**Adipose Is aConserved Dosage-Sensitive Antiobesity Gene**

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**SUMMARY**

*Adipose (Adp)* is an evolutionarily conserved gene isolated from naturally occurring obese flies homozygous for an *adp* mutation. Here we show that the anti-obesity function of *Adp* (worm *Y73E7A.9*, fly *adp*, and murine *Wdtc1*) is conserved from worms to mammals. Further, *Adp* appears to inhibit fat formation in a dosage-sensitive manner. *Adp* heterozygous flies and *Adp* heterozygous mutant mice are obese and insulin resistant, as are mice that express a dominant negative form of *Adp* in fat cells. Conversely, fat-restricted *Adp* transient transgenic mice are lean and display improved metabolic profiles. A transient transgenic increase in *Adp* activity in adult fly fat tissues reduces fat accumulation, indicating therapeutic potential. ADP may elicit these anti-adipogenic functions by regulating chromatin dynamics and gene transcription, as it binds both histones and HDAC3 and inhibits PPARγ activity. Thus *Adp* appears to be involved in an ancient pathway that regulates fat accumulation.

**INTRODUCTION**

Fat-storing tissues play essential roles such as regulating metabolism and lifespan (Bluher et al., 2003; Spiegelman and Flier, 2001). Adipocyte dysfunction underlies obesity and is central to the metabolic derangements in type II diabetes (Must et al., 1999). The dramatic rise in obesity and diabetes have led to a public health crisis that could be addressed by identifying genes that influence adipogenesis and metabolism (Olshansky et al., 2005). In the absence of ligand, NHRs repress transcription by interacting with corepressor molecules (Lazar, 2003; Perissi et al., 2004). Corepressor complexes also regulate non-NHR transcription factors (Rosenfeld et al., 2006; Smith and O’Malley, 2004). Corepressors either contain or recruit histone deacetylases (HDACs), or other chromatin modifying enzymes, that render chromatin structure unfavorable for transcription (Rosenfeld et al., 2006; Smith and O’Malley, 2004). HDAC3 is an important component of the repressive complex that regulates adipogenic genes (Fajas et al., 2002; Fu et al., 2005). Components of the corepressor complex also directly bind histone 2B (H2B) and histone 4 (H4) (Lorain et al., 1998; Magnaghi et al., 1998; Yoon et al., 2005). Gene expression is induced upon exchange of the corepressor complex for a coactivator complex, which often has histone acetyltransferase activity. Thereby chromatin is remodeled into a form accessible for transcriptional activation (Rosenfeld et al., 2006; Smith and O’Malley, 2004). The role of histone modifications in controlling gene expression has been conserved during eukaryotic evolution.

Invertebrate models such as *C. elegans* and *D. melanogaster* are powerful systems for the discovery and analysis of genes critical to human health and disease. These advantages have yet to be fully applied to fat biology in part because of concerns that differences exist between invertebrate and mammalian fat including the observations that worm and fly fat is not stored in dedicated adipocytes but rather in more multifunctional cells (Rosen, 2006). Molecular studies also highlight potential differences (Tong et al., 2000). Other data, however, support the notion that invertebrates may provide useful information on adipocyte biology (Ashrafi et al., 2003; Gronke et al., 2005; McKay et al., 2003; Ruden et al., 2005).

Almost 50 years ago, Winifred Doane hypothesized that climates marked by cycles of famine might select for organisms highly efficient at fat storage to allow for survival during times of limited food. Dr. Doane successfully isolated such an obese *D. melanogaster* mutant, which she ultimately termed *adipose* (*adp*), from a laboratory strain whose origin was Kaduna, Nigeria (Clark and Doane, 1983; Doane, 1960a, 1960b). Recently Doane and colleagues cloned the fly *Adp* gene, which encodes a novel protein conserved as a single copy from flies to humans.
(Hader et al., 2003). The fly *adp* mutation is a 23 base pair deletion predicted to result in premature termination of ADP. ADP contains multiple protein interaction domains (6 WD40, 3 tetratrico peptide repeat [TPR]) and may therefore function as a nexus for a protein complex important in fat biology (Blatch and Lassle, 1999; Hader et al., 2003; Smith et al., 1999). Although Adp has a broad pattern of expression, the primary phenotype observed in *adp* null flies is increased triglyceride storage in the fat body, the fly adipose organ (Hader et al., 2003).

