FROM FLIES TO MICE: DROSOPHILA AS A MODEL SYSTEM TO STUDY FAT BIOLOGY

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To

My Family

To
My Wife, Soohyun Ahn

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FROM FLIES TO MICE: DROSOPHILA AS A MODEL SYSTEM TO STUDY FAT BIOLOGY

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Adipose tissues are found in a wide range of animal species ranging from invertebrates to mammals and are essential for many aspects of the animal life cycle. Fat biology has taken on a new and urgent importance as it is intimately linked to one of the most prevelant and costly health problems of our time - obesity. Model organisms are a powerful resource for the discovery of genes critical to human health and disease. Thus far, the utility of *Drosophila melanogaster* as a model system to study fat biology has not been critically

evaluated. My studies highlight the potential of this system to uncover new genes and pathways that may impact our current understanding of adipose biology.

Hedgehog signals regulate invertebrate and vertebrate development, yet the role of the cascade in adipose development was undefined. I found that fat body specific transgenic activation of Hedgehog signaling inhibited fly fat formation. Conversely, fat body specific Hedgehog blockade stimulated fly fat formation. Strikingly, the anti-adipogenic effect of Hedgehog signaling was conserved in mammalian adipogenic models in both sufficiency and necessity tests. Hedgehog signals elicit this function early in adipogenesis, upstream of PPARgamma, potentially acting as a molecular switch diverting preadipocytes and mesenchymal prescursors away from adipogenesis and towards osteogenesis.

In another study, I investigated the role of *Adipose*, an evolutionarily conserved gene isolated from naturally occurring obese flies. Through gain- and loss-of-function studies in flies, mammalian cell culture, and mice I showed that the Adipose pathway plays a conserved role in obesity and diabetes. Furthermore, I found that Adipose controls the activity of PPARgamma, a central regulator of adipogenesis and the target of the thiazolidinedione class of anti-diabetic drugs. Adipose inhibits PPARgamma function by directly binding Med23, a component of the Mediator Complex, which connects transcription factors with RNA polymerase II.

Taken together, I have shown that both the Hh and Adp pathway have evolutionary conserved functions in fat formation. These results support the idea that Drosophila is a useful system to study adipose biology and discoveries in flies will lead to biological insights relevant to mammals and the treatment of obesity and diabetes.

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LIST OF ABBREVIATIONS

AMPK	AMP activated protein kinase
cDNA	complementary DNA
C/EBPα	CCAAT/enhancer binding protein alpha
CFP	cyan fluorescent protein
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle's Medium
DN	dominant-negative
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
FAS	fatty acid synthase
FBS	fetal bovine serum
FRET	fluorescence energy transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
Glut4	glucose transporter 4
Hh	hedgehog
IP	immunoprecipitation
LPL	lipoprotein lipase
MMLV	Moloney murine leukemia virus

NES	nuclear export signal
NHR	nuclear hormone receptor
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
Osx	osterix
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenymethylsulfonyl fluoride
PPARγ	peroxisome proliferator-activated receptor gamma
Ptc	patched
qPCR	quantative polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase-polymerase chain reaction
Shh	sonic hedgehog
shRNA	short hairpin RNA
Smo	smoothened
SREBP	sterol response element binding protein
TOR	target of rapamycin
TPR	tetratricopeptide repeats
TZD	thiazolidinedione
UAS	upstream activating sequence

UTR	untranslated region
WAT	white adipose tissue
WT	wild-type
YFP	yellow fluorescent protein

Chapter I

Hedgehog signaling plays a conserved role in inhibiting fat formation

Abstract

Hedgehog (Hh) signals regulate invertebrate and vertebrate development, yet the role of the cascade in adipose development was undefined. To analyze a potential function, we turned to *Drosophila* and mammalian models. Fat body specific transgenic activation of Hh signaling inhibited fly fat formation. Conversely, fat body specific Hh blockade stimulated fly fat formation. In mammalian models, sufficiency and necessity tests showed that Hh signaling also inhibits mammalian adipogenesis. Hh signals elicit this function early in adipogenesis, upstream of PPARγ, potentially diverting preadipocytes as well as multipotent mesenchymal prescursors away from adipogenesis and towards osteogenesis. Hh may elicit these effects by inducing the expression of anti-adipogenic transcription factors such as Gata2. These data support the notion that Hh signaling plays a conserved role, from invertebrates to vertebrates, in inhibiting fat formation and highlights the potential of the Hh pathway as a therapeutic target for osteoporosis, lipodystrophy, diabetes, and obesity.

Introduction

Fat storing tissues play essential and conserved roles in invertebrates and vertebrates (McKay et al., 2003; Spiegelman and Flier, 2001). Since the roles of fat are ancient, the regulatory cascades that control fat storage may also be conserved. Although much has been learned about the late stages of adipocyte differentiation, relatively little is known about the conversion of undetermined mesodermal precursors into committed adipocytes (Rosen and Spiegelman, 2000). These still unidentified precursors may generate adipocytes and other mesodermal derivatives such as muscle, blood, and bone (Karsenty, 1998; Mikkola and Orkin, 2002; Molkentin and Olson, 1996). The adipose lineage is located throughout the body in stereotypic patterns and anatomical positions in both invertebrates and vertebrates, supporting the notion that developmental cues might be important for proper formation and function of adipose tissues (Cinti, 2000; Hoshizaki et al., 1994). Such information is often derived from developmental signaling pathways, such as Wnt, BMP, and Hh, that have been shown to be conserved from invertebrates to vertebrates (Cadigan and Nusse, 1997; Graff, 1997; Ingham and McMahon, 2001). Since the roles and anatomical distributions of fat are conserved, it is plausible that conserved developmental signaling pathways control aspects of fat biology.

The Hh pathway regulates the commitment of precursors into a diverse array of cell fates (McMahon et al., 2003). Secreted Hh proteins bind to a cell surface receptor complex consisting of the negatively acting Patched (Ptc) receptors (Ptc1 and Ptc2) and the seven transmembrane receptor Smoothened (Smo). The Hh signal is then conveyed from Smo to the nucleus, affecting gene expression via the ci/Gli family of transcription factors. Despite

the importance of the Hh pathway, its role in adipogenesis is unclear. Extant data support both a pro-adipogenic and an anti-adipogenic role (Buhman et al., 2004; Martin et al., 2002; Spinella-Jaegle et al., 2001; Sweet et al., 1996; van der Horst et al., 2003; Wang et al., 2002; Wu et al., 2004). These results were primarily derived in osteogenic culture conditions and from sufficiency tests, notorious for producing spurious results. Since the potential role of Hh on adipocyte formation has been inferred primarily as a byproduct of its effect on osteogenesis, key tests–including assessing necessity–in adipogenic cell lines, such as 3T3-L1s, might be informative (MacDougald and Lane, 1995).

I found that components of the Hh pathway were expressed in the Drosophila melanogaster fat body, the fly adipose tissue. Activating the Hh pathway decreased fly fat formation. In contrast, fat body-specific transgenic inhibition of Hh signaling increased fat formation. Hh components are also expressed in mammalian fat and their levels responded dynamically to adipogenesis and obesity. I found that Hh activation inhibited 3T3-L1, NIH-3T3, and pluripotent mesenchymal cell adipogenesis while blocking the Hh pathway stimulated mammalian adipogenesis, mirroring the fly results. Hh acts early in the adipogenic cascade, upstream of PPARγ, possibly by altering the potential fates that a precursor cell can adopt. In support of that, I found that Hh signals induced the expression of osteogenic markers in 3T3-L1 adipogenic cells and in pluripotent mesenchymal cells. Mechanistic studies suggest that the Hh signals repress adipogenesis by inducing anti-adipogenic transcription factors such as Gata2 and Gilz (Shi et al., 2003; Tong et al., 2000). In summary, the Hh pathway, in an evolutionarily conserved manner, inhibits fat formation.

Results

Hh signals blocks *D. melanogaster* fat formation

The fat body is the adipose organ of Drosophila melanogaster (Hoshizaki et al., 1994). To study fly fat, I initiated a broad two-component enhancer trap screen (minimal promoter-Gal4, UAS-GFP) selecting lines that expressed GFP in the fat body. One such line had the P-element inserted into the 5' UTR of smo, the Hh receptor, supporting the idea that the Hh cascade may regulate fly fat biology. To further explore this notion, I explanted the larval fat body, extracted RNA, and found that smo, ptc, and ci (Hh transcription factor) were all expressed in developing fly fat (Fig 1.1A). To investigate a potential cell autonomous function for Hh signals in fly fat formation, I specifically activated the Hh pathway in the fly fat body with UAS/Gal4 transgenesis (Brand et al., 1994). For this, I utilized a Dcg-Gal4 strain (kind gift of Dr. Charles Dearolf) that drives transgene expression in the fat body. To activate the Hh pathway, I crossed the Dcg-Gal4 line with a UAS-ci-3P strain containing an active ci (Wang et al., 2000). A potential drawback of this approach is that the Dcg-Gal4 driver is expressed relatively late during fat body formation, after cell proliferation ceases, potentially too late to observe effects on lineage specification. Nonetheless I utilized Dcg-Gal4 as it is the best characterized tool available (Hoshizaki et al., 1994). To visualize the fat body, I used a fat body specific Dcg-GFP reporter. Through a series of assays, I found that increasing Hh signaling reduced the expression of the GFP fat body reporter, fly fat accumulation, the size of the lipid droplets, and the expression of fat body molecular markers–HLH106 (fly SREBP homolog), and fly fatty acid synthase (Rosenfeld and Osborne, 1998) (Fig 1.1B-D). I also increased fat body Hh signaling by generating

transgenic flies that express UAS-hh and UAS-smo under the control of the Dcg-Gal4 driver. To examine the necessity of Hh signaling, I blocked the cascade with fat body transgenesis of dominant negative ci (DNCi) (Wang and Jiang, 2004). In a series of assays this produced the opposite outcomes produced by hh, smo, and ci transgenesis. For example, DNCi transgenesis increased the fluorescent intensity of the GFP fat body reporter (not shown). To quantify the changes induced by activating or inhibiting the cascade, I analyzed the triglyceride content of transgenic flies expressing either the active ci or the DNCi and compared the results to matched control flies. I found that active ci significantly decreased triglyceride accumulation while DNCi significantly increased triglyceride content (Fig 1.1E). These data are consistent with the notion that Hh signals function cell autonomously to decrease fly fat formation and the necessity results support an endogenous role.

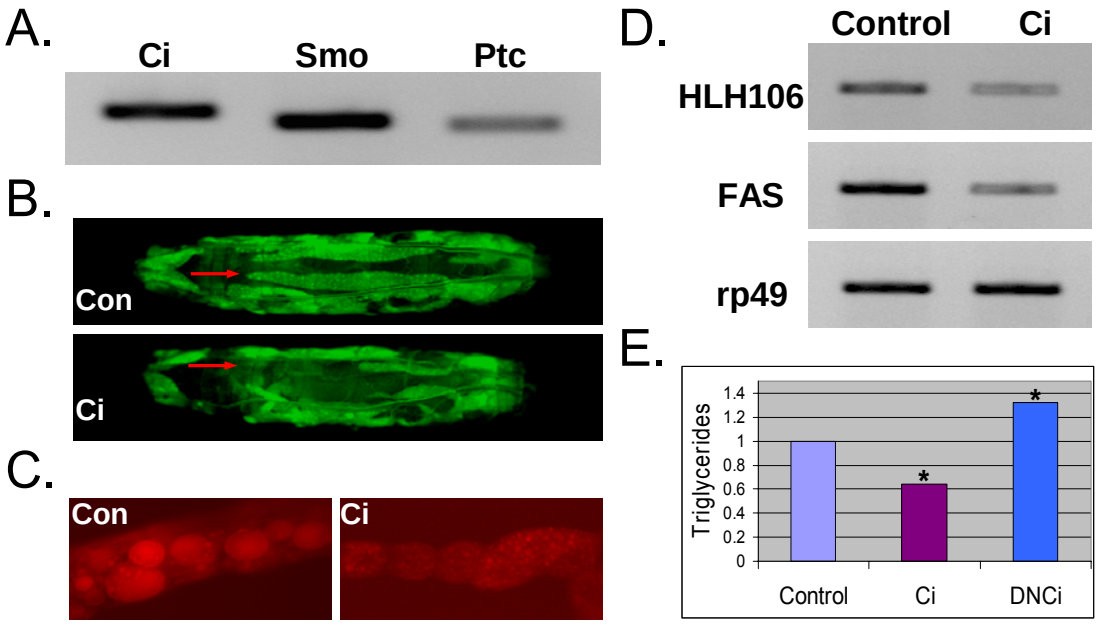


Figure 1.1. The Hh pathway blocks D. melanogaster fat formation.
(A) The fat body was dissected from 200 D. melanogaster 3rd instar larvae and the expression of the indicated components of the Hh pathway was assessed by RT-PCR. **(B-D)** An activated form of ci, the Hh transcription factor, was specifically expressed in the fat body with the Gal4-UAS system and fly fat formation was evaluated with a fat body-specific GFP reporter (B, red arrows indicate loss of dorsal fat), Nile Red, a fat specific stain (C), or molecular analysis of explanted fat bodies (D). HLH106 (fly SREBP) and FAS (fly fatty acid synthase) are fat body markers and rp49 serves as a loading control. **(E)** Triglyceride quantitation shows that active ci inhibits fly fat formation and that dominant negative ci (DNCi) increases fly fat formation. *$p<0.05$ by t-test

Hh cascade components are expressed in mammalian fat

To determine whether the inhibitory role of Hh signaling might be conserved in vertebrates, I evaluated Hh cascade component expression in mouse fat. I found that many of the Hh cascade components, including Smo, the negative regulatory receptors Ptc1 and Ptc2, and the Gli family of Hh transcription factors (ci homologs), were expressed in developing and adult fat tissues based upon RT-PCR, real-time PCR, and in situ hybridizations (not shown). I also examined expression in the widely studied adipogenic model–murine 3T3-LI tissue culture cells (MacDougald and Lane, 1995). These fibroblast-like cells, thought to resemble pre-adipocytes, when placed into appropriate induction media, change into fat-storing cells that express many adipocyte markers. I induced 3T3-LI cells to form adipocytes, extracted RNA during different stages of adipogenesis, and examined Hh pathway component expression. As a control, I analyzed the adipogenic transcription factor PPARγ, and as expected its levels increased (Fig 1.2A). I also found that during adipogenesis the expression of positively acting Hh components (Smo and the Glis) decreased, while expression of the inhibitory receptors, Ptc1 and Ptc2, increased (Fig 1.2A). This dynamic pattern of expression is consistent with the idea that Hh signals might block mammalian adipogenesis.

Hh signals inhibit mammalian adipogenesis

To test the potential role of the Hh pathway in mammalian fat, I induced 3T3-L1s to undergo adipogenesis in the presence of vehicle or recombinant Sonic Hh protein (Shh) and assessed adipogenesis microscopically, with Oil Red O staining, and with triglyceride quantitation. I

found that Hh blocked the morphological changes associated with adipogenesis; that is the cells retained the appearance of uninduced 3T3-L1s, and this was reflected by a marked reduction in Oil Red O staining and triglyceride accumulation (Fig 1.2B, C). I also increased Hh signaling by infecting 3T3-L1s with either a control virus expressing GFP or one expressing SmoA1, an activated form of the Hh receptor Smo (Taipale et al., 2000). Microscopy and Oil Red O stains showed that SmoA1 also reduced adipogenesis (Fig 1.2D). Next I evaluated levels of the adipogenic transcription factors C/EBPα and PPARγ, two genes, aP2 and adipsin, that are thought to mark the terminally differentiated state, and Pref-1, whose expression inversely correlates with adipogenesis (Rosen and Spiegelman, 2000; Sul et al., 2000). As a positive control for Hh activation, I examined expression of Gli1, which, in addition to being a Hh component, is a transcriptional target of the Hh signal (Marigo et al., 1996). As expected, Shh increased Gli1 expression (Fig 1.2E). The molecular analyses also showed that Hh signaling blocked the adipogenic program as evidenced by reduced expression of C/EBPα, PPARγ, aP2 and adipsin and increased Pref-1 expression (Fig 1.2E). Similar results to Shh treatment were obtained with viral overexpression of SmoA1 (Fig 1.2F).

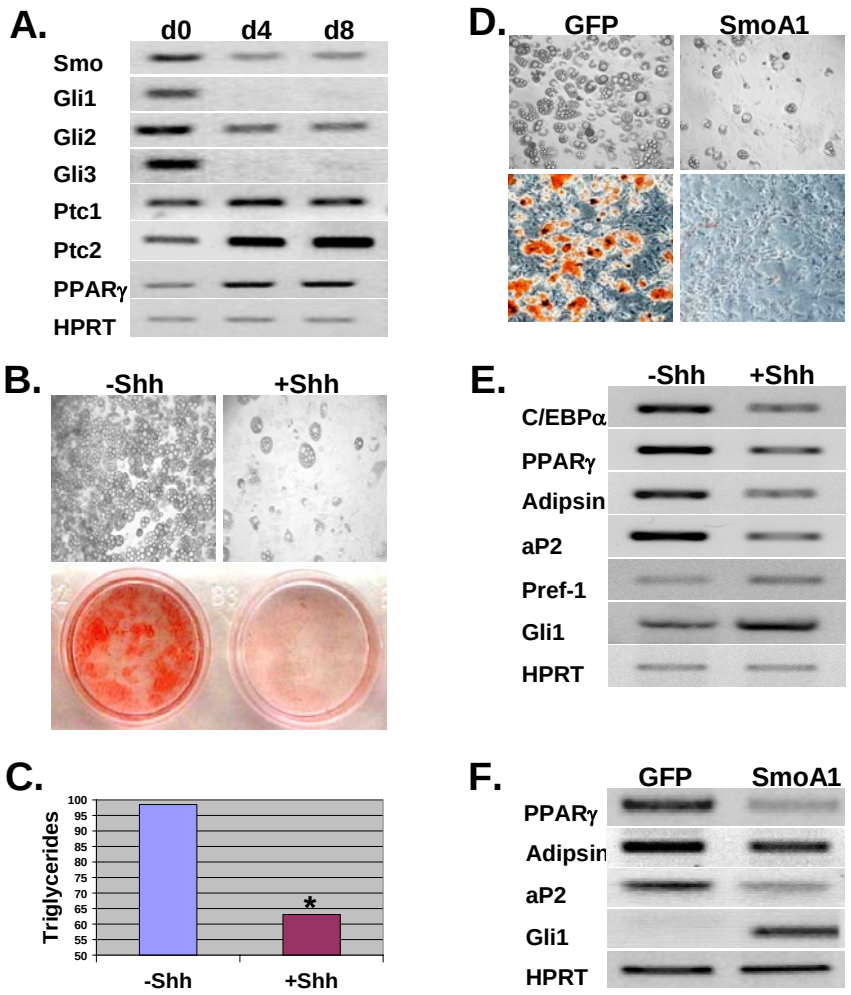


Figure 1.2. The Hh cascade inhibits 3T3-L1 adipogenesis.
(A) 3T3-L1 cells were incubated in adipogenic induction media, RNA extracted on days 0, 4, and 8, and semi-quantitative RT-PCR was done for the indicated transcripts. Expression levels of the positively acting Hh pathway components (Smo, Glis) decrease during adipogenesis, while the levels of the negatively acting components (Ptc1, Ptc2) increase. HPRT serves as a loading control. **(B, C)** 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh protein and fat formation was assessed based upon morphology (B), Oil Red O staining (B), and triglyceride quantitation (C). **(D)** 3T3-L1s were infected with a retrovirus expressing either GFP or SmoA1, an activated form of the Hh receptor Smo. Smo A1 reduced fat formation as scored either morphologically (top) or by Oil Red O staining (bottom). **(E, F)** RNA was extracted from induced 3T3-L1s treated with vehicle or Shh (E) or infected with GFP or SmoA1 expressing virus (F) and semi-quantitative RT-PCR was done for the indicated transcripts. C/EBPα and PPARγ are adipogenic transcription factors; adipsin and aP2 mark differentiated adipocytes. Pref-1 is a preadipocyte marker. HPRT serves as a loading control. Gli1 is a positive control for Hh pathway activation. *p<0.01 by test

Inhibiting Hh signaling increases mammalian adipogenesis

To complement the experiments to test sufficiency, I asked whether blocking Hh signals enhanced adipogeensis in 3T3-L1s. For this, I expressed either GFP or Gli2-ΔC4 (DNGli), a dominant negative form of the Hh transcription factor Gli2 (Sasaki et al., 1999). I found that reducing Hh signaling increased adipogenesis based upon light microscopy, Oil Red O staining, and molecular analyses (Fig 1.3A, B). Pharmacological inhibitors provide a distinct and alternative approach to blocking Hh signaling and provide therapeutic potential. KAAD-cyclopamine (KAAD) is a specific and selective inhibitor of Smo, the positively acting Hh receptor (Chen et al., 2002). To block the Hh pathway, I added either vehicle or KAAD to 3T3-L1 cells during adipogenic induction and found that KAAD, like DNGli, increased adipogenesis (Fig 1.3C).

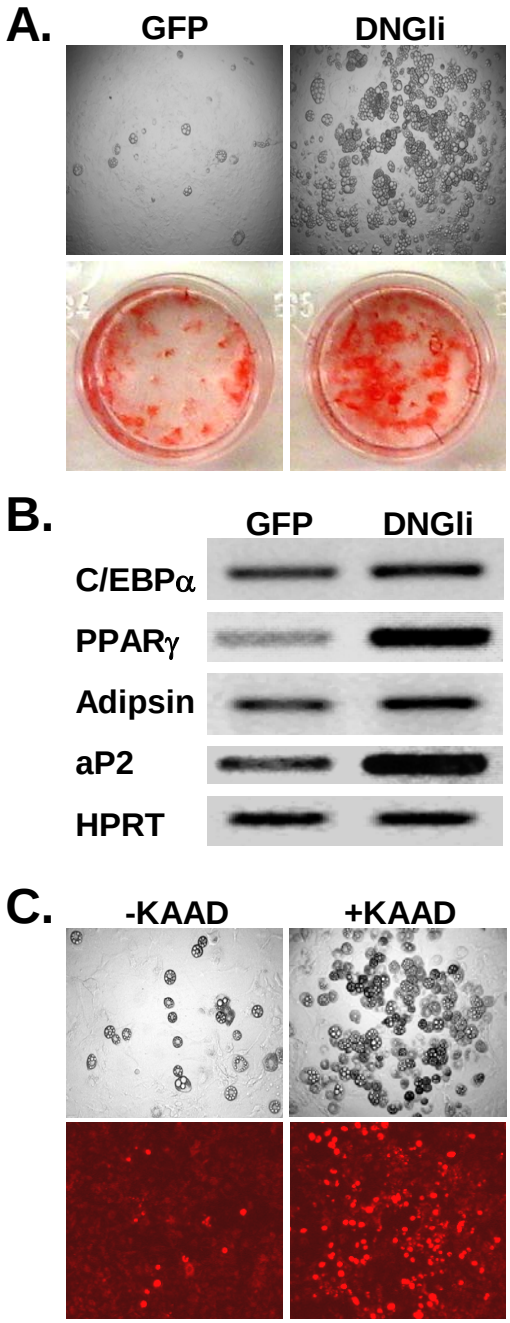


Figure 1.3. Inhibiting the Hh pathway stimulates 3T3-L1 adipogenesis.
(A, B) 3T3-L1s were infected with a retrovirus expressing either GFP or DNGli, a dominant negative form of Gli2, a Hh transcription factor. DNGli increased fat formation as assessed morphologically (A), by Oil Red O staining (A), or by molecular analyses (B). **(C)** 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-KAAD) or KAAD-cyclopamine, a Hh inhibitor. Fat formation was analyzed morphologically (top) and with Nile Red staining (bottom).

Hh inhibits fat formation early in adipogenesis

To begin to understand how Hh signals alter adipogenesis, I attempted to define the Shh-sensitive window of the inhibition of adipogenesis by adding vehicle or Shh to 3T3-L1s at two-day intervals during induction. I observed maximal inhibition of adipogenesis when Shh was added during the first three days of induction, although a slight effect was observed when Shh treatment began on day 4 (Fig 1.4A, B). Relatively little is understood about the mechanisms that regulate the early stages of adipogenesis during which the Hh pathway acts (MacDougald and Lane, 1995). However it is known that this is the time during which mitotic clonal expansion occurs (Tang et al., 2003). Since Hh can act as a proliferative signal in some contexts, it was possible that Hh signals inhibit adipogenesis by stimulating proliferation. To test this possibility, I added vehicle or Shh to 3T3-L1s and quantitated cell number each successive day in order to estimate proliferation; however, cell number was not significantly affected (Fig 1.4C). I also assessed proliferation with a quantitative BrdU assay (Gratzner, 1982). Again, there was no statistically significant difference in cell proliferation and BrdU incorporation between control and Shh-treated samples on any day examined based upon either inspection or quantitation of multiple independent fields in multiple experiments (Fig 1.4D, E). Furthermore, Rb expression and activity, which is tightly regulated by cell cycle, was not altered by Shh (see Fig 1.6A below). These data support the notion that Hh signals do not inhibit adipogenesis by altering proliferation. This lack of effect on cell proliferation in cell culture adipogenesis is in accord with the fly data; the Hh effects observed in flies were elicited independently of cellular proliferation as I activated the Hh pathway in post-mitotic fat body cells.

