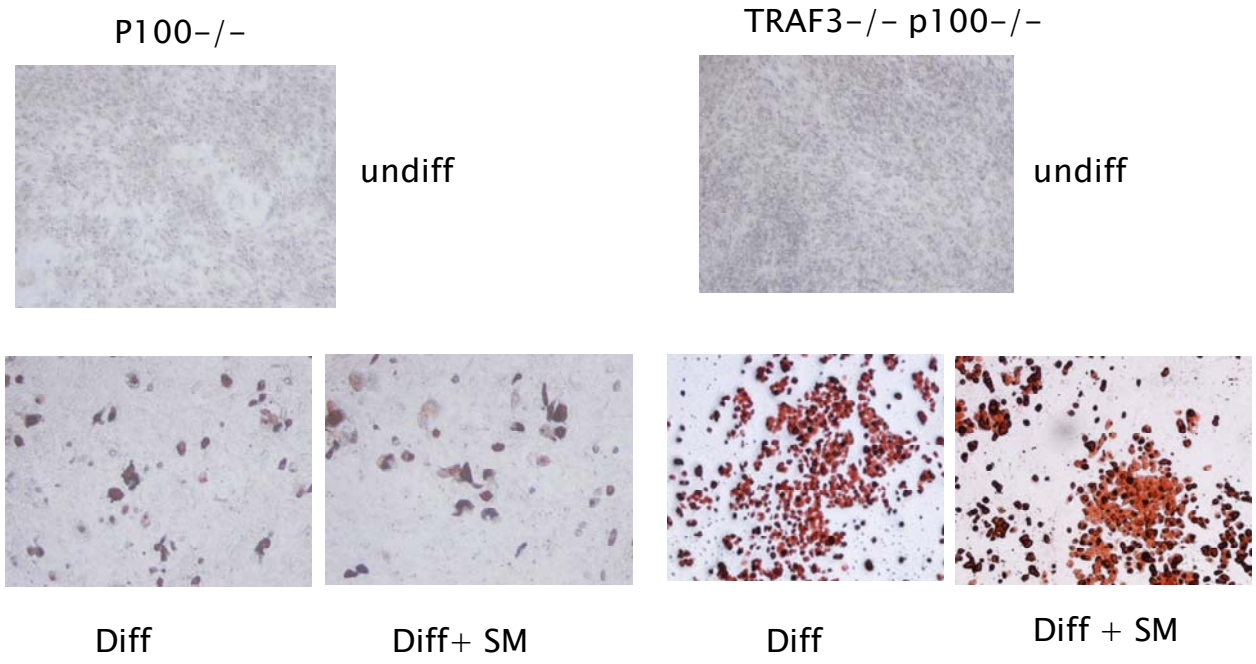

Figure 2.6. SM no longer inhibits adipogenesis in p100-/- and TRAF3-/-p100-/- MEFs.

Adipocyte differentiation of p100-/- and TRAF3-/-p100-/- MEFs without SM and with SM.

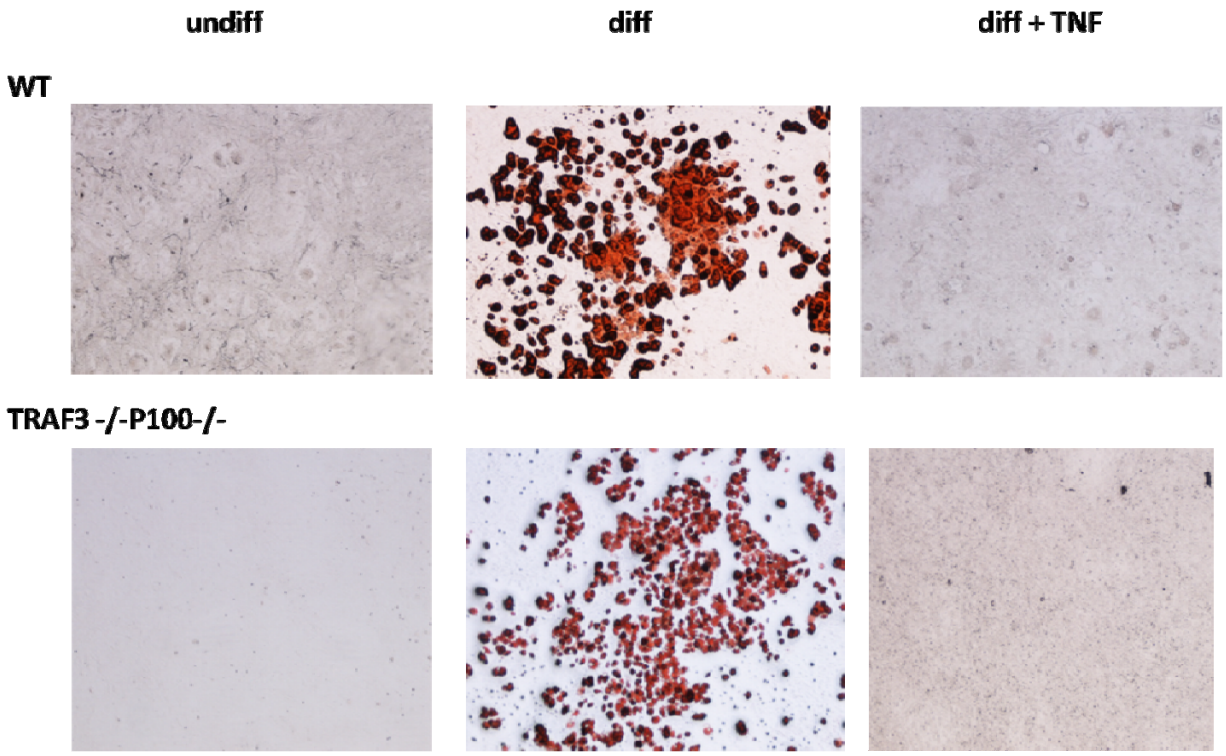

Figure 2.7. Compound loss of TRAF3 and p100 cannot rescue inhibition of TNF on adipogenesis. Differentiation of both WT and DKO MEFs were inhibited by TNFα (20 ng/ml), activator of canonical NF-kB pathway.

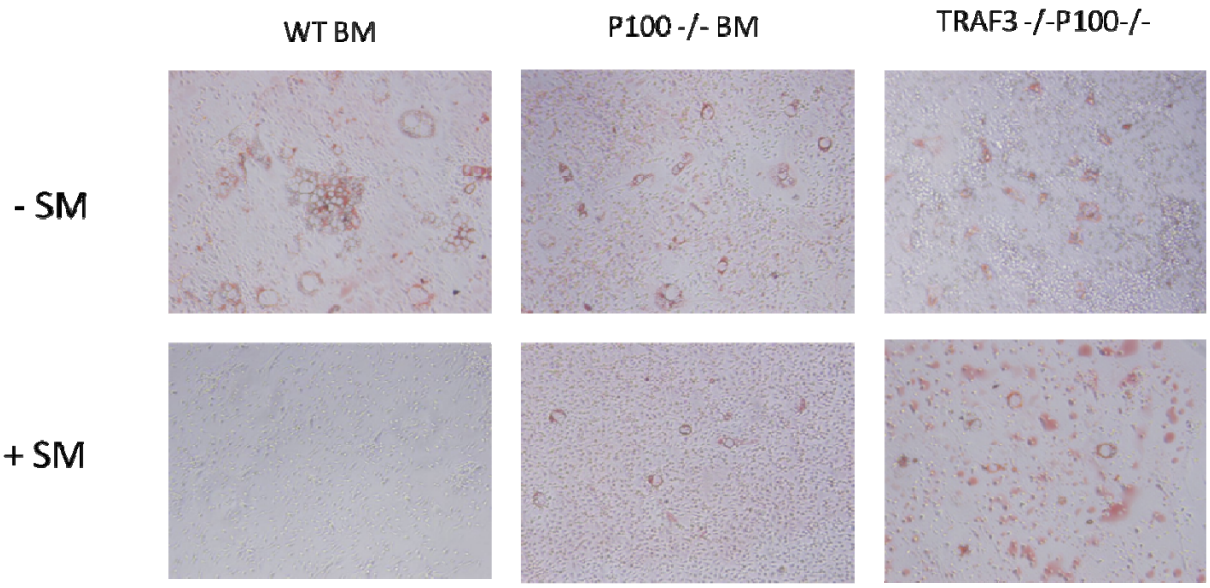

Figure 2.8. SM inhibits only WT BMs. Differentiation of WT, p100-/- and TRAF3-/-p100-/- bone marrow mesenchymal stem cells (BMs) with or without SM.