In worms, we found that *C. elegans* Adp (Y73E7A.9) RNAi increased fat accumulation. Adp heterozygous flies also displayed metabolic phenotypes indicating dosage-sensitivity in fat formation. Transgenic expression of Adp in the fat body reduced fly fat formation even when induced only briefly during adulthood. Murine *Adp* (Wdct1) also blocked fat formation in cell culture adipogenesis systems. Next we generated mice with LacZ inserted into the *Adp* locus. *Adp*⁻/− mutant mice were born at well below the expected Mendelian ratio, making analysis of metabolic phenotypes difficult. However, *Adp*⁻/− mice, like *adp*⁻/− flies, were obese and insulin resistant. Similarly, transgenic mice that expressed a dominant negative *Adp* in fat cells were obese and hyperglycemic. Conversely, transgenic mice that expressed wild-type *Adp* in fat were lean and had improved metabolic parameters. ADP appears to function in the nucleus where it interacts with H2B, H4 and HDAC3, indicating that ADP may be present in a chromatin remodeling complex. PPARγ may be a target of this complex as ADP inhibits PPARγ transcriptional activity. These data are consistent with the notion that Adp is a conserved dosage-sensitive anti-obesity gene that functions in a corepressor complex to regulate gene expression.

**RESULTS**

**Inhibiting Adipose Stimulates Worm Fat Formation**

We designed a genome-wide screen, coupling RNAi, light microscopy and staining with Nile Red, a dye whose fluorescent intensity reflects fat content, to identify genes involved in *C. elegans* fat formation (McKay et al., 2003). We observed that RNAi with a construct targeting the 5′ region of the *C. elegans* Adp homolog (Figure 1A) produced worms with increased fat formation as evidenced by enhanced opacity and increased Nile Red fluorescence (Figure 1B). RNAi with a second construct that targets an independent region of Adp also produced an obese phenotype, indicating specificity (Figure 1B). These data support the idea that Adp has an anti-obesity function in *C. elegans*.

**Adipose Has Dosage-Sensitive Roles in Fly Fat Biology**

*Adipose (adp)* homozygous mutant flies are obese and starvation resistant, but the homozygous state also has negative consequences (Doane, 1960a; Doane, 1960b). For example, *adp* homozygous mutants have decreased mobility compared to *adp* heterozygotes (Figure 1C). Since *adp* heterozygosity might also engender some beneficial effects during famine while minimizing deleterious sequelae (Clark and Doane, 1983; Doane, 1960a; Doane, 1960b), we evaluated the fat biology of *adp*⁻/⁺ flies and found a phenotype intermediate between wild-type and *adp* nulls. In a starvation survival assay, which often correlates with fat content (Djwadan et al., 1998), survival was *adp*⁻/− > *adp*⁻/⁺ > *adp*⁺/⁺ (Figure 1D), consistent with prior observations (Doane, 1960b). Triglyceride quantitation also revealed the same trend (Figure 1E). These data indicate that Adp has dose-dependent effects on fly fat storage.

**Adipose Fat Body Transgenesis Blocks Larval and Adult Fly Fat Storage**

Expression of Adp in fat cells rescued *adp* mutant obesity and reduced fat storage, indicating that its anti-obesity functions are primarily mediated within the fat cell (Hader et al., 2003). We further analyzed this notion by generating transgenic flies that expressed Adp (UAS-Adp) in a fat-restricted manner under the control of the Dcg-Gal4 driver, expressed in late embryonic, larval, and adult fat (Suh et al., 2006). We also introduced a Dcg-GFP fat body GFP transgenic reporter to visualize the fat body in intact animals. We found that fat body transgenic expression of Adp inhibited fat formation based upon reduced opacity, decreased Nile Red and GFP fluorescence, and decreased fat body size (Figures 1F and 1G).

We also examined whether Adp might regulate adult fat biology. For this, we generated two independent fat body RU486-inducible GAL4 strains (FBI-19, FBI-26) in an enhancer trap screen (Roman et al., 2001). Adult flies of these two lines express a UAS-GFP reporter in the fat body when provided RU486 (Figures 1H and 1I). RU486 does not appear to affect the fat biology of FBI flies based upon gross examination, fat body morphology and triglyceride quantitation (Figures 1H, 1J and 1K). We crossed both FBI-GAL4 lines with UAS-Adp flies and randomized adult progeny to 5 days of vehicle or RU486. Gross observations, whole mount Nile Red stains, triglyceride quantitation and starvation survival assays indicated that RU486-treatment reduced FBI-GAL4; UAS-Adp fat accumulation as compared to those treated with vehicle (Figures 1L, 1M, 1N, and 1O). These results indicate that the anti-obesity effects of Adp can operate in adult fat tissues.