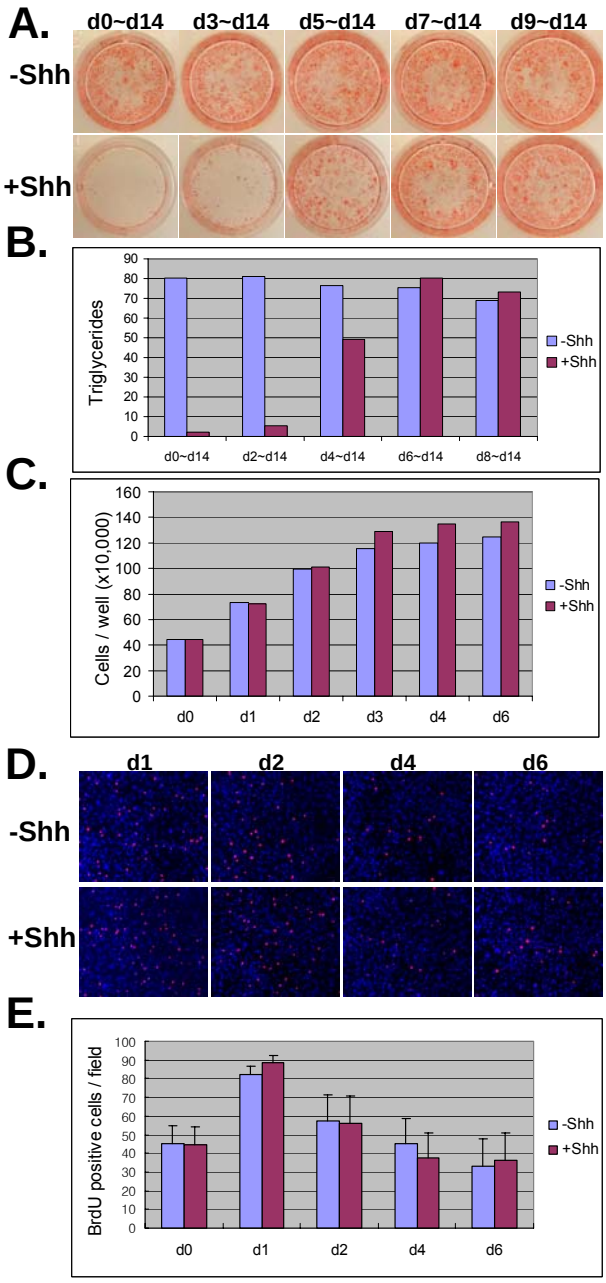


Figure 1.4. The Hh pathway acts early during 3T3-L1 adipogenesis.
(A, B) 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh and fat formation was scored with Oil Red O staining (A) and triglyceride quantitation (B). **(C, D, E)** 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh protein and cellular proliferation was evaluated with cell counts (C) or with a quantitative BrdU assay (D, E). No statistically significant changes were detected at any time point in either assay. BrDu positive cells are pink; DAPI staining in blue (D). Error bars represent SEM.

Hh signaling induces the expression of bone markers in 3T3-L1 cells

Since Hh signals inhibit 3T3-L1 adipogenesis during early time points, it is possible that the Hh pathway blocks progression from preadipocytes to fully differentiated fat, an idea supported by the Hh-dependent increase in expression of Pref-1, which marks preadipocytes (Fig 1.2E). Alternatively, the Shh signals may divert 3T3-L1 cells from adipogenesis and towards alternative fates not of adipocyte lineage. This latter possibility seemed less likely since 3T3-L1s are thought to be preadipocytes already committed toward the adipogenic lineage (MacDougald and Lane, 1995). However, the Hh pathway controls the specification of many mesodermal fates, for example promoting hematopoeisis, myogenesis, and osteogenesis (Gering and Patient, 2005; Li et al., 2004; Wu et al., 2004). To see whether Hh could induce 3T3-L1s toward any of these fates, I incubated 3T3-L1s in vehicle or Shh and analyzed expression of a panel of blood, muscle and bone markers. While no changes were observed in the levels of the blood or muscle markers (not shown), I found that Shh increased the expression levels of all four bone markers tested – two osteogenic transcription factors, Runx2 and Osx, and two markers of osteogenic differentiation, the parathyroid hormone receptor (PTHR) and alkaline phosphatase (ALP) (Fig 1.5A).

Hh inhibits adipogenesis and stimulates osteogenesis in multipotent mesenchymal cells

The data described above are consistent with the idea that Hh signals may regulate the potency of mesenchymal stem cells, promoting osteogenesis and inhibiting adipogenesis. To further explore this notion, I examined the effect of Hh on C3H10T1/2 (10T1/2) cells, a multipotent mesenchymal progenitor cell line that can be induced to form a wide variety of

fates including muscle, cartilage, bone, and fat, and consequently has been proposed to be a mesenchymal stem cell model (Taylor and Jones, 1979). To determine whether Hh retained its anti-adipogenic actions on 10T1/2s, I cultured the cells in adipogenic conditions with vehicle or Shh. Microscopy and molecular analyses showed that Hh blocked 10T1/2 adipogenesis (Fig 1.5B, C). To examine the necessity of Hh signals in blocking 10T1/2 adipogenesis, I cultured 10T1/2s in the presence of Hh inhibitor KAAD or vehicle. I found that KAAD stimulated 10T1/2 adipogenesis (Fig 1.5D). To determine if Hh might be pro-osteogenic to multipotent mesenchymal progenitors, I treated 10T1/2s with either vehicle or Shh and analyzed osteogenic marker expression. Just as in 3T3-L1s, Hh induced 10T1/2 osteogenesis, increasing expression of osteogenic transcription factors and markers of terminal differentiation (Fig 1.5E).

Hh signals function upstream of PPARγ

PPARγ, a master regulator of the adipocyte lineage, begins to be expressed in the time frame in which the Hh pathway acts (Fig 1.4A, B) (Rosen and Spiegelman, 2000) and Hh signals alter PPARγ expression (Figs 2, 3), raising the possibility that Hh acts upstream of PPARγ. To further assess the epistatic relationship between Hh and PPARγ, I infected 3T3-L1 cells with a GFP virus or a virus containing PPARγ Then, I treated the infected cells with Shh and evaluated adipogenesis. I found that PPARγ overcame the Shh-induced adipogenic blockade (Fig 1.5F). I also observed a PPARγ-dependent rescue of the SmoA1-induced inhibition of adipogenesis in NIH-3T3s (Fig 1.5F). These data support the notion that during adipogenesis Hh signals function upstream of PPARγ.

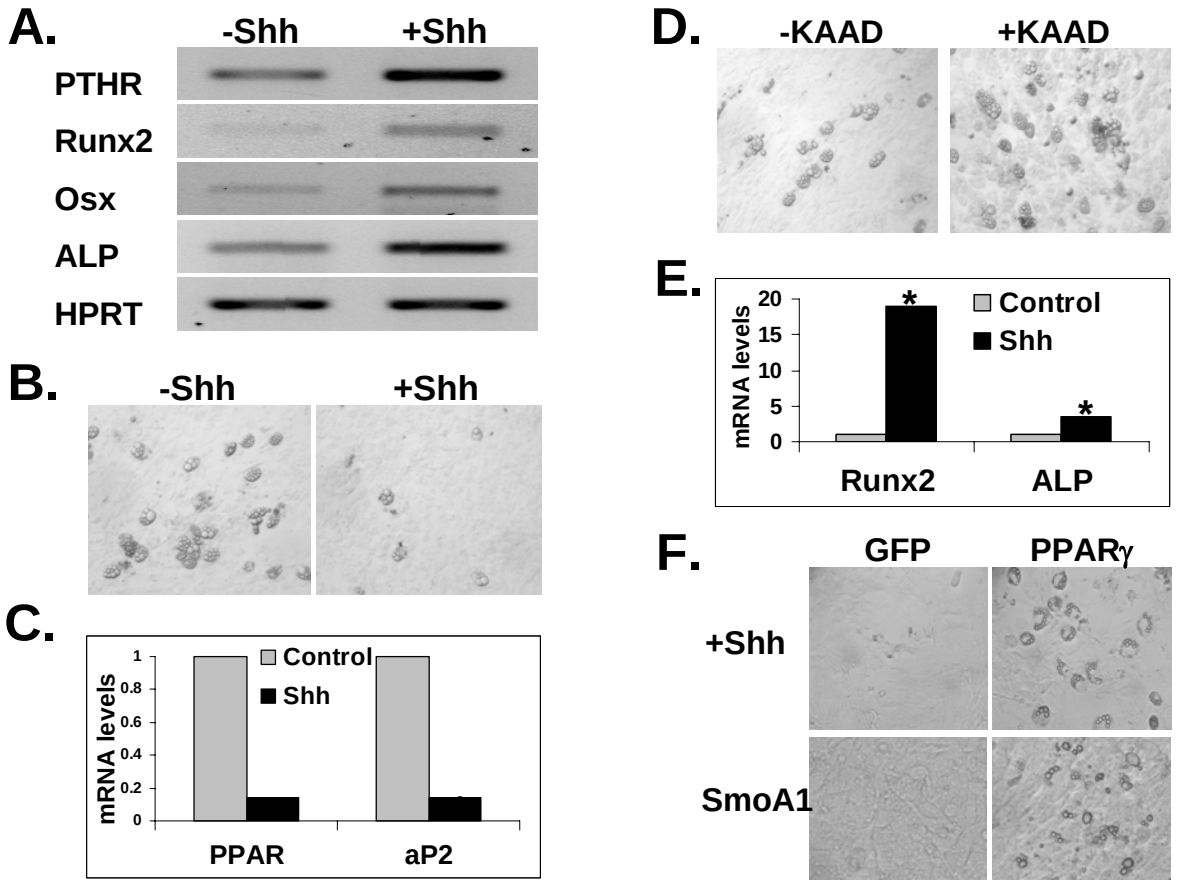


Figure 1.5. Hh signaling is anti-adipogenic and pro-osteogenic in 3T3-L1s and 10T1/2 pluripotent mesenchymal cells, and functions upstream of PPARγ.
(A) Hh signals induce the expression of bone markers in 3T3-L1s. 3T3-L1s were induced to form adipocytes in the presence of vehicle (-Shh) or Shh and the levels of the indicated osteogenic markers were assessed with semi-quantitative RT-PCR. HPRT serves as a loading control. **(B, C)** 10T1/2 multipotent mesenchymal cells were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh, which inhibited adipogenesis as scored morphologically (B) or with real-time PCR analysis (C). **(D)** 10T1/2s were incubated in vehicle (-KAAD) or KAAD (Hh antagonist) and morphology showed that KAAD stimulated adipogenesis. **(E)** 10T1/2s were incubated in adipogenic induction media with vehicle or Shh and osteogenic markers were assessed with real-time PCR. ß-actin serves as a loading control for real-time PCR (C, E). **(F)** 3T3-L1s were infected with a virus encoding either GFP or PPARγ and Shh protein was added throughout induction. PPARγ reversed the Shh-dependent inhibition of adipogenesis (top). NIH-3T3 cells expressing SmoA1, an activated form of the Hh receptor Smo, were infected with a virus encoding either GFP or PPARγ, and PPARγ rescued the SmoA1 blockade of adipogenesis (bottom). *p<0.01 by test

Hh signals require Gata to block adipogenesis

To further examine the mechanisms whereby the Hh pathway might regulate PPARγ expression and hence adipogenesis, I analyzed the relevant literature and identified four genes Rb, Gata2, Gata3, and Gilz that conformed to two criteria: 1) they are reported to regulate either PPARγ expression or adipogenesis and 2) they can be regulated by Hh signals.

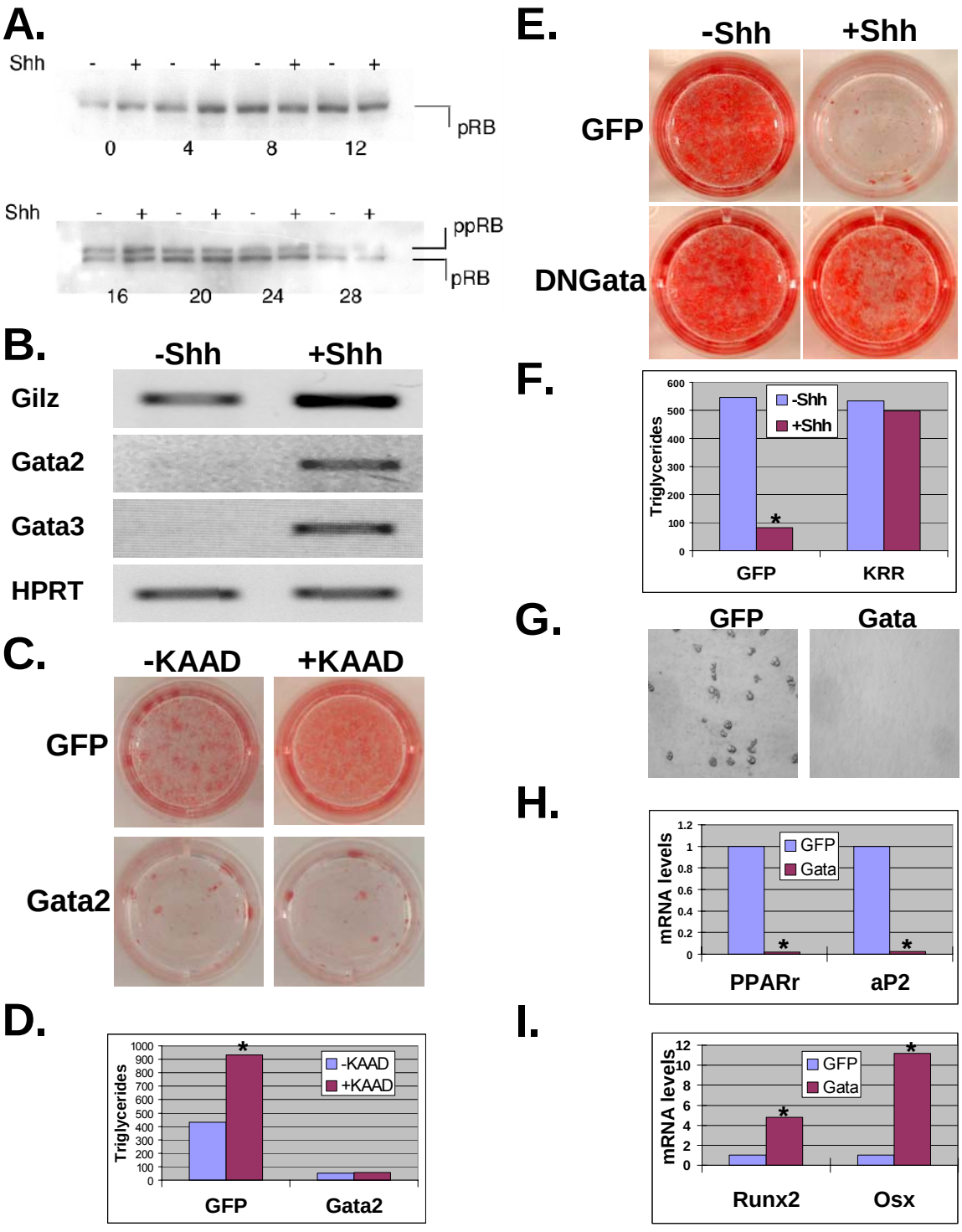
Rb, via cell cycle-dependent and -independent means, regulates adipogenesis and Hh signals can lead to the phosphorylation and inactivation of Rb (Chen et al., 1996; Duman-Scheel et al., 2002; Fajas et al., 2002; Hansen et al., 2004; Kenney and Rowitch, 2000). To test whether Hh inhibits adipogenesis by regulating the expression or phosphorylation (i.e. activity) levels of Rb, I incubated 3T3-L1s with vehicle or Shh, harvested cells at time zero and every four hours until 28 hours, and assessed Rb content and phosphorylation status by Western blotting in which the phosphorylated, inactive form of Rb migrates more slowly. By this test, it appeared that the Hh pathway did not alter the expression levels or activity of Rb (Fig 1.6A).

The Hh pathway controls specific neural fates by increasing the expression of Gata2 (Craven et al., 2004). Gata2 and Gata3 block adipogenesis by binding to and inhibiting the PPARγ promoter/enhancer (Tong et al., 2000). Gilz is a transcription factor that inhibits adipogenesis and microarray data indicate that Hh can induce Gilz in mesenchymal stem cells (Ingram et al., 2002; Shi et al., 2003). So it was plausible that Hh signaling inhibits fat formation by increasing the expression of the anti-adipogenic transcription factors Gata2, Gata3 and/or Gilz. To test this possiblity, I incubated 3T3-L1s with vehicle or Shh and

evaluated Gata2, Gata3, and Gilz levels. As expected, after adipogenic induction Shh increased expression of all three (Fig 1.6B).

These data suggest that Shh might elicit its anti-adipogenic effects by regulating Gata expression. If so, forced expression of Gata should block the effects of KAAD, a Hh inhibitor. To test this, I infected 3T3-L1 cells with a GFP or Gata2 virus and then placed the cells into adipogenic induction media containing vehicle or KAAD. In this assay, Gata2 inhibited the ability of KAAD to stimulate adipogenesis (Fig 1.6C, D). I also evaluated whether inhibiting Gata2/3 could prevent the Shh-dependent adipogenic blockade by infecting 3T3-L1s with GFP or a dominant negative Gata3 (DNGata) that blocks Gata2 and Gata3 (Smith et al., 1995). I induced the GFP or DNGata cells to become adipocytes in the presence of vehicle or Shh and found that inhibiting Gata2/3 overcame the Shh adipogenic block (Fig 1.6E, F). So Shh appears to function upstream of Gata factors to elicit its anti-adipogenic actions.

I also assessed Gata function in 10T1/2 cell fate specification by infecting 10T1/2s with a GFP or Gata virus. As in 3T3-L1s, Gata blocked 10T1/2 adipogenesis (Fig 1.6G, H) and reversed KAAD-stimulated adipogenesis (not shown), supporting the idea that Gata functions downstream of Hh for anti-adipogenesis. Since Gata factors are required for the anti-adipogenic functions of Hh, it seemed plausible that Gatas might also have Shh's pro-osteogenic actions. To test this, I examined 10T1/2s expressing GFP or Gata for levels of osteogenic markers. Molecular analysis showed that Gata stimulated 10T1/2 osteogenesis as evidenced by the increased expression of the osteogenic transcription factors Runx2 and Osx (Fig 1.6I).


A.
Shh
- + - + - + - +
pRB
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Shh
- + - + - + - +
ppRB
pRB
16 20 24 28
B.
-Shh
+Shh
Gilz
Gata2
Gata3
HPRT
C.
-KAAD
+KAAD
GFP
Gata2
D.
Triglycerides
-KAAD
+KAAD
GFP
Gata2
E.
-Shh
+Shh
GFP
DNGata
F.
Triglycerides
-Shh
+Shh
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KRR
G.
GFP
Gata
H.
mRNA levels
GFP
Gata
PPARr
aP2
I.
mRNA levels
GFP
Gata
Runx2
Osx

Figure 1.6. The Hh cascade requires Gata factors to inhibit fat formation.**(A)** 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-) or Shh (+), cell lysates were harvested at the indicated hours after Shh addition, and subjected to SDS-PAGE and Western blotting with anti-Rb antibodies. The phosphorylated and inactive Rb protein migrates slower than non-phosphorylated Rb and the levels of both forms are unaffected by Shh. **(B)** 3T3-L1 cells were induced to form adipocytes in the presence of vehicle or Shh and the levels of the indicated anti-adipogenic transcription factors were assessed with semi-quantitative RT-PCR. HPRT serves as a loading control. **(C, D)** 3T3-L1s infected with a retrovirus expressing either GFP or Gata2 were incubated in adipogenic induction media containing either vehicle (-KAAD) or KAAD (Hh antagonist). Based upon Oil Red O staining (C) and triglyceride quantitation (D), Gata2 inhibits adipogenesis even when the Shh pathway was blocked. **(E, F)** 3T3-L1 cells were infected with a GFP or a dominant negative Gata (DNGata) virus, incubated in adipogenic induction media containing either vehicle or Shh, and adipogenesis was assessed by Oil Red O staining (E) or triglyceride quantitation (F), both show that Gata inhibition reverses the Shh-dependent adipogenic blockade. **(G, H)** 10T1/2 multipotent mesenchymal cells were infected with a virus encoding either GFP or Gata, incubated in adipogenic induction media and adipogenesis was scored microscopically (G) and molecularly (H). **(I)** C3H10T1/2s expressing GFP or Gata were adipogenically induced and RNA was extracted. Real-time PCR showed that Gata induced the expression of the osteogenic transcription factors, Runx2 and Osx. ß-actin serves as a loading control for real-time PCR (H, I). *$p<0.01$ by test

Hh signals are altered in mouse models of obesity

The fly and cell culture data support the idea that the Hh pathway might be relevant to mammalian fat biology. Since changes in expression levels induced by physiological or pathophysiological cues often indicate functional importance, I examined potential changes in adipose tissue expression of the Hh signaling components produced by either genetic or diet-induced obesity (DIO). For DIO, I randomized 5-week old C57BL/6J littermates to four months of either normal or high fat chow, extracted RNA from fat depots, and examined expression with real-time PCR. I found that DIO significantly decreased expression levels of Smo, Gli1, Gli2, and Gli3 (Fig 7A), the four positively acting cell autonomous components of the Hh cascade. I also examined expression in fat tissues from 6-month old Ob/Ob genetically obese mice compared to matched controls and again detected an obesity-dependent decrease in Smo, Gli1, Gli2, and Gli3 expression (Fig 7B). The concordant expression patterns in two obesity models support the idea that Hh signals may be germane to fat pathophysiology.

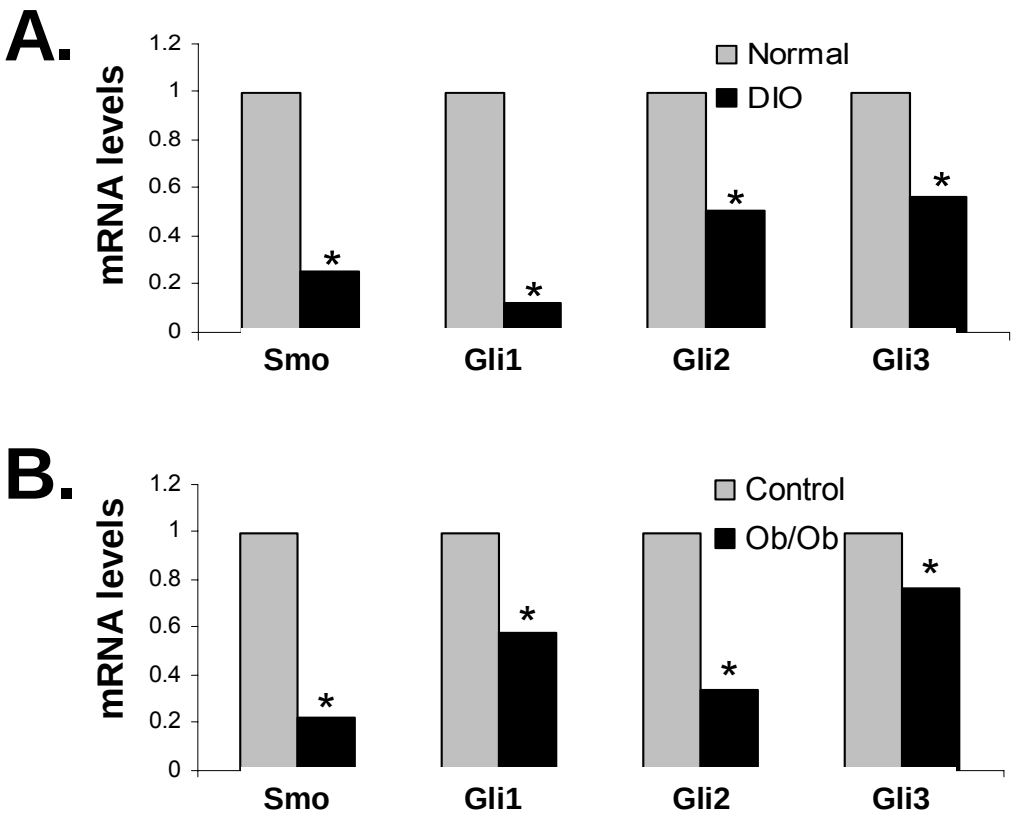

Figure 1.7. Murine obesity regulates the Hh pathway.
A) Matched littermates were fed four months of normal or high fat chow (DIO) and adipose depot gene expression was analyzed with real-time PCR, which demonstrated a statistically significant decrease in Smo, Gli1, Gli2, and Gli3 expression in DIO fat. n = 4. **B)** Smo and Gli1, Gli2, and Gli3 levels are reduced in genetically obese (Ob/Ob) fat depots compared to controls. n = 4. ß-actin serves as a loading control for real-time PCR. *P<0.05

Discussion

Adipose tissues play crucial roles in many biological processes and the ability to store fat is found over a vast evolutionary distance. Homologous molecules control aspects of worm and mammalian fat formation, so the mechanisms underlying fat biology, such as those that might be important in human diseases, may well be conserved (Ashrafi et al., 2003; McKay et al., 2003). Signaling pathways, such as BMP, Wnt and Hh, are conserved, not only in terms of the components that constitute the pathways, but also often conserved in their biological roles (Cadigan and Nusse, 1997; Graff, 1997; Ingham and McMahon, 2001). Wnt and BMP signals regulate mammalian adipogenesis, however the role of the Hh pathway was undefined (Ross et al., 2000; Sottile and Seuwen, 2000). Since a variety of small molecule activators and inhibitors of the Hh cascade have been developed (Bak et al., 2003) and because diseases of fat tissues, such as lipodystrophy, obesity, and diabetes, are major causes of morbidity and mortality, identifying a potential role for the Hh pathway in adipose biology could have therapeutic implications.

In this study, I provided evidence that the Hh pathway might play a conserved role in fat biology. I found that Hh pathway components were expressed in the fly fat body. Hh pathway components were also expressed in developing and adult mouse fat and their levels were regulated by adipogenesis and obesity. The function of the Hh pathway also appeared to be conserved as sufficiency and necessity tests support the notion that the Hh pathway blocks fly and mammalian fat formation.

Mechanistic studies showed that Hh signals blocked the early steps of adipogenesis, presumably after mitotic clonal expansion. Consistent with that temporal placement, epistasis

studies support the idea that Hh acts upstream of PPARγ. I also found that Hh signals increased the expression levels of three anti-adipogenic genes: Gata2, Gata3, and Gilz, (Ingram et al., 2002; Shi et al., 2003; Tong et al., 2000). This expression appeared to be functionally significant as Hh signals required Gata to elicit anti-adipogenic actions. So it is possible that Hh signals inhibit adipogenesis at least in part by regulating Gata expression.

Based upon the timing and epistasis results, it seemed plausible that Hh signals keep 3T3-L1s as preadipocytes or divert the cells to alternative fates. Molecular analyses support both notions; I observed an increase in the expression of Pref-1, a preadipocyte marker, and also of bone markers. I also found that Hh was anti-adipogenic and pro-osteogenic in C3H10T1/2 pluripotent mesenchymal cells. Gata appeared to have similar functions to Hh, but this observation does not exclude the possibility that other mechanisms downstream of Hh exist. It appears that the Hh cascade induces an anti-adipogenic program leading to down-regulation of the key adipogenic transcription factor PPARγ as well as a pro-osteogenic program inducing expression of osteogenic transcription factors such as Runx2. So Hh may act upon an unidentified population of mesenchymal stem cells to promote osteogenesis at the expense of adipogenesis. If so, the increase in fat and decrease in bone mass observed with aging might be accounted for by a reduction in Hh signals or may be reversed or prevented by activating the cascade.

