Hedgehog signaling plays a conserved role in inhibiting fat formation

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Summary

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 inhibits 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 toward osteogenesis. Hh may elicit these effects by inducing the expression of antiadipogenic 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 highlighting 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 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 proadipogenic and an antiadipogenic 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 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 lines, such as 3T3-L1s, might be informative (MacDougald and Lane, 1995).

We 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. We 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, we 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 antiadipogenic 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 block *D. melanogaster* fat formation

The fat body is the adipose organ of *Drosophila melanogaster* (Hoshizaki et al., 1994). To study fly fat, we initiated a broad two-component enhancer trap screen (minimal promoter-Gal4, UAS-GFP) selecting lines that expressed GFP in the fat body

(J.M.S. and J.M.G., unpublished data). One such line had the P element inserted into the 5′UTR of *smo*, the Hh receptor (data not shown), supporting the idea that the Hh cascade may regulate fly fat biology. To further evaluate that notion, we explanted the larval fat body, extracted RNA, and found that *smo*, *ptc*, and *ci* (Hh transcription factor) were all expressed in developing fly fat (Figure 1A). To investigate a potential cell autonomous function for Hh signals in fly fat formation, we specifically activated the Hh pathway in the fly fat body with *UAS/Gal4* transgenesis (Brand et al., 1994). For this, we utilized a *Dcg-Gal4* strain that drives transgene expression in the fat body. To activate the Hh pathway, we 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, we utilized *Dcg-Gal4* as it is the best-characterized tool available (Hoshizaki et al., 1994). To visualize the fat body, we ingressed a fat body-specific *Dcg-GFP* reporter (J. Peters, J.M.S., and J.M.G., unpublished data). Through a series of assays, we 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) (Figures 1B–1D). We 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. Activating the Hh pathway with either of these genes produced the same outcomes as *ci* transgenesis establishing the generality of the effects of the Hh pathway on fly fat formation (data not shown). To examine the necessity of Hh signaling, we 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 (data not shown). To quantify the changes induced by activating or inhibiting the cascade, we analyzed the triglyceride content of transgenic flies expressing either the active *ci* or the DNCi and compared the results to matched control flies. We found that active *ci* significantly decreased triglyceride accumulation while DNCi significantly increased triglyceride content (Figure 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.

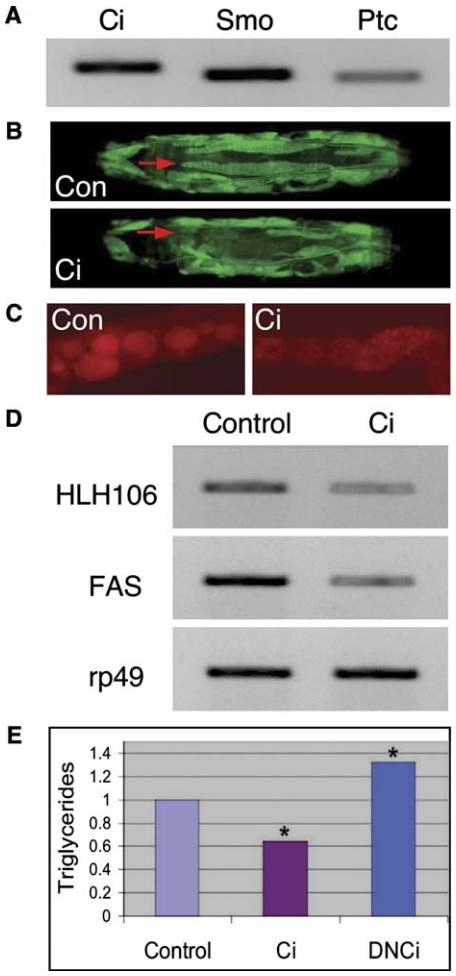

Figure 1. The Hh pathway blocks *D. melanogaster* fat formation

A) The fat body was dissected from 200 *D. melanogaster* third 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. *$p < 0.05$

Hh cascade components are expressed in mammalian fat

To see whether the inhibitory role might be conserved from invertebrates to vertebrates, we evaluated Hh cascade component expression in mouse fat. We 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 (data not shown). We 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 preadipocytes, when placed into appropriate induction media, change into fat-storing cells that express many adipocyte markers. We induced 3T3-LI cells to form adipocytes, extracted RNA during different stages of adipogenesis, and examined Hh pathway-component expression. As a control, we analyzed the adipogenic transcription factor PPARγ, and as expected, its levels increased (Figure 2A). We 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 (Figure 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, we induced 3T3-L1s to undergo adipogenesis in the presence of vehicle or Sonic Hh protein (Shh) and assessed adipogenesis