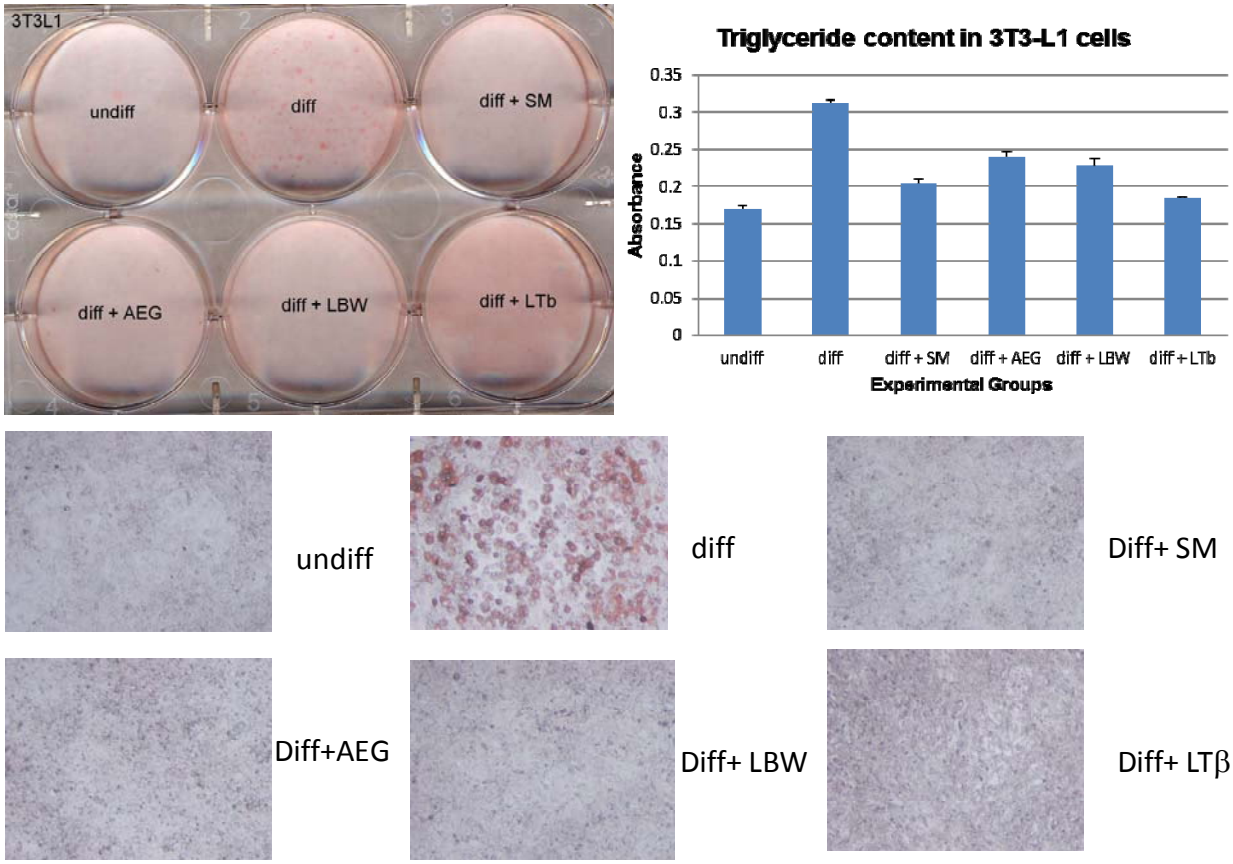

Figure 2.9. SM inhibits 3T3-L1 adipogenesis. Adipocyte differentiation of 3T3-L1 cells with different Smac mimetic; SM (100nM), LBW242 (300nM), AEG (300nM), and agonistics αLTβR (2ug/ml). Quantitative amount of triglycerides in each well is obtained by eluting Oil Red O in each well with 100% isopropanol and Triglycerides absorbance is measured at wavelength 500nm.

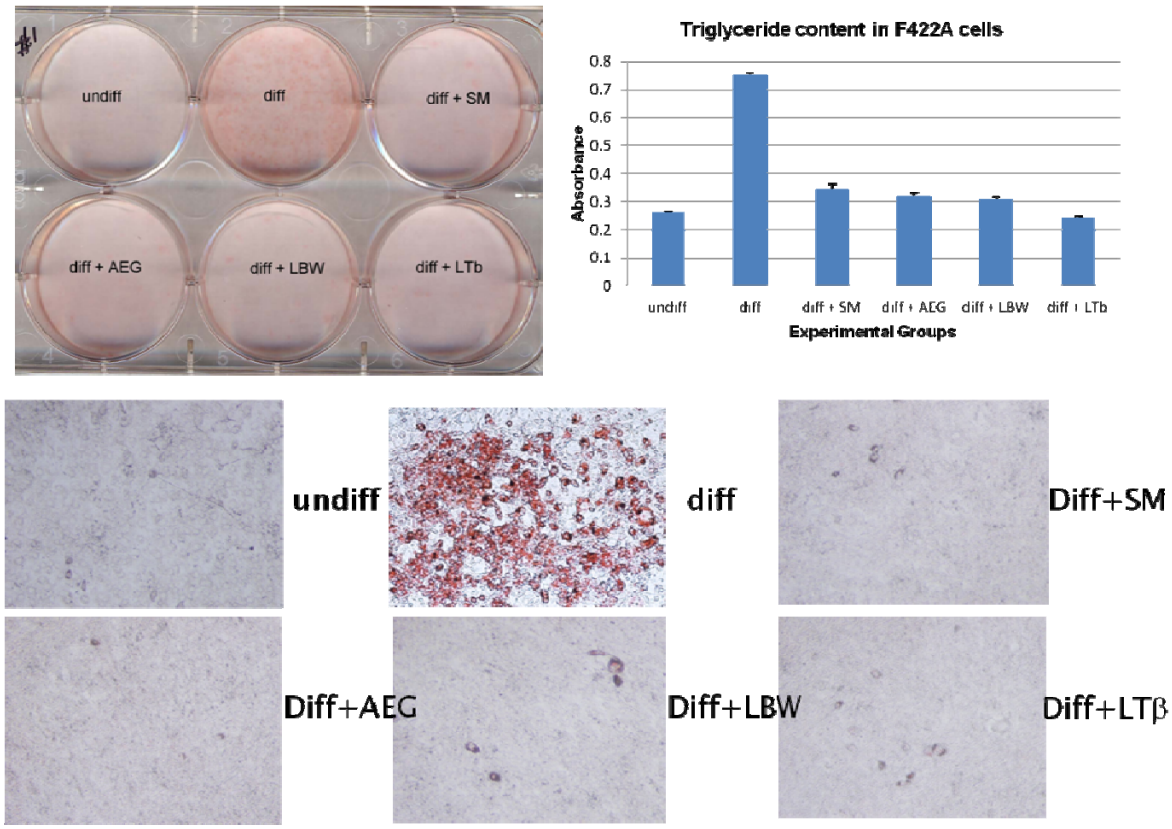

Figure 2.10. SM inhibits adipogenesis in 3T3-F422A cells. Adipocyte differentiation of 3T3-F422A cells with different Smac mimetic; SM (100nM), LBW242 (300nM), AEG (300nM), and agonistics αLTβR (2ug/ml). Triglycerides absorbance is measured at wavelength 500nm.

