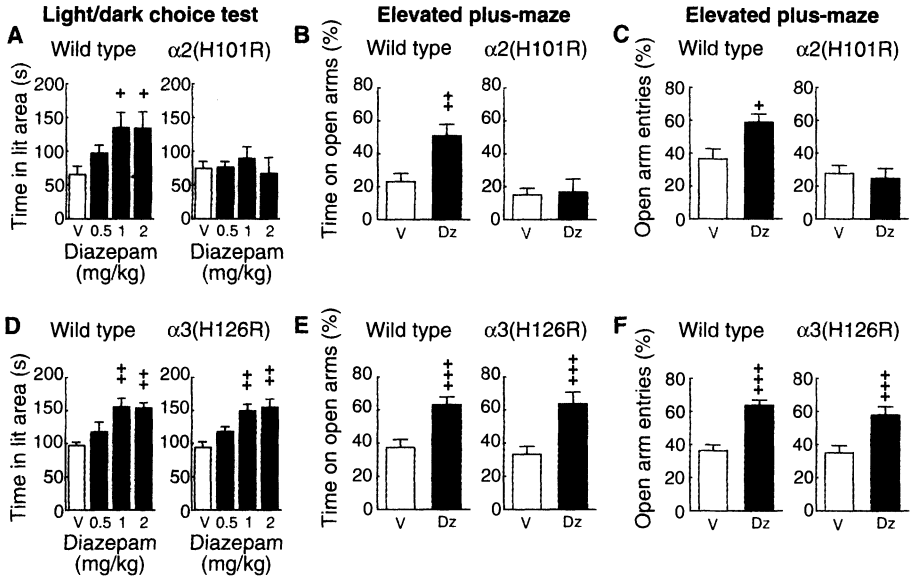

Fig. 4. Behavioral assessment of anxiolytic-like action of diazepam in α2(H101R) and α3(H126R) mice relative to wild-type mice. **(A)** Light/dark choice test. Diazepam dose-dependently increased the time spent in the lit area in wild-type mice [$F(3,36) = 3.14$, $P < 0.05$] but not in α2(H101R) mice [$F(3,36) = 0.32$, not significant] ($n = 10$ mice per group). **(B and C)** Elevated plus-maze. Diazepam (2 mg/kg) increased the percentage of time spent on the open arms and the number of entries on the open arms in wild-type mice ($P < 0.01$ and $P < 0.05$ versus vehicle) but not in α2(H101R) mice [$F(1,32) = 4.31$ and $F(1,32) = 4.76$, $P < 0.05$, respectively] ($n = 8$ to 10 mice per group). **(D)** Light/dark choice test. Both wild-type and α3(H126R) mice displayed a dose-dependent increase in the time spent in the lit area [$F(1,70) = 14.74$, $P < 0.001$, $n = 9$ or 10 mice per group]. **(E and F)** Elevated plus-maze. Diazepam (2 mg/kg) increased the percentage of time spent on the open arms and the number of entries on the open arms to the same extent in wild-type and α3(H126R) mice [$F(1,36) = 26.52$ and $F(1,36) = 37.31$, $P < 0.001$, respectively] ($n = 10$ mice per group). Results are given as means ± SEM. +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$ (Dunnett's or Fisher's pairwise post hoc comparisons or Fisher's exact tests). V, vehicle; Dz, diazepam. The light-dark choice test was carried out as described (*11*) with an illumination of 500 lux. Mice were given vehicle or increasing doses of diazepam (0.5, 1, and 2 mg/kg orally). The elevated plus-maze was performed according to Lister (*12*) under an indirect dim-light illumination (< 10 lux). Vehicle or diazepam were administered 30 min before testing.

responses to diazepam in the light/dark choice test ($P < 0.01$ versus vehicle) (Fig. 4D) and in the elevated plus-maze ($P < 0.001$ versus vehicle) (Fig. 4, E and F). These results indicate that the anxiolytic action of diazepam in wild-type mice does not involve interaction with α3 $GABA_A$ receptors.

The anxiolytic-like action of diazepam is selectively mediated by the enhancement of GABAergic transmission in a population of neurons expressing the α2 $GABA_A$ receptors, which represent only 15% of all diazepam-sensitive $GABA_A$ receptors (*13*). The α2 $GABA_A$ receptor–expressing cells in the cerebral cortex and hippocampus include pyramidal cells that display very high densities of α2 $GABA_A$ receptors on the axon initial segment, presumably controlling the output of these principal neurons (*14, 15*). Our findings indicate that the α2 $GABA_A$ receptors are highly specific targets for the development of future selective anxiolytic drugs.