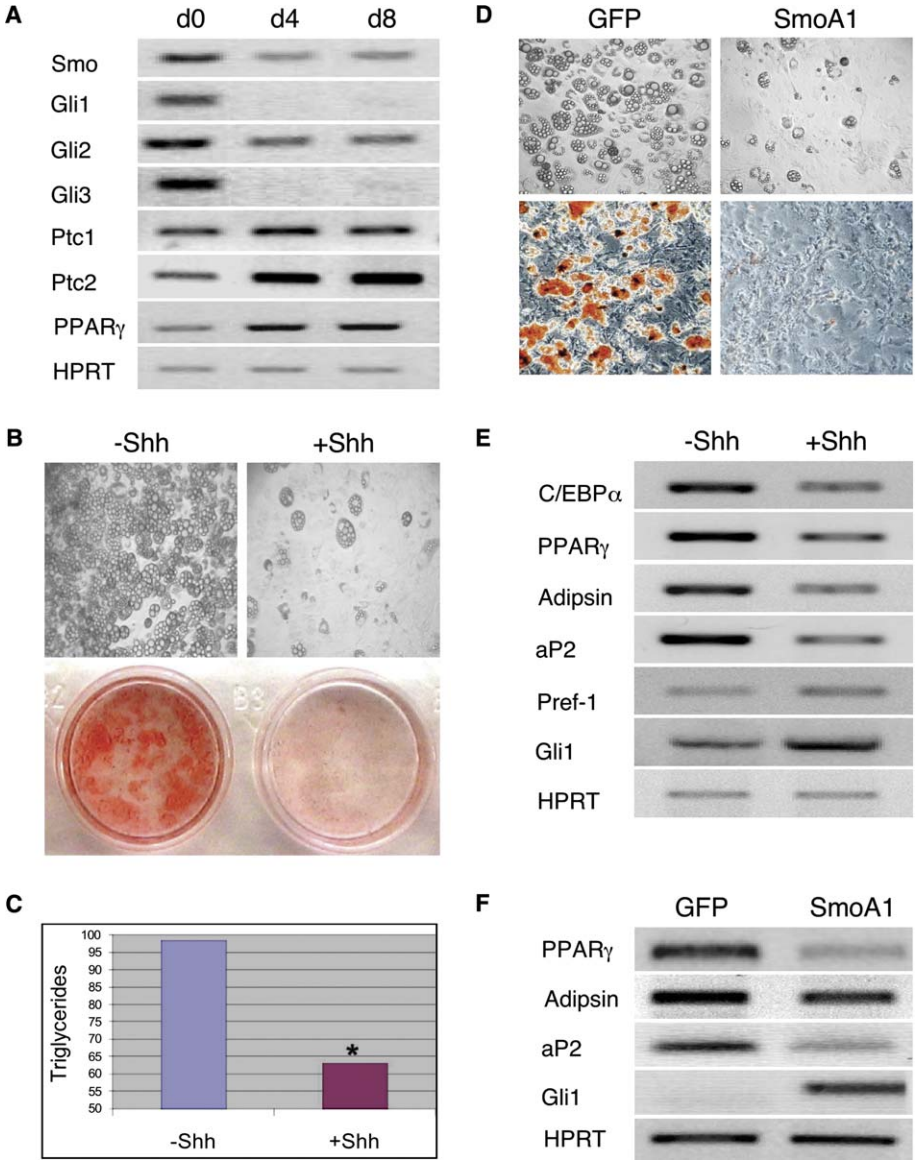

Figure 2. The Hh cascade inhibits 3T3-L1 adipogenesis

A) 3T3-L1 cells were incubated in adipogenic induction media, RNA extracted on days 0, 4, and 8, and semiquantitative 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 and 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**). *$p<0.01$.

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 and F) RNA was extracted from induced 3T3-L1s treated with vehicle or Shh (**E**) or infected with GFP or SmoA1 expressing virus (**F**) and semiquantitative 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.

microscopically, with Oil Red O staining, and with triglyceride quantitation. We 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 (Figures 2B and 2C). We also increased Hh signaling by infecting 3T3-L1s with a control GFP virus or one containing 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 (Figure 2D). Next, we 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, we 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 (Figure 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 (Figure 2E). Similar results were obtained with SmoA1 (Figure 2F). We also found that Shh and SmoA1 inhibited NIH-3T3 adipogenesis (data not shown).

Inhibiting Hh signaling increases mammalian adipogenesis

To complement the sufficiency results, we undertook necessity tests, blocking Hh signals in 3T3-L1s. For this, we expressed either GFP or Gli2-ΔC4 (DNGli), a dominant-negative form of the Hh transcription factor Gli2 (Sasaki et al., 1999). We found that reducing Hh signaling increased adipogenesis based upon light microscopy, Oil Red O staining, and molecular analyses (Figures 3A and 3B). 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, we added either