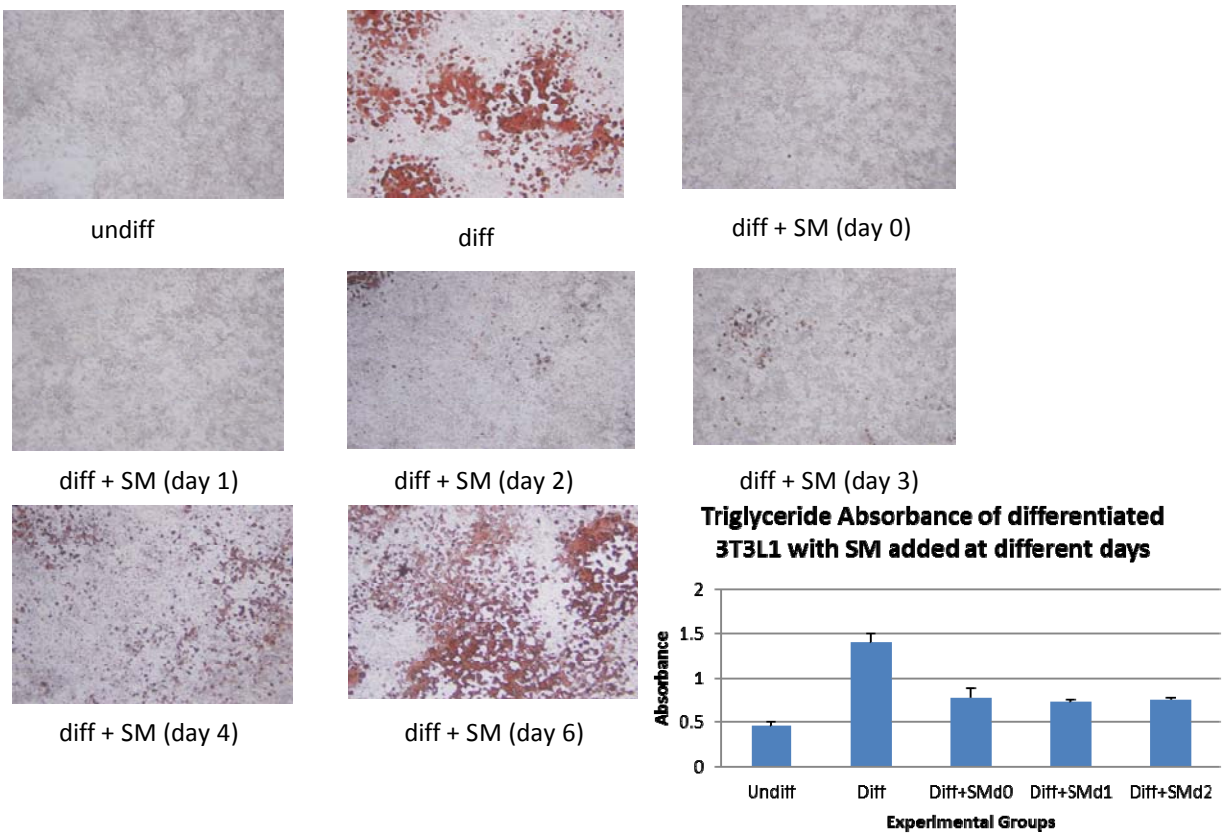

Figure 2.11. Time course of SM inhibition in 3T3-L1 adipogenesis. SM is added to culture media at indicated days. SM inhibition is best if added during first two days of adipogenesis.

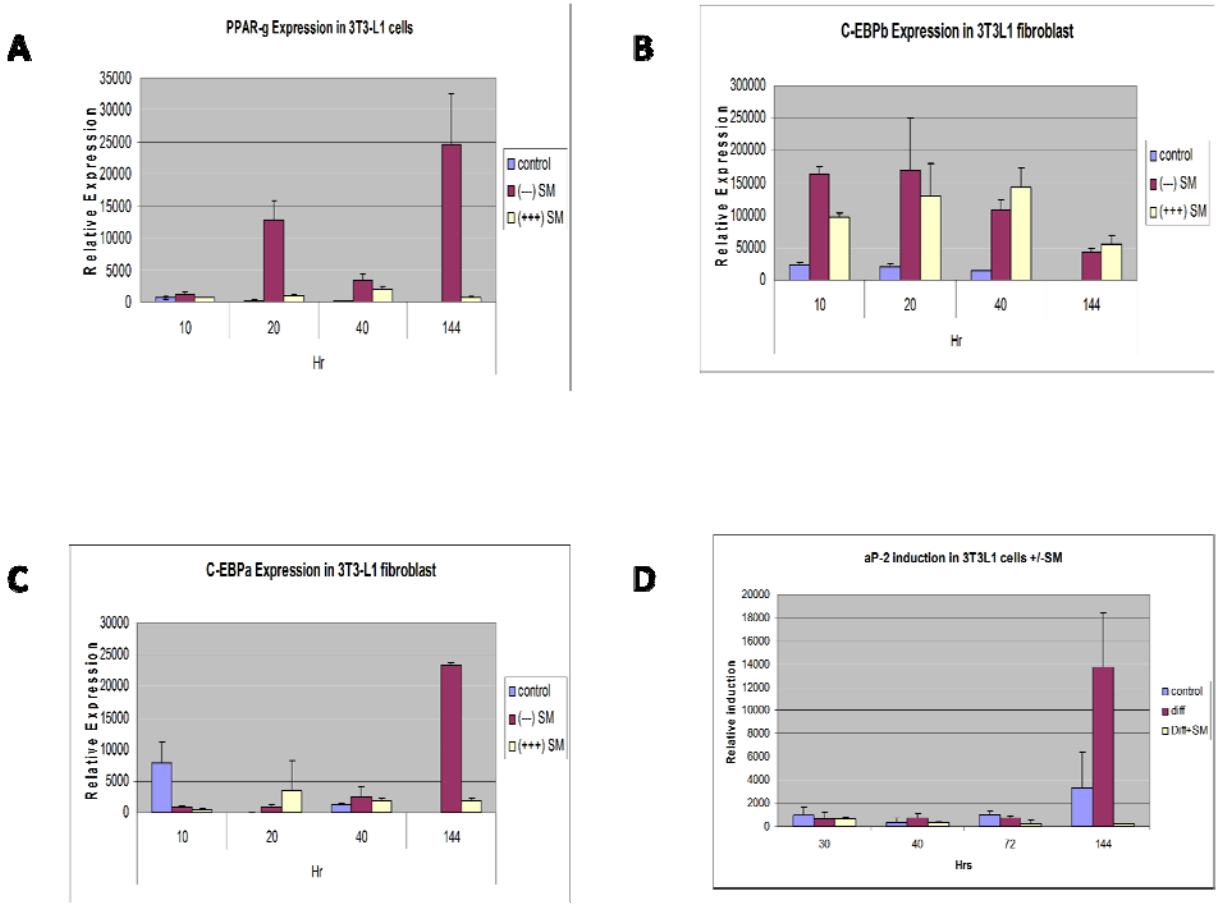

Figure 2.12. PPARγ and C/EBPα expression decreased in 3T3-L1 adipocytes treated with SM. QPCR analysis of PPARγ (A), C/EBPβ (B) C/EBPα (C), and aP-2 (D) expression in undifferentiated, differentiated, and Smac treated 3T3-L1 cells.