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8. Cultured hippocampal pyramidal cells were chosen as a model system to confirm that the pharmacological properties of recombinant mutant α2 $GABA_A$ receptors can also be demonstrated for $GABA_A$ receptors in mutant mice.
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Function of GATA Transcription Factors in Preadipocyte-Adipocyte Transition

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Genes that control the early stages of adipogenesis remain largely unknown. Here, we show that murine GATA-2 and GATA-3 are specifically expressed in white adipocyte precursors and that their down-regulation sets the stage for terminal differentiation. Constitutive GATA-2 and GATA-3 expression suppressed adipocyte differentiation and trapped cells at the preadipocyte stage. This effect is mediated, at least in part, through the direct suppression of peroxisome proliferator–activated receptor γ. GATA-3–deficient embryonic stem cells exhibit an enhanced capacity to differentiate into adipocytes, and defective GATA-2 and GATA-3 expression is associated with obesity. Thus, GATA-2 and GATA-3 regulate adipocyte differentiation through molecular control of the preadipocyte-adipocyte transition.

In vertebrates, adipose tissue is critical for energy storage and release, as well as for endocrine homeostasis (*1, 2*). The two general classes of fat cells in mammals, brown and white, have different functions. White adipose tissue (WAT) stores excess energy in the form of triglyceride and releases free fatty acids during caloric deficiency. Brown adipose tissue (BAT), on the other hand, can dissipate energy through thermogenesis. The

coordinated action of the peroxisome proliferator–activated receptor (PPAR) γ (*3*) and the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (*4*) regulates the adipocyte differentiation program. Subsequent to C/EBPβ and C/EBPδ expression during differentiation of adipocytes, C/EBPα and PPARγ production is stimulated (*5*). There is a positive feedback loop between PPARγ and C/EBPα; both factors induce the expression of the other (*6*). This synergy drives the expression of a complex gene program that is necessary for the generation and maintenance of the adipogenic phenotype (*1, 2*). However, little is known about the commitment of pluripotent stem cells into adipogenic lineages and the genes that control the transition from preadipocytes to adipocytes. To identify factors critical at these early stages, we examined whether the genes necessary for the formation of the *Drosophila melanogaster* fat body, a homolog of mammalian adipose tissue and liver, are conserved in mammals.

The *Drosophila serpent* (*srp*) gene is critical for fat body formation (*7, 8*) and belongs to the GATA family of transcription factors, all of which share highly conserved zinc-finger DNA binding domains and bind specifically to a consensus DNA sequence (A/T)GATA(A/G) (*9–11*). To study their potential biology in mammalian adipogenesis, we used Northern blot analysis to examine the patterns of expression of GATA genes in murine adipose tissues. Of the six GATA factors investigated, adipose expression was only evident for GATA-2 and GATA-3 (Fig. 1A). Screening of adipocyte-derived libraries did not reveal any novel GATA isoforms in fat. Both GATA-2 and GATA-3 were predominantly present in the WAT. This striking expression pattern was confirmed in several additional strains of mice [see Web fig. 1 (*12*)].

Adipose tissue contains mature adipocytes, adipocyte precursors, and other cell types, such as vascular endothelial or smooth muscle cells and macrophages. To determine the source of GATA expression, we fractionated adipose tissue into mature adipocytes and the stromal-vascular fraction. Both GATA-2 and GATA-3 are expressed preferentially in the stromal-vascular fraction, which contains adipocyte precursors (Fig. 1B). Because GATA factors are expressed in WAT but not in BAT and are not found in other sites that are rich in nonadipogenic cell types present in the stromal-vascular fraction,