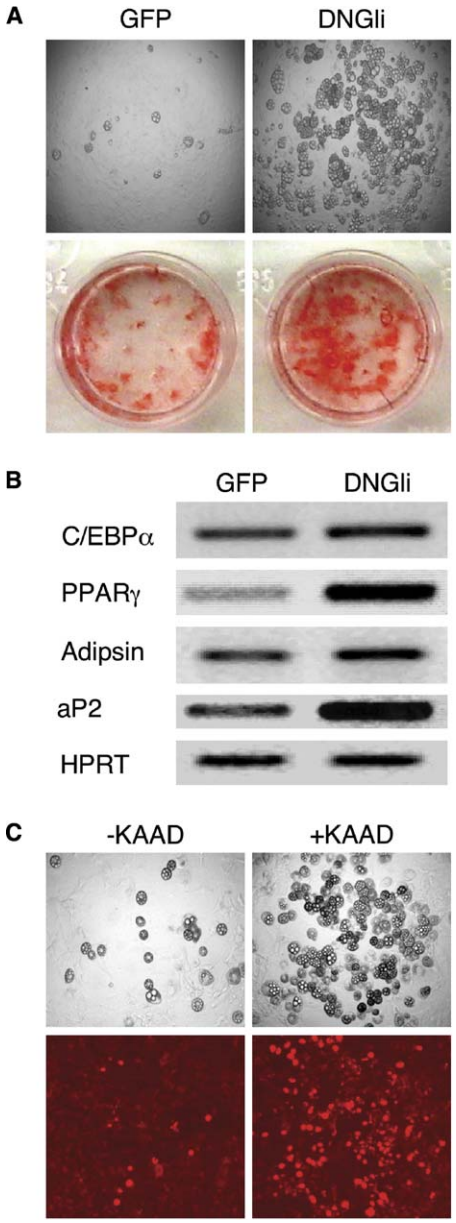

Figure 3. Inhibiting the Hh pathway stimulates 3T3-L1 adipogenesis

A and 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).

vehicle or KAAD to 3T3-L1 cells during adipogenic induction and found that KAAD, like DNGli, increased adipogenesis (Figure 3C).

Hh inhibits fat formation early in adipogenesis

To begin to understand how Hh signals alter adipogenesis, we attempted to define the Shh-sensitive window by adding vehicle or Shh to 3T3-L1s at 2-day intervals during induction. We observed maximal inhibition of adipogenesis when Shh was added during the first 3 days of induction, although a slight effect was observed when Shh treatment began on day 4 (Figures 4A and 4B). 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 when mitotic clonal expansion occurs (Tang et al., 2003). Since Hh can sometimes act as a proliferative signal, it was possible that Hh signals inhibit adipogenesis by stimulating proliferation. To test this, we added vehicle or Shh to 3T3-L1s and counted cells each successive day as an estimate of proliferation; however, cell number was not significantly affected (Figure 4C). The small and statistically insignificant change observed in the latter time points was not detected in all experiments. We 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 (Figures 4D and 4E). As a further test of cell cycle control, we evaluated Rb expression and activity and found that neither was altered by Shh (see Figure 6A below). These data support the notion that Hh signals do not inhibit adipogenesis by altering proliferation. This lack of effect on cell culture is in accord with the fly data; the Hh effects observed in flies were elicited independently of cellular proliferation as we activated the Hh pathway in postmitotic fat-body cells.

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 Pref-1 expression (Figure 2E). Alternatively, the Shh signals may divert 3T3-L1 cells from adipogenesis and toward alternative fates. 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, we incubated 3T3-L1s in vehicle or Shh and analyzed expression of a panel of blood, muscle and bone markers. While we did not observe any changes in the levels of the blood or muscle markers (data not shown), we did find 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) (Figure 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, we examined the effects 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 as such has been proposed to be a mesenchymal stem cell model (Taylor and Jones, 1979). To determine whether Hh retained its antiadipogenic actions on