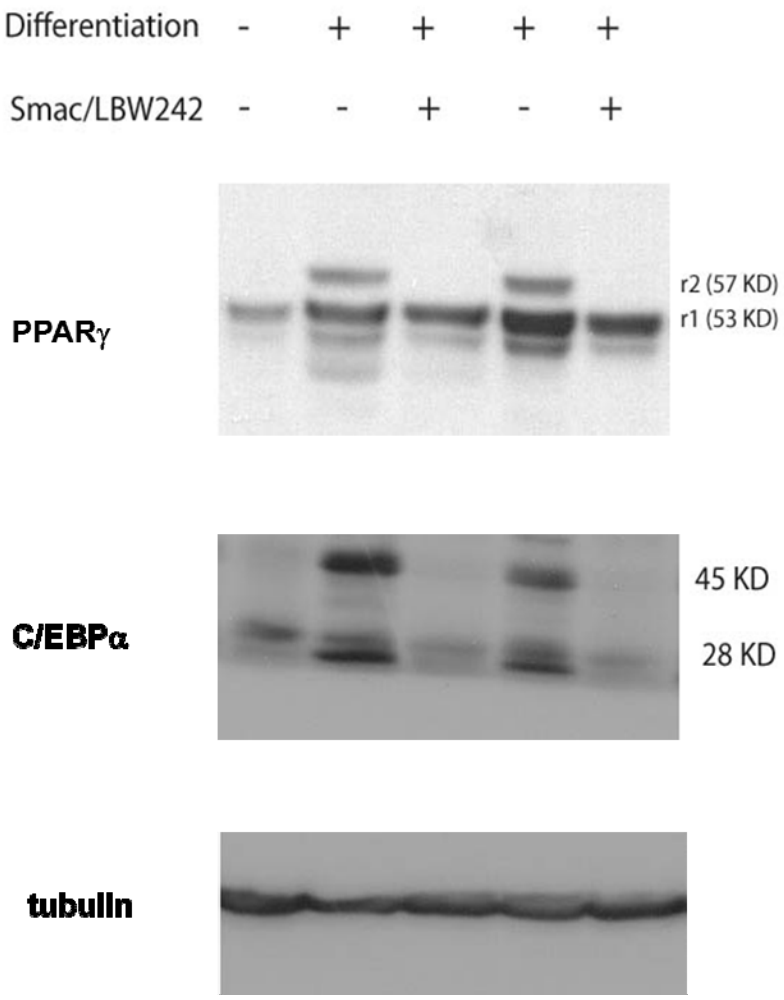

Figure 2.13. 3T3-L1 adipocytes treated with SM exhibit decreased protein expression of PPARγ and C/EBPα. Western Blot analysis of differentiated 3T3-L1 cells with or without SM.

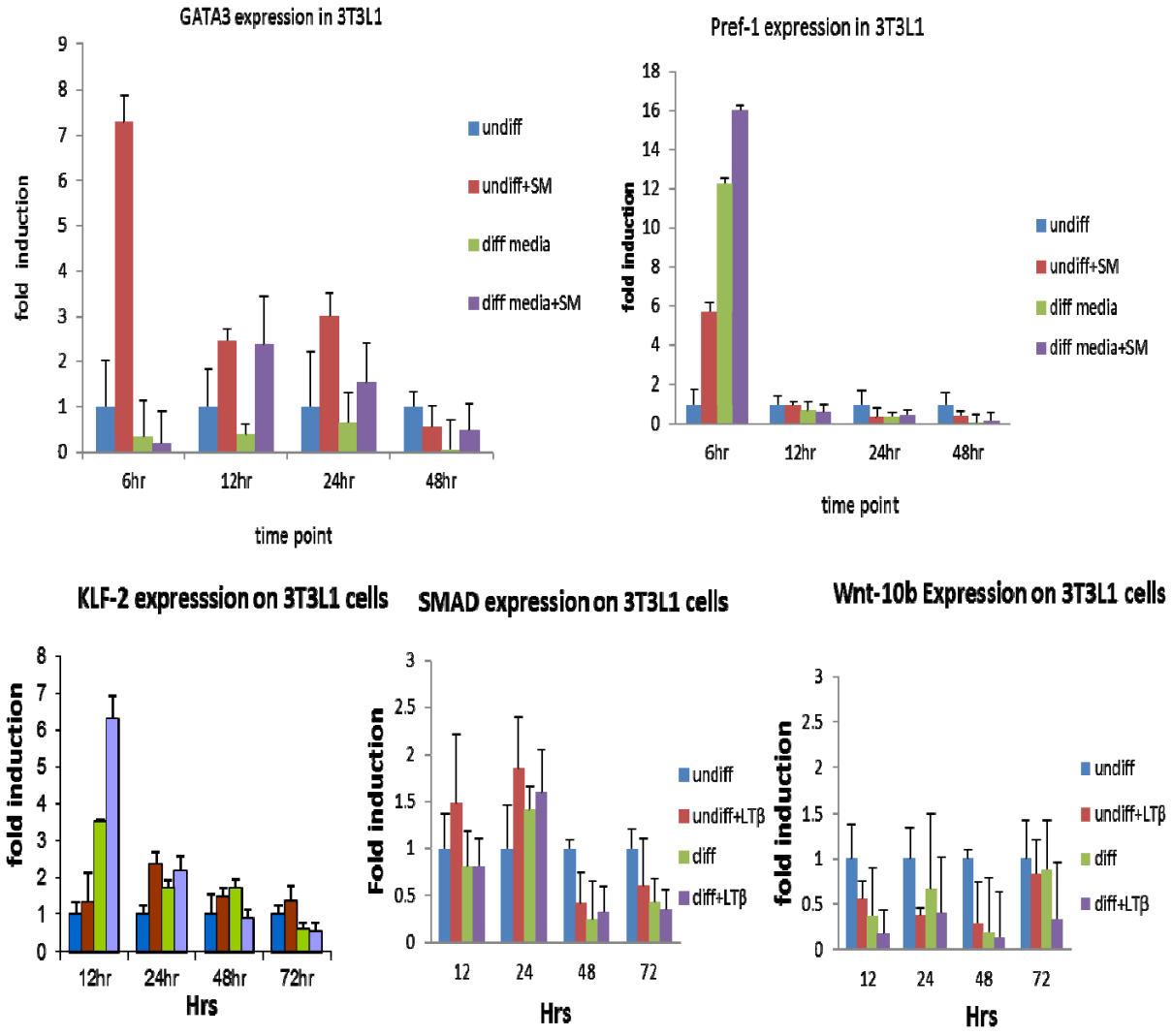

Figure 2.14. SM inhibits down-regulation of GATA3 expression in differentiated 3T3-L1 adipocytes. Expression of activators of adipogenesis, Pref-1 and inhibitors of adipogenesis GATA3, KLF-2, SMAD and Wnt10b.