Taken together, the results support the notion that the Hh pathway inhibits fat formation in a conserved manner from invertebrates to mammalian cellular models. Signaling pathways and receptors, especially seven transmembrane receptors such as Smo,

are often amenable to therapeutics as demonstrated by the identification of small molecules that can either activate of inhibit the Hh pathway (Bak et al., 2003; Chen et al., 2002; Taipale et al., 2000). My studies suggest that the Hh pathway might be an appropriate target for the treatment of fat and bone related disease such as lipodystrophy, obesity, diabetes and osteoporosis. The potential therapeutic value of Hh pathway modulators in the treatment of such disease in whole organisms awaits further evaluation.

Materials and Methods

Retrovirus production and infection.

Recombinant retroviruses (pMX, gift of Gary Nolan, and pLNCX2, Clontech) were generated by calcium phosphate transfection of the retroviral constructs into Phoenix packaging cells. Media was changed the day after transfection and viral supernatant was harvested at 48 and 72 hrs post-transfection. Viral supernatant was passed through a 0.2 uM filter, polybrene added to a final concentration of 8 μg/ml, and applied on successive days to pre-confluent 3T3-L1, NIH3T3 and C3H10T1/2 cells.

Cell culture and adipocyte differentiation.

Mouse NIH-3T3 fibroblasts, C3H10T1/2 pluripotent mesenchymal cells, and 3T3-L1 preadipocytes were purchased from the American Type Culture Collection and maintained in growth media (DMEM with 10% calf serum, 10 units/ml penicillin, 10 μg /ml streptomycin) at 37 °C in 5% CO2. Cells were passaged before confluence and discarded after 10 passages. Media changes were performed every other day during cell maintenance and adipogenesis. 3T3-L1 cells were induced to form adipocytes as described (MacDougald and Lane, 1995; McKay et al., 2003). Briefly, post-confluent 3T3-L1 cells were induced to form adipocytes by placing cells in induction media (growth media supplemented with 1 μg/ml insulin) and further maintained in induction media until analysis. For C3H10T1/2 and NIH-3T3 adipogenesis, post-confluent cells were placed in growth media containing 250 nM dexamethasone, 0.5 mM isobutyl-methyl-xanthine and 1 μg/ml insulin for 2 (C3H10T1/2) or 4 (NIH-3T3) days and then maintained in growth media supplemented with 1 μg/ml insulin

until analysis. Recombinant Shh (R&D systems) dissolved in 2% BSA or an equal volume of 2% BSA was added to the cells at the indicated times. Shh was added to cells at a final concentration of 300 ng/ml. KAAD-cyclopamine (Toronto Research Chemicals Inc.) dissolved in DMSO or an equal volume of DMSO was added to the cells at the indicated times. KAAD-cyclopamine was added to cells at a final concentration of 3.6 μM.

Analysis of lipid accumulation.

Lipid droplets in differentiated adipocytes were stained with Oil Red O and extracted stain was quantified as described previously (McKay et al., 2003; Ramirez-Zacarias et al., 1992). Briefly, monolayer cells were washed with PBS, fixed in buffered formalin, incubated in freshly prepared Oil Red O solution (four parts water mixed with six parts 0.5% Oil Red O in isopropanol) for 30 min, and washed several times with water to remove excess stain. Oil Red O was extracted from stained cells with isopropanol and absorbance (600nm) was measured to quantify stain. To quantify triglyceride levels, flies or cells were lysed in 0.5% SDS/PBS and triglyceride content was measured using the Infinity Triglyceride Reagent (Sigma) following manufacturer's instructions. Protein concentrations used to normalize triglyceride content were measured with a BCA protein assay kit (Pierce).

RNA extraction and RT-PCR.

Total RNA from flies, mouse perigonadal fat pads or cultured cells was extracted with Trizol (Invitrogen), DNAse I-treated, and reverse-transcribed using random hexamers and MMLV reverse transcriptase (Invitrogen) to obtain cDNA. Gene expression was

analyzed using either semi-quantitative RT-PCR (McKay et al., 2003) or real-time PCR using SYBR Green Master Mix reagent (Applied Biosystems, 7500 Real-Time PCR System). For the semiquantitative assays, two concentrations of cDNA template were used to demonstrate that the reaction conditions were semi-quantitative. Real-time PCR values for gene expression were normalized over beta-actin expression.

BrdU proliferation assay.

Cells were incubated in growth media containing BrdU (10 μg/μl) for two hours on the indicated day of differentiation. Then the cells were rinsed in PBS and fixed in 4% paraformaldehyde. Immunocytochemistry using anti-BrdU was performed to identify labeled cells and DAPI staining identified nuclei. BrdU positive cells were counted from 10 random fields and averaged for analysis.

Cell lysate preparation and Western blot.

Monolayer cells were washed twice with ice-cold PBS, scraped into 0.2 ml of lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 100 mM NaF, 0.2 mM Na-orthovanadate, 0.5% NP-40, 1.5 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, 10 mM benzamidine, 1 μg/ml pepstatin A, 10.5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and incubated for 20 min on ice with intermittent vortexing. After a brief centrifugation, the supernatant was mixed with Laemmli sample buffer and resolved by SDS-polyacrylamide (10%) gel electrophoresis. Resolved proteins were blotted

onto a PVDF membrane (Immobilon-P, Millipore) and proteins were detected with ECL (Amersham Corp.). Rb antibodies were from BD biosciences.

Fly experiments.

The Gal4/UAS system (Brand et al., 1994) was utilized for tissue-specific activation or inhibition of the Hh pathway. 3rd instar larval fat bodies were dissected in PBS under a microscope and lipid content of fat bodies was analyzed by Nile Red staining as described (McKay et al., 2003) and triglyceride assays as described above. Fat body gene expression levels were analyzed by semi-quantitative RT-PCR as described above.

Mouse studies.

Pure inbred C57BL/6J mice and pure inbred C57BL/6J Ob/Ob mice were purchased from the Jackson labs. Mice were housed in a 12:12 light:dark cycle and chow and water were provided ad libitum. For diet-induced obesity I mated the inbred C57BL/6J mice and then randomized 5-week old C57BL/6J littermates to four months of either normal (4% fat, Teklad) or high fat chow (60% fat, Research Diets). After four months on the appropriate diet, RNA was extracted from identical fat depots from all mice in the cohorts and molecular analyses were done as described above. Ob/Ob genetically obese mice and matched controls were fed normal chow and at six months of age, gene expression was analyzed in identical fat depots explanted from all mice in the cohort as described above. Veterinary care was provided by the Division of Comparative Medicine. All animals were maintained under the

guidlines of the U.T. Southwestern Medical Center Animal Care and Use Committee according to current NIH guidelines.

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Chapter II

Adp is a Conserved, Dosage-sensitive, Cell Autonomous Anti-obesity Gene

Abstract

The rapidly increasing prevalence of obesity and its sequelae are a major public health problem. Genes that function within fat cells are likely to contribute to obesity, yet such genes have been difficult to identify. Adipose (Adp) is an evolutionarily conserved gene isolated from naturally occurring obese flies homozygous for the *adp* mutation. Here I show that Adp function is conserved from flies to mammals and that it functions as a dosage-sensitive, cell autonomous anti-obesity gene. Adp heterozygous flies are obese and display intermediate levels of fat between wild-type and Adp nullizygous flies. Adp heterozygous mutant mice are also obese and insulin resistant, whereas the fat-specific expression of a dominant negative Adp protein produces mice that are obese and glucose intolerant. Conversely, fat-specific Adp transgenic mice are lean and display improved metabolic profiles compared to controls. These data demonstrate that Adp plays an evolutionarily conserved role in inhibiting fat formation and highlight the potential of the Adp pathway as a therapeutic target for obesity, diabetes and lipodystrophy.

Introduction

An essential feature of life is the ability to acquire or generate the requisite energy to sustain the animal life cycle. The evolution of multicellularity and then tissue specification was aided by the development of dedicated energy stores often harbored as fat, which is required for the life cycle of essentially all higher organisms. Many of the roles of fat storing tissues, from energy homeostasis to control of lifespan, are conserved from invertebrates to vertebrates, so it is plausible that the underlying mechanisms may also be related (Bluher et al., 2003; Campbell and Dhand, 2000; Hwangbo et al., 2004; Rosen and Spiegelman, 2000; Spiegelman and Flier, 1996). This may include genes, often termed "thrifty", designed to maximize energy storage as needed to handle the common threat of famine or reduced food availability (Doane, 1960a; Hader et al., 2003). Genes may also have evolved to restrain fat accumulation, as excess fat might be deleterious in times of plenty (Doane, 1960a). Thus endemic natural cycles of feast and famine may have selected for genes that regulate energy homeostasis in lower animals and orthologous genes may function similarly in mammals. Identifying genes with conserved functions may help identify central components of metabolism and may lead to important insights in our understanding and treatment of relevant human diseases such as diabetes.

Obesity and diabetes are caused by a complex and still poorly understood interaction between behavior, environment, and genetics. The genetic predispositions to both conditions are very strong and many genomic susceptibility intervals have been

uncovered in mice and humans, yet relatively few genes that predispose to either condition have been identified (Rankinen et al., 2006). Given the large number of identified intervals, it is possible that a collection of polymorphisms that only mildly affected gene function are important in determining susceptibility to obesity or diabetes. Although most genes that underlie human obesity remain unknown, genes that underlie the rare cases of homozygous recessive obesity have been found and these genes normally function in the hypothalamus to suppress appetite (Farooqi et al., 2003; Montague et al., 1997). However, it appears clear that the genes underlying the rare inherited syndromes do not account for the preponderance of human obesity. Notably, there is evidence suggesting the importance of monogenic mutations of obesity in humans or mice that function in the fat cell *per se*, such as ones that are found in the PPARγ gene (Cecil et al., 2006; Deeb et al., 1998; Lehrke and Lazar, 2005; Picard et al., 2004) . Unfortunately, such cell autonomous anti-obesity (or anti-adipogenic) genes have been difficult to identify. In contrast, the majority of well established pro-adipogenic genes (e.g., C/EBP, SREBP, and PPARγ) act in the fat cell *per se* (Rosen and Spiegelman, 2000; Smas and Sul, 1995). Therefore, identifying genes that function autonomously in adipose cells to restrain fat accumulation hold forth the promise of a mechanistic understanding of obesity, and, as a result, the discovery of potential therapeutic targets.

Fruit flies are an important and powerful invertebrate model system for the discovery and analysis of genes critical to human health and disease (Chien et al., 2002; Reiter et al., 2001; Ruden et al., 2005). These advantages have yet to be fully applied to

fat biology, in part, because of concerns that important differences may exist between fly and human fat. This includes the observation that in Drosophila fat is stored in the fat body, which is not a dedicated adipocyte but rather also has liver-like functions and plays a central role in immunity. Molecular studies also highlight potential differences between fat storing tissues in flies and mammals. For example, the fly gene *srp*, a Gata class transcription factor, is a pro-adipogenic factor required for fly fat formation, yet mammalian Gata homologs inhibit, rather than stimulate, mammalian cell culture adipogenesis (Tong et al., 2000). Further, flies lacking C/EBP or SREBP, key mammalian adipogenic transcription factors, have relatively normal fat storage (personal observations, data not shown). Yet flies do contain homologs for many key components of mammalian lipid storage and metabolism, including lipogenic and lipolytic enzymes, neuropeptides, and serotonergic and insulin signaling pathways. Additionally, I have shown in the previous chapter (Chapter I) that at least one signaling mechanism, hedgehog (Hh) cascade inhibition of fat formation, is conserved between fly fat and mammalian adipogenic cell culture models. Although conserved functions were shown for Hh pathway, which has effects on virtually all tissues, it is not clear if this is a dedicated role for fat biology and whether flies can serve as a suitable model. Notably, no gene has yet been discovered based upon a fat or lean fly that has then been shown to have the homologous effect *in vivo* in mammals. The discovery of such a gene would support the idea that fly and mammalian fat storage may be related at the mechanistic level and would strengthen the idea that the *D. melanogaster* model can be exploited to help combat the epidemic of obesity and diabetes.

Natural populations provide an enormous resource of potential mutants or polymorphisms that affect various developmental and physiological processes. Over 40 years ago, Winifred Doane hypothesized that climates marked by repetitive cycles of famine might select for organisms that are highly efficient at fat storage to allow for survival during times of limited food. In Kaduna, Nigeria, Dr. Doane successfully isolated just such an obese D. melanogaster mutant she termed Adipose (Adp)(Doane, 1960a). Recently, Brönner and colleagues cloned the fly Adp gene, which encodes a novel protein conserved as a single copy from flies to humans, consisting of six WD40 and three TPR motifs and as such appears to be a scaffold or docking protein (Hader et al., 2003). WD40 and TPR domains, defined by the arrangement and conservation of specific amino acids, mediate protein-protein interactions and proteins that contain such domains often regulate assembly and function of multi-protein complexes, many of which control signal transduction or gene expression (D'Andrea and Regan, 2003; Smith et al., 1999). In flies, fat-specific Adp transgenesis rescued $Adp^{-/-}$ obesity and decreased wild-type fly fat storage (Hader et al., 2003), implying that the endogenous function of Adp is to restrain fat accumulation within the fat cell. Identification of such cell autonomous anti-obesity genes has been quite uncommon, indicating that Adp, if conserved, might provide important and potentially clinically relevant insights into human fat biology.

In times of plenty, homozygosity of the Adp mutation, and its attendant obesity, can confer some potential negative consequences resulting in reduced fitness, e.g., decreased flying ability (Doane, 1960a), much as obesity-predisposing

polymorphisms/mutations do in humans. I hypothesized that heterozygosity at the Adp locus might produce an intermediate increase in fat accumulation, offering benefits to the population in times of famine, while mitigating some of the deleterious consequences of homozygosity. In support of this hypothesis, I found that Adp heterozygotes display metabolic phenotypes intermediate between wild-type and Adp homozygous mutant flies. Since haploinsufficiency often indicates that a molecule is a key regulator of the process of interest (Favor, 1995; Rankinen et al., 2006) and because Adp possessed several interesting features–a primary structure that is consistent with the notion that it may be a node of information, an evolutionary conserved gene found in single copy from flies to humans, and potential fat autonomous anti-obesity function – I decided to assess whether the inhibitory role on fat formation was conserved from flies to mammalian systems. In both sufficiency and necessity tests, I found that Adp inhibited mammalian cell culture adipogenesis: expressing Adp blocked mammalian cell culture adipogenesis and inhibiting Adp with RNAi stimulated adipogenesis. To extend the data to a relevant *in vivo* mammalian model, I generated mutant mice with LacZ inserted into the Adp locus. Strikingly, Adp heterozygous mutants, like Adp heterozygous flies, were obese and insulin resistant compared to their wild-type littermates. I also generated aP2-Adp fat-specific transgenic mice that had a marked reduction in fat accumulation, in spite of normal food intake, and improved metabolic parameters such as increased glucose sensitivity. Conversely, mice with fat-specific inhibition of Adp function were obese and hyperglycemic. Thus Adp is the first example of a conserved anti-obesity gene that functions in a cell-autonomous and haploinsufficient manner.

Results

Adp has Dosage-Sensitive Roles in Fly Fat Biology

Adp homozygous mutant flies are obese and starvation resistant, but the homozygous state also has some negative consequences (Doane, 1960a). For example, the Adp homozygous mutants have decreased mobility compared to wild-type flies (not shown). So it seemed plausible that Adp heterozygosity might engender some beneficial effects while minimizing deleterious sequelae. Although it is unusual to identify such haploinsufficient phenotypes, they often indicate importance of the gene in the process of interest, so I evaluated the fat biology of Adp heterozygous flies with a battery of tests, including triglyceride measures, molecular marker analyses, and starvation survival, done as I described (See Fig 1.1 and Material and Methods, Chapter I), and found a phenotype intermediate between wild-type and Adp nulls. For example, in a starvation survival assay, which correlates with fat content (Djawdan et al., 1998), survival was $Adp^{-/-}$ > $Adp^{+/-}$> $Adp^{+/+}$ (Fig 2.1A). Triglyceride quantitation also revealed the same trend with the starvation assay, as an inverse relationship between fat content and Adp gene dose was observed (Fig 2.1B). These data show that Adp has dose-dependent effects on fly fat storage.

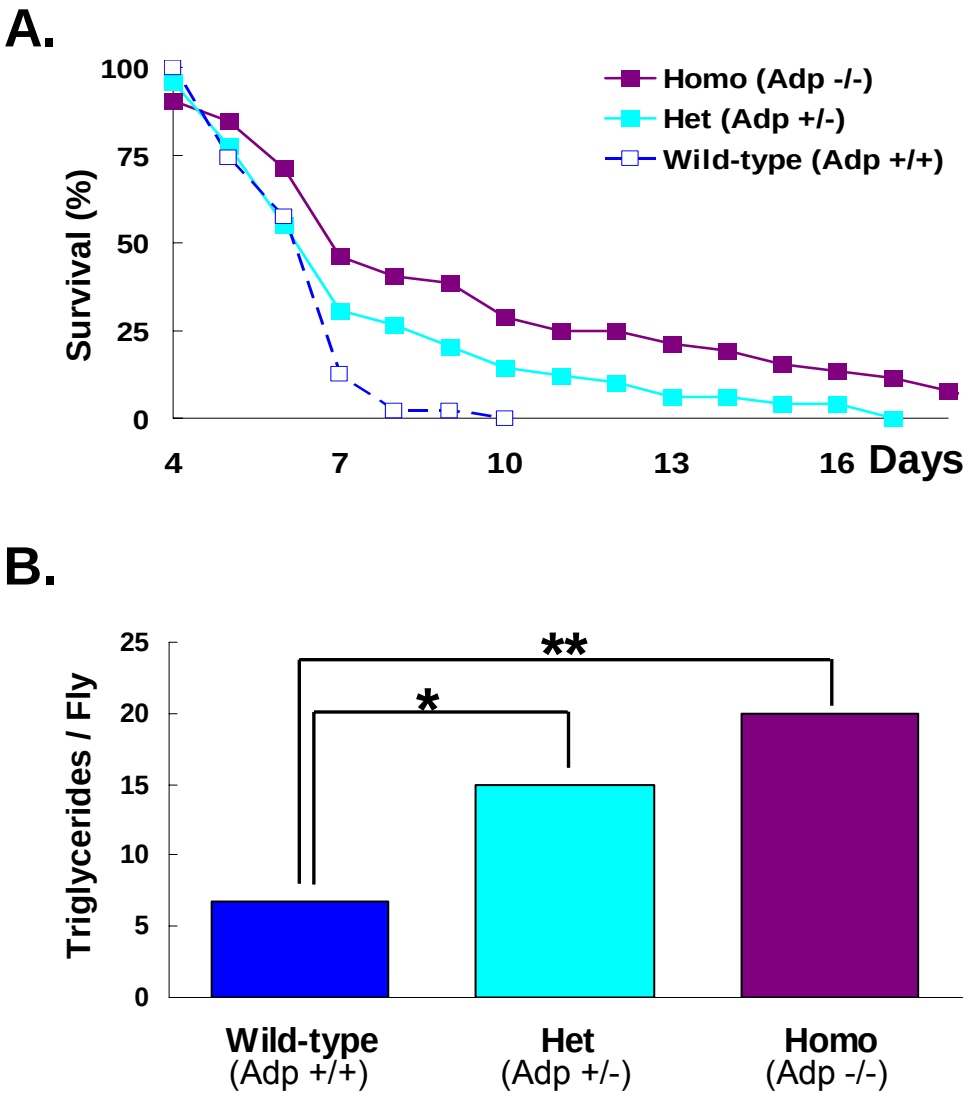


Figure 2.1. Adp inhibits fly fat accumulation in a dosage-sensitive manner. **(A)** Well-fed $Adp^{+/+}$ wild-type, $Adp^{+/-}$ heterozygous (Het), and $Adp^{-/-}$ homozygous (Homo) adult sibling flies (n > 100 per genotype, experiment was repeated 3 times) were deprived of food and survival was plotted versus time. The survival curves show a haploinsufficient response to starvation. **(B)** Triglycerides were extracted and quantified from well-fed adult $Adp^{+/+}$ wild-type, $Adp^{+/-}$ heterozygous, and $Adp^{-/-}$ homozygous mutant flies (n = 10 per genotype, experiment was repeated 4 times). The results demonstrate that $Adp^{+/-}$ heterozygotes have an intermediate level of fat accumulation between wild-type and obese $Adp^{-/-}$ homozygotes. *$p \leq 0.05$; **$p \leq 0.01$ by t-test.

Adp Inhibits Murine Adipogenesis

Database searches revealed that mice and humans contain a single structural homolog of Adp, which if functionally conserved, might act as a mammalian "anti-obesity" gene. Both NIH3T3s and 3T3-L1s, a clonal derivative of NIH3T3 cells, are murine tissue culture models of adipogenesis and in the presence of the appropriate hormonal milieu can differentiate from fibroblasts to fat storing cells that express a comprehensive set of adipocyte markers. To examine whether Adp has a role in mammalian adipogenesis, I infected 3T3-L1 cells with retroviruses expressing either murine Adp or a GFP control. When incubated in adipogenic induction media, the control GFP cells became lipid-laden adipocytes as evidenced by morphology, fat-specific stains and triglyceride quantitation (Fig 2.2A, B). In contrast, the Adp cells accumulated very few fat droplets, retained pre-adipocyte morphology and the fat-specific stains and triglyceride quantitation showed that Adp blocked adipogenesis (Fig 2.2A, B). Furthermore, Adp inhibited the expression of a panel of adipogenic markers including the adipogenic transcription factors PPARγ and C/EBPα, aP2, which marks fully differentiated adipocytes, and two adipokines, adipsin and leptin, that are key components of mature fat cell function (Fig 2C)(Rosen and Spiegelman, 2000; Smas and Sul, 1995). I also detected a commensurate increase in expression of Pref1, whose expression inversely correlates with adipogenesis (Fig 2.2C)(Sul et al., 2000). Adp also strongly inhibited NIH-3T3 adipogenesis (Fig 2.2D, E). Taken together, these data are consistent with the idea that Adp inhibits mammalian cell culture adipogenesis.

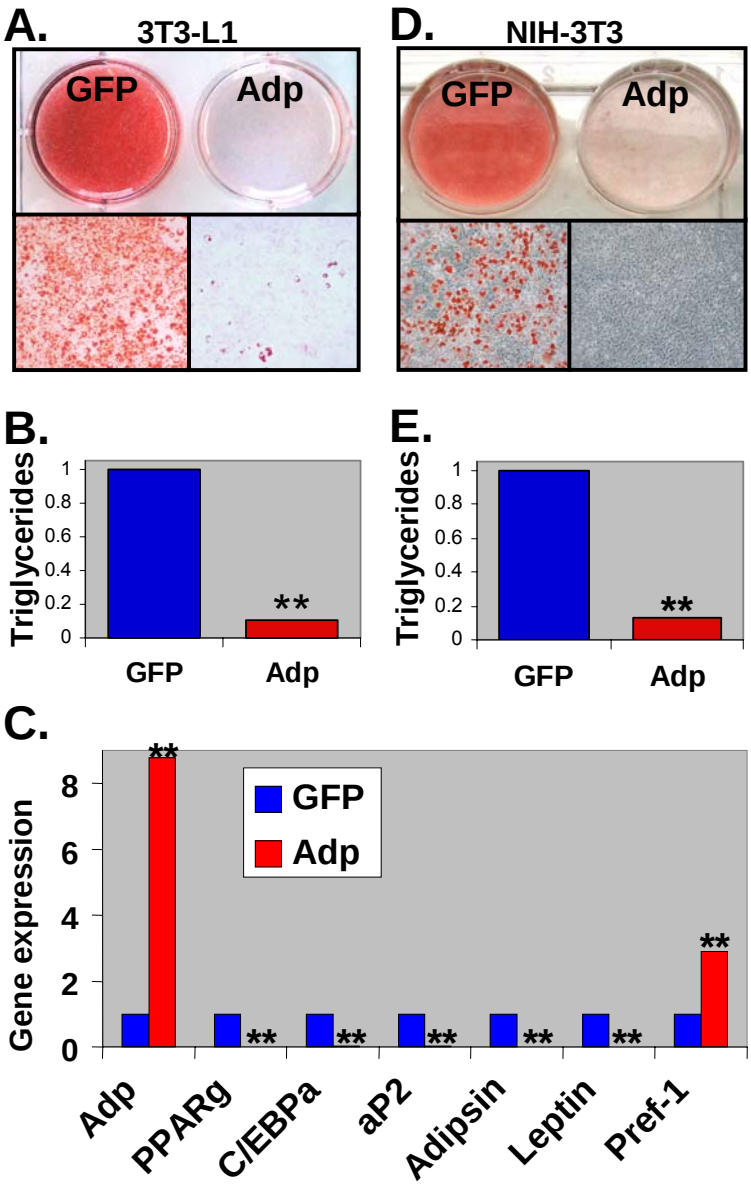


Figure 2.2. Adp blocks murine adipogenesis.
3T3-L1 cells **(A-C)** and NIH-3T3 cells **(D, E)** were infected with a virus encoding GFP or Adp, adipogenically induced and adipogenesis was evaluated with **(A, D)** Oil Red O staining (Fat stains red; low magnification upper panel, high magnification lower panel), **(B, E)** triglyceride quantitation, and **(C)** molecularly. For the molecular analysis, RNA was extracted from induced 3T3-L1s infected with a GFP or Adp expressing virus and real-time PCR was done for the indicated transcripts. C/EBPα and PPARγ are adipogenic transcription factors; aP2 marks differentiated adipocytes, adipsin and leptin are adipokines expressed by mature adipocytes. Pref-1 is a preadipocyte marker. **p ≤ 0.01 by t-test

Inhibiting Adp Stimulates Murine Adipogenesis

To begin to examine the endogenous role that mAdp might play in mammalian adipogenesis, I turned to necessity tests. For this, I generated, with methods I described in Chapter I, NIH-3T3 cells that expressed control or Adp RNAi and confirmed that Adp RNAi specifically reduced mAdp expression (Fig 2.3C). Strikingly, reducing endogenous Adp provoked the entire program of adipogenesis, as assessed by Oil Red O staining, triglyceride quantitation, and molecular analyses (Fig 2.3A-C). Taken together, these data suggest that Adp is a molecular switch for mammalian cell culture adipogenesis, inhibiting the adipogenic program when expressed, and releasing the adipogenic program when its expression is reduced, mirroring the fly phenotypes and supporting the idea of cell autonomous anti-obesity functions.