**Adipose Inhibits Murine Fat Formation**

To begin to characterize mammalian Adp, we evaluated its relative expression in a variety of mouse tissues using qPCR. We found that murine Adp, like fly Adp (Hader et al., 2003), is widely expressed (Figure 2A). Attempts to generate high-titer specific antibodies against murine ADP have been unsuccessful. 3T3-L1 cells are a murine cell culture adipogenic model that can be induced to differentiate from preadipocytes to fat-storing cells that express adipocyte markers (Rosen and Spiegelman, 2000). Adp was expressed in 3T3-L1 cells (Figure 2B) with highest levels in preadipocytes (Figure 2B), although in a few cases the levels were similar. Adp expression was also enriched in
Figure 1. Inhibiting Adipose Stimulates Worm and Fly Fat Formation

(A) Alignment of worm (ce), fly (dm), mouse (m) and human (h) ADP protein. WD40 domains: light blue rectangles, TPR domains: orange rectangles. The percentages refer to comparison with hADP.

(B) Progeny of control or Adp RNAi worms were examined with bright field microscopy in which increased opacity indicates more fat storage. Fat content was also assessed with Nile Red, a lipid-specific dye. Worms are at the same developmental stage.

(C) Mobility of male adp+/C0 (Het) and adp/C0/C0 (Homo) flies was assessed in a negative geotaxis assay. Mobility is plotted as the inverse of time (seconds) required for flies to crawl a fixed distance.

(D) Adp+/+ wild-type, adp+/C0 (Het), and adp/C0/C0 (Homo) male adult flies (n > 100) were deprived of food and survival was plotted.

(E) Triglycerides were quantified from well-fed adult male adp+/+ wild-type, adp+/C0 (Het), and adp/C0/C0 (Homo) flies (n = 10 per genotype, averages from 4 experiments plotted).

(F) Larvae expressing a fat body GFP reporter either alone (control) or with Adp fat body transgenesis were photographed under bright field (left) or GFP fluorescence (right) microscopy.

(G) Fat body explants of larvae described in F were stained with Nile Red and photographed with bright field (left) or fluorescence (right) microscopy.

(H, I) Adult fat body inducible (FBI)-GAL4; UAS-GFP male reporter flies were randomized to vehicle (VEH) or inducer (RU486) and then the whole fly (H) or fat body explants (I) were photographed. Wings and legs were removed in H for imaging.

(J, K) Single transgenic FBI-19 male adult flies were treated with either vehicle or RU486 and photographed with light microscopy (J) or subjected to triglyceride quantitation and levels were normalized to vehicle-treated flies (K) (n = 10 per treatment, averages from 5 experiments plotted). Wings and legs were removed in J for photography.

(L) Adult FBI-19-Adp male flies were treated with vehicle or RU486 and then photographed. Arrow indicates abdomen and the fat body, which appears smaller after RU486 treatment. Wings and legs were removed for imaging.

(M) Flies described in L were stained with Nile Red and whole mounts were photographed.

(N) Triglyceride quantification of male adult FBI-19-Adp and FBI-26-Adp flies treated with vehicle or RU486. Values were normalized to vehicle or RU486-treated single transgenic controls (n = 10 per treatment, averages from 5 experiments plotted).

(O) Day 7 starvation survival of male adult FBI-19-Adp and FBI-26-Adp flies treated with vehicle or RU486 (n > 50). Similar results obtained for females in analyses performed in C-E and H-O. *p < 0.05; **p < 0.01; N/S not significant by t test. Error bars indicate standard error of the mean (SEM).
the stromal-vascular fraction of murine adipose depots, thought to contain preadipocytes (Figure 2C) (Poznanski et al., 1973; Soukas et al., 2001). We also evaluated Adp expression in murine fat depots in response to genetic and diet-induced obesity or fasting. We found that Adp levels decreased in both forms of obesity and increased upon fasting (Figure 2D), suggesting that Adp may regulate mammalian fat biology.

To investigate a potential role for Adp in mammalian adipogenesis, we first cloned murine Adp from a 3T3-L1 cDNA library. We then infected 3T3-L1 s with virus encoding GFP or Adp. When adipogenically induced, the GFP cells became lipid-laden adipocytes as evidenced by fat-specific stains and triglyceride quantitation (Figures 2F and 2G). In contrast, the Adp cells
retained preadipocyte morphology and accumulated only trivial amounts of lipid (Figures 2F and 2G). Further, Adp inhibited the expression of a panel of adipogenic markers (Figure 2H). Expression of Pref-1, whose levels inversely correlate with adipogenesis, increased (Figure 2H). Adp also inhibited NIH 3T3 adipogenesis (Figure 2I). To test specificity, we examined the effect of Adp on MC3T3-E1 cell culture osteogenesis. However, based upon Von Kossa stain and molecular analyses, Adp did not alter osteogenesis (Figures 2J and 2K).