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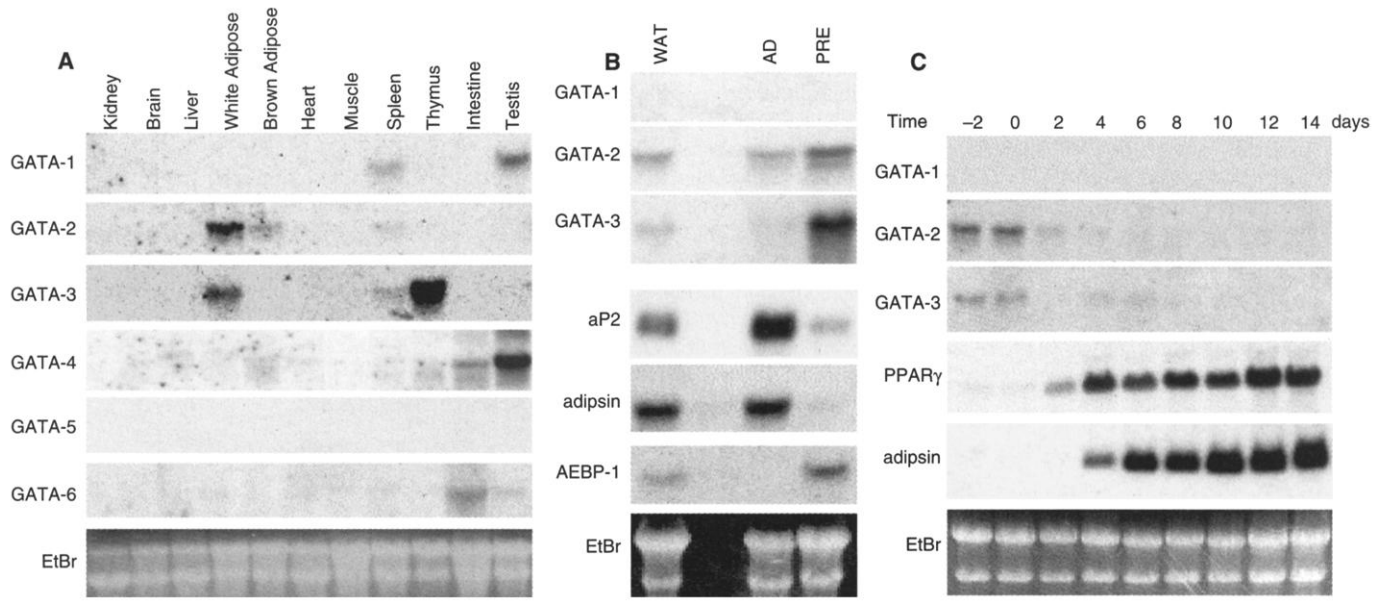

Fig. 1. Expression of GATA factors in adipose tissue and preadipocytes. (**A**) Total RNA was extracted from tissues of male mice, and the expression of GATA transcription factors was determined by Northern blot analysis, as described (*22*). (**B**) Mouse WAT was separated into mature adipocytes (AD) and the stromal-vascular fraction (PRE), as described (*23*). The expression of GATA transcription factors and control genes expressed in adipocytes (adipsin and aP2) and preadipocytes (AEBP-1) are examined by Northern blot analysis. (**C**) 3T3-F442A cells were cultured to confluency (day 0), and adipocyte differentiation was induced. Plates of cells were collected for RNA isolation and examination of gene expression before the induction of differentiation and at 2-day intervals thereafter. Ethidium bromide (EtBr) staining was shown as a control for loading and integrity of RNA.

Fig. 2. Effect of constitutive GATA-2 and GATA-3 expression on adipocyte differentiation. GATA transcription factors were expressed in 3T3-F442A cells with a retroviral expression system, as described (*22*), and treated with insulin (5 μg/ml) for a week to stimulate differentiation. The extent of adipocyte differentiation was determined by Oil Red O staining.

their source in adipose tissue is likely to be preadipocytes. Furthermore, GATA-2 and GATA-3 mRNA expression was readily detected in pure populations of cultured 3T3-F442A preadipocytes but was down-regulated upon differentiation of these cells into adipocytes (Fig. 1C). Identical results were also obtained in the 3T3-L1 preadipocyte cell line (*13*). This differentiation-dependent down-regulation of GATA expression is not due to contact growth inhibition in confluent preadipocytes, because cells cultured in nonpermissive conditions for adipogenesis maintained expression of GATA-2 and GATA-3, even after reaching confluence.