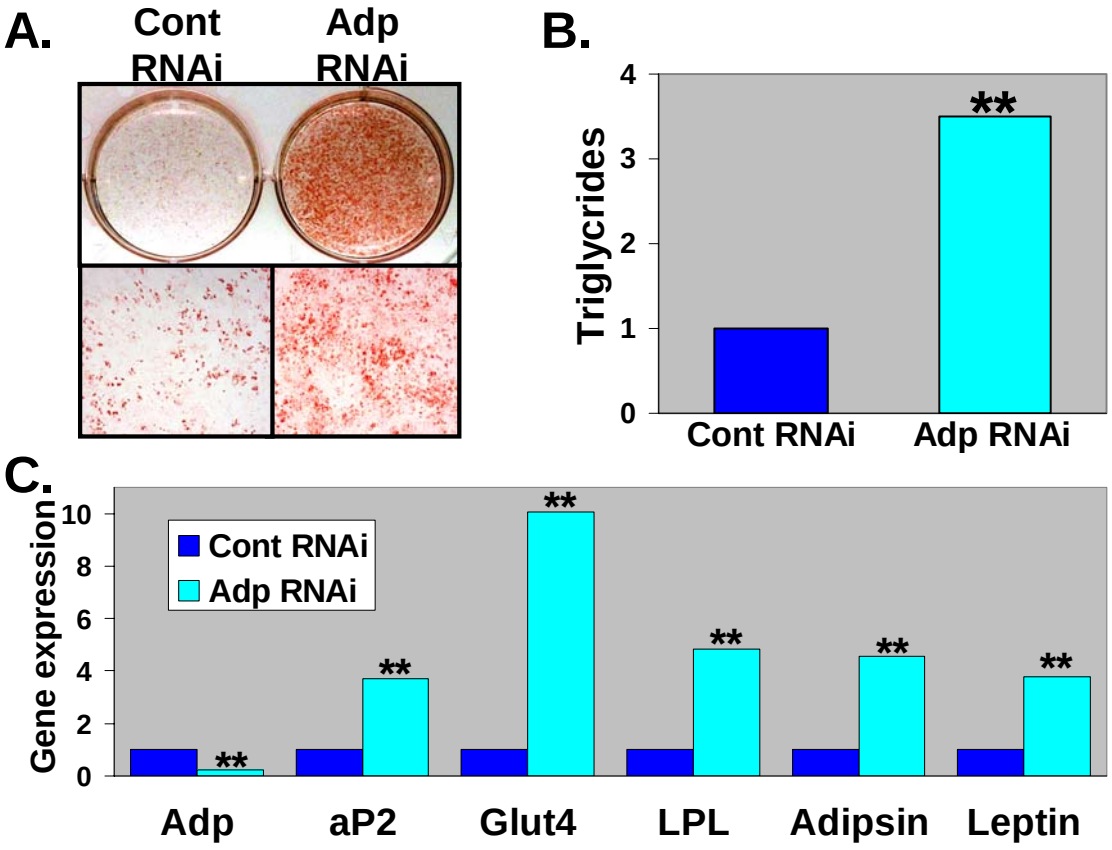


Figure 2.3. Inhibiting Adp stimulates mammalian adipogenesis.
NIH-3T3 fibroblastic cells containing either a control shRNA (Cont RNAi) or an Adp shRNA (Adp RNAi) cassette were evaluated for adipogenesis with **(A)** Oil Red O staining, **(B)** triglyceride quantitation, and **(C)** gene expression analyses as in Figure 2.2. aP2, glucose transporter 4 (Glut4), and lipoprotein lipase (LPL) are components of differentiated adipocyte function; adipsin and leptin are adipokines expressed by mature adipocytes. **p < 0.01 by t-test

Adp$^{+/-}$ Heterozygous Mice are Obese and Insulin Resistant

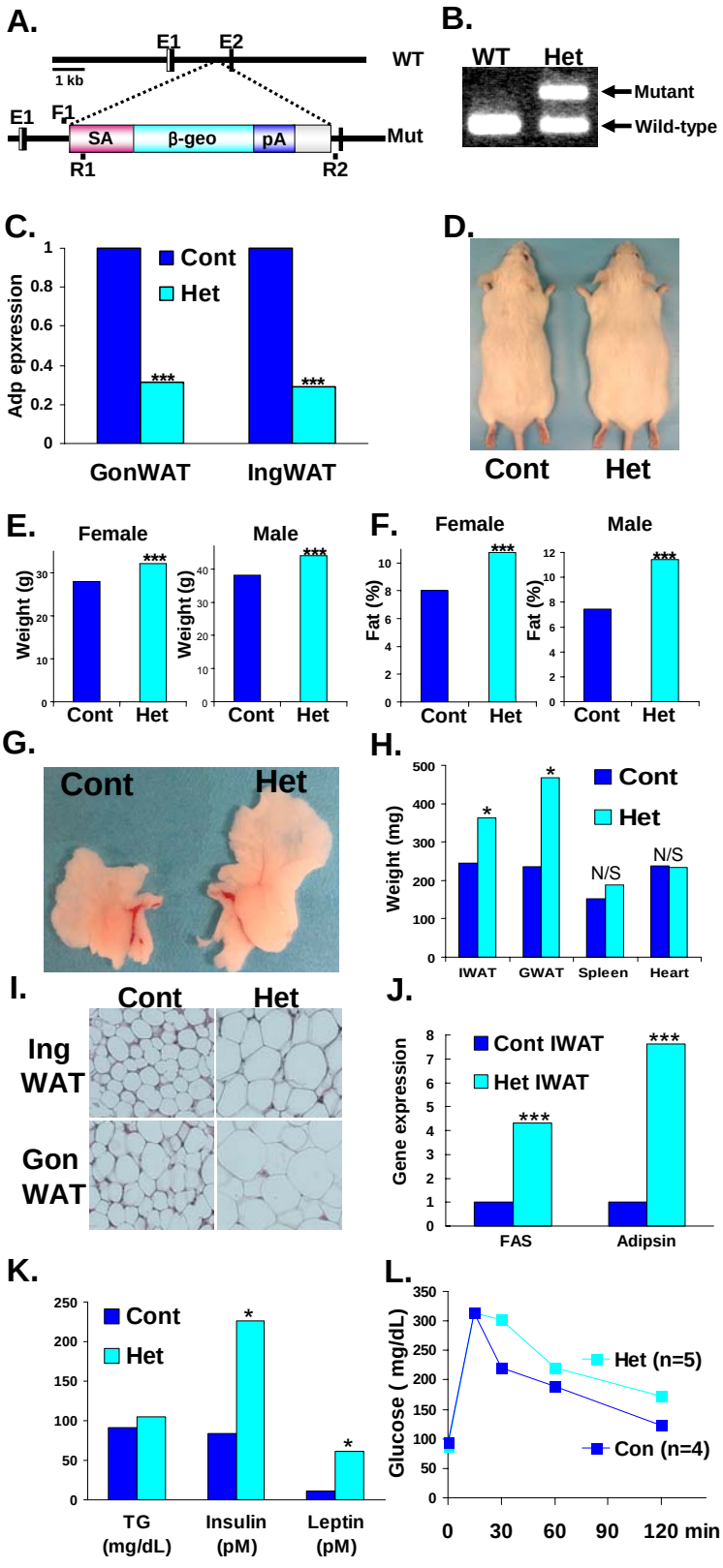
To test the relevance of the fly and mammalian cell culture data to mammalian biology, I explored whether Adp functioned similarly in mice. To disrupt the mAdp gene, I obtained ES cells in which a splice acceptor-LacZ-Neo resistance cassette (ß-geo in Fig 2.4A) was inserted into the Adp locus at a position that should produce an early truncation of the Adp protein at amino acid 15 (Fig 2.4A). Southern blots, PCR analyses, and direct sequencing of the insertion junctions (via inverse PCR) all showed that the allele was targeted appropriately (Fig 2.4B, not shown). The recombinant ES cells were injected into C57BL/6J (B6) blastocysts and the mutant allele was transmitted through the germline, establishing an Adp mutant strain (Fig 2.4B). As expected, Adp expression, based upon qPCR analyses, is significantly reduced in Adp heterozygotes consistent with the creation of a loss-of-function mutant Adp allele (Fig 2.4C). The expression levels of Adp in adipose tissues was consistently reduced by more than half in the Adp heterozygotes (Fig 2.4C), which may increase the likelihood of a haploinsufficient phenotype as observed in flies (Fig 2.1). To try to eliminate potential confounding effects from modifier alleles that are prevalent in metabolic studies, I bred the Adp mutation 7 generations (F7) into ICR, C57BL/6J and 129T2Sv/J backgrounds. I reasoned that any conclusions drawn from the mouse data would be more reliable and generally translatable to other mammals (e.g., humans) if I obtained similar results in divergent strains. While backcrossing, I set aside and analyzed several litters (F2s, F3s, F4s, F5s, and F6s) of Adp+/- heterozygous mutants, separating males and females into individual cohorts as a window into possible trends. Strikingly, in every one of these small cohorts I detected an

increase in weight and fat content, based upon NMR quantitation and evaluation of fat pad explants (not shown). In most cohorts, the difference in fat content reached statistical significance and when all cohorts were combined the significance was high, indicating that Adp heterozygosity may confer an obese phenotype in mice, as in flies. In addition, the data also suggest that the effect is robust as it was observed in a range of genetic backgrounds.

To critically substantiate these findings in relatively pure genetic backgrounds, I analyzed large male and female cohorts expanded from mice backcrossed into the ICR genetic background for at least 7 generations. Upon examination of these large-scale cohorts, I found that male and female $Adp^{+/-}$ heterozygotes were obese compared to wild-type littermate controls based upon physical examination, body weights, NMR fat content quantitation, as well as the gross appearance and weights of explanted fat depots (Fig 2.4D-H). This is not a general phenomenon, as other organs, such as the heart or spleen, weigh the same as the corresponding control tissues (Fig 2.4H). Histology of the fat pads demonstrated that the Adp heterozygotes had significantly larger adipocytes than controls as is often observed in human obesity (Fig 2.4I). I also quantitated adipocyte gene expression in RNA extracted from explanted fat depots. The Adp heterozygotes displayed significantly higher gene espression levels of adipocyte markers including the the PPARγ targets fatty acid synthase (FAS) and Adipsin (Fig 2.4J). Leptin is an adipokine whose blood levels often correlate with fat content. Consistent with the obese phenotype, serum leptin levels were significantly elevated in Adp heterozygotes (Fig 2.4K). I also quantified the levels of triglyceride and insulin in blood removed from Adp heterozygotes

and littermate controls. Although triglyceride levels approximated controls, I found that insulin levels were elevated more than two-fold, indicating that the obese phenotype produced secondary metabolic consequences including insulin resistance (Fig 2.4K). To determine whether the insulin resistance altered glucose homeostasis, I performed dynamic testing. In glucose tolerance tests, the Adp heterozygotes had a trend towards hyperglycemia, however the increase often did not reach statistical significance (Fig 2.4L).

Notably, there were no significant differences in the general behavior or activity levels between control and Adp heterozygotes suggesting that the obese phenotype is not a result of decreased physical activity. The identical trend of Adp heterozygous mice being obese compared to control mice was also observed in the 129 genetic background (not shown), supporting the generality of the findings across different genetic backgrounds. Thus, Adp heterozygosity in flies and mice produces an obese phenotype supporting the notion that Adp is an evolutionary conserved regulator of adipose biology.


A.
E1
E2
WT
1 kb
E1
F1
SA
β-geo
pA
Mut
R1
R2
B.
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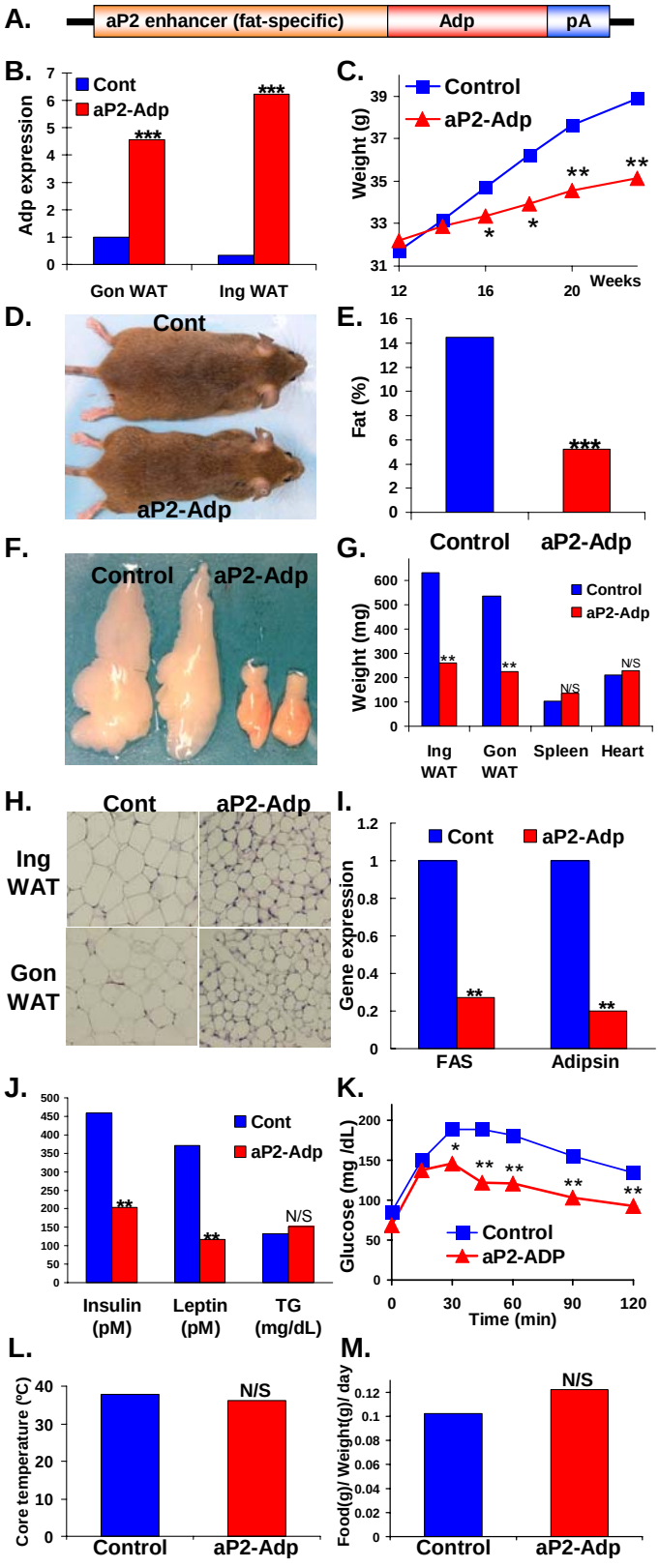
Het
Mutant
Wild-type
C.
Adp epxression
Cont
Het
***
GonWAT
IngWAT
D.
Cont
Het
E.
Female
Male
Weight (g)
***
Cont
Het
F.
Female
Male
Fat (%)
***
Cont
Het
G.
Cont
Het
H.
Weight (mg)
Cont
Het
*
N/S
IWAT
GWAT
Spleen
Heart
I.
Cont
Het
Ing WAT
Gon WAT
J.
Gene expression
Cont IWAT
Het IWAT
***
FAS
Adipsin
K.
Cont
Het
*
TG (mg/dL)
Insulin (pM)
Leptin (pM)
L.
Glucose (mg/dL)
Het (n=5)
Con (n=4)
0
30
60
90
120 min

Figure 2.4. Adp heterozygous mutant mice are obese and insulin resistant. **(A)** Schema of the wild-type (WT) Adp locus and mutant (Mut) Adp allele. Splice Acceptor (SA) and the lacZ-neomycin selectable marker (ß-geo) cassette were inserted between the first (E1) and second coding exon (E2) and disrupt gene expression starting from the second exon. Open box is 5' UTR and filled boxes are coding regions. PCR genotyping primers (F1, R1, R2) are illustrated. **(B)** Molecular analysis on genomic DNA from pups of a wild-type (WT) by $Adp^{+/-}$ heterozygous (Het) mating show that the Adp mutation is passed through the germline, thus establishing an Adp mutant line. **(C)** RNA was extracted from perigonadal (Gon) and inguinal (Ing) white adipose tissues (WAT) and real-time PCR quantitation of Adp expression demonstrates that mice harboring a heterozygous (Het) insertion have significantly reduced expression of Adp compared to wild-type control littermates (cont), supporting the notion that the mutation is a loss-of-function allele. **(D)** Photograph of representative wild-type (left) and $Adp^{+/-}$ heterozygous mice (right) at 4 months of age shows that $Adp^{+/-}$ heterozygotes are fatter than wild-type littermates. **(E)** Average weights and **(F)** average fat content as assessed by NMR of littermate matched female (control n = 10 mice, $Adp^{+/-}$ Het n = 12) and male (control n = 15, $Adp^{+/-}$ Het n = 20) cohorts are shown. **(G)** Photograph of representative perigonadal white adipose tissue (WAT) explants from sibling wild-type (left) and Adp heterozygotes (right). **(H)** Average weights of inguinal (I) and perigonadal (G) WAT and indicated organs. **(I)** Histological analyses of inguinal (Ing) and perigonadal (Gon) WAT show that $Adp^{+/-}$ heterozygous adipocytes are larger than wild-type adipocytes. **(J)** Real-time quantitation of gene expression in wild-type and $Adp^{+/-}$ heterozygous inguinal (I)_WAT demonstrates that Adp heterozygosity significantly increases expression of adipocyte markers. Fatty acid synthase (FAS) and adipsin are markers of mature adipocytes. **(K)** Analyses of plasma of 4-month old mice show that Adp heterozygosity significantly increases both leptin and insulin levels while triglycerides levels are not significantly changed. **(L)** Glucose tolerance tests on 4-month old $Adp^{+/-}$ heterozygotes and sibling controls show a trend towards hyperglycemia that does not reach statistical significance. *$p<.05$, ***$p \leq 0.005$ by t-test, N/S not significant

Fat-Specific Adp Transgenic Mice are Lean and Insulin Sensitive

To further explore Adp biology and to complement the necessity tests, I examined the consequences of overexpressing Adp selectively in adipocytes by placing Adp under the control of the 5.4 kB aP2 promoter/enhancer (Fig 2.5A), the best characterized fat-specific promoter (Ross et al., 1990). I generated transgenic founders twice, once in a B6/D2F background and again on a pure B6 background. qPCR analyses showed that the aP2-Adp mice had significantly higher levels of Adp in fat depots (Fig 2.5B), but no increased expression in examined non-adipose tissues. I followed 8 cohorts with serial weights and, as illustrated by one representative cohort, by 16 weeks, the weight of the aP2-Adp transgenics began to diverge from their wild-type littermate controls (Fig 2.5C). From 18 weeks on, the decreased weight reached statistical significance and this was reflected in their appearance (Fig 2.5C, D). At 24 weeks, I analyzed fat content with NMR and found that the Adp transgenics had significantly less body fat than controls (Fig 2.5E). These findings were confirmed by gross examination and weights of adipose depots (Fig 2.5F, G). The weights of other organs were unaffected (Fig 2.5G). The decreased fat mass was reflected in histological analyses demonstrating significantly smaller adipocytes compared to controls (Fig 2.5H). Molecular analyses showed that the levels of adipose molecular markers were also significantly reduced in the transgenic adipose depots (Fig 2.5I). Similarly, plasma leptin levels, which typically reflect fat mass, were markedly reduced in the aP2-Adp mice (Fig 2.5J). Because the degree of fat reduction in the Adp transgenics approached that of lipodystrophic models [61, 62], I explored whether these mice might manifest the hypertriglyceridemia, insulin resistance,

and hyperglycemia seen in lipodystrophy (Fig 2.5J). Although triglyceride levels were unaffected, insulin levels were significantly reduced in the aP2-Adp mice. Further, glucose tolerance tests (GTT) showed that the aP2-Adp mice had significantly lower blood glucose levels than controls (Fig 2.5K). Another intriguing observation was that aP2-Adp mice have a slightly higher rate of food consumption (0.12 gm food/wt/day versus 0.1 gm food/wt/day) and roughly equal activity levels and body temperature compared to controls (Fig 2.5L,M), so the leanness of these mice does not appear secondary to any of these factors. Notably, the adipocyte-restricted expression of Adp produces leanness on both mixed and pure backgrounds (not shown), similar to what was observed for the mouse Adp loss-of-function studies, indicating the robustness and potential general implications of my findings.


A.
aP2 enhancer (fat-specific)
Adp
pA
B.
Cont
aP2-Adp
Adp expression
Gon WAT
Ing WAT
C.
Control
aP2-Adp
Weight (g)
Weeks
D.
Cont
aP2-Adp
E.
Fat (%)
F.
Control
aP2-Adp
G.
Control
aP2-Adp
Weight (mg)
Ing WAT
Gon WAT
Spleen
Heart
N/S
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Gon WAT
I.
Cont
aP2-Adp
Gene expression
FAS
Adipsin
J.
Cont
aP2-Adp
Insulin (pM)
Leptin (pM)
TG (mg/dL)
N/S
K.
Glucose (mg /dL)
Control
aP2-ADP
Time (min)
L.
Core temperature (°C)
N/S
Control
aP2-Adp
M.
Food(g)/ Weight(g)/ day
N/S
Control
aP2-Adp

Figure 2.5. Fat-specific Adp transgenic mice are lean and insulin sensitive. (**A)** Illustration of the aP2-Adp transgene construct. (**B**) Real-time PCR quantitation of Adp levels in RNA extracted from gonadal (Gon) and inguinal (Ing) white adipose tissues (WAT) of aP2-Adp transgenic mice and littermate controls (n = 8) shows that the transgenic line expresses significantly more Adp in WAT than do controls. (**C**) Weight curves of male aP2-Adp transgenic mice and littermate controls (n = 8) show that the aP2-Adp mice become significantly lighter than controls. (**D**) Photograph of a representative control (top) and aP2-Adp transgenic (bottom), shows that the latter is thinner. (**E)** NMR fat content analyses show that the aP2-Adp transgenic mice have significantly less fat than control littermates (n = 8). (**F)** Photograph of representative inguinal WAT depots from control and aP2-Adp transgenic mice. (**G)** Average weights of inguinal (Ing) and perigonadal (Gon) WAT depots as well as the indicated organs from aP2-Adp transgenic mice and littermate controls. (n = 8) (**H)** Histological sections of inguinal (top, Ing) and perigonadal (bottom, Gon) WAT depots of control and aP2-Adp transgenic mice show that the latter have smaller adipocytes. **(I)** Real-time PCR molecular analysis of RNA harvested from WAT depots shows that aP2-Adp transgenesis significantly reduces adipocyte marker expression. FAS fatty acid synthase **(J)** Analyses of plasma taken from overnight fasted aP2-Adp transgenic mice and littermate controls shows that insulin and leptin levels are significantly reduced in the aP2-Adp transgenics while triglyceride levels are roughly equal (n = 6). (**K)** Glucose tolerance tests on 4-month old aP2-Adp transgenic mice and control littermates show that fat-specific Adp transgenesis significantly lowers blood glucose levels. **(L)** The core temperature of control and aP2-Adp littermates was measured and the average temperature was plotted. **(M)** Control and aP2-Adp littermates were acclimatized and then food intake was measured for one week and averaged for the cohort (n = 8). *$p<0.05$, **$p<0.01$, *** $p<0.005$; N/S not significant

Adp Structure Function Analyses

To critically examine cell autonomous anti-obesity functions in vivo, I needed to block Adp action specifically in adipocytes. One possible way to achieve this effect is to express a dominant negative form of Adp in fat cells. Adp is a novel protein, with several protein-interaction motifs (6WD40s, 3TPRs) (Fig 2.6A); so generating a dominant negative Adp required that I first identify the salient regions for Adp function. As a first step, I attempted to characterize the structure-function relationship of the Adp protein in cell culture adipogenesis models. I divided murine Adp into several pieces designed around the protein interaction domains (Fig 2.6A) and assayed their function in both 3T3-L1 and NIH-3T3 cells, both in which full-length mAdp reduces (Figs 2.2) and Adp RNAi stimulates adipogenesis (Figs 2.3). Many deletion constructs were inactive and all constructs that inhibited adipogenesis contained the TPR domains (Fig 2.6A). Strikingly, expressing just the carboxyl-portion of Adp containing two WD40 domains (termed AdpC1 or C1) stimulated fat formation as assessed by Oil Red O staining of mature adipocytes (Fig 2.6B). As Adp inhibits adipogenesis, these results suggested that AdpC1 functions as a dominant negative, a notion I evaluated by expressing AdpC1 with varying doses of full-length Adp. I found that the wild-type Adp rescued the AdpC1 effect in a dose-dependent fashion (Fig 2.6C). I also expressed full-length Adp with increasing amounts of AdpC1 and again found a competitive relationship (Fig 2.6D). Taken together, these data support the idea that AdpC1 acts in a dominant negative manner, potentially providing a tool to dissect tissue-specific functions of Adp function *in vivo* .