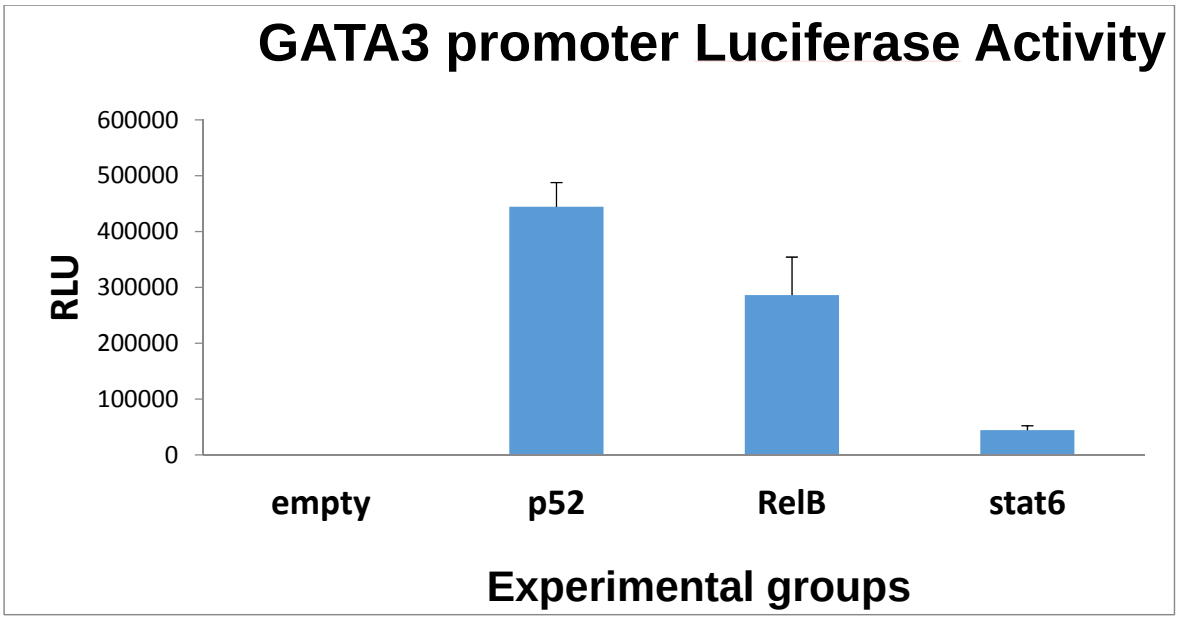

Figure 2.15. Type II NF-κB subunits can activate GATA3 promoter. GATA3 promoter luciferase assay with non-canonical NF-κB subunits; p52 and Rel B. Stat6 plasmid is used as positive control and empty pcDNA3 is used as negative control.

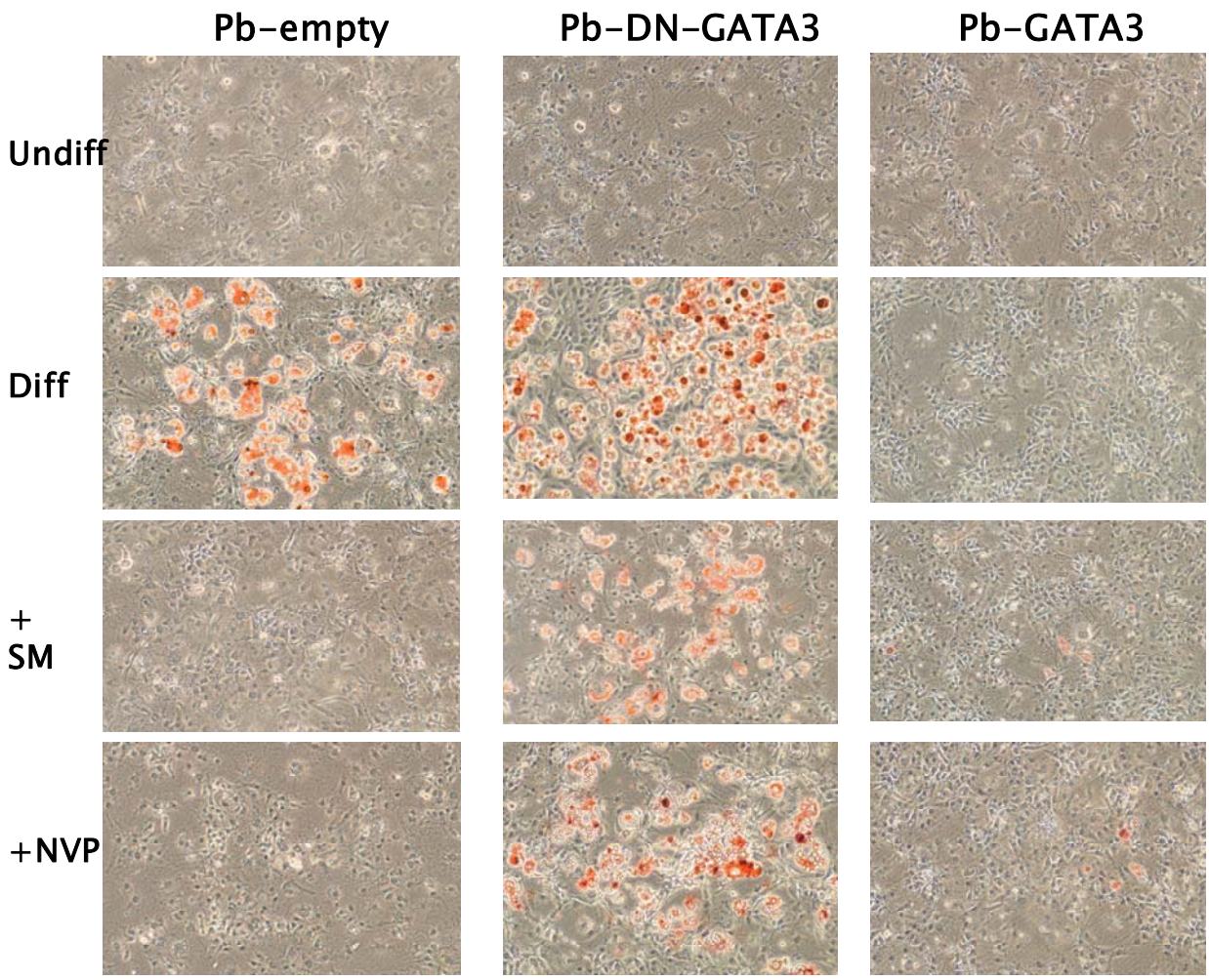

Figure 2.16. SM no longer inhibits adipogenesis in 3T3-L1 cells over-expressed with dominant negative GATA3. Differentiation of 3T3-L1 cell lines overexpressed with empty pBabe, pBabe-KRR (DN) and pBabe-GATA3 without Smac mimetics and with SM or NVP.