To determine whether Adp could inhibit murine fat formation, we placed it under the control of the 5.4kb ap2 promoter/enhancer (Figure 3A), which drives expression in murine adipocytes, although it can be expressed in some macrophages (Makowski et al., 2001; Ross et al., 1990). We generated several independent lines of ap2-Adp transgenics which had the same phenotype. qPCR analyses showed that ap2-Adp mice expressed higher levels of Adp in fat (Figure 3B). We first studied mice after backcrossing into an ICR background. By 16 weeks the weight of the ap2-Adp mice began to diverge from their wild-type littermates and this was reflected in their appearance (Figures 3C and 3D). At 24 weeks, NMR and fat depot analyses showed that the Adp transgenics had significantly less body fat than controls (Figures 3E, 3F, and 3G). The weights of other organs were unaffected (Figure 3G). Histological analyses revealed that ap2-Adp mice had significantly smaller adipocytes than controls (Figure 3H). Plasma analyses showed reduced levels of leptin and insulin in the ap2-Adp mice (Figure 3I). Glucose tolerance tests (GTT) showed that the ap2-Adp mice had significantly lower blood glucose levels than controls (Figures 3J and 3K). Besides, expression levels of Adp in adipose tissues, based upon qPCR analyses, were reduced by more than half in the Adp heterozygotes (Supplemental Figure S3). We bred the Adp mutation 7 or 8 generations (F7, F8) into the ICR background and then intercrossed F7 or F8 ICR Adp heterozygotes. However, Adp homozygotes were born at markedly reduced ratios compared to Mendelian predictions (Figure 6B). Although some Adp homozygotes were obese, their scarcity precluded statistically significant analyses and adult phenotypes observed in the few mice that escaped embryonic lethality may reflect secondary rather than primary effects.

Next we generated mice with a splice acceptor-LacZ-Neo resistance cassette inserted into the Adp locus at a position that should produce an early truncation of the Adp protein at amino acid 15 (Figure 6A). Expression levels of Adp in adipose tissues, based upon qPCR analyses, were reduced by more than half in the Adp heterozygotes (Supplemental Figure S3). We bred the Adp mutation 7 or 8 generations (F7, F8) into the ICR background and then intercrossed F7 or F8 ICR Adp heterozygotes. However, Adp homozygotes were born at markedly reduced ratios compared to Mendelian predictions (Figure 6B). Although some Adp homozygotes were obese, their scarcity precluded statistically significant analyses and adult phenotypes observed in the few mice that escaped embryonic lethality may reflect secondary rather than primary effects.

Since AdpC1 appeared to act as a functional antagonist of Adp, it provided a potential tool to dissect Adp function in vivo. For this, we generated several aP2-AdpC1 transgenic and non-transgenic B6:D2F founders (Figure 5A). Although aP2-AdpC1 transgenic mice had similar weights as the control founders, NMR analysis showed that the aP2-AdpC1 mice had increased fat content (Figures 5B and 5C). Further, the transgenic WAT depots weighed more than controls whereas non-adipose organs showed no differences (Figure 5D). Histological studies showed that the aP2-AdpC1 adipocytes were larger than control adipocytes (Figure 5E). Food intake and body temperature were not significantly altered (Figure 5F and Supplemental Figure S2). In GTTs, the aP2-AdpC1 had higher blood glucose levels (Figure 5G).

**Adipose Heterozygous Mice Are Obese**

Next we generated mice with a splice acceptor-LacZ-Neo resistance cassette inserted into the Adp locus at a position that should produce an early truncation of the Adp protein at amino acid 15 (Figure 6A). Expression levels of Adp in adipose tissues, based upon qPCR analyses, were reduced by more than half in the Adp heterozygotes (Supplemental Figure S3). We bred the Adp mutation 7 or 8 generations (F7, F8) into the ICR background and then intercrossed F7 or F8 ICR Adp heterozygotes. However, Adp homozygotes were born at markedly reduced ratios compared to Mendelian predictions (Figure 6B). Although some Adp homozygotes were obese, their scarcity precluded statistically significant analyses and adult phenotypes observed in the few mice that escaped embryonic lethality may reflect secondary rather than primary effects.