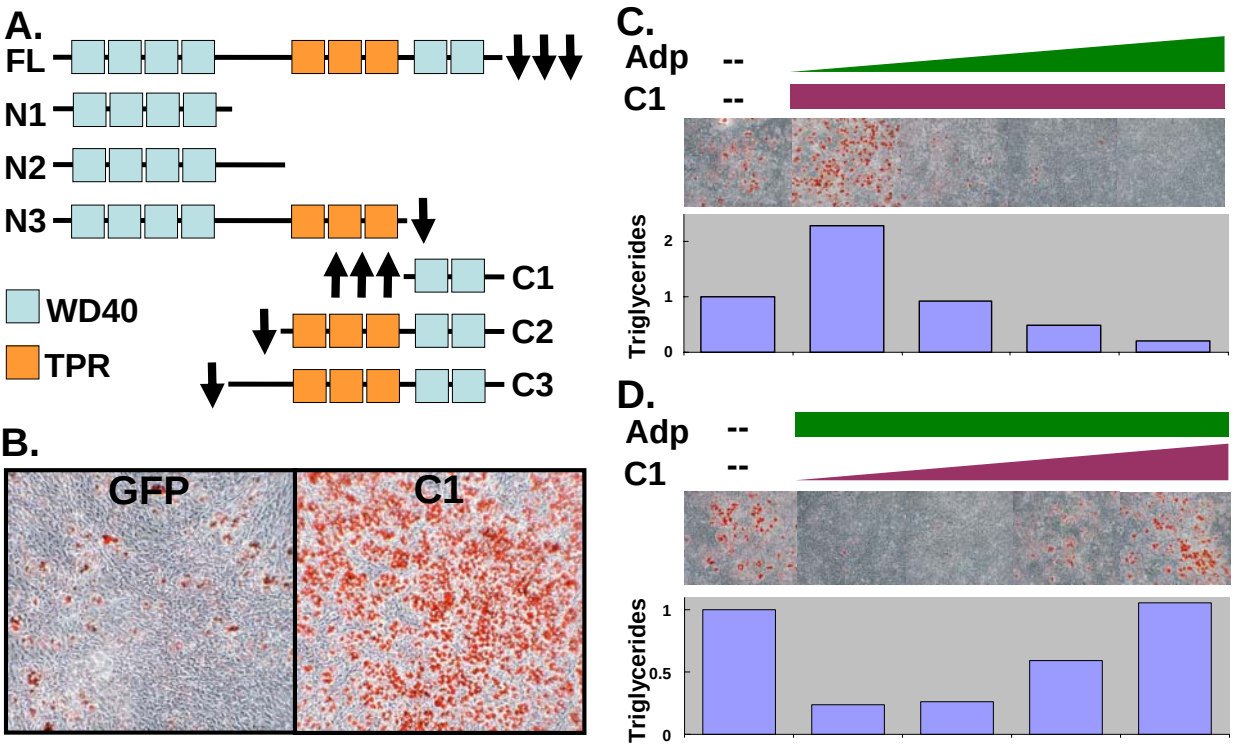

Figure 2.6. Adp structure-function analyses show that the C-terminal region functions as a dominant negative.
(A) Cartoon showing Adp structure, the series of deletion mutants, and their effects on both 3T3-L1 and NIH3T3 cell culture adipogenesis models. FL = full length Adp. Black arrows indicate effect on adipogenesis. (↓) indicates inhibition and (↑) indicates promotion of adipogenesis. WD40 domains: light blue rectangles, TPR domains: orange rectangles. **(B)** NIH-3T3 cells were infected with viruses encoding either GFP or AdpC1 and fat accumulation was assessed with Oil Red O staining, which showed that AdpC1 stimulates 3T3-L1 adipogenesis. **(C)** GFP (--), Adp, and AdpC1 were introduced into 3T3-L1s and based upon Oil Red O stains (top) and triglyceride quantitation (bottom) Adp rescues the AdpC1 effect in a dosage-dependent manner. Green shows increasing concentration of Adp while C1 (purple) is kept constant. **(D)** C1 competes with Adp. Schema as in C, except in this case Adp is held constant while AdpC1 levels are varied.

Fat-Specific Adp Blockade Produces Obesity and Hyperglycemia

To determine whether fat-specific blockade of Adp function causes obesity, I generated aP2-AdpC1 transgenic (Fig 2.7A) and non-transgenic founders and evaluated expression levels of the transgene and fat accumulation. I found that the aP2-AdpC1 mice had a significant increase in fat content as evidenced by NMR analyses (Fig 2.7B, C). Examination of fat depots showed that the aP2-AdpC1 explants were heavier than controls (Fig 2.7D, E). Subgroup analyses demonstrated a dose-dependent relationship with the mice that expressed the most AdpC1 having the highest fat content based upon NMR and fat pad explant analyses (Fig 2.7B-E). Histology also showed that the aP2-AdpC1 adipocytes were larger than control adipocytes (Fig 2.7F). To evaluate glucose metabolism, I subjected aP2-AdpC1 mice and littermate controls to dynamic glucose tolerance testing. I found that the aP2-AdpC1 had significantly higher blood glucose levels than controls (Fig 2.7G). Secondary causes including food intake and body temperature did not account for the increased fat content (Fig 2.7H, I).

My observations also indicate that the aP2-AdpC1 founders with high transgene expression were fatter than Adp heterozygotes, although this is based upon historical studies rather than direct pair-wise comparisons. This difference may be due to elimination of consequences of loss of Adp in other tissues or because the AdpC1 blocks Adp function to a greater extent than Adp heterozygosity as might be expected of a dominant negative form. Since fat content was more severely affected in the aP2-AdpC1 mice compared to the Adp heterozygotes, it seemed plausible that metabolic parameters might also be worsened in the aP2-AdpC1. To test this possiblity, I subjected aP2-AdpC1

mice and littermate controls to dynamic glucose tolerance testing. I found that the aP2-AdpC1 had significantly higher blood glucose levels and this also paralleled transgene expression with the highest expressers having the highest blood glucose levels. This suggests that Adp may participate in the coordinated signaling of adipocytes and the metabolic network. Taken together, these data show that Adp functions in a dosage-sensitive and cell autonomous manner to restrain fat accumulation.

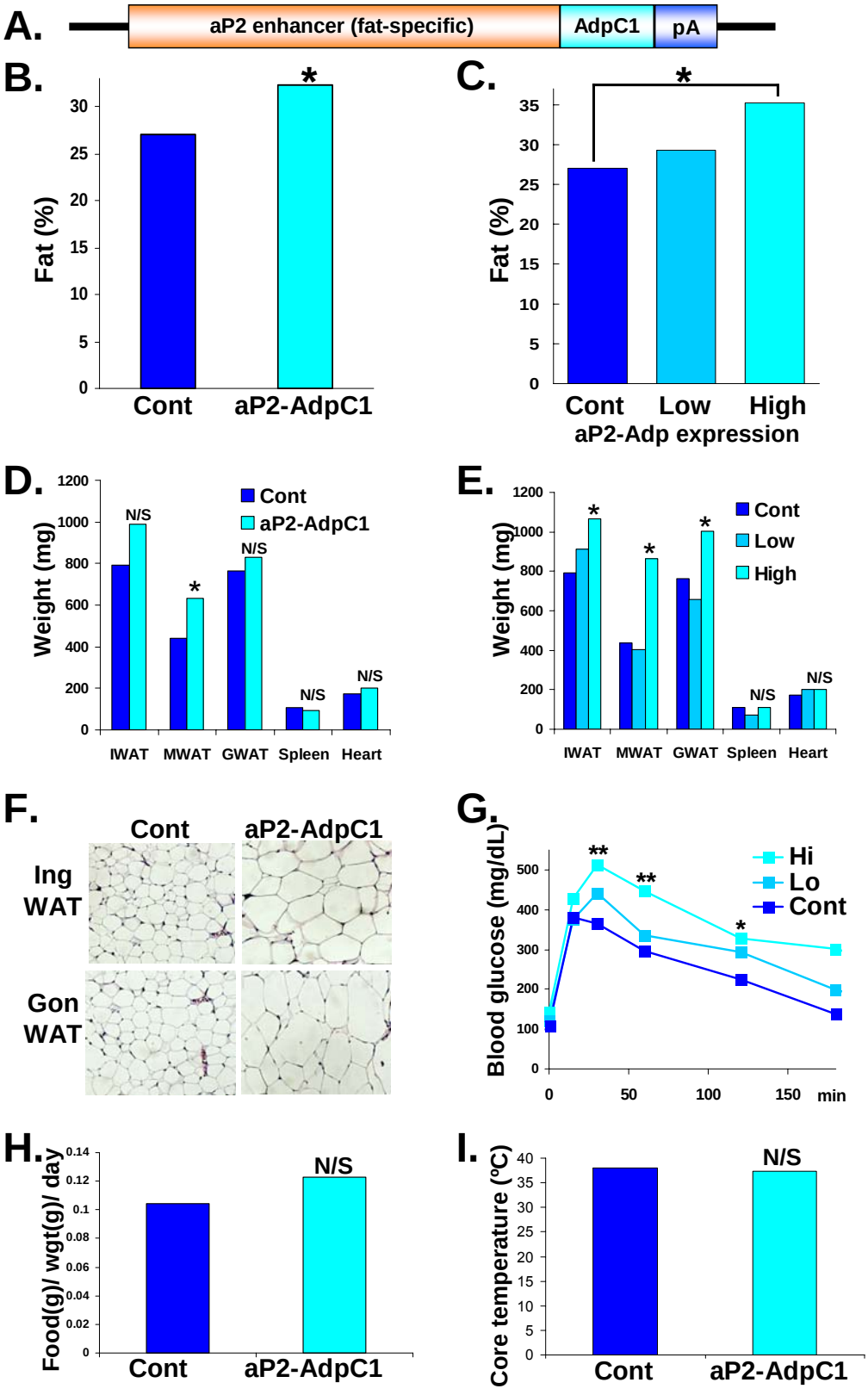
A.
aP2 enhancer (fat-specific)
AdpC1
pA
B.
Fat (%)
Cont
aP2-AdpC1
C.
Fat (%)
Cont
Low
High
aP2-Adp expression
D.
Weight (mg)
Cont
aP2-AdpC1
N/S
IWAT
MWAT
GWAT
Spleen
Heart
E.
Weight (mg)
Cont
Low
High
IWAT
MWAT
GWAT
Spleen
Heart
F.
Cont
aP2-AdpC1
Ing WAT
Gon WAT
G.
Blood glucose (mg/dL)
Hi
Lo
Cont
min
H.
Food(g)/ wgt(g)/ day
N/S
Cont
aP2-AdpC1
I.
Core temperature (ºC)
N/S
Cont
aP2-AdpC1

Figure 2.7. Fat-specific blockade of Adp produces murine obesity and hyperglycemia.
(**A**) Diagram of the aP2-AdpC1 construct. (**B)** NMR analyses show that aP2-AdpC1 mice have significantly higher levels of fat than littermate controls (n = 10). (**C**) Based upon NMR analyses, fat content is proportionate to amount of AdpC1 expressed in the adipocytes, as scored by qPCR analyses of explanted fat depots. (**D**) Average weights of inguinal (I), mesenteric (M), and perigonadal (Gon) WAT depots as well as the indicated organs from aP2-AdpC1 transgenic mice and littermate controls. (n = 6) (**E**) The increase in weight of the individual fat depots is statistically significant only in the mice that have higher levels of the AdpC1 expression. (**F**) Histological analyses of inguinal (Ing) and perigonadal (Gon) WAT show that aP2-AdpC1 adipocytes are enlarged compared to controls **(G)** Glucose tolerance testing shows that hyperglycemia is induced by AdpC1 in a manner related to its expression levels. **(H)** After acclimatization, food intake of control and aP2-AdpC1 littermates was measured for one week and averaged for the cohort (n = 10). **(I)** The core temperature of control and aP2-AdpC1 littermates was measured, averaged, and plotted (n = 10). In C, E, and G, Hi is the average of the two highest AdpC1 expressors, Lo is the average of the next two highest expressors, and the controls do not contain the transgene. *$p<0.05$, **$p<0.01$, N/S not significant

Discussion

The ability to store fat was acquired early in evolution and is essential for survival of most, if not all, multi-cellular animals. Fat-storing tissues regulate energy balance and metabolic homeostasis, provide the resources for metamorphosis, migration, hibernation, and other processes, protect against mechanical and thermal trauma, and are important for reproduction and lifespan in a range of invertebrates and vertebrates (Bluher et al., 2003; Hwangbo et al., 2004; Kloting and Bluher, 2005; Spiegelman and Flier, 1996). Since the biological functions of fat storing tissues are ancient, the molecular mechanisms that underlie fat biology may also be conserved. Few studies have thus far been directed towards understanding invertebrate fat formation and no gene had yet been discovered based upon an altered fat phenotype in invertebrates and been shown to have an analogous *in vivo* role in mammals. Furthermore, some evidence suggests that the molecular mechanisms that regulate fly and mammalian fat biology may not be conserved (Tong et al., 2000). So it is not yet clear if the tremendous strengths of invertebrate models can be exploited to help combat the epidemic of obesity and diabetes. Since Drosophila provides many experimental advantages, it is important to determine whether the fly is a suitable model for the study of fat biology and, if so, to then exploit the fly to help characterize fat biology potentially providing insights that may help people who suffer negative consequences from obesity, diabetes, and other fat-related conditions.

Adipose flies were isolated as a naturally occurring obese variant when the mutation is present in the homozygous state (Doane, 1960a). Adp encodes an evolutionarily conserved protein consisting of multiple protein interaction domains that may be a nidus or scaffold for a multiprotein complex that regulates fat biology, a notion further supported by studies described in the following chapter (Chapter III). Adp not only has a conserved structure, but also has striking functional conservation between fly and mammalian systems. For example, loss of Adp function leads to increased adipose formation in flies, in mammalian tissue culture and in mice. Conversely, increasing Adp activity blocks fat formation in flies, cell culture and mice. These data support the idea that the fly and mammalian Adp orthologs have similar biological roles.

The hedgehog (Hh) cascade, whose role in adipogenesis was examined in the previous chapter (Chapter I), was the first pathway shown to have a conserved role in fat biology from flies to mammalian tissue culture models; however, no definitive *in vivo* studies have yet been reported to establish whether the pathway may have an endogenous role in mammalian fat biology. Given the key role that signaling pathways play in virtually all cell fate decisions, it is not unexpected that the Hh cascade regulates fat development in a conserved manner. However, Adp is quite different from the Hh pathway in that Adp does not have broad pleiotropic roles like Hh but rather appears more dedicated to fat biology—Adp heterozygous and homozygous flies and Adp heterozygous mice have as the primary-if not only-phenotype increased fat stores.

Since haploinsufficient phenotypes often indicate the importance of the gene in the process of interest and because homozygous Adp mutant flies suffer some negative

consequences from obesity (i.e. difficulty flying), I evaluated the possibility that Adp heterozygotes might also have abnormal fat accumulation. I found, initially in flies and later in mice, that Adp heterozygotes of either species were obese. Such autosomal dominant phenotypes caused by loss-of-function mutations are uncommon and typically occur in genes that are key regulators of the process under study (Favor, 1995; Goldstein and Brown, 1979). For example, PPARγ heterozygous mutant mice are resistant to diet-induced obesity, supporting PPARγ's critical role in fat biology (Kubota et al., 1999; Lehrke and Lazar, 2005; Meirhaeghe et al., 2000; Mueller et al., 2002; Rosen et al., 1999). Of note, characteristics inherent in dosage-sensitive genes may make them better and more amenable drug targets as has been found for PPARγ and the thiazolidinedione (TZD) class of anti-diabetes therapies (Lehmann et al., 1995; Lehrke and Lazar, 2005). With striking similarities to PPARγ, in both sufficiency and necessity tests, Adp demonstrates dosage-sensitive effects on fat accumulation and glucose homeostasis, so the Adp pathway might provide targets for diabetes and obesity therapeutics. Altering Adp dose by 50% throughout the mouse (or fly) specifically alters adipose tissues and apparently does not lead to other side effects. Therefore, the Adp pathway may provide a large therapeutic window for new treatments. A potentially significant disadvantage is that Adp lacks recognizable catalytic motifs, consisting primarily of protein interaction domains, making it less amenable as a druggable target. However, the components of the pathway through which Adp works might be appropriate candidates. Identification of such components are potentially made easier both by the strengths of fly genetics, such as modifier screens exploiting the extant Adp mutant and transgenic flies, and by the

primary structure of Adp. Although the presence of multiple protein interaction domains and the absence of known catalytic domains make it difficult to target Adp by drugs, it is consistent with Adp being an organizing node for assembly of a multiprotein complex. As such, Adp may provide a tool to identify interacting proteins that may work in concert with Adp, for example as bait in a yeast two hybrid screen (see Chapter III).

Polymorphisms in dosage-sensitive genes may represent risk factors for disease. The recent explosion of obesity is largely attributed to changes in behavior and environment including the modern sedentary lifestyle and ready access to inexpensive and calorie-dense foods (Allison et al., 1999; Campbell and Dhand, 2000; Spiegelman and Flier, 2001). Yet these factors might not contribute to the dramatic increase in body weight unless coupled with a fertile genetic basis. One reason that humans might harbor a large number of genes that when slightly altered predispose to obesity (with negative consequences) in times of plenty is that these same polymorphisms might confer a competitive advantage during famine, which was a frequently encountered condition throughout human history (Neel, 1999a, b). If so, a species may contain many polymorphisms to handle harsh environmental conditions, although these same polymorphisms might be detrimental under current times of plenty. Isolated or combined polymorphisms that slightly alter activity or expression levels in dosage-sensitivity genes might account for the high prevalence of overweight individuals as has been supported by genetic linkage studies (Rankinen et al., 2006). Since Adp was originally identified as a naturally occurring homozygous mutant, heterozygosity or polymorphisms that lead to slight reductions in the activity of Adp, or genes like Adp, might also exist in human

populations as natural variants, just as observed in the fly. Notably Adp lies within a well-recognized susceptibility interval for obesity and diabetes conserved in both rodents and humans (Rankinen et al., 2006). Since Adp is sensitive to dose, subtle changes in the promoter or the open reading frame of Adp, if present in high frequency within human populations, may be a major contributor to the high incidence of obesity. Such genetic changes could also be present in other components of the Adp pathway as suggested by the studies in the following chapter (Chapter III).

Adp appears to act in a cell autonomous manner to restrain fat accumulation. In flies and mice, fat-restricted Adp expression reduced fat accumulation and this is also observed in cell culture. These data show that Adp can act within fat cells to inhibit fat storage, but they did not demonstrate that the loss-of-function obesity phenotypes observed in Adp mutant flies and mice were due to functions of Adp in fat cells *per se*. The cell culture loss-of-function studies showed that Adp can have cell autonomous anti-obesity functions, at least in cell culture. To attempt to directly examine cell autonomy in vivo, I generated mice that express a dominant negative form of Adp in an adipocyte restricted manner. This fat-specific Adp inhibition produced obesity and hyperglycemia in proportion to levels of transgene expression. Taken together, these data support the notion that Adp acts in a cell autonomous manner. Mechanistic insights into Adp function also strongly substantiate the notion that Adp acts in a cell autonomous manner (see Chapter III).

To date, it has been difficult to identify genes that function *in vivo* within adipocytes to inhibit fat formation although several lines of evidence indicate that such

genes contribute to human obesity. Identifying molecules such as Adp that function within adipocytes to restrain fat accumulation might be a significant advance for several reasons. First, understanding the biology of such genes should lead to new findings regarding the function of adipocytes and this notion is supported by the findings described in the next chapter (Chapter III). Second, understanding pathways that function autonomously in fat cells may yield targets that are amenable to new therapeutic strategies and possibly without prohibitive side effects. Notably,many widely used type II diabetes therapies (i.e. TZDs) elicit their actions in adipocytes, supporting this idea (Kintscher and Law, 2005; Lehmann et al., 1995; Lehrke and Lazar, 2005). Further, identification of such cell autonomous anti-obesity genes may substantiate the notion held by many-if not the majority of overweight people, who feel that overeating is not the root of the problem but rather that it is their "metabolism." Although this idea is often pandered and many impugn character as the main issue, some evidence, including results from my studies described here, support aspects of this notion. For example, the identification of the Adp mutant phenotype seems to support the obese patient's idea, as Adp mutant mice have relatively normal caloric intake and activity, yet are obese secondary to a defect in the fat cell *per se*. That is, a mutation that alters energy partitioning, rather than overeating, can underlie the propensity to significant weight gain. So the discovery of such genes and drugs that modify their actions may help to relieve the suffering associated with obesity and diabetes.

Material and Methods

Fly experiments

The *adp*^{60} mutant stock was a gift from Dr. Winifred Doane. *w*^{1118} flies were used as controls. Newly eclosed flies reared under identical conditions were collected and further cultured for another week under well-fed conditions by addition of yeast paste before analysis. Starvation assays were performed by subjecting flies of each sex to starvation with an unlimited supply of water. Death was scored daily. Starvation assays were performed at least three times per experiment and survival curves were plotted. For triglyceride quantitation, fly lysates were prepared by grinding groups of 10 flies that were sex and age matched in ice-cold 0.5% SDS/PBS followed by a 30 min. incubation at 65ºC. Heat-inactivated lysates were cleared of debris by a brief centrifugation at 4ºC and triglyceride content was measured as described below. Six replicates were used per genotype per sex for triglyceride measurements. Triglyceride content was measured using the Infinity Triglyceride Reagent (ThermoElectron) following manufacturer's instructions

Plasmids

Murine Adp (NM_199306**)** was cloned into the pMX retroviral expression vector (generous gift of Dr. Gary Nolan) generating pMX-Adp. pMX-AdpC1 was generated by PCR amplification and subsequent cloning of the two C-terminal WD40 repeats of murine Adp into the pMX vector. For shRNA, three hairpins targeting different regions of Adp were cloned into the mU6-neo shRNA plasmid (generous gift of David Turner) and tested for knock-down efficiency by real-time PCR. The two hairpins with the highest knock-down efficiency were used for further experiments. All clones were sequence verified.

Retrovirus production and infection

Standard methods were used to generate recombinant retroviruses and viral transduction to 3T3-L1 and NIH3T3 cells were as previously described (see Chapter I).

Cell culture and adipocyte differentiation

Mouse NIH-3T3 fibroblasts and 3T3-L1 preadipocytes were purchased from the American Type Culture Collection and maintained in growth media (DMEM with 10% calf serum, 10 units/ml penicillin, 10 μg/ml streptomycin) at 37 °C in 5% CO_2. Cells were passed before confluence and discarded after 10 passages. Media changes were performed every other day during cell maintenance and adipogenesis. 3T3-L1 and NIH-3T3 cells were induced to undergo adipogenesis as previously described (see Chapter I).

Analysis of lipid accumulation

Lipid droplets in differentiated adipocytes were stained with Oil Red O as previously described (see Chapter I). To quantify triglyceride levels, flies or cells were lysed in 0.5% SDS/PBS and triglyceride content was measured using the Infinity Triglyceride Reagent (ThermoElectron) following manufacturer's instructions. Protein concentrations used to normalize triglyceride content were measured with a BCA protein assay kit (Pierce).

RNA extraction and RT-PCR

Total RNA from mouse tissues or cultured cells was extracted with Trizol (Invitrogen), DNAse I-treated, and reverse-transcribed using random hexamers and M-MLV-reverse transcriptase (Invitrogen) to obtain cDNA. Gene expression was analyzed with real-time PCR using SYBR Green Master Mix reagent (Applied Biosystems, 7500

Real-Time PCR System). Real-time PCR values for gene expression were normalized over ß-actin expression. Primer sequences are available upon request.

Adp mutant mice

Adp mutant gene trap ES cell line RRF015 was purchased from BayGenomics. RRF015 ES cells contain an insertion of the pGT2lxf exon trap vector, which is comprised of a splice acceptor followed by β-geo (lacZ - neomycin selectable marker gene fusion), between the 1st and 2nd coding exon of mouse Adp. The RRF015 ES cells were expanded, genomic DNA was extracted and the gene trap insertion disrupting the Adp locus was confirmed as recommended by Baygenomics (please refer to URL http://baygenomics.ucsf.edu/protocols/comp1/Genotyping_BayGenomics_Mice.pdf).
Next, the confirmed Adp mutant ES cells were injected into C57BL/6J blastocysts to produce chimeric mice. The chimeras were bred to ICR, B6 and 129T2/SvJ strains for germline transmission. Heterozygous mice were backcrossed into each background for > 6 generations. PCR amplification of genomic DNA from tail biopsies was used for routine genotyping using a combination of the following primers:
5'-ACCACCTTCCATCCTAACTACACAA-3',
5'-ACTTCCGGAGCGGATCTCAAACTCT-3',
5'-GCCGGTTGTAAGCTACCATATGGAT-3'.
The mutant allele generates a 320 bp PCR product and the wild type allele produces a 220 bp PCR product.

Transgenic mice

aP2-Adp and aP2-AdpC1 transgenic constructs were generated by cloning mouse full-length Adp or the AdpC1 fragment downstream of the 5.4 kb aP2 enhancer (Generous gift of Bruce Spiegelman). Constructs were linearized and microinjected into fertilized eggs of B6D2F1/J or C57BL/6J genetic strain. Transgenic founders were further bred and backcrossed as for the Adp mutant mice to establish relatively pure lines or used directly for animal studies when in the inbred C57BL/6J genetic strain.

Plasma analyses

Overnight fasted mice were euthanized and blood was drawn by cardiac puncture into EDTA-coated tubes. After a brief centrifugation plasma samples were collected and aliquots were kept at -80°C until analysis was performed. Insulin and Leptin were measured by ELISA (Linco Research Inc., MENDO-75K-02). Non-esterified fatty acids (Wako Chemicals), cholesterol (ThermoElectron) and triglycerides (ThermoElectron) were measured following manufacturer's guidelines.

Mouse studies

Mice were housed in a 12:12 light:dark cycle and chow and water were provided *ad libitum*. Total body fat mass of overnight fasted mice was measured by NMR spectroscopy on the Minispec mq spectrometer (Bruker). For glucose tolerance tests, overnight fasted mice received an intraperitoneal injection of 75 mg/ml glucose-PBS solution (1.5 g/kg body weight). Tail blood was drawn at various intervals and blood glucose levels were measured with a TrueTrack glucometer (CVS). Body weight was recorded weekly and food intake was recorded daily for 5 consecutive days. Mouse tissues were harvested and weighed before freezing at -80°C for RNA extraction or fixed in formalin for paraffin embedding. 8μm sections were obtained and sections were hematoxylin and eosin stained for histological examination. Veterinary care was provided by the Division of Comparative Medicine. All animals were maintained under the guidlines of the U.T. Southwestern Medical Center Animal Care and Use Committee according to current NIH guidelines.

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Chapter III

Adp Regulates PPARγ Function via

The Mediator Complex

Abstract

Adp regulates fat and glucose homeostasis in a fat-autonomous manner, implying that Adp is involved in the intimate functional and clinically relevant relationship of adipocytes and metabolism. I undertook a series of studies to unravel the mechanisms underlying the observed *in vivo* anti-obesity and anti-diabetes effects of Adp. I found that Adp inhibits the activity of PPARγ, a key adipogenic transcription factor and the target of the thiazolidinedione class of diabetes therapies. Adp may inhibit PPARγ action through direct interactions with Med23, a component of the Mediator Complex that connects PPARγ to RNA polymerase II. Complementing the stimulatory role identified for the Med1-containing Mediator Complex on both PPARγ action and adipogenesis, I find that the Med23 Mediator subunit inhibits both PPARγ function and mammalian adipogenesis, similar to the action of Adp. Notably, Adp requires Med23 to elicit its anti-obesity and PPARγ inhibitory functions. These data support the notion that the Mediator complex can be both pro-and anti-adipogenic depending upon subunit composition. The role of the Med23 antiadipogenic Mediator Complex appears conserved as Med23 mutant flies demonstrate dosage-sensitive obesity, just like Adp mutants. These data identify a new mechanism to regulate PPARγ activity, enhance the understanding of adipocyte biology, and may lead to the development of rational therapeutic strategies for the treatment of obesity and/or diabetes.