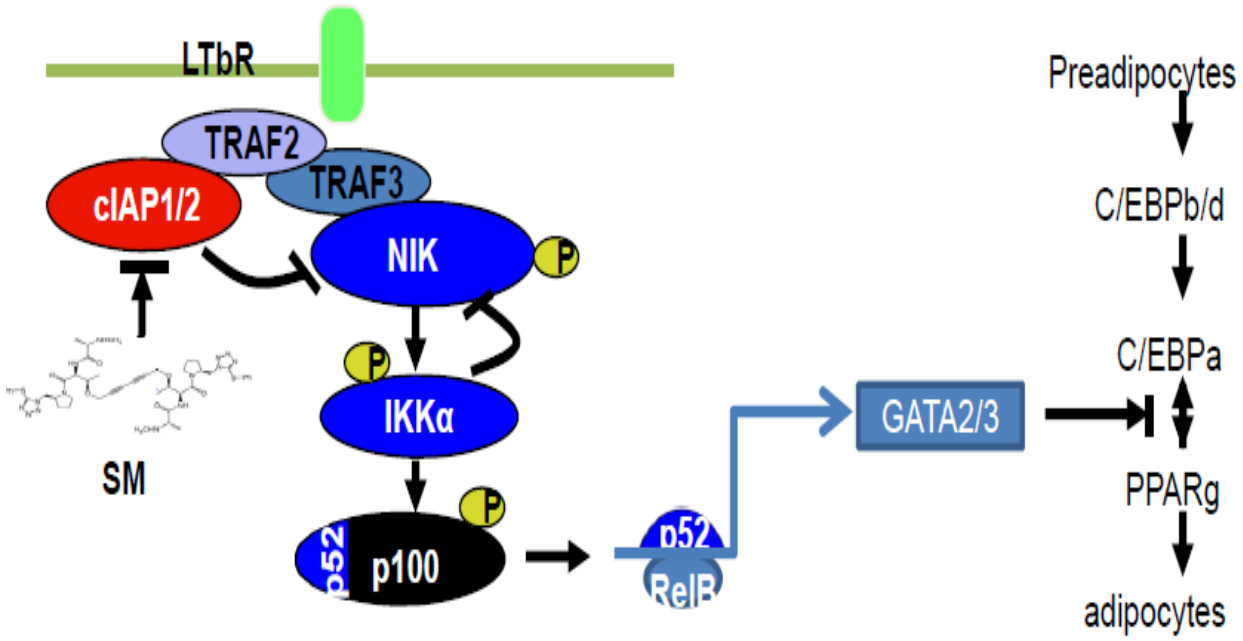

Figure 2.17. Model: Potential Role of TRAF3 in adipogenesis

CHAPTER 3

Effect of TRAF3 knockout in glucose metabolic pathways

It has been shown that TRAF3$^{-/-}$ mice have greatly reduced serum glucose levels and elevated serum corticosterone. However, these abnormalities were rescued in TRAF3-/- P100-/- mice (He et al., 2006). From microarray data, we have found that TRAF3-/- liver express 2.7 fold increase in lactose dehydrogenase 2 (Ldh2) and 5 fold increase in leptin receptor (LepR) compared to those of WT. Our real-time PCR analysis has also confirmed that TRAF3-/- liver express higher level of not only Ldh2 and LepR genes but also gluconeogenesis genes such as PEPCK and Pdk1. On the other hand, expression of fatty acid oxidation genes such as carnitine palmitoyltransferase 1A (Cpt1a) and AOX is much lower in TRAF3-/- liver compared to those of WT littermates (Fig 3.1). This shows that glucose is utilized extremely fast in TRAF3-/- mice to produce ATP as quickly as possible. In addition, TRAF3-/- have higher insulin sensitivity though low blood glucose level. Here, we have found that unstimulated TRAF3-/- MEFs have higher expression of pAKT as well as pGSK3α and phosphorylation of AKT and GSK3α is increased even with low insulin stimulation (Fig 3.2). This phenomenon is also found in 3T3-L1 cells treated with SM since treatment with SM causes increase in phosphorylation of AKT and GSK3α (Fig 3.3). However, there is no difference in Glut4 expression in both mRNA and protein level in TRAF3-/- MEFs as well as 3T3-L1 cells treated with SM, relative to their WT counterparts.

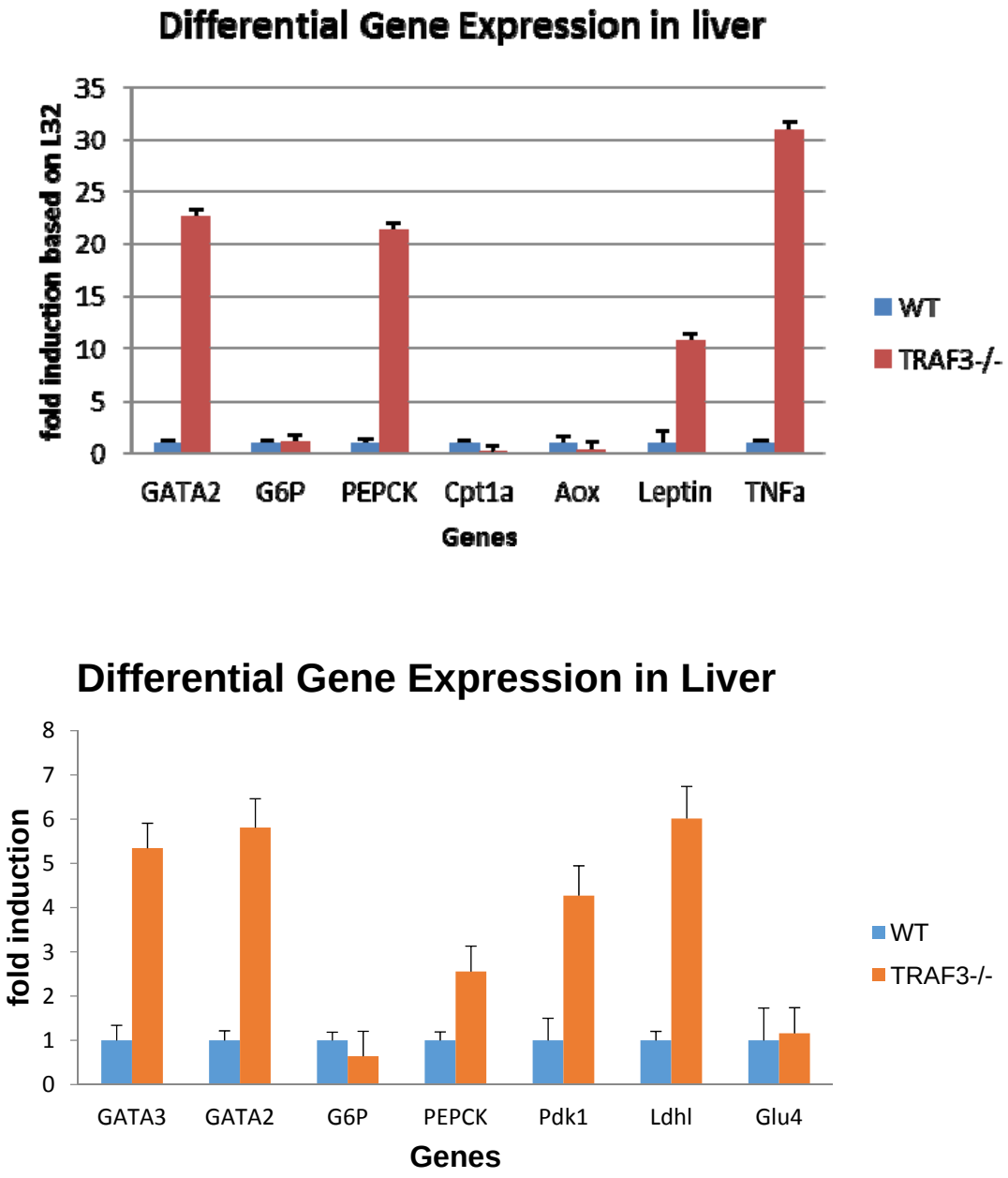

Figure 3.1. TRAF3-/- liver express higher level of genes involved in gluconeogenesis and lower level of genes involved in fatty acid oxidation. Liver were isolated from 7 days old WT and TRAF3-/- mice and fresh frozen for RNA isolation.