**Adipose Functions in the Nucleus**

We explored ADP subcellular distribution by incorporating an N-terminal GFP-tag (Figure 7A). We first confirmed that GFP-Adp remained functional in inhibiting 3T3-L1 adipogenesis (Figure 7B). Then we expressed GFP-Adp and examined subcellular distribution with 5 μm Z-series confocal microscopy. In low, medium, and high expressing cells, GFP-Adp was located in both the cytosol and nucleus with higher levels in the cytosol (Figure 7C). We
also GFP tagged the dominant negative AdpC1 domain (Figure 7D); GFP-AdpC1 stimulated adipogenesis like untagged AdpC1 (Figure 7E). Z-series confocal microscopy showed that GFP-AdpC1 was also expressed in both the cytosol and nucleus with nuclear levels greater than cytosolic levels (Figure 7F).
To identify the compartment in which ADP elicits its anti-adipogenic actions, we targeted ADP either to the nucleus, with a nuclear localization signal fusion (NLS-Adp), or away from the nucleus, with a nuclear export sequence fusion (NES-Adp) (Figures 7G and 7I). NLS-Adp blocked adipogenesis while NES-Adp did not and in some conditions even stimulated adipogenesis, like a dominant negative Adp (Figures 7H and 7J). Thus it is likely the nuclear fraction of ADP functions to inhibit adipogenesis.

Adipose Binds Histones and HDAC3

We performed a yeast two-hybrid screen with full-length murine Adp and a mouse library, and we isolated multiple independent clones of histone 2B (H2B). We tested whether this interaction occurred in mammalian cells and also tested histone 4 (H4), since other WD40 proteins interact with this pair of histones (Li et al., 2000; Magnagni et al., 1998; Yoon et al., 2005). We found that FLAG-ADP co-immunoprecipitated with either myc-H2B or myc-H4 (Figure 7K). Several proteins that interact with H2B and H4 function in transcriptional corepressor complexes that contain HDAC3, a regulator of adipocyte biology (Fajas et al., 2002; Fu et al., 2005; Guenther et al., 2000; Li et al., 2000; Yoon et al., 2003; Yoon et al., 2005). We therefore examined whether ADP interacts with HDAC3, and found that these two molecules co-purify. (Figure 7L). These data indicate that H2B, H4 and HDAC3 may exist in complexes that contain ADP and raise the possibility that Adp functions as a transcriptional corepressor.

Next, we attempted to address the functional relevance of ADP and HDAC3 interactions. For this, we infected 3T3-L1 s with GFP or Adp virus, adipogenically induced and treated the cells with vehicle or the HDAC inhibitors sodium butyrate and 4-phenyl butyrate, which are limited as they inhibit multiple HDAC family members (Jung, 2001). The HDAC inhibitors significantly reversed the Adp-dependent blockade of adipogenesis (Figures 7M and 7N), suggesting that Adp functions upstream of histone deacetylation during adipogenesis.

If ADP does function in a corepressor complex, it may inhibit key adipogenic transcription factors such as C/EBPs, PPARγ, and SREBP. To investigate this possibility, we assessed the effect of ADP on C/EBP-, PPARγ-, and SREBP-dependent transcriptional reporters. We found that ADP inhibited the action of PPARγ, but not the other factors (Figure 7Q, Supplemental Figure S4). Conversely, Adp RNAi significantly stimulated PPARγ reporter expression (Figure 7P). These results were consistent with the prior observations that PPARγ target gene expression was altered by Adp levels in cell culture adipogenesis (Figures 4–7).
Next, we examined PPARγ target gene expression in fat depots of the Adp heterozygous and the aP2-Adp transgenic mouse models. The levels of expression of all PPARγ targets were increased in Adp heterozygous fat and reduced in aP2-Adp transgenic fat, which may result from a direct effect on PPARγ action or a secondary response to altered fat accumulation (Figure 7Q and 7R).

DISCUSSION

The search to identify molecules that underlie fat accumulation and glucose homeostasis has taken on additional urgency due to the recent dramatic increase in obesity and diabetes (Campbell and Dhand, 2000; Must et al., 1999). Mammalian adipocytes store large amounts of lipid and secrete a variety of hormones that regulate feeding behavior and metabolism (Friedman and Halaas, 1998). The ability to store fat was acquired early in evolution and is essential for survival of multi-cellular animals. Adp encodes an evolutionarily conserved protein that displays functional conservation between invertebrate and mammalian systems. Loss of Adp function leads to increased fat accumulation in worms, flies, mammalian tissue culture, and mice. Conversely, increasing Adp activity blocks fat formation in flies, mammalian cell culture and mice. In the fly model, a brief induction of Adp activity in adult fat tissues reduced fat accumulation. If this phenotypic response is conserved to mammals, the Adp pathway could be a rational target for obesity and diabetes therapies.