Introduction

Fat storing tissues play a variety of essential roles, such as regulating energy balance, protecting against trauma and cool temperatures, and controlling lifespan (Bluher et al., 2003; Lehrke and Lazar, 2005; Meirhaeghe et al., 2000; Spiegelman and Flier, 1996, 2001). Adipocyte dysfunction underlies lipodystrophy and obesity, and is central to the metabolic derangements in Type II diabetes (Lehrke and Lazar, 2005; Spiegelman and Flier, 1996). The dramatic rise in obesity and diabetes with their attendant negative health sequelae have led to a new and important public health crisis. Since understanding the mechanisms of fat accumulation offers therapeutic promise, identifying genes that influence adipogenesis and metabolism is an important task.

Adipogenesis, the differentiation of pluripotent precursor cells into mature adipocytes, is regulated by the adipogenic transcription factors C/EBP, SREBP (aka ADD1) and most notably PPARγ (Ge et al., 2002; Lehrke and Lazar, 2005; Smas and Sul, 1995; Spiegelman and Flier, 1996). PPARγ is a nuclear hormone receptor (NHR) that is a master regulator of adipogenesis. The C/EBP family of basic-leucine zipper transcription factors regulates adipogenesis in part by controlling PPARγ expression. SREBP (ADD1) is a pro-adipogenic basic helix-loop-helix transcription factor that controls expression of the fatty acid biosynthetic machinery and is important in activating PPARγ. A series of *in vitro* and *in vivo* studies have demonstrated that PPARγ is required for fat formation. Beyond developmental effects, PPARγ is critically important in adult fat biology (Cecil et al., 2006; Imai et al., 2004; Kintscher and Law, 2005; Kubota et al.,

1999; Lehmann et al., 1995; Rosen et al., 1999). For example, in mature adipocytes PPARγ regulates lipid and glucose metabolism and is the target of the thiazolidinedione (TZD) family of diabetes therapies taken by millions of patients with type II diabetes (Kintscher and Law, 2005; Lehmann et al., 1995; Lehrke and Lazar, 2005). Because of these facts and since TZDs produce side effects, such as weigth gain and edema, understanding the regulation of PPARγ function, especially in a tissue-specific manner, is an active, clinically relevant research area (Kintscher and Law, 2005; Rosen and Spiegelman, 2000).

A central challenge in fat biology is to elucidate the mechanisms whereby external and internal cues regulate gene expression via the key adipogenic transcription factors such as PPARγ. A potential candidate for this central role is the Mediator Complex. The Mediator is a conserved transcriptional regulatory complex consisting of >25 proteins (Kornberg, 2005; Malik and Roeder, 2005). The mammalian Mediator was identified as a nuclear hormone receptor (NHR) coactivator, but further studies demonstrated its role in the function of many non-NHR transcription factors including C/EBP and SREBP (Kornberg, 2005; Malik and Roeder, 2005). Studies from a variety of systems show that the Mediator integrates and communicates external information to transcription factors and is also a main connection between transcription factors and the general transcriptional machinery (i.e. RNA Pol II). The Mediator functions as either a co-activator or a co-repressor depending on cellular context and upon which subunits are present in the Mediator Complex (Ito et al., 2002; Ito and Roeder, 2001; Kornberg, 2005; Malik and Roeder, 2005; Mo et al., 2004). Thus, the Mediator is ideally positioned to

regulate coordinated gene transcription, either in a positive or negative manner, involved in a variety of biological processes. Strikingly, all three of the key adipogenic transcription factors can, in some contexts, associate with the Mediator, implying that the Mediator may in fact play a critical role in the regulation of their actions (Ge et al., 2002; Mo et al., 2004; Stevens et al., 2002; Wallberg et al., 2003; Yang et al., 2006). For example, the Med1 Mediator subunit directly binds to NHRs including PPARγ, and PPARγ requires Med1 to stimulate adipogenesis (Belakavadi and Fondell, 2006; Ge et al., 2002). Biochemical studies have also shown that C/EBPß binds with the Mediator in a MAPK-dependent manner and this association is thought to be important in C/EBPß activity (Mo et al., 2004). However, the functional consequences of this interaction in adipogenesis are unknown (Mo et al., 2004). Recently, Med15 mutant worms have been shown to contain decreased fat stores likely, at least in part, due to Med15 affecting worm SREBP function (Taubert et al., 2006; Yang et al., 2006). Thus, accumulating evidence supports a role for the Mediator in stimulating fat formation. Since the Mediator often inhibits the same processes that it activates, it seems plausible that a yet uncharacterized anti-adipogenic form of the Mediator Complex also exists.

The adipose (Adp) gene was discovered as a naturally occurring mutation that produces an obese fruit fly (Doane, 1960a, b; Hader et al., 2003). Adp encodes an evolutionarily conserved protein that contains multiple protein interaction domains (6 WD40, 3 TPR) and may therefore be a nexus or scaffold for multiprotein complex assembly (D'Andrea and Regan, 2003; Hader et al., 2003; Smith, 2004; Smith et al., 1999). In flies and mice, loss of Adp function leads to a dose-dependent increase in

adipose tissue (see Chapter II). Further, adipose-specific Adp transgenesis produces lean flies and mice whereas inhibiting Adp in fat cells leads to obesity indicating that Adp restrains fat formation in an adipocyte autonomous manner (see Chapter II). Adp may be involved in the adipose-glucose metabolic connection, as adipose-specific Adp transgenic mice display increased glucose sensitivity while mice lacking Adp function in fat cells are insulin resistant and hyperglycemic (see Chapter II). Identification of such conserved, haploinsufficient cell autonomous anti-obesity genes is unusual and suggests that studies with Adp may provide important and potentially medically relevant insights into fat biology.

I embarked upon studies to elucidate how Adp regulates fat biology and glucose metabolism. I found that Adp may elicit adipocyte autonomous anti-obesity (anti-adipogenic) effects by inhibiting PPARγ transcriptional activity. Adp was found to directly bind Med23, a component of the Mediator Complex which functions as a link between transcription factors and RNA polymerase II. Furthermore, Adp required Med23 for its ability to inhibit adipogenesis and PPARγ function. Thus, the regulation of PPARγ activity may occur via the Adp binding partner, Med23. The ability of Adp to interact with a putative Med23-PPARγ-Mediator Complex may depend on regulated cytoplasmic-nuclear translocation of Adp. Previous reports demonstrate that the Med1 Mediator stimulates PPARγ function and is required for adipogenesis (Ge et al., 2002). I extend and complement those data and show that the Mediator component Med23 inhibits both PPARγ function and adipogenesis. To determine whether the mammalian cell culture findings are relevant to an intact organism, I examined Med23 mutant flies. Notably,

Med23 null flies are obese and Med23 heterozygotes display an intermediate level of fat formation between wild-type and Med23 null flies, demonstrating haploinsufficiency for fat accumulation, mirroring Adp. So the accumulated *in vivo* and cell culture data support the idea that an anti-fat Mediator exists. Taken together, the data support the idea that Adp is an important regulator of fat biology and one that might lie in a evolutionarily conserved and therapeutically accessible pathway.

Results

Adp Localizes to the Cytosol but Functions in the Nucleus: Potential Cyto-Nuclear Translocation

To begin to understand the cellular basis of Adp function, I explored its subcellular distribution by N-terminally GFP-tagging Adp (Fig 3.1A). I first confirmed that GFP-Adp remained functional in inhibiting 3T3-L1 adipogenesis (Fig 3.1B), indicating that the GFP tag does not interfere with Adp function or its normal subcellular localization. Then, I expressed GFP-Adp and examined its subcellular distribution with 5 μm Z-series confocal microscopic sections. In low, medium, and high expressing cells, GFP-Adp was located in both the cytosol and nucleus with significantly higher levels in the cytosol (Fig 3.1C). I also GFP tagged the dominant negative AdpC1 domain (see Chapter II) which consists of the two WD repeats within the carboxy-terminus of Adp (Fig 3.1A). GFP-AdpC1 was also functionally intact since it stimulated adipogenesis in NIH3T3 cells like untagged AdpC1 (Fig 3.1D). Z-series confocal microscopy showed that GFP-AdpC1, like GFP-Adp, was expressed in both cytosolic and nuclear compartments with greater levels in the nucleus than in the cytoplasm (Fig 3.1E). These data raise the intriguing possibility that Adp may be subject to dynamic cytoplasmic-nuclear translocation which may be involved in the cellular regulation of Adp activity.

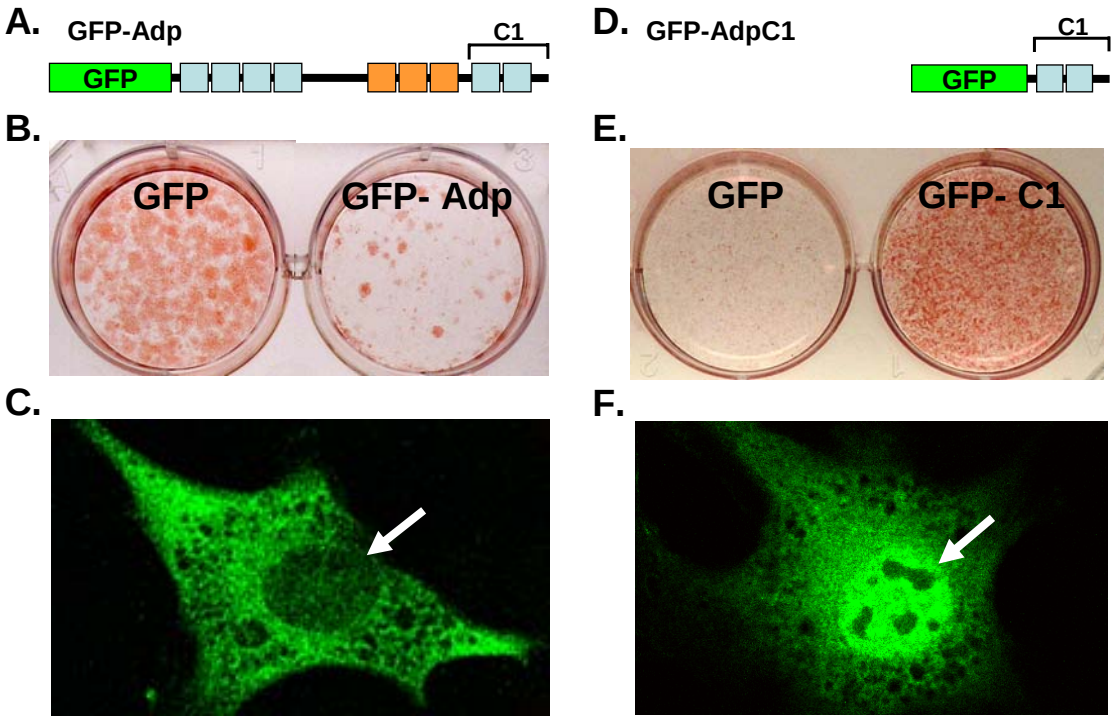


Figure 3.1. Adp is located in both the cytoplasm and nucleus.
(A, D) Cartoons illustrating the (A) GFP-Adp and (D) GFP-AdpC1 chimeras. WD40 domains: light blue rectangles, TPR domains: orange rectangles, GFP: green rectangles. **(B)** 3T3-L1 cells were infected with viruses containing either GFP or GFP-Adp and adipogenically induced. Based upon Oil Red O staining (red), GFP-Adp blocks adipogenesis just like Adp. **(C)** GFP-Adp was introduced into cells and its localization was assessed with 5um confocal Z series microscopy, which showed that GFP-Adp is present in cytosol > nucleus (white arrow). **(E)** NIH3T3 cells were infected with GFP or GFP-AdpC1 and adipogenesis was assessed with Oil Red O stains, which showed that GFP-AdpC1 stimulates 3T3-L1 adipogenesis, maintaining AdpC1 dominant negative action. **(F)** 5um confocal Z series photograph shows that GFP-AdpC1 is present in nucleus (white arrow) > cytosol.

The observations suggesting potential cytoplasmic-nuclear translocation prompted me to investigate the compartment in which Adp actually elicits its anti-adipogenic actions. Thus, I targeted Adp to the nucleus by fusing a strong nuclear localization signal (NLS) to the amino terminus of Adp (Fig 3.2A, NLS-Adp). Notably NLS-Adp blocked adipogenesis (Fig 3.2B) (in several assays, the NLS-form appeared more active than wild-type Adp, not shown), suggesting that Adp exerts its anti-adipogenic actions in the nucleus. Further support for this notion derives from studies with a nuclear export sequence Adp fusion (NES-Adp) designed to target Adp away from the nucleus (Fig 3.2C). Remarkably, NES-Adp not only failed to block adipogenesis but actually stimulated adipogenesis, apparently functioning as a dominant negative Adp (Fig 3.2D). Thus, although GFP-Adp is located in the cytosol to a greater extent than the nucleus, it is likely the nuclear fraction of Adp functions to inhibit adipogenesis. Taken together, these data raise the possibility that nuclear entry and exit could serve as a mechanism to regulate the anti-adipogenic function of Adp. Further support for this model derives from two recent reports describing WD40 family members translocating from the cytosol to nucleus and inhibiting the transcriptional activity of NHRs (Kino et al., 2005; Zhang et al., 2006).

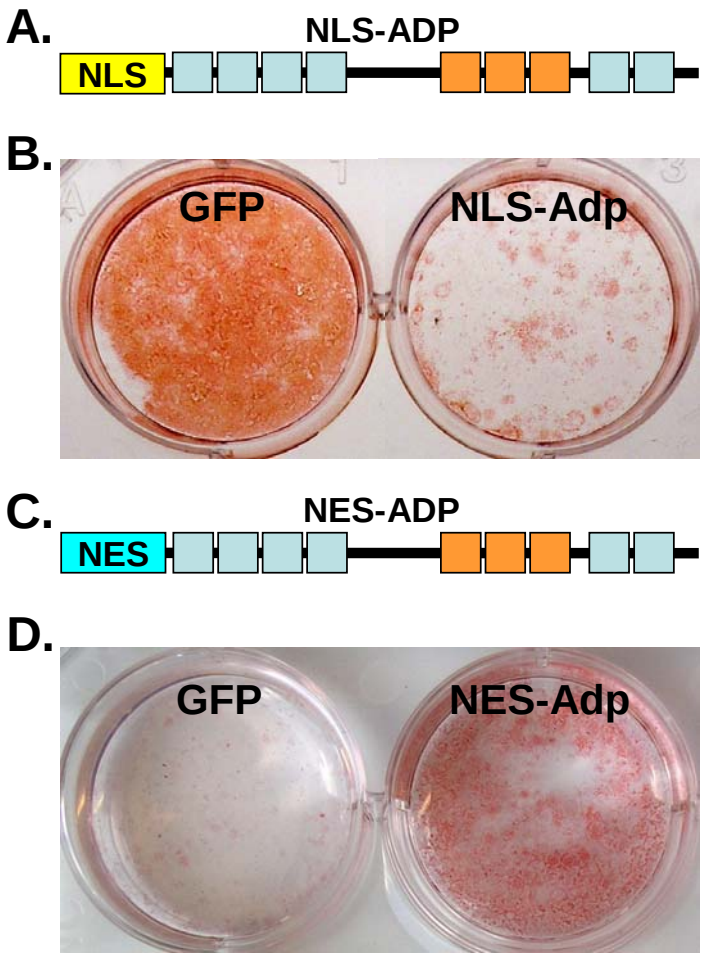


Figure 3.2. Adp functions in the nucleus.
(A, C) Cartoons showing the (A) nuclear localization signal (NLS)-Adp fusion protein expression construct and the (C) nuclear export signal (NES)-Adp chimera. NLS: yellow rectangle, NES: aqua rectangle, Adp as in Figure 3.1. (**B)** 3T3-L1s were infected with GFP or NLS-Adp and adipogenesis was assessed with Oil Red O staining, which showed that NLS-Adp blocked adipogenesis, just as Adp does. (**D)** 3T3-L1s were infected with a GFP or NES-Adp virus and later stained with Oil Red O, which showed that NES-Adp stimulated adipogenesis, functioning like a dominant negative Adp.

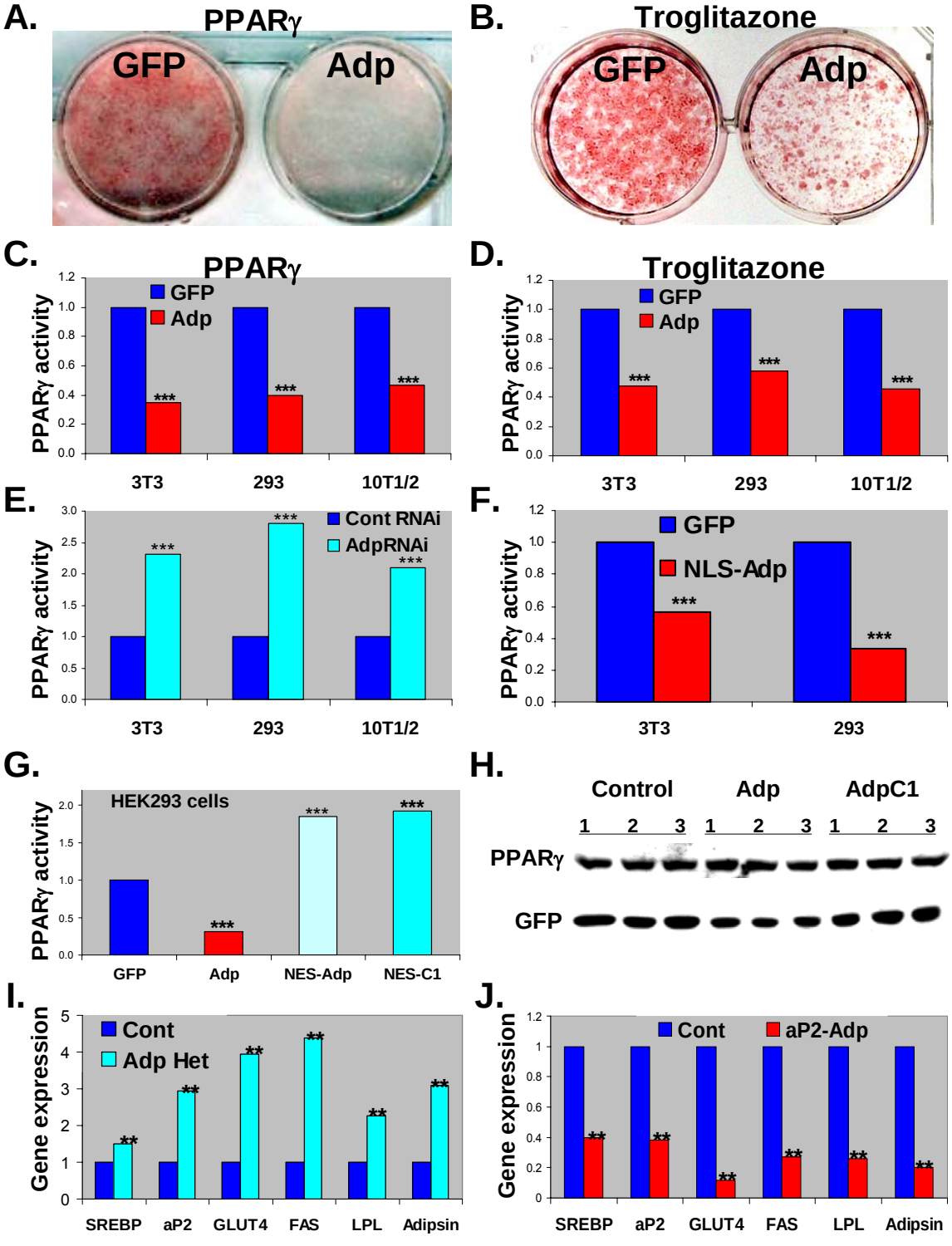
Adp Inhibits PPARγ Function

If Adp does translocate from the cytoplasm to nucleus to inhibit a transcription factor analogous to previous reports describing mechanisms of the WD repeat family of transcriptional repressors, PPARγ, an NHR and the key pro-adipogenic transcription factor, is a potential target. To attempt to clarify the relationship between Adp and PPARγ, I turned to functional studies. For this, I infected 3T3-L1 cells with viruses that express PPARγ with either GFP or Adp, and evaluated adipogenesis. In this assay, Adp inhibited PPARγ-stimulated adipogenesis (Fig 3.3A). Adp also blocked the ability of a TZD class of PPARγ agonist, troglitazone, to stimulate adipogenesis, suggesting specificity for PPARγ transactivation activity (Fig 3.3B). These data are consistent with the notion that Adp inhibits PPARγ function.

To determine whether Adp regulates PPARγ activity, I turned to a heterologous system and assessed the effect of Adp on a PPARγ-dependent transcriptional reporter containing multimerized PPARγ response elements coupled to firefly luciferase (PPRE-Luc). To test whether Adp directly controls PPARγ activity, independently of altered adipogenesis or endogenous PPARγ expression, I combined transient assays, forced expression of PPARγ, and non-adipogenic conditions or cells. For this, I transfected PPRE-Luc, renilla luciferase expression plasmid (transfection control), PPARγ expression plasmids, and either GFP or Adp expression plasmids into NIH-3T3, HEK293 and C3H10T1/2 cells and quantified luciferase levels which serve as a readout for PPARγ transactivation activity. In all three cell lines, Adp significantly inhibited PPARγ-dependent luciferase activity (Fig 3.3C). In addition, Adp inhibited the increase in

activity induced by the PPARγ agonist troglitazone, supporting specificity of the Adp effect (Fig 3.3D). In contrast, inhibiting Adp activity with Adp RNAi significantly stimulated PPARγ reporter expression in all three cell lines (Fig 3.3E). I also examined the effects of targeting Adp in and out of the nucleus. The nuclear-targeted form of Adp, NLS-Adp, inhibited PPARγ-dependent activation (Fig 3.3F), similar to its action on adipogenesis, while the nuclear excluded NES-Adp, which stimulates adipogenesis, significantly increased PPARγ activity (Fig 3.3G). Similarly, the dominant negative AdpC1 also stimulated PPARγ activity (Fig 3.3G). As specificity controls, I also evaluated whether Adp could alter the activity of a series of control transcription factors including a Zn finger, a MEF, a bHLH, and two NHRs, but I did not observe any effect (not shown). To address the possibility that Adp alters PPARγ levels, which could secondarily affect PPARγ activity, I repeated the studies but in this case I evaluated PPARγ protein levels in response to control, Adp or AdpC1 using Western blot; however, PPARγ protein levels were unchanged (Fig 3.3H). In co-immunoprecipitations, even under the least stringent conditions, I did not detect direct binding between Adp and PPARγ (not shown), decreasing the likelihood that Adp and PPARγ directly associate. To check whether the inhibitory action of Adp on PPARγ might be relevant *in vivo*, I quantified the expression levels of PPARγ target genes in fat pads excised from $Adp^{+/+}$ wild-type controls, $Adp^{+/-}$ heterozygotes, and aP2-Adp transgenic mice (See Chapter II for description of these mice). The levels of expression of all PPARγ targets were increased in Adp heterozygous fat and reduced in aP2-Adp transgenics, which may indicate a direct effect on PPARγ action or, alternatively, a secondary response to the

alterations in fat accumulation (Fig 3.3I, J). Taken together, these data support the notion that Adp blocks PPARγ transcriptional activity, which may underlie its ability to regulate mammalian fat formation and glucose homeostasis.


A.
PPARγ
GFP
Adp
B.
Troglitazone
GFP
Adp
C.
PPARγ
PPARγ activity
GFP
Adp
3T3
293
10T1/2
D.
Troglitazone
PPARγ activity
GFP
Adp
3T3
293
10T1/2
E.
PPARγ activity
Cont RNAi
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3T3
293
10T1/2
F.
PPARγ activity
GFP
NLS-Adp
3T3
293
G.
HEK293 cells
PPARγ activity
GFP
Adp
NES-Adp
NES-C1
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Adp
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1 2 3 1 2 3 1 2 3
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SREBP
aP2
GLUT4
FAS
LPL
Adipsin
J.
Gene expression
Cont
aP2-Adp
SREBP
aP2
GLUT4
FAS
LPL
Adipsin

Figure 3.3. Adp inhibits PPARγ function.
(**A**) 3T3-L1s were co-infected with viruses encoding PPARγ and either GFP or Adp and adipogenesis was assessed with Oil Red O staining, which showed that Adp inhibits PPARγ-dependent adipogenesis. (**B**) 3T3-L1s expressing either GFP or Adp were adipogenically induced with the TZD PPARγ agonist troglitazone (1μg/ml). Adipogenesis was scored with Oil Red O stains, which showed that Adp blocks TZD-stimulated adipogenesis. (**C**) GFP or Adp along with PPARγ, a PPRE-luc reporter, and a renilla luciferase loading control were transfected into the indicated cell lines and luciferase activity was measured. Then the measurements were standardized with the renilla control. These normalized values, termed PPARγ activity, show that Adp significantly inhibited PPARγ function in all tested cell lines. (**D**) GFP or Adp and PPARγ, a PPRE-luc reporter, and renilla luciferase control were introduced into the indicated cell lines in the presence of troglitazone (1μg/ml) and the normalized levels of luciferase show that Adp inhibits TZD-stimulated PPARγ activity (**E)** The indicated cell lines, expressing control RNAi or Adp RNAi together with PPARγ, PPRE-luc, and renilla luciferase control, were analyzed for luciferase activity. The standardized results demonstrate that Adp RNAi stimulates PPARγ function. Study as in C except with control or Adp RNAi. (**F)** Luciferase levels, done as in C-E, were measured in cells expressing either NLS-Adp or GFP. The normalized results show that NLS-Adp, like wild-type Adp, significantly inhibits PPARγ activity. (**G)** Based upon a PPRE-luc study done as above, targeting Adp to the cytosol and away from the nucleus leads to a significant increase in PPARγ activity, which contrasts to wild-type Adp which reduces PPARγ function. (**H)** HEK293 cells were transfected in triplicate with PPARγ and GFP (loading control) and empty vector (control), Adp, or AdpC1, with conditions identical to C-G. Then, rather than assessing luciferase activity, we evaluated PPARγ (top panel) and GFP (bottom panel) protein expression with Western blots, which showed that PPARγ protein levels were unchanged by Adp or AdpC1. (**I, J)** RNA was extracted from fat pads dissected from control, Adp heterozygous, and aP2-Adp transgenic mice and levels of the indicated PPARγ target genes were assessed with real-time PCR. The results show that reducing Adp leads to significantly higher expression of the PPARγ targets while increasing Adp represses PPARγ target gene expression. Glut4 glucose transporter 4, FAS fatty acid synthase, LPL lipoprotein lipase $^{**}p < 0.01$, $^{***} p < 0.005$

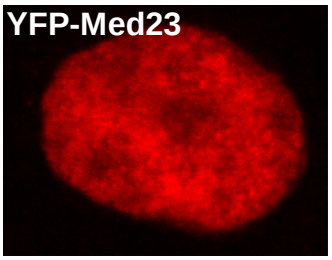
Adp Binds the Mediator Subunit Med23

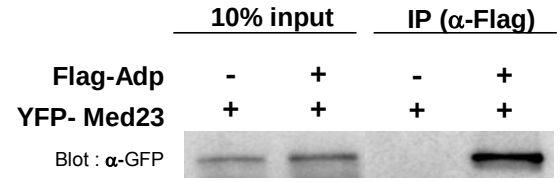
To better understand Adp's mechanism of action and the Adp-PPARγ functional interaction, I characterized proteins that bind Adp in a yeast two-hybrid screen. I focused on one interactor, Med23, as a plausible candidate Adp interactor (Fig 3.4A) because Med23 is a component of the Mediator Complex that is known to regulate PPARγ activity, because Med23 localizes to the nucleus (Fig 3.4B), the subcellular compartment in which the functional interaction between Adp and PPARγ appears to occur (Figs 3.1-3), and because Med23, like Adp, regulates transcription. Structure-function analyses showed that the carboxyl terminal Adp regulatory C1 region strongly interacted with Med23 (Fig 3.4A) consistent with the functional role I found for this domain in previously described experiments (Figs 3.1, 3, and Chapter II). To further assess the potential interaction between Adp and Med23, I transfected HEK293 cells with Flag-AdpC1 and YFP-Med23, immunoprecipitated with an anti-flag antibody and probed a Western blot of the precipitates with an anti-GFP antibody that also recognizes YFP. In the absence of the tagged Adp construct, Med23 was not present in the immunoprecipitate (Fig 3.4C). However, when the tagged Adp construct was included, Med23 was readily detected in the immunoprecipitate (Fig 3.4C). Myc-AdpC1 and YFP-Med23 also co-immunoprecipitated (not shown). To determine whether Adp and Med23 interact in living mammalian cells and with spatial resolution, I evaluated fluorescent resonance energy transfer (FRET) (Karpova et al., 2003). In this context, FRET relies on the intimate juxtaposition of two fluorescent tags for the FRET effect. Since the two fluorescent tags must be close enough to detect fluorescence transfer, I selected AdpC1

for the initial studies since this portion of Adp contains the Med23 interactions domain and because it is much smaller than full-length Adp, and so may overcome the proximity issues that can limit FRET. For FRET, I transfected cells with CFP-AdpC1 and YFP-Med23 and used the photobleaching method in which a signal ≥5% indicates a significant positive interaction. With this approach, I detected a strong FRET signal of 11%, and the interaction between AdpC1 and Med23 was only observed in the nucleus, indicating specificity of the detected interaction (Fig 3.4D).