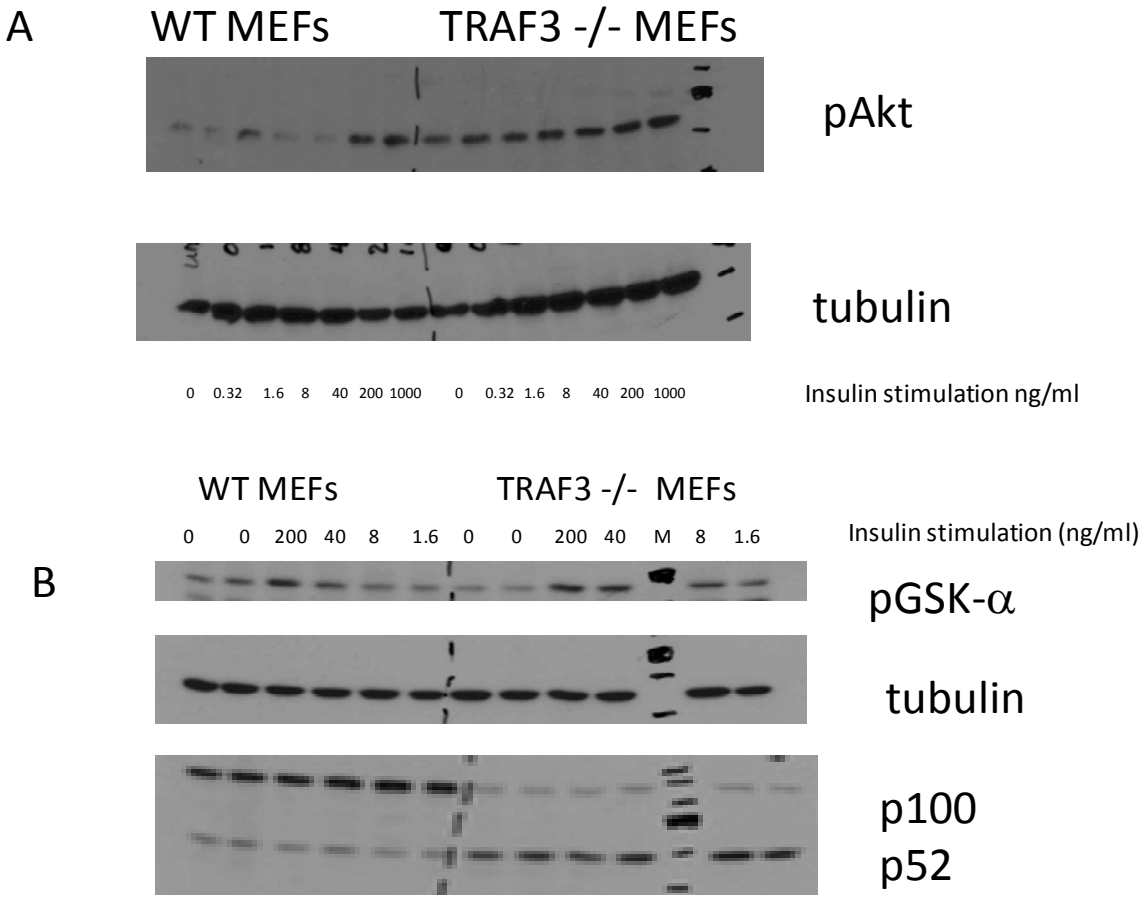

Figure 3.2. Insulin sensitivity increased in TRAF3-/- MEFs. Western Blot Analysis of pAKT and pGSK3α in WT and TRAF3-/- MEFs treated with various insulin concentrations.

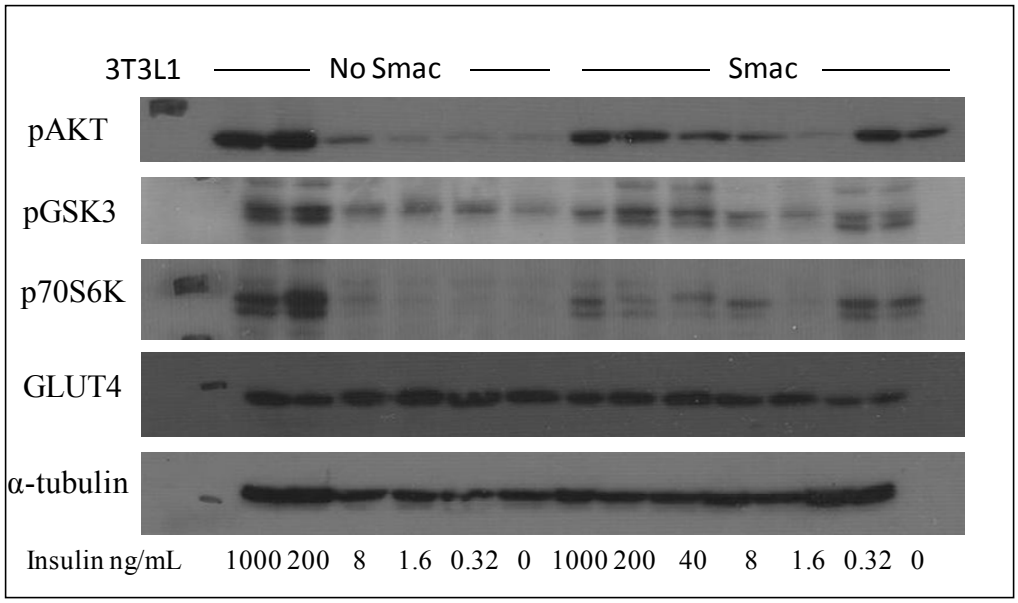

Figure 3.3. Insulin sensitivity increased in 3T3-L1 cells treated with SM. Western blot analysis of pAKT, pGSK3α, p70S6K and Glut4 in insulin stimulated 3T3-L1 cells with or without SM.

CHAPTER 4

Conclusion

Obesity is a major health concern in the United States. According to 2003-2004 National Health and Nutrition Examination Survey (NHANES), 66 percent of U.S. adults and 17 percent of children and adolescents are either overweight or obese. The bone marrow cells are pleiotropic to be differentiated into fibroblasts, chondrocytes, myocytes, adipocytes and osteoblasts. Thus, it is evident that there are regulators to govern the cell fate switching, and particularly in normal bone continuous osteoblastogenesis is maintained while adipogenesis appears suppressive. It is known that adipogenesis is abnormally enhanced in the bone marrow in osteoporotic or elder states and under chronic treatments of a PPARγ agonist for diabetes (Takada et al., 2007).

Currently, the development of pharmacological inhibitors of cIAPs has been a major goal in advancing cancer therapeutics since cIAPs antagonize activated caspases and are often amplified in human cancers. Two reports using two different second generation SMAC mimetic compound LBW242 (Chauhan et al., 2007, Weisberg et al., 2007) showed that inhibition of cIAPs can activate NF- B noncanonically. Our lab have found that TRAF3 recruits NIK and TRAF2 recruits E3 ubiquitin ligases, cellular inhibitor of apoptosis (cIAP) 1 and 2, to TRAF3/NIK and promote NIK ubiquination and degradation. On the other hand, Smac mimetics can prevent NIK degradation and initiates constitutive processing of type II NF-κB (Zarnegar et al., 2008).

In addition, we have demonstrated that loss of TRAF3 results in constitutive type II NF-κB activities and TRAF3 null mice have greatly reduced serum glucose level and elevated serum

corticosterone level. However, these defects are rescued by compound loss of type 2 NF-κB p100 gene (He et al., 2007). Here, we have shown that TRAF3-/- mice have little or no BAT and WAT and compound loss of TRAF3 and p100 also rescue the phenotype. In addition, we demonstrated that constitutive type II NF-κB inhibits adipogenesis by upregulating GATA3 causing metabolic abnormality in TRAF3 null mice.