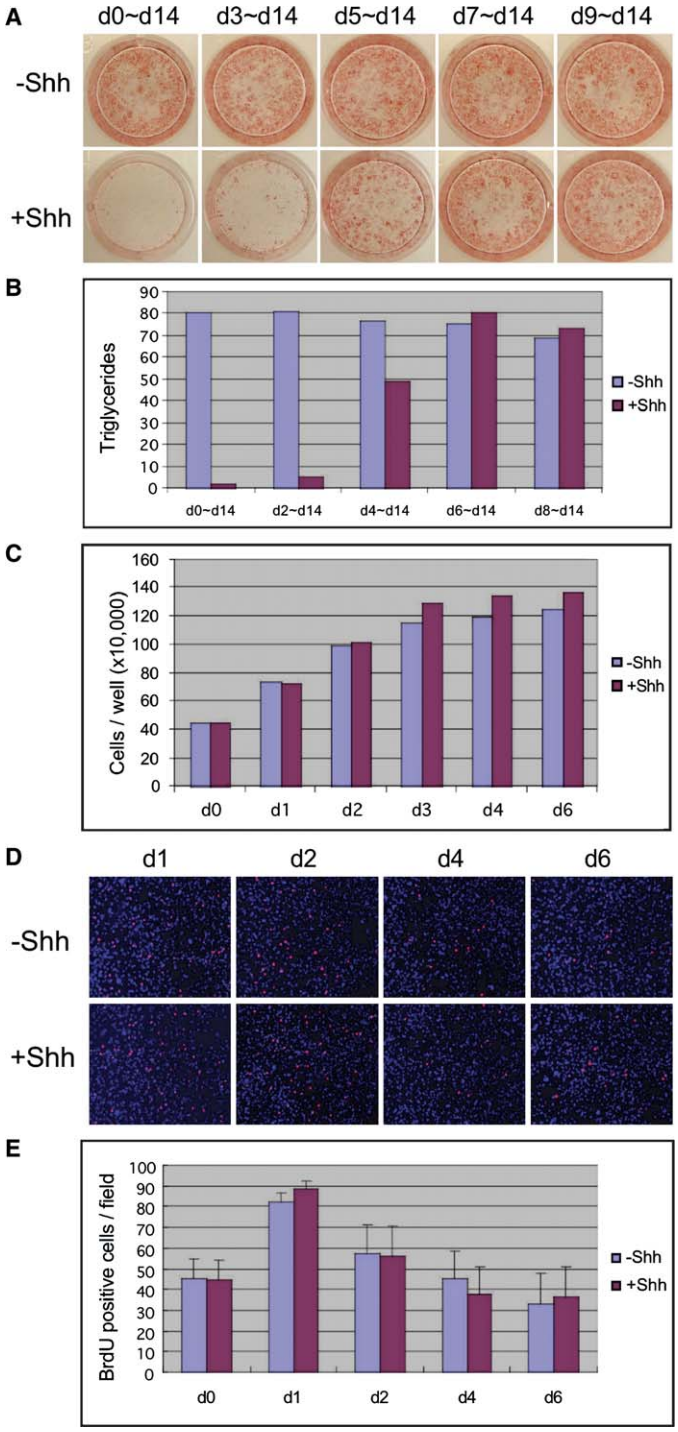

Figure 4. The Hh pathway acts early during 3T3-L1 adipogenesis, independently of the cell cycle

A and 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–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** and **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.

10T1/2s, we cultured the cells in adipogenic conditions with vehicle or Shh. Microscopy and molecular analyses showed that Hh blocked 10T1/2 adipogenesis (Figures 5B and 5C). To examine necessity, we cultured 10T1/2s in the Hh inhibitor KAAD or vehicle. We found that KAAD stimulated 10T1/2 adipogenesis (Figure 5D). To determine if Hh might be pro-osteogenic to multipotent mesenchymal progenitors, we 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 (Figure 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 (Figures 4A and 4B) (Rosen and Spiegelman, 2000) and Hh signals alter PPARγ expression (Figures 2 and 3), raising the possibility that Hh acts upstream of PPARγ. To further assess the epistatic relationship between the two, we infected 3T3-L1 cells with a GFP virus or a virus-containing PPARγ. Then, we treated the infected cells with Shh and evaluated adipogenesis. We found that PPARγ overcame the Shh-induced adipogenic blockade (Figure 5F). We also observed a PPARγ-dependent rescue of the SmoA1-induced inhibition of adipogenesis in NIH-3T3s (Figure 5F). These data support the notion that during adipogenesis, Hh signals function upstream of PPARγ.

Hh signals require Gata to block adipogenesis

To further examine the mechanisms whereby the Hh pathway might regulate PPARγ expression and hence adipogenesis, we 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, we incubated 3T3-L1s with vehicle or Shh, harvested cells at time zero, and every 4 hr until 28 hr, and assessed Rb content and phosphorylation status by Western blotting in which the phosphorylated, inactive form of Rb migrates slower. By this test, it appeared that the Hh pathway did not alter the expression levels or activity of Rb (Figure 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, we incubated 3T3-L1s with vehicle or Shh and evaluated Gata2, Gata3, and Gilz levels. After adipogenic induction, Shh increased expression of all three (Figure 6B). Next, we examined whether Shh could regulate their expression during early adipogenesis, the relevant time frame (Figure 4). Based upon real-time PCR analyses, it appeared that Shh significantly