The conservation of Adp function appears to extend to dosage-sensitive phenotypes. Adp heterozygous flies...
have intermediate levels of fat accumulation between wild-type and homozygous mutants, and Adp heterozygous mice are also obese. Such autosomal dominant phenotypes caused by loss-of-function mutations are uncommon and can occur in genes that are key regulators of the process under study, such as the rate limiting enzyme in a metabolic pathway (Wilkie, 1994). Other WD40 repeat proteins also display haploinsufficient phenotypes (Hirot-sune et al., 1998; Li and Roberts, 2001; Sidow et al., 1999; Zhang et al., 2004). This feature of WD40 proteins is thought to stem from their role as organizing centers of protein complexes.

**Figure 6. Adp Heterozygous Mutant Mice Are Obese and Insulin Resistant**

(A) Schema of the wild-type (WT) Adp locus and the mutant (Mut) Adp allele. Splice Acceptor (SA) and the lacZ-neomycin (β-geo) cassette were inserted and disrupt gene expression from the second exon (E2). The location of the genotyping primers (F1, R1, R2) are illustrated.

(B) Genomic DNA was extracted from pups of multiple Adp+/− by Adp−/− intercrosses and genotyped for the presence of Adp mutant and wild-type alleles.

(C) Average weights of littermate matched female (control n = 10 mice, Adp Het n = 12) and male (control n = 15, Adp, Het n = 20) cohorts.

(D) Photograph of representative wild-type (Cont) and Adp+/− mice (Het).

(E) 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.

(F) Photograph of representative perigonadal white adipose tissue (WAT) explants from sibling wild-type (Cont) and Adp+/− (Het).

(G) Average weights of inguinal (I) and perigonadal (G) WAT and indicated organs of wild-type and Adp heterozygous litters.

(H) Histological analyses of inguinal (Ing) and perigonadal (Gon) WAT.

(I) Plasma of control and Adp heterozygous cohorts was analyzed for leptin, insulin and triglyceride levels.

(J) GTTs of Adp heterozygous and control siblings (n = 8). Analyses in panels C-J done on 4-month old mice.

*p < 0.05, **p < 0.01, ***p < 0.005 by t test. N/S not significant. Error bars indicate SEM.
Figure 7. ADP Functions in the Nucleus and Interacts with a Corepressor Complex

(A) GFP-Adp chimera. WD40 domains: light blue rectangles, TPR domains: orange rectangles, GFP: green rectangle.
(B) 3T3-L1 s were infected with virus containing either GFP or GFP-Adp, adipogenically induced and Oil Red O stained.
(C) GFP-Adp was introduced into cells and its localization was assessed with confocal microscopy. White arrow indicates nucleus.
(D) GFP-AdpC1 chimera.
(E) NIH 3T3 s were infected with GFP or GFP-AdpC1 and adipogenesis was assessed with Oil Red O staining.
(F) Confocal microscopic photograph of GFP-AdpC1 expressing cells. White arrow designates nucleus.
(G) Nuclear localization signal (NLS)-Adp chimera. NLS: yellow rectangle.
(H) 3T3-L1 s were infected with virus encoding GFP or NLS-Adp, adipogenically induced, and stained with Oil Red O.
(I) Nuclear export signal (NES)-Adp chimera. NES: aqua rectangle.
(J) 3T3-L1 s were infected with GFP or NES-Adp virus, cultured in media supplemented with insulin, and then stained with Oil Red O.
(K) HEK293 s were transfected with a FLAG vector, a myc vector, FLAG-Adp, myc-H2B, or myc-H4 as indicated. After the cells were lysed, part of the homogenates was removed (input) and the remainder immunoprecipitated (IP) with anti-myc antibody. The resultant immunoprecipitates were subjected to Western blots (WB) against the FLAG tag (top panel) or the myc tag (bottom panel). 10% of the homogenate was also evaluated with Western blots using the indicated antibodies.
Cell Metabolism

Adp is a conserved antiobesity gene

Worms, flies, mice and humans all contain only one copy of Adp. ADP contains 9 protein interaction domains (6 WD40, 3 TPR) and no predicted catalytic domains. Although WD40 proteins have other functions, a common theme is that they act as transcriptional corepressors through interactions with (and connections to) transcription factors, histones, and histone-modifying enzymes (Cerna and Wilson, 2005; Chen and Courey, 2000; Perissi et al., 2004; Yoon et al., 2005). ADP associates with H2B, H4, and HDAC3 raising the possibility that ADP functions in a transcriptional corepressor complex (Guenther et al., 2002; Fu et al., 2005). PPARγ has been identified as a target of the HDAC3 corepressor and we find that ADP levels can influence PPARγ activity in various contexts. The ability of ADP to regulate PPARγ function may account for at least part of Adp’s anti-adipogenic role and supports the notion that Adp may act in a therapeutically relevant pathway.