A.

	Med23	Adp
Adp	++	---
AdpC1	++++	---
Lam	n/d	---
ZnF	---	---





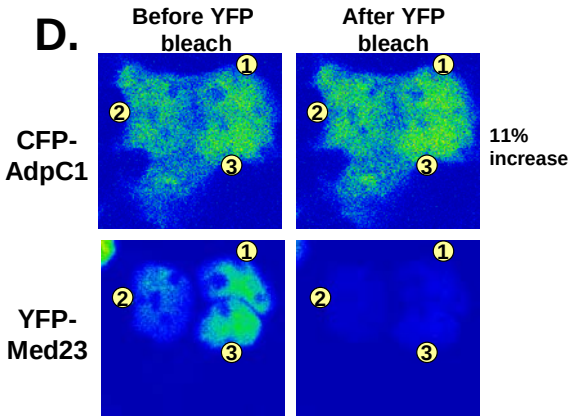


Figure 3.4. Adp binds to the Mediator subunit Med23.
(**A**) Summary of yeast two hybrid data showing that Med23 binds to Adp and AdpC1. Lamin (Lam) and a zinc finger transcription factor (ZnF) are negative controls. n/d not tested. (**B**) YFP-Med23 was expressed in cell culture and YFP fluorescence (Red), visualized with fluorescence microscopy, was only detected in the nucleus. (**C**) Flag-tagged Adp and YFP-Med23 were transfected into cells, and after cell lysis and immunoprecipitation with an anti-flag antibody the resulting immunoprecipitates were subjected to Western blotting to identify the presence of YFP-Med23, which was only detected in the immunoprecipitate in the presence of Adp. Western blotting of the cell lysates (10% input) shows that both samples contain roughly equal levels of YFP-Med23. (**D**) CFP-AdpC1 and YFP-Med23 were introduced into cells and photos were taken on a Zeiss LSM 510 Confocal Microscope before and after YFP photobleaching. The 11% increase in CFP signal after YFP photobleaching demonstrates FRET. 1, 2, and 3 label three different nuclei, all of which display FRET between Adp and Med23.

Med23 Inhibits Adipogenesis and PPARγ Function

To examine a potential role of Med23 in adipogenesis, I infected C3H10T1/2 cells with a virus encoding GFP, Adp, or Med23 and incubated the cells in adipogenic induction media. Notably, Med23, like Adp, blocked adipogenesis (Fig 3.5A, B). Since Adp and Med23 interact and since both inhibit adipogenesis, it seemed plausible that Med23 might inhibit PPARγ activity as I observed for Adp (Fig 3.3). To evaluate this possibility, I transfected mammalian cells with PPRE-luc, renilla luciferase transfection control, PPARγ expression plasmid, and either GFP, Adp, or Med23 expression plasmids, and after 48 hours assessed luciferase levels. I found that in all tested cell lines, Med23, like Adp, significantly reduced PPARγ-dependent luciferase activity (Fig 3.5B). The finding that Med23 blocked adipogenesis and PPARγ activity is opposite to the result anticipated from the Med1 data showing enhanced adipogenesis and PPARγ stimulation (Ge et al., 2002). So it is possible that the Med23-containing Mediator has antagonistic function to the identified pro-adipogenic, pro-PPARγ Mediator. Alternatively, the Med23 results I obtained may be due to squelching. Although either interpretation supports the idea that Med23 regulates adipogenesis, providing potential mechanistic explanations for Adp action, it does not clarify whether Med23 is pro- or anti-adipogenic. To attempt to determine the endogenous role of Med23 in mammalian adipogenesis, I turned to necessity tests and inhibited Med23 (and Adp) function with RNAi and evaluated fat formation. I found that inhibiting Med23 function with RNAi stimulated adipogenesis, similar to Adp RNAi (Fig 3.5C-E). Next, I reduced Med23 levels with Med23 RNAi and evaluated PPARγ function with the PPRE-lux assays described above (Fig 3.3). Notably

reducing Med23 levels with RNAi significantly stimulated PPARγ activity (Fig 3.5G). Thus Med23, like Adp, inhibits adipogenesis and PPARγ function, which supports the existence of an anti-adipogenic, anti-PPARγ Mediator.

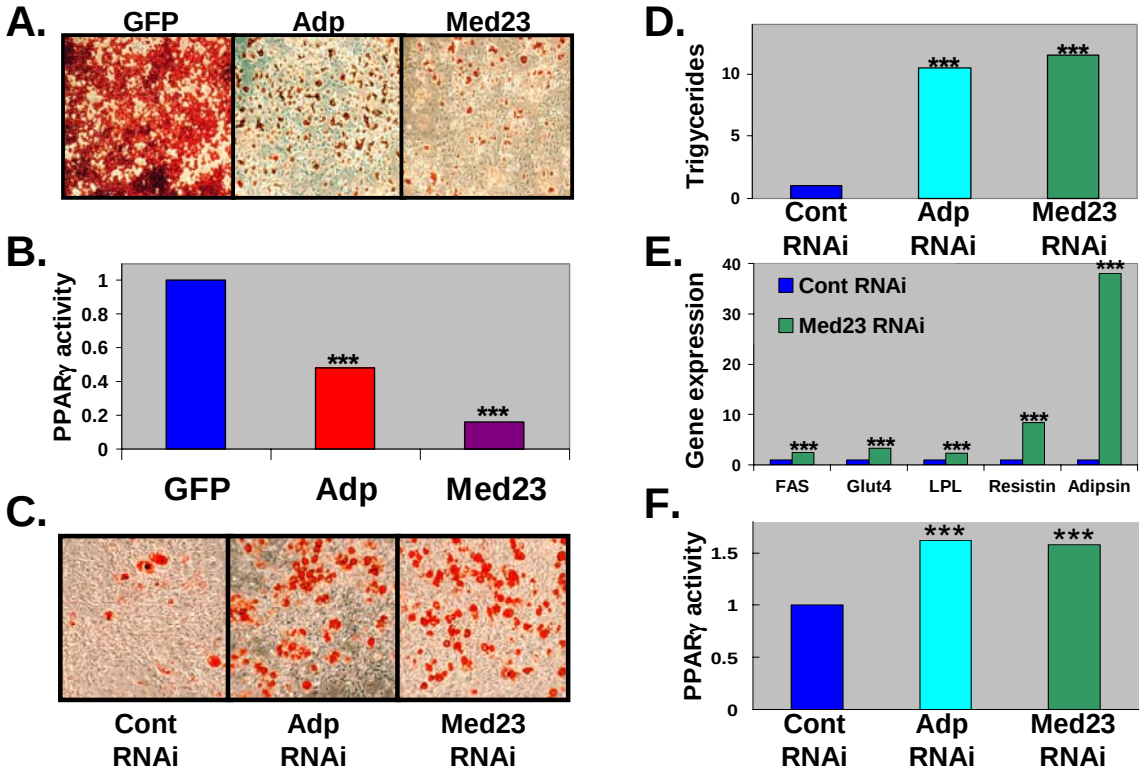


Figure 3.5. Med23 inhibits adipogenesis and PPARγ activity. (**A**) C3H10T1/2 cells were infected with viruses encoding GFP, Adp, or Med23 and then induced to form adipocytes. Oil Red O staining shows that Adp and Med23 block adipogenesis. (**B**) HEK293 cells were transfected with GFP, Adp, or Med23 together with PPARγ, PPRE-Luc, and renilla luciferase plasmid, and normalized luciferase levels (PPARγ activity) was assessed as in Figure 3.3. PPARγ activity were significantly reduced by both Adp and Med23. (**C-E**) NIH3T3 cells were generated that express control RNAi or Med23 RNAi, then adipogenesis was scored with (C) Oil Red O staining, (D) triglyceride quantitation, and (E) real-time PCR molecular analysis of the indicated PPAR□ target genes: glucose transporter 4 (Glut4), fatty acid synthase (FAS), and lipoprotein lipase (LPL) are key components of differentiated adipocyte function; adipsin and resistin are adipokines expressed by mature adipocytes. (**F**) HEK293 cells expressing control RNAi, Adp RNAi, or Med23 RNAi and PPARγ, PPRE-Luc, and renilla luciferase were assayed for PPARγ activity as in B. *** $p<0.005$

Adp Requires Med23 to Inhibit both Adipogenesis and PPARγ action

Since Adp and Med23 directly bind and have strikingly similar functions in mammalian adipogenesis and PPARγ regulation, it seemed plausible that the two molecules might interact in a functionally relevant manner. So I evaluated possible synthetic effects of Adp and Med23 on adipogenesis by expressing Adp and Med23 at modest levels separately and together in C3H10T1/2 cells. I found that both Adp and Med23 blocked adipogenesis, as judged by Oil Red O stain, and apparently in a cooperative manner (Fig 3.6A). This lends some support to the idea that the Adp-Med23 interaction has functional consequences. Similar cooperative interactions were obtained in NIH3T3s. Since Med23 inhibits adipogenesis in a synthetic manner with Adp (Fig 3.6A), I tested whether Adp requires Med23 for adipogenic inhibition by expressing Adp in NIH3T3s in which Med23 function was reduced with RNAi. I found that the ability of Adp to block adipogenesis was inhibited by Med23 RNAi (Fig 3.6B, C). Next I determined whether Adp requires Med23 for its ability to inhibit PPARγ activity using the PPRE-luc reporter system described above. I found that reducing Med23 function with Med23 RNAi inhibited the ability of Adp to block PPARγ activity (Fig 3.6D). Taken together, these data are consistent with the notion that Adp elicits its anti-adipogenic and anti-PPARγ actions via Med23

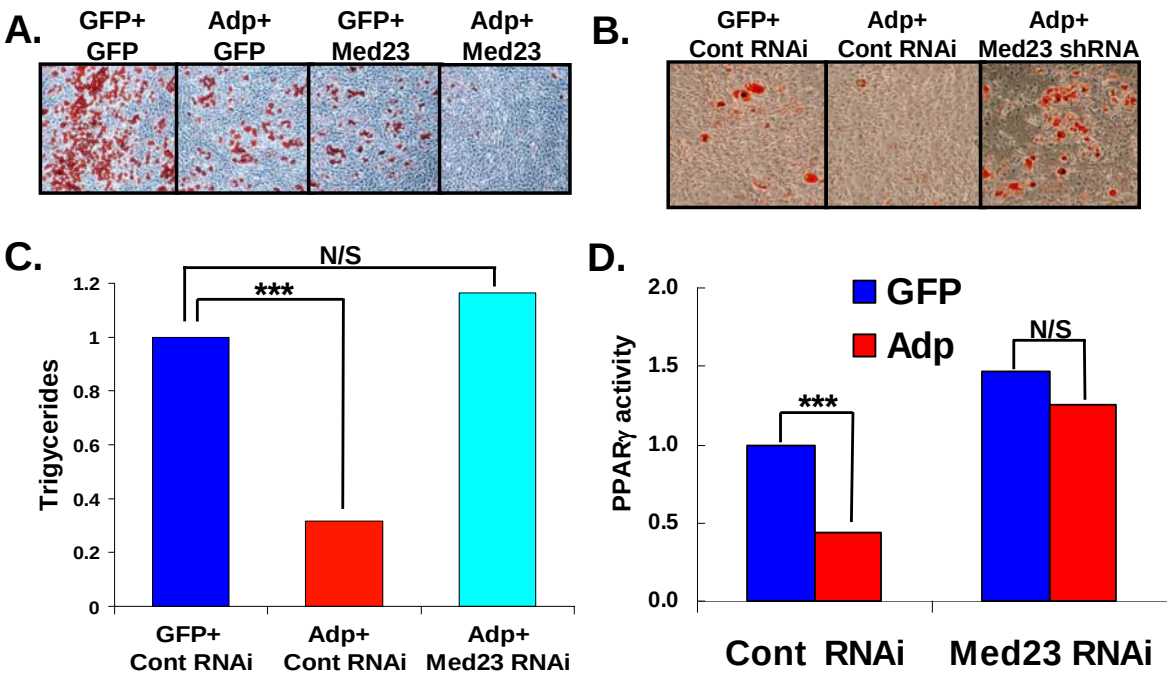


Figure 3.6. Adp requires Med23 to inhibit adipogenesis and PPARγ function. **(A)** C3H101/2 cells were infected with viruses encoding GFP + GFP, Adp + GFP, Med23 + GFP, or Adp + Med23, induced to form adipocytes, and adipogenesis was evaluated with Oil Red O staining. **(B, C)** NIH3T3s expressing GFP and control RNAi, Adp and control RNAi, and Adp and Med23 RNAi. The Adp-dependent inhibition of adipogenesis, scored by (B) Oil Red O staining and (C) triglyceride quantitation, was blocked by loss-of-Med23 function. **(D)** HEK293 cells were transfected with GFP or Adp with either control RNAi or Med23 RNAi together with PPARγ, PPRE-Luc, and renilla luciferase control plasmid. PPARγ activity, measured as in Figures 3.3 and 3.5, was significantly inhibited by Adp but the Adp-dependent blockade was lost when Med23 levels were reduced with RNAi. ***$p < 0.005$, N/S not significant

dMed23 Restrains Fly Fat Formation in a Dosage-Sensitive Manner

To determine whether the Med23 anti-adipogenic cell culture data could be extended to an intact organism, I turned to the fly, the system in which the anti-obesity actions of Adp was initially uncovered. I obtained flies in which the fly Med23 (dMed23) gene was disrupted by a P-element insertion (Fig 3.7A). I then quantified expression of dMed23 message in wild-type and heterozygotes and found that dMed23 expression levels were approximately 50% of wild-type in the heterozygotes supporting the notion that the insertion is a dMed23 loss-of-function allele (Fig 3.7B). The presence of a balancer chromosome in 100% of adult flies indicated that the P-insertion was homozygous lethal and thus precluded direct analysis of the entire allelic series. Further evaluation to identify the timing of homozygous lethality revealed that the dMed23 homozygotes survived until the second larval instar stage. Therefore, I first compared, in adults, dMed23 wild-type to dMed23 heterozygotes, and then, in second instar larvae, dMed23 heterozygotes to dMed23 homozygotes in several tests directed towards fly fat biology as I described previously (see Chapter I and II). In these assays, the dMed23 heterozygotes displayed an obesity phenotype intermediate between wild-type and dMed23 homozygotes (Fig 3.7C-F). For example, dMed23 heterozygotes, like Adp heterozygotes, are resistant to starvation, which in flies strongly correlates with increased fat stores (Fig 3.7C). Consistent with that, the dMed23 heterozygotes contained significantly higher levels of fat than wild-type flies (Fig 3.7D). Next, I explanted, examined and stained the larval fat bodies of dMed23 heterozygous and homozygous mutants. I found that $dMed23^{-/-}$ homozygotes, compared to $dMed23^{+/-}$ heterozygotes, had

significantly larger fat droplets and increased Nile Red fat-specific staining, whose fluorescent intensity is proportionate to fat content, strongly suggesting that they have increased lipid stores (Fig 3.7E). To further examine lipid content in a quantitative assay, I directly measured triglyceride levels and found that the dMed23$^{-/-}$ homozygotes contained significantly higher levels of triglycerides than did dMed23$^{+/-}$ heterozygotes, supporting a haploinsufficient role of Med23 in fly fat storage (Fig 3.7F). Thus, Med23 inhibits fly fat formation in a dosage-sensitive manner, mirroring Adp (see Chapter II, Fig 2.1).

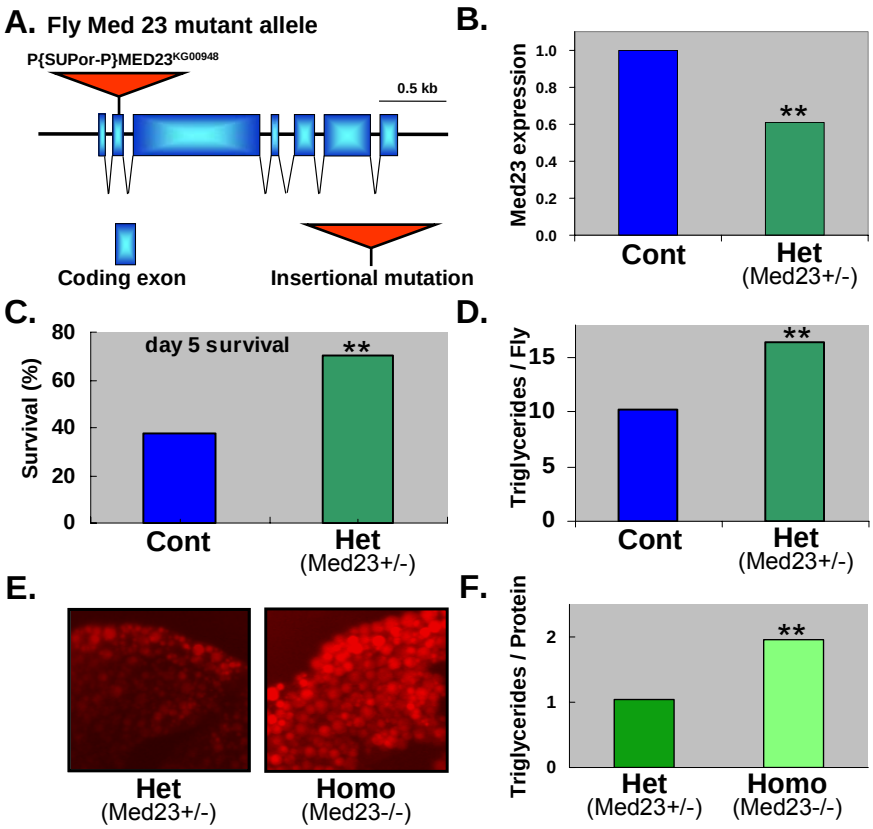


Figure 3.7. Med23 mutant flies display dosage-sensitive obesity.
(**A**) Schematic of the P-element insertion into the Med23 locus. (**B**) Med23 expression levels were quantified from RNA harvested from wild-type and Med23 heterozygote sibling flies (n = 100 per genotype) with real-time PCR. Med23 levels are significantly reduced by the Med23 insertion. (**C**) Well-fed control and Med23 heterozygous siblings (n = 80 per genotype) were deprived of food and starvation survival, an excellent indicator of fly fat storage, was evaluated 5 days after food removal. Med23 heterozygotes were significantly resistant to starvation compared to controls. (**D**) Triglycerides were extracted from wild-type and Med23 heterozygous sibling flies (n = 40 per genotype) and quantitation showed that Med23 heterozygotes have significantly more fat than controls. (**E**) Fat bodies were dissected from identically reared Med23 heterozygous and homozygous siblings at the second instar stage, stained with Nile Red, a fat-specific fluorescent dye whose emission intensity is proportionate to fat content, and visualized under identical conditions with a fluorescent dissecting microscope. Med23 homozygotes had a larger more intensely staining lipid droplets compared with Med23 heterozygotes and the Med23 homozygotes. (**F**) The fat content of identically reared sibling Med23 heterozygotes and homozygotes (n = 50 per genotype) was assessed with a triglyceride assay, showing that the homozygotes have significantly more fat stores than heterozygotes. **p < 0.01

Discussion

Higher animals store energy as fat and, consistent with its central role in many physiological processes, fat-storing tissues are required for the life cycle of many higher organisms (Bluher et al., 2003; Rosen and Spiegelman, 2000; Spiegelman and Flier, 1996, 2001). Alterations in fat accumulation lead to important human diseases, including diabetes, and current predictions suggest that obesity is becoming one of the most important and preventable causes of human morbidity and mortality (Allison et al., 1999; Campbell and Dhand, 2000; Lehrke and Lazar, 2005; Must et al., 1999; Olshansky et al., 2005; Spiegelman and Flier, 1996, 2001). Therefore, elucidating the molecular mechanisms that underlie fat accumulation and glucose homeostasis is an important task. The accumulated data suggests that Adp, and one of its binding partners Med23, are important and conserved regulators of fly and mammalian fat biology and ones that appear to function in a pathway amenable to therapeutic interventions. So studies on the Adp pathway might lead to a better understanding of the molecular mechanisms that underlie fat biology and may identify targets for the rational design of drugs to treat people with lipodystrophy, obesity, and diabetes.

Through cellular, biochemical, molecular, and genetic approaches, I attempted to garner insights about the mechanisms of Adp action. The data obtained thus far are consistent with the idea that Adp may elicit anti-obesity effects by inhibiting the transcriptional activity of PPARγ, a key adipogenic transcription factor and a target of widely used anti-diabetes therapies (Lehrke and Lazar, 2005; Spiegelman and Flier, 1996,

2001). Such regulation may occur via Med23 and the Mediator Complex, and involve a cytoplasmic-nuclear translocation. Currently, there are limitations in drawing strong conclusions supporting the latter cell biological notions, especially the idea of a regulated shuttling, as the data are primarily derived from fusion constructs rather than endogenous protein. Antibodies directed against Adp would be a significant advance, but my attempts to generate antibodies of sufficient quality for cell biological studies have been thus far unsuccessful. Nonetheless, several lines of evidence support the idea that Adp is located in both the nucleus and cytoplasm. The primary structure of Adp lacks recognizable motifs such as putative transmembrane domains, signal sequences, nuclear localization signals, etc. that are often present in non-cytoplasmic proteins, so the initial notion was that Adp was cytosolic. However, confocal microscopic imaging of a GFP-tagged version of Adp indicated that Adp was located in both the nucleus and the cytosol, with significantly more in the latter compartment. It is possible that the tagged-Adp was mislocalized, however, the tagged construct had wild-type activity supporting the notion of appropriate localization. Further, the AdpC1 domain was predominantly nuclear, supporting the idea that Adp contains information to direct it to the nucleus. The observations that Adp binds Med23, a nuclear protein, and that this interaction is functionally relevant also support the idea that Adp functions in the nucleus. The forced subcellular targeting studies with either an NLS or NES modified Adp further substantiate the idea that the nucleus is where Adp elicits its anti-adipogenic function. These data highlight the potential importance of regulated Adp cytosolic/nuclear partitioning. Interestingly, there is precedence for similar behavior of two other WD40-

containing proteins, Gβ subunit and TBLR1, which translocate from the cytosol to the nucleus to inhibit NHRs (Kino et al., 2005; Zhang et al., 2006).

PPARγ is a central regulator of adipocyte development, maintenance, and function and is the target of the widely used thiazolidinedione (TZD) class of anti-diabetes therapies (Kintscher and Law, 2005; Lehmann et al., 1995; Lehrke and Lazar, 2005). Given the predominant role of PPARγ in adipocyte biology and glucose metabolism, much effort has been directed towards understanding its regulation. Several factors (e.g., PGC-1, Rb, Med1) that increase PPARγ activity have been identified (Ge et al., 2002; Lehrke and Lazar, 2005; Wallberg et al., 2003). My studies suggest that Adp may counterbalance these factors and inhibit PPARγ function. The ability of Adp, which contains six WD40 repeats, to inhibit transcription is consistent with previous reports showing that a primary function of WD40 repeat containing proteins is to repress transcription factor activity (Kino et al., 2005; Perissi et al., 2004; Zhang et al., 2006). My initial studies suggested that Adp-dependent inhibition of PPARγ does not appear to be due to a direct physical interaction between the two proteins. So I began to unravel the pathway through the identification of novel interactors of Adp, and found that the Adp interaction partner Med23 also inhibited PPARγ function and adipogenesis. The ability of Adp to inhibit PPARγ function may account for at least part of Adp's anti-adipogenic role and supports the notion that the Adp cascade acts in a therapeutically relevant pathway.