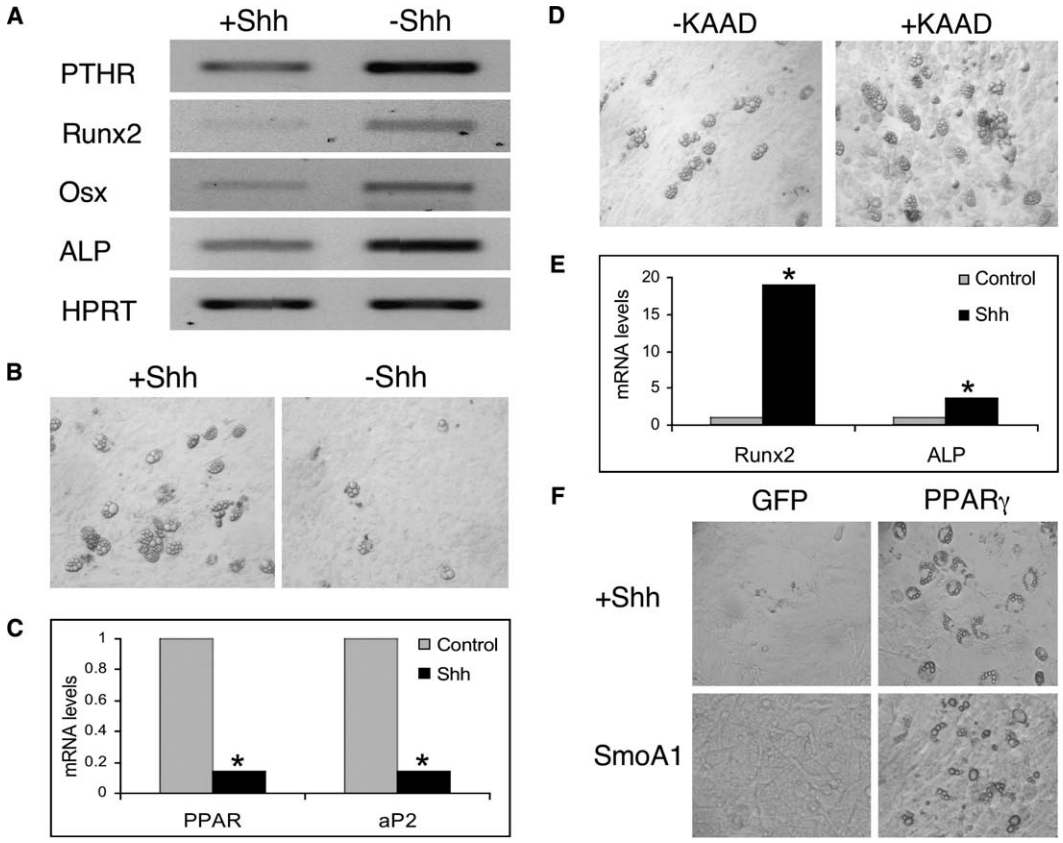

Figure 5. Hh signaling is antiadipogenic and pro-osteogenic in 3T3-L1s and 10T1/2 pluripotent mesenchymal cells and functions upstream of PPARγ

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 semiquantitative RT-PCR. HPRT serves as a loading control.

B and 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. β-actin serves as a loading control for real-time PCR (**C** and **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). *$p < 0.01$

altered Gata2 expression at both 12 and 24 hr (data not shown). We also detected a moderate increase in Gata3 expression at 12, but not 24, hr, and no change in Gilz levels at 12 or 24 hr (data not shown).

These data suggest that Shh might elicit its antiadipogenic effects by regulating Gata expression. If so, forced expression of Gata should block the effects of KAAD, a Hh inhibitor. To test this, we 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 (Figures 6C and 6D). We 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). We 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 (Figures 6E and 6F). Therefore, Shh appears to function upstream of Gata factors to elicit its antiadipogenic actions.

We 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 (Figures 6G and 6H) and reversed KAAD-stimulated adipogenesis (data not shown), supporting the idea that Gata functions downstream of Hh for antiadipogenesis. Since Gata factors are required for the antiadipogenic functions of Hh, it seemed plausible that Gatas might also have Shh's pro-osteogenic actions. To test this, we 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 (Figure 6I).

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, we examined potential changes in adipose tissue expression of the Hh signaling components produced by either genetic or diet-induced obesity (DIO). For DIO, we 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. We found that DIO significantly decreased expression levels of Smo, Gli1, Gli2, and Gli3 (Figure 7A), the four positively acting cell autonomous components of the Hh cascade. We 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 (Figure 7B). The concordant expression patterns in two obesity models support the idea that Hh signals may be germane to fat pathophysiology.

Discussion

Adipose tissues play crucial roles in many biological processes and the ability to store fat is found over a wide evolutionary distance. Homologous molecules control aspects of worm and mammalian fat formation, so 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 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 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, we provide evidence that the Hh pathway might play a conserved role in fat biology. We 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. We also found that Hh signals increased the expression levels of three antiadipogenic 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 antiadipogenic 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; we observed an increase in the expression of Pref-1, a preadipocyte marker, and also of bone markers. We also found that Hh was antiadipogenic and pro-osteogenic in C3H10T1/2 pluripotent mesenchymal cells. Gata appeared to have similar functions to Hh, but this does not exclude the possibility that other mechanisms downstream of Hh exist. It appears that the Hh cascade induces an antiadipogenic 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 modulate the Hh pathway (Bak et al., 2003; Chen et al., 2002; Taipale et al., 2000). So our data support the notion that the Hh pathway might be an appropriate target for drugs to treat lipodystrophy, obesity, and diabetes.

Experimental procedures

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 hr post-transfection. Viral supernatant was passed through a 0.2 uM filter, polybrene added to a final concentration of 6 μg/ml, and applied on successive days to preconfluent 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 37ºC in 5% CO_2. 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, postconfluent 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, postconfluent 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).