Although the mechanism by which Adp reduces mammalian fat storage might involve inhibiting PPARγ, no PPARY ortholog has yet been identified in flies. So despite the conservation of primary structure and biological function, it is possible that part of Adp’s fat inhibitory mechanisms evolved in concert with the formation of dedicated adipocytes present in higher organisms. Since flies and other invertebrates contain fat but no clear PPARγ homolog, it is likely that Adp functions in a more primordial protein complex, for example the corepressor complex, that functioned to restrain fat accumulation before PPARγ existed.

Invertebrates and vertebrates store fat, which in turn controls many diverse aspects of the life cycle. However, a burgeoning epidemic of obesity and diabetes affects millions and is altering our health care system. Therefore, elucidating the molecules that underlie fat accumulation and glucose homeostasis is an important task. The functional conservation from worms to mammals implies that ADP may be a component of an ancient fat regulatory pathway.

EXPERIMENTAL PROCEDURES

Worm Studies

Two non-overlapping RNAi constructs targeting C. elegans Adp were generated by PCR amplification of cDNA isolated from wild-type (N2) worms. RNAi and Nile Red stains were as described (McKay et al., 2003).

Fly Stocks

The adp(T) mutant was a gift of Dr. Doane (Clark and Doane, 1983). Newly eclosed flies reared under identical conditions were cultured for a week with abundant yeast paste before analysis. UAS-Adp stocks were a gift of Dr. Kuhlein (Hader et al., 2003) and the Dcg-GAL4 fat body driver was described previously (Su et al., 2006). Dcg-GFP fat body reporter flies were generated by P-element mediated transformation of a plasmid with ~10 kb upstream of the ATG of the Drosophila collagen gene (Dcg) fused to GFP. FBl-GAL4 lines were generated by mobilizing the p[Switch] element (gift of Dr. Davis) and screening for lines with specific GFP expression in the fat body after feeding 200 μM RU486 (Sigma) (Roman et al., 2001). FBl-GAL4 drivers were crossed to either UAS-GFP or UAS-Adp and the resulting 5-day old adult progeny, or the salient controls, were fed yeast containing vehicle (1.6% ethanol) or 200 μM RU486 for 5 days.

Fly Starvation and Triglyceride Assays

Adult flies emerging from uncrowded cultures on the same day were collected and cultured for another week under well-fed conditions. Food was removed from cultures of ~100 flies of each sex while providing unlimited water and death was scored daily. Triglyceride quantitation was performed as described (Su et al., 2006).

Fat Body Visualization and Nile Red Staining

Larvae or adult flies were briefly immersed in methanol prior to microscopic analysis or dissection. For Nile Red, whole flies or dissected fat bodies were fixed in formalin, permeabilized in 0.2% Triton X-100 solution and stained with Nile Red.

Retrovirus Production and Infection

Standard methods (Su et al., 2006) were used to generate recombinant retroviruses and to virally transduce 3T3-L1, NIH 3T3 and MC3T3-E1 cells.

Cell Culture, Adipogenic and Osteogenic Differentiation

Mouse NIH 3T3 fibroblasts, 3T3-L1 preadipocytes, MC3T3-E1 preosteoblast cells, and C3H10T1/2 cells were purchased from ATCC and maintained in DMEM or α-MEM [for MC3T3-E1] with 10% calf serum. 3T3-L1 and NIH 3T3 cells were induced to undergo adipogenesis as described (McKay et al., 2003; Su et al., 2006). NIH 3T3 cells were cultured on gelatin coated plates, induced for 6 days in insulin, dexamethasone, and IBMX, and then documented after 2 weeks of culture in 10% FBS and insulin. MC3T3-E1 cells were osteogenically differentiated as described (Wang et al., 1999). Sodium butyrate and 4-phenyl butyrate (Calbiochem) were resuspended in sterile water.

RNA Extraction, CDNA Synthesis, and qPCR

Total RNA from mouse tissues 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). Gene expression was measured through real-time PCR analysis

(L) HEK293s were transfected with a FLAG vector, a myc vector, FLAG-HDAC3, or myc-Adp. The cells were lysed, part of the homogenates was removed and the remainder divided and immunoprecipitated with antibodies directed against either the myc or FLAG epitopes. The resultant immunoprecipitates were subjected to Western blotting against the other tag (top two panels). The homogenates were also directly evaluated with Western blots as indicated. Bottom panel is the myc immunoprecipitates Western blotted with myc antisera.