By understanding exact mechanism(s) of inhibition of adipocyte differentiation and glucose metabolism caused by constitutive activation of type II NF-KB, we will be able to use derivatives of SM to decrease fat accumulation, reduce adipogenesity and increase insulin sensitivity. These drug can be indirectly apply to treating diabetes patients since obesity can lead to diabetes and type II diabetes is caused by decrease in insulin sensitivity. Furthermore, we can develop drugs other than SM that can inhibit master regulators that promote adipogenesis with fewer side effects.

CHAPTER 5

Materials and Methods

Reagents

Smac mimetic (SM) was a gift from Dr. Xiaodong Wang (University of Texas Southwestern Medical Center). Smac mimetic LBW242 and NVP-LCI 161-MX-9 (NVP) were generous gift from Novartis. Agonistic LTβR antibody was purchased from Alexis Biochemicals. Rosiglitzone and Cycloheximide was purchased from Sigma. Overexpression experiments were done using PEI. Monoclonal anti-mouse TNFR I (R&D Systems, Minneapolis, MN).

Mice

TRAF3-/- and TRAF3-/-p100-/- mice have described previously (He et al., 2006). Targeted disruption of the ***TRAF3*** and ***NFκB2*** alleles has been previously described (Xiu Y., Cheng G et al., 1996). All mice were maintained and bred under specific pathogen-free conditions in the University of California, Los Angeles CHS and Public Health mouse facilities, and experiments were conducted within the parameters of our protocol approved by the Animal Research Committee.

Various tissues were collected, embedded in tissue sections with TissueTek O.C.T (Sakura Fine Tek, CA) and frozen with dry ice. Frozen tissue slides (4 microns) were cut at Translational Path Core Laboratory (TPCL) under UCLA Pathology and Laboratory Medicine and slides are stored in -80.C. The slides were stained with hematoxylin and eosin (H & E) at TPCL.

Cell culture

MEFs were isolated from Day 15 embryos. MEFs and 3T3 cell lines were propagated and differentiated according to the protocol described previously (12). In brief, 3T3-L1 cells were propagated in DMEM (Corning Cellgro) culture media (CM) containing 10% FBS (Omega

Scientific) and penicillin-streptomycin (100 U/ml each) and allowed to reach confluence (*day -2*). After 2 days (*day 0*), the medium was changed to differentiation media (CM containing 5ug/ml insulin, 1μM dexamethasone (Biovision, Mountain View, CA), and 0.5 mM 3-isobutyl-1-methylxanthine (MIX) (Sigma; Saint Louis, MO). Two days later (*day 2*), the medium was switched to CM containing 5ug/ml insulin and maintained in this media. 3T3-F422A cell line are obtained from Reue Lab and differentiated with CM containing 5ug/ml insulin and 0.5mM MIX at *day 0* and change every 2 day with CM containing only 5ug/ml insulin. MEFs were differentiated in CM with MIX and 1μM rosiglitazone (BRL) at day 0 and 2 days later (day 2), the medium was switched to CM containing 5ug/ml insulin and 1uM BRL and maintained in this media. LTβR antibody and different types of Smac mimetic were added at *day 0* and every 2 days when CM is changed. HEK 293Tcells were maintained in CM supplemented with 5% FBS.

Mesenchymal Stem Cell Differentiation

Murine BMCs were generated by flushing bone marrow cells from the femurs and tibias of mice and lysing red blood cells with ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Gibco Life Technologies). Plate 1x10^6 bone marrow cells per well in 6 well plate in MesenCult Basal Media with Mouse Mesenchymal Stem Cells Stimulatory Supplements (MS media) (Stem Cell Technologies). Grow for 5 days in MS media and then differentiate with MS media with 1μM BRL and then change media every 3 days with MS media + 1μM BRL.

Oil O-red Staining

Cells were stained with Oil Red O as described previously (Janderova, et al., 2003). In brief, dishes were washed twice with phosphate-buffered saline and fixed with 10% buffered formalin

for at least 1 h at room temperature. Cells were then stained for half hour at room temperature with a working filtered oil red O solution (6 parts Oil Red O stock- 0.7 g of oil red O in 200 ml of isopropyl alcohol and 4 parts dH_2O), washed four time with water, and visualized under microscope (10x). The slides were brought to room temperature, fix in 10% formalin for 10 min, and rinse three times with tap water. Then, slides are soak in 60% isopropanol for 10 min, stain with working Oil Red O working solution for 30 min and then rinse in 60% isopropanol until clear. Rinse three times with tap water and stain with Meyer's Hematoxylin for 2 min. After rinsing three times with tap water, air dry slides and visualize under microscope.

Quantitative PCR

RNA was isolated using TRIzol reagent (Invitrogen) and converted to cDNA using iScript (Bio-Rad). Quantitative PCR (Q-PCR) analysis was performed using the iCycler thermocycler. All data are presented as relative mRNA transcript levels as normalized to 18s and L32 values. Primer sequences were previously published (Tontonoz P lab, Reue K lab).

Immunobloting and Western Blot Analysis

6-well plates of 3T3-L1 cells after treatment were washed twice with PBS and collected in 1 ml of 0.5mM PBS-EDTA. Cells were lysed in a modified radioimmune precipitation (mRIPA) buffer, containing 0.5% (vol/vol) NP-40, 0.1% (wt/vol) Na-Deoxycholate, and no SDS, followed by brief sonication using the Misonix Sonicator 3000. Protease inhibitor cocktail (Sigma) was included in all lysates. The lysates are mixed 1:1 with SDS loading buffer (50 mM Tris-Cl, pH 6.8, 1% (vol/vol) beta-mercaptoethanol, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.1% (wt/vol) bromophenol blue) and then boiled for 5 min. Thirty micrograms (μg) of total protein

were fractionated by 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes (Immobilon-P Millipore) and immunoblotted with antibodies according to the manufacturer's recommended instructions. Antibodies were obtained from following companies: TRAF3 (M20), C/EBPβ (Santa Cruz); C/EBPα, PPARγ, p100/p52, GATA2 (Cell Signaling); GATA3 (Abcam 32858).

Luciferase Reporter Assays

Fugene 6 (Roche Diagnostics, Germany) were used for all HEK 293T transfections. PGL3-empty/ pGL3-GATA3 promoter is generous gift from Dr. Chen Dong (MD Anderson Cancer Center, Houston, TX). Stat-6 promoter is a gift from Dr. Dubinett (UCLA). Cells were transfected with the specified constructs, along with a Firefly luciferase pGL3-basic reporter plasmid and Renilla luciferase for normalization. Cells were lysed and luciferase values were quantified on a luminometer by Dual-Luciferase Reporter Assay System from Promega (Madison, WI).

Overexpression Experiments

3T3-L1 and F-422A cells were reconstituted with stably expressing full-length GATA3 or dominant negative GATA2/3 (KRR) pBABE-plasmids containing a puromycin selection marker by selection in complete media in the presence of 2ug/mL puromycin. pCDNA-GATA3 and pcDNA-KRR are gifts from Dr. Astar Winoto (UC Berkeley). All reconstituted cell lines were generated as described previously (He et al). Briefly, HEK 293T cells were transfected with Moloney murine leukemia virus-ΨA helper construct plus either pBABEpuro alone or the indicated pBABEpuro construct using Polyethylenimine (PEI) (Sigma). 3T3 cell lines were then

infected with the filtered 293T cell supernatants with polybrene (American Bioanalytical), followed by selection with 2.5 ug/ml of puromycin.

Statistical Analysis

Data were presented as means ± S.E.M. Differences between two groups were analyzed with two-tailed Student's t test and differences between three or more groups were analyzed with one-way ANOVA. $P < 0.05$ was considered statistically significant.

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