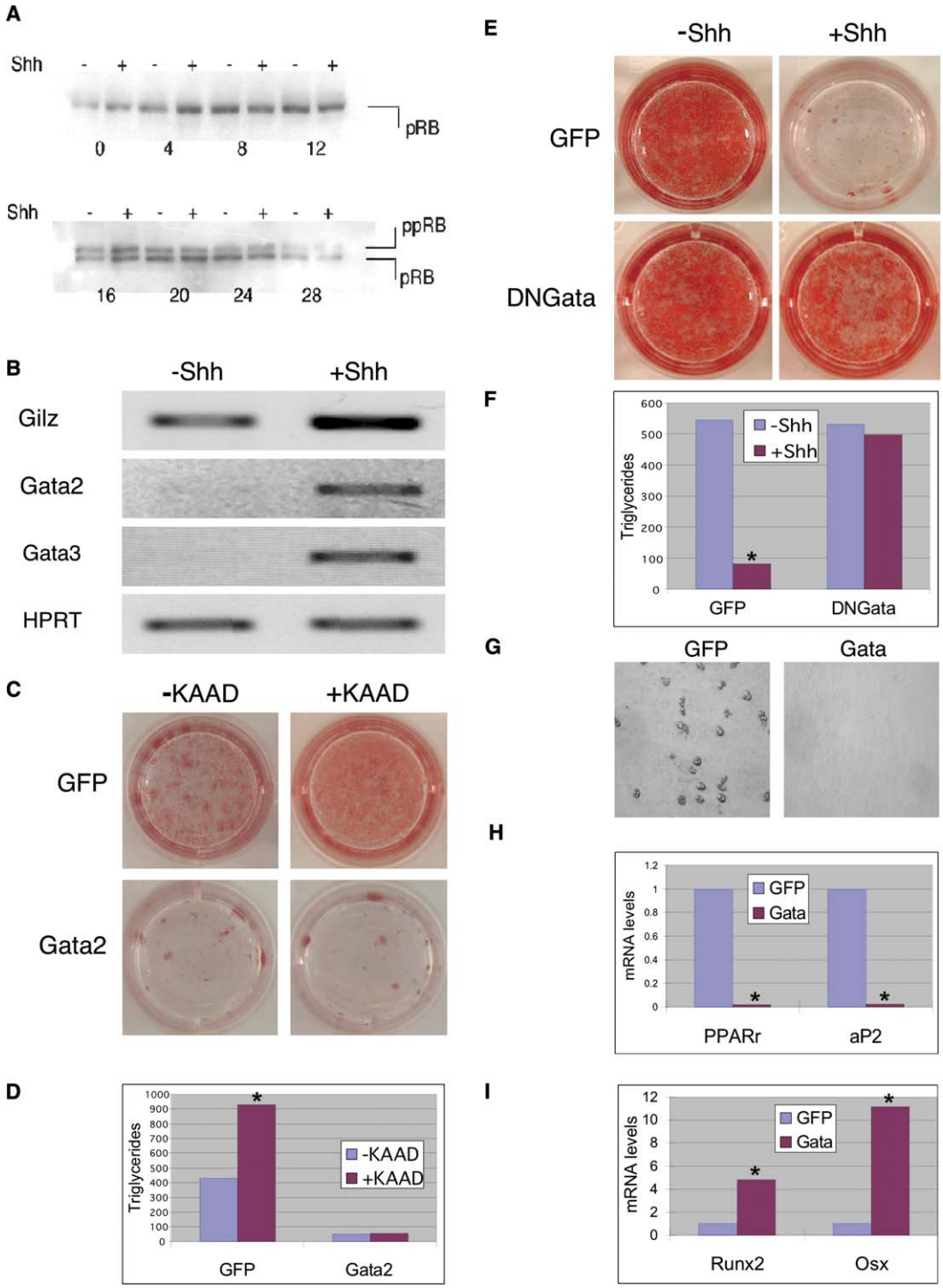

Figure 6. The Hh cascade requires Gata factors to inhibit fat formation

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 nonphosphorylated 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 antiadipogenic transcription factors were assessed with semiquantitative RT-PCR. HPRT serves as a loading control.

C and 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 and 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.

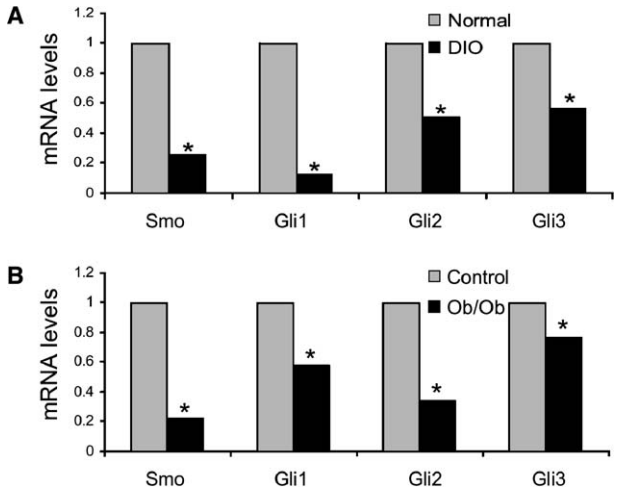

Figure 7. Murine obesity regulates the Hh pathway

A) Matched littermates were fed 4 months with 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. β-actin serves as a loading control for real-time PCR. *$p < 0.05$

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 M-MLV-reverse transcriptase (Invitrogen) to obtain cDNA. Gene expression was analyzed using either semiquantitative 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 semiquantitative. Real-time PCR values for gene expression were normalized over β-actin expression. Primer sequences are available upon request.