(M) 3T3-L1 expressing either GFP or Adp were adipogenically induced in the presence of vehicle (VEH) or 1.5 mM of HDAC inhibitors sodium butyrate (NaB) or 4-phenylbutyrate (4-PB), and then stained with Oil Red O.

(N) The Oil Red O stain of cells in M was extracted and quantified by measuring absorbance (620nm).

(O) PPARγ luciferase assay: GFP or Adp along with PPARγ, a PPRE-luc reporter, and a renilla luciferase control were transfected into the indicated cell lines and luciferase activity was measured and then standardized with renilla. These normalized values were plotted as PPARγ activity.

(P) The indicated cell lines, expressing control RNAi or Adp RNAi together with PPARγ, PPRE-luc, and renilla, were assayed for luciferase activity as in O.
using SYBR Green Master Mix reagent (Applied Biosystems, 7500 Real-Time PCR System). Real-time PCR values for gene expression were normalized over endogenous β-actin expression. Real-time primer sequences are available upon request.

Analysis of Cell Culture Lipid Accumulation
Lipid droplets were stained with Oil Red O; Oil Red O content was measured spectrophotometrically as previously described (McKay et al., 2003; Suh et al., 2008). To quantify triglyceride levels, cells were lysed in 0.5% SDS/PBS and triglyceride content was measured using the Infinity Triglyceride Reagent (ThermoElectron). Protein concentrations used to normalize triglyceride content were measured with a BCA protein assay (Pierce).

Cell Culture RNAi
The indicated cell lines were transfected with either control mU6neo shRNA plasmid or mU6neo-Adp shRNA plasmids that contain the appropriate hairpins to reduce the expression of murine Adp. Stable clones were selected in 400 µg/ml G418 for one week and then the remaining G418-resistant stable clones were pooled together for further experiments. shRNA mediated knockdown of the targeted message was determined by qPCR.

Transgenic Mice
aP2-Adp and aP2-AdpC1 transgenic constructs were generated by cloning mouse Adp or the AdpC1 fragment downstream of the 5.4 kb aP2 enhancer (gift of Dr. Spiegelman). Transgenic founders were further bred to establish lines (aP2-Adp), backcrossed two generations into ICR, and then intercrossed or used directly (aP2-AdpC1) for the studies.

Adp Mutant Mice
Adp mutant gene trap ES cell line RRF015, containing an insertion between the 1st and 2nd coding exons, was purchased from BayGenomics. We confirmed the insertion site and injected the Adp mutant ES cells into C57BL/6J blastocysts to produce chimeric mice. The chimeras were bred to ICR strains and heterozygous mice were backcrossed into ICR for > 6 generations.

Mouse Studies
Mice were housed in a 12:12 light:dark cycle, Chow (Teklad) and water were provided ad libitum unless otherwise noted. Body weight was recorded weekly. Total body fat mass of overnight fasted mice was measured by NMR spectroscopy on the Minispec mq spectrometer (Bruker). Food intake and core body temperature were recorded daily for 5 consecutive days in the week prior to sacrifice. Mouse tissues were harvested and weighed before freezing at ~80°C for RNA extraction or fixed in formalin for paraffin embedding. For histology, 8 µm sections were stained with hematoxylin and eosin. Ob mutant and C57BL/6J mice were purchased from Jackson Laboratories. For GTTs, overnight fasted mice received a 1.5 g glucose/kg body weight intraperitoneal injection of 75 mg/ml glucose-PBS solution. Tail blood was drawn at the indicated intervals and blood glucose levels were measured with a TrueTrack glucometer. For diet-induced obesity (DIO), we randomized 5-week old inbred C57BL/6J littermates to either normal (4% fat, Teklad) or high fat (60% fat, Research Diets) chow. After four months, the identical fat depots were explanted from all mice in both cohorts and RNA was extracted and analyzed for gene expression with qPCR as described above. For genetic obesity, ob/ob mice and control littermates were provided normal chow (4% fat, Teklad) and at six months of age fat pad gene expression was analyzed as described. Fat depot gene expression was also analyzed in 5-month old inbred C57BL/6J littermates randomized to either ad libitum food or to a 24 hr fast with water provided. Veterinary care was provided by the Division of Comparative Medicine. All animals were maintained under the guidelines of the U.T. Southwestern Medical Center Animal Care and Use Committee according to NIH guidelines.

Yeast Two Hybrid
The yeast two hybrid screen was done as we described (Peters et al., 1999). Full-length murine Adp was cloned into the pGBK7T7 bait vector (BD Biosciences) and transformed into L40 yeast. 1 X 10^5 clones of a mouse library (pGAD) were screened and several in-frame, independent isolates of H2B were obtained.

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/3/195/DC1/

REFERENCES