We then hypothesized that GATA-2 and GATA-3 are potential preadipocyte markers and play important roles in the regulation of adipocyte differentiation. To test this, we constitutively expressed individual GATA factors in 3T3-F442A preadipocytes with a retroviral expression system (*14*) and assessed their ability to differentiate into adipocytes. Forced expression of GATA-2 and GATA-3 inhibited adipocyte differentiation of 3T3-F442A cells, as determined by Oil Red O staining of intracellular lipid droplets (Fig. 2A) and examination of molecular markers of adipogenesis [Web fig. 2 (*12*)]. The expression of PPARγ, Glut4, the adipocyte fatty acid binding protein aP2, and adipsin was suppressed by GATA, as compared to differentiated controls, and the expression of Pref-1 (*15*) and AEBP-1 (*16*) was maintained at levels comparable to those observed in undifferentiated preadipocytes [Web fig. 2 (*12*)]. Thus, the GATA-expressing cells were trapped at the preadipocyte stage.

Next, we began exploring potential mechanisms of the effect of GATA factors on adipocyte differentiation. Because of the central role of PPARγ in adipogenesis, we examined the 0.6-kb proximal PPARγ2 promoter region (*17*) and observed several potential GATA binding sites. We then tested the ability of GATA-2 or GATA-3 to directly regulate the transcriptional activity of PPARγ2. Both GATA factors significantly suppressed (approximately fivefold) the activity of the 0.6-kb PPARγ2 promoter (Fig. 3A). In the same experimental setting, estrogen receptor response element–driven luciferase activity was enhanced by GATA-2, indicating the specificity of the GATA-mediated suppression of PPARγ2 promoter (*13*). The carboxyl and amino zinc fingers of GATA factors are involved in DNA binding (*18*) and protein-protein interactions (*19*). Deletion of both zinc-finger domains abolished the suppression of PPARγ2 promoter activity by both GATA factors (Fig. 3A). Deletion of the carboxyl zinc finger of either GATA-2 or GATA-3 also significantly but incompletely reversed their ability to suppress PPARγ2 promoter. A similar but smaller effect was observed upon deletion of the amino zinc finger. Thus, both zinc fingers are necessary, albeit with different potency, for the GATA factors to fully interact with and suppress the activity of PPARγ2 promoter.

To test whether this GATA activity requires direct interaction with the PPARγ2 promoter, we studied the proximal PPARγ2 promoter region by deletion analysis and demonstrated that a 370–base pair (bp) fragment (nucleotides –361 to +9) retains basal promoter activity and is suppressed by GATA factors. To determine if and at which sites GATA factors interact directly with this promoter fragment, we performed deoxyribonuclease (DNase) I footprinting experiments (Fig. 3B) combined with elecrophoretic mobility shift assays (Fig. 3C) using recombinant GATA-2 and GATA-3 proteins (*20*). DNase I footprinting revealed two GATA binding sites at positions –112 and –1. Sequence-dependent GATA binding to these sites was further demonstrated by the formation of specific DNA-protein complexes in electrophoretic mobility shift assays (Fig. 3C). The formation of GATA protein-DNA complexes was prevented by the addition of a specific competitor DNA fragment with known GATA binding site from the mouse α1-globin promoter (Fig. 3C). This complex was supershifted by specific antibodies to GATA, and glutathione *S*-transferase (GST) alone had no capacity to interact with the DNA targets used in these experiments, thus confirming specificity (*13*).

We next introduced point mutations into these two GATA binding sites within the 370-bp promoter region. These mutations completely abolished GATA binding, as demonstrated by electrophoretic mobility shift assays (Fig. 3C). We then transfected NIH 3T3 cells with a luciferase reporter gene driven by the 370-bp PPARγ2 promoter har-