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 $MgCl_2$, 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 semiquantitative RT-PCR as described above. Primer sequences are available upon request.

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 we 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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References

Ashrafi, K., Chang, F.Y., Watts, J.L., Fraser, A.G., Kamath, R.S., Ahringer, J., and Ruvkun, G. (2003). Genome-wide RNAi analysis of Caenorhabditis elegans fat regulatory genes. Nature *421*, 268–272.

Bak, M., Hansen, C., Tommerup, N., and Larsen, L.A. (2003). The Hedgehog signaling pathway–implications for drug targets in cancer and neurodegenerative disorders. Pharmacogenomics *4*, 411–429.

Brand, A.H., Manoukian, A.S., and Perrimon, N. (1994). Ectopic expression in Drosophila. Methods Cell Biol. *44*, 635–654.

Buhman, K.K., Wang, L.C., Tang, Y., Swietlicki, E.A., Kennedy, S., Xie, Y., Liu, Z.Y., Burkly, L.C., Levin, M.S., Rubin, D.C., and Davidson, N.O. (2004). Inhibition of Hedgehog signaling protects adult mice from diet-induced weight gain. J. Nutr. *134*, 2979–2984.

Cadigan, K., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes Dev. *11*, 3286–3305.

Chen, J.K., Taipale, J., Cooper, M.K., and Beachy, P.A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. *16*, 2743–2748.

Chen, P.L., Riley, D.J., Chen, Y., and Lee, W.H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev. *10*, 2794–2804.

G and 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. β-actin serves as a loading control for real-time PCR (**H** and **I**). *$p < 0.01$

Cinti, S. (2000). Anatomy of the adipose organ. Eat. Weight Disord. *5*, 132–142.

Craven, S.E., Lim, K.C., Ye, W., Engel, J.D., De Sauvage, F., and Rosenthal, A. (2004). Gata2 specifies serotonergic neurons downstream of sonic hedgehog. Development *131*, 1165–1173.

Duman-Scheel, M., Weng, L., Xin, S., and Du, W. (2002). Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature *417*, 299–304.

Fajas, L., Egler, V., Reiter, R., Hansen, J., Kristiansen, K., Debril, M.B., Miard, S., and Auwerx, J. (2002). The retinoblastoma-histone deacetylase 3 complex inhibits PPARgamma and adipocyte differentiation. Dev. Cell *3*, 903–910.

Gering, M., and Patient, R. (2005). Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. Dev. Cell *8*, 389–400.

Graff, J. (1997). Embryonic patterning: To BMP or not to BMP that is the question. Cell *89*, 171–174.

Gratzner, H.G. (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science *218*, 474–475.

Hansen, J.B., Jorgensen, C., Petersen, R.K., Hallenborg, P., De Matteis, R., Boye, H.A., Petrovic, N., Enerback, S., Nedergaard, J., Cinti, S., et al. (2004). Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. Proc. Natl. Acad. Sci. USA *101*, 4112–4117.

Hoshizaki, D.K., Blackburn, T., Price, C., Ghosh, M., Miles, K., Ragucci, M., and Sweis, R. (1994). Embryonic fat-cell lineage in Drosophila melanogaster. Development *120*, 2489–2499.

Ingham, P.W., and McMahon, A.P. (2001). Hedgehog signaling in animal development: paradigms and principles. Genes Dev. *15*, 3059–3087.

Ingram, W.J., Wicking, C.A., Grimmond, S.M., Forrest, A.R., and Wainwright, B.J. (2002). Novel genes regulated by Sonic Hedgehog in pluripotent mesenchymal cells. Oncogene *21*, 8196–8205.

Karsenty, G. (1998). Transcriptional regulation of osteoblast differentiation during development. Front. Biosci. *3*, d834–d837.

Kenney, A.M., and Rowitch, D.H. (2000). Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol. Cell. Biol. *20*, 9055–9067.

Li, X., Blagden, C.S., Bildsoe, H., Bonnin, M.A., Duprez, D., and Hughes, S.M. (2004). Hedgehog can drive terminal differentiation of amniote slow skeletal muscle. BMC Dev. Biol. *4*, 9.

MacDougald, O.A., and Lane, M.D. (1995). Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem. *64*, 345–373.

Marigo, V., Johnson, R.L., Vortkamp, A., and Tabin, C.J. (1996). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. Dev. Biol. *180*, 273–283.

Martin, P.L., Lane, J., Pouliot, L., Gains, M., Stejskal, R., Smith, S.Y., Galdes, A., and Green, J. (2002). Increase in adipose and total body weight, but not in lean body mass, associated with subcutaneous administration of sonic hedgehog-Ig fusion protein to mice. Drug Dev. Res. *57*, 107–114.