The cellular and *in vivo* mouse adipose phenotypes appear consistent with a role for Adp and Med23 in PPARγ regulation. Notably, both Adp and Med23 play a

conserved role in flies and mammalian systems in restraining fat accumulation (also see Chapter II). Although the mechanism through which Adp and Med23 reduce mammalian fat storage might involve inhibiting PPARγ, no PPARγ ortholog has yet been identified in flies. So despite the conservation of primary structure and biological function, it is possible that Adp inhibits fly and mammalian fat formation through distinct mechanisms or that PPARγ is not the conserved aspect. Although Adp inhibits PPARγ action, the two molecules do not appear to directly interact but rather are more likely to function in a multi-protein complex, possibly containing Adp, Med23, the Mediator, and PPARγ. Since flies and other invertebrates contain fat but do not appear to have a PPARγ homolog, it is possible that Adp may interact directly with a more primordial protein complex, such as the Mediator, that may have functioned to restrain fat accumulation before PPARγ existed. The data that Med23 mutant flies have increased fat stores is consistent with that idea as are recent reports showing that Med15 mutant worms have altered fat accumulation (Taubert et al., 2006; Yang et al., 2006). Once a nuclear hormone receptor, such as PPARγ evolved, it may have co-opted an extant pathway involving Adp and the Mediator to allow for its optimal regulation. It is also possible that the observed inhibition of PPARγ is an *in vitro* activity that does not reflect the biological role that Adp plays in mammalian fat biology. That is, non-PPARγ-dependent mechanisms are biologically relevant and these unidentified molecules, in conjunction with Adp, control fat formation and glucose homeostasis. In either scenario, it seems possible that the direct binding partners of Adp may also play a conserved role in fat biology as seems to be the case for Med23 at least in flies and mammalian cell culture

models. Since Adp primarily consists of known protein interaction modules, Adp should serve as a useful platform to initiate protein interaction or genetic screens aimed at identifying additional partners that participate in the Adp pathway.

To further understand how Adp inhibits adipogenesis and/or PPARγ function, I attempted to characterize proteins that interact with Adp. A series of studies support the idea that Med23 interacts with Adp in a functionally important manner. Yeast two-hybrid, co-IP, and FRET studies indicate that Adp and Med23 directly interact and that the interaction occurs in the nucleus of living cells. Not only do the two molecules interact, but Med23 and Adp also produced remarkably similar functional outcomes in gain- and loss-of-function studies in cell culture and flies, including dosage-sensitive inhibition of fly fat formation. Further, overexpression of both Adp and Med23 inhibited PPARγ activity. The interaction between Med23 and Adp appears functionally relevant in synthetic and epistatic tests, which showed that Adp requires Med23 to inhibit adipogenesis and PPARγ action. Taken together the data support the idea that Adp and Med23 function in a common biological and biochemical pathway and one that when mutated could predispose to obesity and diabetes.

Med23 is part of the Mediator, a multisubunit complex that regulates transcription, either activating or inhibiting the transcription factor and target gene expression (Belakavadi and Fondell, 2006; Kornberg, 2005; Malik and Roeder, 2005). My data indicate that Med23 plays a conserved role from flies to mammalian models in blocking fat formation and that Med23 inhibits PPARγ function. These results complement the previously identified pro-fat role for the Med1 (mammalian) and Med15

(worm) Mediator subunits (Ge et al., 2002; Taubert et al., 2006; Yang et al., 2006). Med1 is required for mammalian adipogenesis and stimulates PPARγ function, which is consistent with the known role of Med1 as the direct contact between NHRs and the Mediator. Since the Mediator exhibits inhibitory and stimulatory functions on the same promoters depending upon cellular context it is possible that both a pro-adipogenic (and pro-PPARγ complex) and an anti-adipogenic (and anti-PPARγ) form exist (Belakavadi and Fondell, 2006; Ge et al., 2002; Malik and Roeder, 2005; Mo et al., 2004; Stevens et al., 2002). In such a model (Fig 3.8), an Adp-associated Med23 containing Mediator inhibits adipogenesis, potentially in part by reducing PPARγ action. Signals, such as adipogenic stimuli and PPARγ ligands, may alter the Mediator, for example shifting Adp localization from the nucleus to the cytosol and minimizing Med23 inhibitory action, thereby converting the Mediator from anti-adipogenic to pro-adipogenic functions.

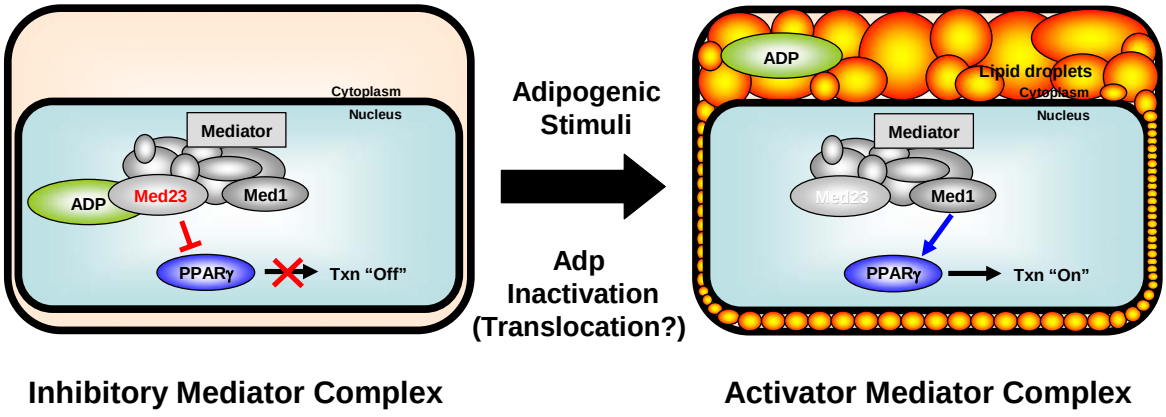


Figure 3.8. Model of Adp pathway function.
Under non-adipogenic conditions (left panel), Adp is located in the nucleus where, via an interaction with Med23 and the Mediator Complex, PPARγ function is inhibited. Pro-adipogenic stimuli translocate Adp from the nucleus to the cytosol, thereby altering the Mediator from a PPARγ inhibitory to a PPARγ stimulatory form (right panel), thus generating fat-containing adipocytes.

Previous studies indicate that Med23 can regulate transcriptional responses of receptor tyrosine kinase (RTK) signals in part through a signal-induced association with transcription factors. C/EBPß is a transcription factor that has many actions including pro-adipogenic functions (Mo et al., 2004). C/EBPß functions early in adipogenesis in part by increasing PPARγ expression (Rosen and Spiegelman, 2000; Smas and Sul, 1995). At baseline, C/EBPß is a repressor, but RTK signals convert C/EBPß to an activator, apparently at least in part by inducing C/EBPß to bind to the Mediator through Med23 (Mo et al., 2004). Consistent with the reported role of Med23 on C/EBPß activity, I also found that Med23 stimulates C/EBPß reporter activity in cell culture (not shown). The findings that C/EBPß is proadipogenic and that C/EBPß requires Med23, at least in heterologous systems, could indicate that Med23 will stimulate adipogenesis. It is difficult to reconcile these data with the series of sufficiency, necessity, and biochemical studies that I have performed that support the idea that Med23 inhibits fly fat formation, mammalian adipogenesis, and PPARγ function. The differences may be a consequence of cellular and environmental context as the results with Med23 and C/EBPß were obtained in non-adipogenic cells under non-adipogenic conditions. That is, Med23 can inhibit C/EBPß in some settings but not during adipogenesis. Alternatively, it is possible that the ability of Med23 to activate C/EBPß and inhibit PPARγ is dependent on post-translational modification or association with additional molecules, so the two functions can occur in the same cells but under different conditions, cell states, or timing.

Diverse animal phyla from invertebrates to vertebrates store fat, which in turn controls and supports multiple aspects of the life cycle such as metabolic homeostasis,

thermal and traumatic protection, and lifespan. However, a burgeoning epidemic of obesity and diabetes endangers millions and is altering the landscape of our health care system (Allison et al., 1999; Must et al., 1999; Olshansky et al., 2005). Adp is a conserved anti-obesity gene that functions in a cell autonomous manner. The data obtained thus far are consistent with the idea that Adp may elicit anti-obesity effects by inhibiting the transcriptional activity of PPARγ, a key adipogenic transcription factor and a target of anti-diabetes therapies. Such regulation may occur via Med23 and the Mediator Complex and may involve a regulated cytoplasmic-nuclear translocation. This implies that Adp may function to integrate and link environmental cues to gene expression, especially those that are important in energy homeostasis, such as PPARγ. Also noteworthy is the fact that the putative function of Adp serving as a link between external environmental cues and Mediator dependent transcription predates the appearance of a functional PPARγ, suggesting Adp may be part of a rather ancient nutrient sensing pathway. Therefore, elucidating the upstream signals and additional components of the Adp pathway is an important task with broad implications ranging from basic scientific to clinical aspects of understanding fat biology and energy homeostasis.

Material and Methods

Plasmids

Murine Med23 (NM_027347), and murine PPARγ (NM_011146) were cloned into the pMX retroviral expression vector (generous gift of Dr. Gary Nolan) generating pMX-Med23 and pMX-PPARγ respectively. pMX-NLS-Adp was constructed by cloning the large T antigen nuclear localization signal (DPKKKRKV) N-terminal to full-length mouse Adp. Likewise, pMX-NES-Adp was constructed by cloning the human protein kinase inhibitor α nuclear export signal (NSNELALKLAGLDINKTE) N-terminal to full-length mouse Adp. For shRNA vectors, three hairpins were cloned into the mU6 shRNA plasmid. All three hairpins were tested for knock-down efficiency by real-time PCR and two hairpins with the highest efficiency were used for further experiments. Both Adp and Med23 hairpin sequences were designed to target both mouse and human message. Retroviral expression plasmids were all cloned into pMX vector. Flag epitope and GFP/YFP/CFP-tagged versions of Adp, Adp, AdpC1 and Med23 were generated by subcloning appropriate fragments into pCMV-Flag 7.1 (Sigma) or pCS2+-GFP/YFP/CFP - N – terminal vectors.

Retrovirus production and infection

Standard methods were used to generate recombinant retroviruses and viral transduction to 3T3-L1, NIH3T3 and C3H10T1/2 cells were as described (see Chapter I).

Cell culture and adipocyte differentiation

Mouse NIH-3T3 fibroblasts, C3H10T1/2 pluripotent mesenchymal cells, and 3T3-L1 preadipocytes were purchased from the American Type Culture Collection and maintained in growth media (DMEM with 10% calf serum, 10 units/ml penicillin, 10 μg/ml streptomycin) at 37 °C in 5% CO_2. Cells were passed before confluence and discarded after 10 passages. Media changes were performed every other day during cell maintenance and adipogenesis. 3T3-L1, C3H10T1/2 and NIH-3T3 cells were induced to undergo adipogenesis as previously described (see Chapter I).

Analysis of lipid accumulation

Lipid droplets in differentiated adipocytes were stained with Oil Red O as described previously (see Chapter I). To quantify triglyceride levels, flies or cells were lysed in 0.5% SDS/PBS and triglyceride content was measured using the Infinity Triglyceride Reagent (ThermoElectron) following manufacturer's instructions. Protein concentrations used to normalize triglyceride content were measured with a BCA protein assay kit (Pierce).

RNAi: Stable shRNA cell lines

The indicated cell lines were transfected with either the control parent mU6-neo shRNA plasmid or mU6-neo shRNA plasmids that contain the appropriate hairpins to reduce the expression of Adp (see Chapter II) or Med23 (see above). Stable clones were

selected in 400μg/ml G418 for one week then the remaining G418-resistant stable clones were pooled together for further experiments. shRNA mediated knockdown of the targeted message was determined by qPCR.

RNA extraction and quantitative real-time RT-PCR

Total RNA from mouse tissues, flies, or cultured cells was extracted with Trizol (Invitrogen), RNase-free DNAse I-treated, and reverse-transcribed using random hexamers and M-MLV-reverse transcriptase (Invitrogen) to obtain cDNA. Gene expression was measured through real-time PCR analysis using SYBR Green Master Mix reagent (Applied Biosystems, 7500 Real-Time PCR System). Real-time PCR values for gene expression were normalized over endogenous ß-actin expression. All real-time primer sequences were validated for specificity and efficacy prior to use.

Luciferase assays

tk-PPRE3X-luc plasmid (generous gift of Dr. Ron Evans) was used as a reporter for PPARγ transcriptional activation. A pRL-cytomegalovirus (CMV) vector (Promega Corp.) expressing the *Renilla* luciferase gene served as an internal control, and all results were normalized against this control. Transfections were performed in 24-well plates using Fugene 6 (Roche) and transfections typically consisted of 20 ng tk-PPRE3X, 5 ng pRL-CMV, 50 ng pMX-PPARγ2, and 200 ng effector or control expression vector. 36-48 hrs. post-transfection, cell lysates were harvested and luciferase assays were performed with the Dual Luciferase Reporter Assay System (Promega Corp.) following the

manufacturer's guidelines. Luciferase activity was measured using the POLARstar OPTIMA plate reader (BMG labtech).

Immunoprecipitation and Western blots

Fugene6 was used to transfect HEK293 cells with the indicated plasmids. For immunoprecipitation, transfected cells were resuspended in IP buffer (50mM Hepes, pH 7.5, 200mM KCl, 0.1% NP40, 5% glycerol, 1mM phenylmethylsulfonyl fluoride, 1X protease inhibitor cocktail), sonicated and cleared of cell debris. Cell lysates were then incubated with M2 beads (Sigma) for 1 hr. in IP buffer at 4ºC, followed by three washes with RIPA buffer. Proteins were eluted from beads by boiling in SDS-PAGE sample buffer. Eluted proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes and proteins were detected with anti-Flag (Sigma) or anti-GFP (Clontech), cross-reacts with YFP, monoclonal antibodies using the ECL kit (Amersham).

FRET analysis

HEK293 cells were co-transfected with equal amounts of pCS2+CFP-AdpC1 and pCS2+YFP-Med23 mammalian expression plasmids. Between 24-48 hours after transfection, samples were collected, washed with PBS, fixed with 4% paraformaldehyde, and mounted on coverslips. I used the acceptor photobleaching method on a Zeiss LSM510 confocal microscope for FRET analyses. In this protocol, I bleached the acceptor (YFP) channel by scanning a region of interest using the 514 argon laser line at 100% intensity. Before and after the bleach, 2 images were collected to assess changes in

donor (CFP) fluorescence. The FRET efficiency (E_f) was calculated using the formula $E_f = (I_2-I_b-I_1-I_b) \times 100/(I_2-I_b)$, where I_n is the CFP intensity at the n^{th} time point and I_b is the background CFP intensity (Karpova et al., 2003).

Fly experiments

The *adp*^{60} mutant stock was a kind gift from Dr. Winifred Doane. The fly Med23 mutant stock, P(SUPor-P)MED23^{KG00948}, was obtained from the Bloomington Stock Center. The P-element insertion of the Med23 mutant stock was confirmed via genomic PCR and sequencing. The P(SUPor-P)MED23^{KG00948} insertion allele is late larval lethal and maintained over the CyO balancer. The CyO balancer was exchanged to CyO, P(ActGFP)JMR1 balancer, which expresses GFP in the larval gut in order to unambiguously distinguish Med23 homozygous larvae from heterozygous larvae. *w*^{1118} flies were used as controls. To analyze fat phenotypes, newly eclosed flies reared under identical conditions were collected and further cultured for another week under well-fed conditions by addition of yeast paste. Nile red staining of explanted fat tissues was done as previously described (see Chapter I). Starvation assays were performed by subjecting ~ 80 flies of each genotype to starvation with an unlimited supply of water and dead flies were scored daily. Starvation assays were performed at least three times and representative data is shown. Whole fly lysates were prepared by grinding cohorts of flies or larvae in ice-cold 0.5% SDS/PBS followed by a 30 min. incubation at 65ºC to inactivate endogenous lipase activity. Heat-inactivated lysates were cleared of debris by a

brief centrifugation at 4ºC and triglyceride content was measured. Six replicates were used per genotype per sex for triglyceride measurements.

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Chapter IV

Future Perspectives

I have undertaken a series of studies to identify genes and pathways that are of importance to the development and function of adipose tissues. While doing so, I have attempted to assess the potential of Drosophila as a genetically tractable model system to aid in the research of adipose biology. Most, if not all, of the current body of knowledge in adipose biology has orginated from mammalian studies, and have produced many exciting discoveries related to the development, function and diseases of adipose tissue. These include important genes such as the C/EBPs, PPARγ, leptin and resistin. On the other hand, invertebrate model systems, such as Drosophila, to study adipose biology have been largely overlooked. Since fat storing tissues are found in a wide range of animals it seems plausible that the underlying molecular mechanisms that govern the development and function of fat may be conserved. Therefore, the use of invertebrate model systems may hasten the discovery of new genes and pathways involved in fat formation and function that may be conserved across distant animal phyla.

The roles of both the Hh and Adp pathway in adipose tissues were first identified in fruit flies and further studies thus far imply an analogous role in mammalian systems. These initial results support the notion that flies can be a valuable model for the study of adipose biology. However, further efforts to define these pathways at both the molecular and whole organism level will be required in order to evaluate the significance of these findings within the framework of our current understanding of adipose biology and its related diseases. In the remaining sections, I will discuss some of the directions in to which my current work may lead in the future.

Future studies on the Hh pathway and adipose biology

In Chapter I, I show data supporting a conserved role of Hh pathway in fat formation. I found that Hh pathway components were expressed in both the fly fat body and mammalian fat tissues. Furthermore, the function of the Hh pathway also appeared to be conserved as sufficiency and necessity tests support the notion that the Hh pathway blocked fat formation in both organisms. While the importance of the Hh pathway in fat formation was demonstrated *in vivo* using the Drosophila system, it is still not clear whether the same will be true in a mammalian animal model. The Hh pathway has been an intense area of research due to its importance in many developmental processes and clinical relevance to disease such as cancer (Bak et al., 2003; Ingham and McMahon, 2001). As a result, many genetic and pharmacological tools have been developed to manipulate the pathway, both in the whole organism and in a tissue-specific manner. These tools provide us with a means to critically evaluate the potential of Hh pathway manipulation in the treatment of adipose tissue related disease such as obesity and diabetes. For example, the Hh pathway activity can be modulated by using conditional alleles of either gain- or loss-of-function Smo in conjunction with a fat-specific Cre, such as aP2-Cre, which expresses Cre recombinase under the control of the fat-specific 5.4kb murine aP2, fatty acid binding protein, promoter/enhancer (Long et al., 2001; Mao et al., 2006). In these genetic models, Hh pathway activity could be either up-regulated or down-regulated in an adipose tissue-specific manner from embryonic stages throughout life, and thus may reveal the consequence of sustained modulation of Hh signaling in adipose tissue. If the *in vitro* effects of Hh signaling on adipogenesis are conserved in a whole mouse model, then activation of Hh signaling in mouse adipose tissues would be predicted to produce a lean mouse and the inhibition of Hh signaling would produce an obese mouse. Another distinct, and more clinically relevant, approach to

modulate Hh signaling *in vivo* is by the use of pharmacological agents that block or activate Hh signaling. For example, KAAD-cyclopamine acts as a potent and specific antagonist of Hh signaling via its properties to inhibit Smo activity (Bak et al., 2003). Conversely, a Hh signaling agonist, purmorphamine, binds and activates Smo signaling (Sinha and Chen, 2006). It will be interesting to investigate the possibility that pharmacological modulation of Hh signaling in adult mice may prevent or alleviate obesity and the associated changes in metabolism, such as decreased insulin sensitivity. One major concern would be that the delivery method of these drugs will most likely be systemic and may lead to unwanted side-effects. Nevertheless, these experiments will be an important step towards assessing, in principle, whether the Hh pathway can serve as a valid therapeutic target in the treatment of obesity.

Future studies on the Adp pathway and adipose biology

Loss-of-function of *Adipose* causes obesity in *D. melanogaster*. In chapters II and III, I showed that the anti-obesity function of Adp is conserved from flies to mammals and that Adp may act through regulating the activity of PPARγ via the Mediator Complex. Still, many questions regarding the Adp pathway remain unanswered. One important question to be addressed is the elucidation of the upstream signals that regulate Adp activity. Since, the Adp pathway is conserved and plays a critical role in adipose tissues, which are central to whole organism energy homeostasis, one may reason that the upstream regulatory pathway(s) may also be evolutionarily conserved and involved in energy sensing and partitioning. Several such pathways have been identified to date, such as the TOR pathway, AMPK pathway and insulin pathway (Marshall, 2006; Sarbassov et al., 2005). Studies of the relationship between Adp and these pathways are aided by a variety of experimental paradigms involving both genetic and

pharmacological tools. Interestingly, a recent report shows that the TOR pathway inhibits PPARγ transcriptional activity, independent of the TOR signaling leading to decreased protein synthesis, suggesting a direct effect of TOR signaling on PPARγ activity (Kim and Chen, 2004). However, the intermediaries between TOR signals and PPARγ remain unidentified. Since both Adp and TOR signaling seems to play a role in the same processes, i.e. inhibition of PPARγ activity and energy homeostasis, and since both pathways are conserved, it seems plausible that the two may be linked at the molecular level. If so, this would shed light on not only how Adp may be regulated, but also on how TOR signaling directly controls its transcriptional targets, which is a poorly understood process relative to TOR signaling and the translational control of global protein synthesis.

Although I my data shows that Adp affects PPARγ activity through Med23, a Mediator Complex subunit, it is still not clear what, if any, other mediator components may interact with Adp and play a role in the Adp pathway leading to the suppression of PPARγ activity. Using relatively straightforward techniques, it should be possible to delineate the interactions, both physically and functionally, between Adp and other mediator subunits, just as I have done with Med23 in Chapter III. These studies will provide a framework to understand the Adp-Mediator-PPARγ axis at the molecular level. One interesting outcome from such studies would be to see whether there are mediator subunits that regulate fat formation independent of Adp. Such results would suggest that the Mediator Complex itself may be the major node to integrate signaling from upstream pathways involved in energy homeostasis, and Adp may participate in one of the many input signaling pathways. The Mediator Complex, being the major physical and functional link between various transcription factors and RNA polymerase II, is adequately positioned to carry out a role in coordinating global gene expression in response to energy-sensing signal

transduction pathways. In addition to studies aimed at elucidating the details of Adp-Mediator interactions, it will be important to understand the exact mechanism which PPARγ activity is modulated as a result of the proposed Adp-Mediator signals. The Mediator has been shown to be capable of directly regulating transcription by controlling the rate of transcription initiation of RNA polymerase II complexes bound to native promoters, and thus the Mediator may convey all the effects of Adp by directly modulating RNA polymerase II at PPARγ target gene promoters. Alternatively, there may be proteins that are not part of the Mediator Complex that play a role in altering PPARγ activity. Important candidates to test would include chromatin remodeling factors, such as protein complexes containing histone deacetylases or histone acetyl transferase family members, which play critical roles in controlling transcription factor activity (Xu et al., 1999). Detailed mechanistic studies of the Adp pathway and its components may identify new therapeutic targets for the treatment of obesity and related disease and may lead to a better understanding of how cells and tissues respond to energy-sensing signals.

Adp seems to be able to counter obesity in both invertebrates and vertebrates by blocking adipogenesis. The fly and mouse models of Adp function I have described in Chapter II had alterations in Adp levels in adipose tissues throughout the life of the organism, and, therefore, it is not yet clear what the consequences, if any, of modifying Adp levels later in adult life might be. Determining the late effects of Adp *in vivo* will be a critical test in gauging whether the Adp pathway can be a viable therapeutic target for the treatment of obesity. Both flies and mice can be used to generate genetic models to test the effects of adult stage activation of Adp. Recently, in flies, a GAL4-progesterone receptor chimera has been utilized to create a P-element enhancer trap, named Gene-Switch, that can be used for regulated spatio-temporal expression of UAS-driven effector genes (McGuire et al., 2004). Through mobilization of a Gene-Switch P-element,

I have created a collection of fat body enhancer trap lines (unpublished results) that can be used for driving UAS-Adp expression in adult fat bodies in a temporally controlled fashion by feeding the flies RU486, a progesterone receptor ligand that induces translocation of the GAL4-progesterone receptor chimera and allows for the GAL4 portion of the chimeric protein to activate integrated UAS transgenes. There are several methods in mice that allow for inducible transgene expression in adult stages. One such inducible method has been developed for adipose tissues in mice, the aP2-Cre-ER^{T2} transgenic mice, that employs a tamoxifen-dependent Cre-ER^{T2} recombination system, which, under the control of the aP2 fat-specific enhancer, is expressed in a adipose tissue-restricted pattern (Imai et al., 2004). In this system, similar to the Gene-Switch system in flies, the Cre recombinase-estrogen receptor chimeric protein translocates to the nucleus only in the presence of tamoxifen and can then act to recombine loxP sequences. A targeting construct for the ROSA26 locus that contains the Adp cDNA preceded by a loxP-neomycin-stop-loxP cassette (Soriano, 1999) can be created using standard cloning techniques. Mice that harbor the Cre-inducible Adp cassette targeted to the ROSA26 locus can be generated by homologous recombination in ES cells followed by blastocyst injection to create chimeric mice. The Cre-inducible Adp allele in conjunction with the aP2- Cre-ER^{T2} transgene can be used to overexpress Adp specifically in adult fat by tamoxifen-controlled induction of the Cre-inducible Adp allele. The development of fat-specific inducible mouse models of Adp expression may provide important data on the potential value of the Adp pathway as a therapeutic target.

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VITAE

Jaemyoung Suh, the son of Sang-kee Suh and Kyoung-sook Chung, was born in Taegu, Kyoungbook Province, Korea on Feburary 13th, 1971. In the fall of 1971, he moved to the United States with his family. In 1972, after a brief stay in Detroit, Michigan, he moved to Philadelphia, Pennsylvania. In 1976, he moved back to Livonia, Michigan. In 1981, he returned to Korea with his family, settling in Changwon, Kyoungnam Province. He graduated from Changwon High School in February 1989. In 1990, he entered the Department of Biology, College of Science, Yonsei University in Seoul, Korea. He spent the summer of 1992 performing undergraduate research with Dr. Claude Desplan at the Rockefeller University, New York. He received the degree of Bachelor of Science with a major in Biology from Yonsei University in February 1994. In March 1994 he entered the Graduate School in the Department of Biology at Yonsei University and completed his master course in the laboratory of Dr. In Kwon Chung. He received the degree of Master of Science with a major in Microbiology in February 1996. In September 1996 he enlisted in the Republic of Korea Army where he served as a specialist and attained a final rank of Sergeant. In November 1998 he was discharged upon completion of mandatory military service. In September 1999 he moved to Dallas, Texas to enter the Ph.D. program at the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center. He joined the laboratory of Dr. Jonathan Graff in May 2000. He graduated with a Ph.D. degree in December, 2006. He married Soohyun Ahn in June 1995 and has two sons, Timothy Jungmin Suh, 10, and James Jungho Suh, 5.

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