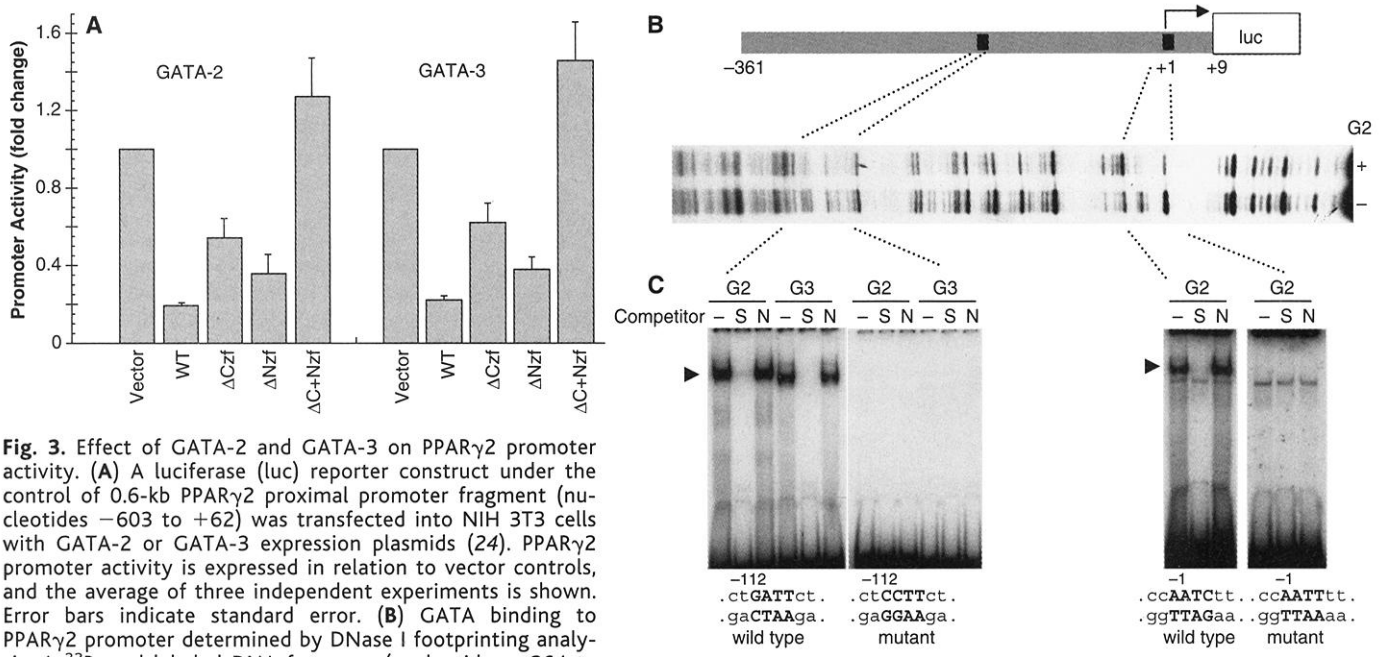

Fig. 3. Effect of GATA-2 and GATA-3 on PPARγ2 promoter activity. (**A**) A luciferase (luc) reporter construct under the control of 0.6-kb PPARγ2 proximal promoter fragment (nucleotides −603 to +62) was transfected into NIH 3T3 cells with GATA-2 or GATA-3 expression plasmids (*24*). PPARγ2 promoter activity is expressed in relation to vector controls, and the average of three independent experiments is shown. Error bars indicate standard error. (**B**) GATA binding to PPARγ2 promoter determined by DNase I footprinting analysis. A ^{32}P–end labeled DNA fragment (nucleotides −264 to +62) was incubated at 25°C with GST–GATA-2 fusion protein by using the Footprinting System (Promega, Madison, WI). The two sites of interaction are indicated by the dotted lines. (**C**) Interaction of GATA-2 (G2) and GATA-3 (G3) with DNA fragments containing GATA sites at −112 and −1 (nucleotides −119 to −85 and −22 to +9, respectively), as shown by electrophoretic mobility shift assays (*25*). For each protein-DNA reaction, specific (S) or nonspecific (N) DNA competitors were also included.

boring mutations in GATA binding sites at positions −112 and −1. The activity of the 370-bp fragment of PPARγ2 promoter lacking both GATA-binding sites was not suppressed by GATA factors (106 versus 100% in the presence or absence of GATA). Introduction of mutations into either site, alone, was insufficient to prevent GATA-mediated suppression (*13*). Thus, GATA-2 and GATA-3 bind directly to two specific sites on the proximal PPARγ promoter and negatively regulate its basal transcriptional activity.