McKay, R.M., McKay, J.P., Avery, L., and Graff, J.M. (2003). C. elegans. A Model for Exploring the Genetics of Fat Storage. Dev. Cell *4*, 131–142.

McMahon, A.P., Ingham, P.W., and Tabin, C.J. (2003). Developmental roles and clinical significance of hedgehog signaling. Curr. Top. Dev. Biol. *53*, 1–114.

Mikkola, H.K., and Orkin, S.H. (2002). The search for the hemangioblast. J. Hematother. Stem Cell Res. *11*, 9–17.

Molkentin, J.D., and Olson, E.N. (1996). Defining the regulatory networks for muscle development. Curr. Opin. Genet. Dev. *6*, 445–453.

Ramirez-Zacarias, J.L., Castro-Munozledo, F., and Kuri-Harcuch, W. (1992). Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry *97*, 493–497.

Rosen, E.D., and Spiegelman, B.M. (2000). Molecular regulation of adipogenesis. Annu. Rev. Cell Dev. Biol. *16*, 145–171.

Rosenfeld, J.M., and Osborne, T.F. (1998). HLH106, a Drosophila sterol regulatory element-binding protein in a natural cholesterol auxotroph. J. Biol. Chem. *273*, 16112–16121.

Ross, S., Hemati, N., Longo, K., Bennett, C., Lucas, P., Erickson, R., and MacDougald, O. (2000). Inhibition of adipogenesis by Wnt signaling. Science *289*, 950–953.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development *126*, 3915–3924.

Shi, X., Shi, W., Li, Q., Song, B., Wan, M., Bai, S., and Cao, X. (2003). A glucocorticoid-induced leucine-zipper protein, GILZ, inhibits adipogenesis of mesenchymal cells. EMBO Rep. *4*, 374–380.

Smith, V.M., Lee, P.P., Szychowski, S., and Winoto, A. (1995). Gata-3 dominant negative mutant. J. Biol. Chem. *270*, 1515–1520.

Sottile, V., and Seuwen, K. (2000). Bone morphogenetic protein-2 stimulates adipogenic differentiation of mesenchymal precursor cells in synergy with BRL 49653 (rosiglitazone). FEBS Lett. *475*, 201–204.

Spiegelman, B.M., and Flier, J.S. (2001). Obesity and the regulation of energy balance. Cell *104*, 531–543.

Spinella-Jaegle, S., Rawadi, G., Kawai, S., Gallea, S., Faucheu, C., Mollat, P., Courtois, B., Bergaud, B., Ramez, V., Blanchet, A.M., et al. (2001). Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. J. Cell Sci. *114*, 2085–2094.

Sul, H.S., Smas, C., Mei, B., and Zhou, L. (2000). Function of pref-1 as an inhibitor of adipocyte differentiation. Int. J. Obes. Relat. Metab. Disord. *24* (*Suppl 4*), S15–S19.

Sweet, H.O., Bronson, R.T., Donahue, L.R., and Davisson, M.T. (1996). Mesenchymal dysplasia: a recessive mutation on chromosome 13 of the mouse. J. Hered. *87*, 87–95.

Taipale, J., Chen, J.K., Cooper, M.K., Wang, B., Mann, R.K., Milenkovic, L., Scott, M.P., and Beachy, P.A. (2000). Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature *406*, 1005–1009.

Tang, Q.Q., Otto, T.C., and Lane, M.D. (2003). Mitotic clonal expansion: a synchronous process required for adipogenesis. Proc. Natl. Acad. Sci. USA *100*, 44–49.

Taylor, S.M., and Jones, P.A. (1979). Multiple phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell *17*, 771–779.

Tong, Q., Dalgin, G., Xu, H., Ting, C.N., Leiden, J.M., and Hotamisligil, G.S. (2000). Function of GATA transcription factors in preadipocyte-adipocyte transition. Science *290*, 134–138.

van der Horst, G., Farih-Sips, H., Lowik, C.W., and Karperien, M. (2003). Hedgehog stimulates only osteoblastic differentiation of undifferentiated KS483 cells. Bone *33*, 899–910.

Wang, G., Amanai, K., Wang, B., and Jiang, J. (2000). Interactions with Costal2 and suppressor of fused regulate nuclear translocation and activity of cubitus interruptus. Genes Dev. *14*, 2893–2905.

Wang, G., and Jiang, J. (2004). Multiple Cos2/Ci interactions regulate Ci subcellular localization through microtubule dependent and independent mechanisms. Dev. Biol. *268*, 493–505.

Wang, L.C., Nassir, F., Liu, Z.Y., Ling, L., Kuo, F., Crowell, T., Olson, D., Davidson, N.O., and Burkly, L.C. (2002). Disruption of hedgehog signaling reveals a novel role in intestinal morphogenesis and intestinal-specific lipid metabolism in mice. Gastroenterology *122*, 469–482.

Wu, X., Walker, J., Zhang, J., Ding, S., and Schultz, P.G. (2004). Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. Chem. Biol. *11*, 1229–1238.