To test whether the negative regulation of PPARγ activity is important for the GATA-induced suppression of adipocyte differentiation, we exogenously expressed PPARγ to rescue the inhibitory effects of GATA factors on adipocyte differentiation in 3T3-F442A cells. Coexpression of PPARγ with GATA-3 in 3T3-F442A cells resulted in substantial but incomplete reversal of GATA-induced suppression of adipogenesis as determined by Oil Red O staining of the cells (Fig. 4A) and by the expression of adipocyte markers such as aP2 and adipsin (Fig. 4B). Similar results were also obtained in cells coexpressing GATA-2 and PPARγ (*13*). Thus, GATA-induced suppression of PPARγ activity is, at least in part, responsible for diminished differentiation capacity and defective expression of most adipogenic genes, although complete expression of the adipocyte phenotype in these cells may involve additional GATA-modulated pathways.

If GATA expression is required at the preadipocyte commitment stage, then cells lacking GATA factors might be impaired in their ability to differentiate. On the other hand, if GATA factors control the preadipocyte-adipocyte transition, the lack of GATA should result in accelerated differentiation into adipocytes. To address these possibilities directly, we examined the adipocyte differentiation capacity of the pluripotent embryonic stem (ES) cells lacking both functional copies of the GATA-3 gene (*21*). In vitro differentiation experiments demonstrated an enhanced capacity of the GATA-3–deficient ES cells to form mature adipocytes, in comparison to wild-type controls under minimally permissive hormonal conditions (Fig. 5A). In three independent experiments, a substantially higher number of GATA-3$^{-/-}$ embryoid bodies displayed adipogenesis, as compared to wild-type cells (50 versus 18%). Furthermore, the number of differentiated cells and the extent of adipocyte differentiation on each embryoid body were also markedly enhanced in the GATA-3$^{-/-}$ ES cells (Fig. 5A). Consistent with this extensive differentiation, the expression levels of adipocyte markers were also significantly elevated in GATA-3–deficient ES cells [Web fig. 3 (*12*)]. Therefore, GATA-3 is not required for the lineage commitment of preadipocytes in culture, but instead functions as a negative regulator of the preadipocyte-to-adipocyte transition.

If a lack of GATA factors promotes adipogenesis, increased adiposity might be associated with defects in the expression and/or function of these genes. To begin to test this,

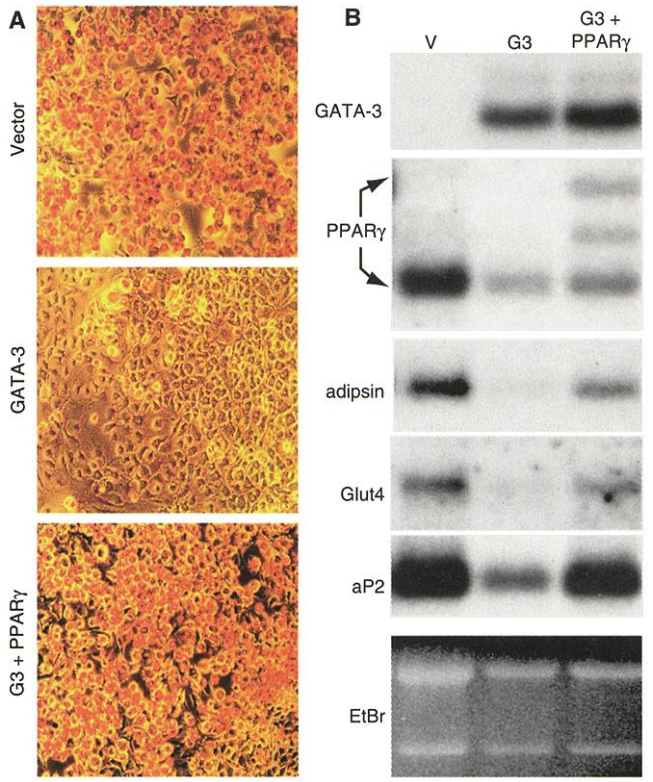

Fig. 4. Reversal of GATA-induced suppression of adipogenesis by expression of exogenous PPARγ. 3T3-F442A cells were infected with empty retroviral vectors (V) or vectors expressing GATA-3 (G3) and both GATA-3 and PPARγ (G3+PPARγ). Infected cells were selected with both hygromycin and puromycin treatment and differentiated into adipocytes with insulin (5 μg/ml) and 0.1 μM of PPARγ activator BRL49653 for 6 days. The extent of differentiation was revealed by (**A**) Oil Red O staining and (**B**) mRNA levels of adipocyte-specific genes (aP2, Glut4, and adipsin). For PPARγ, arrows indicate exogenous (upper) and endogenous (lower) transcripts.

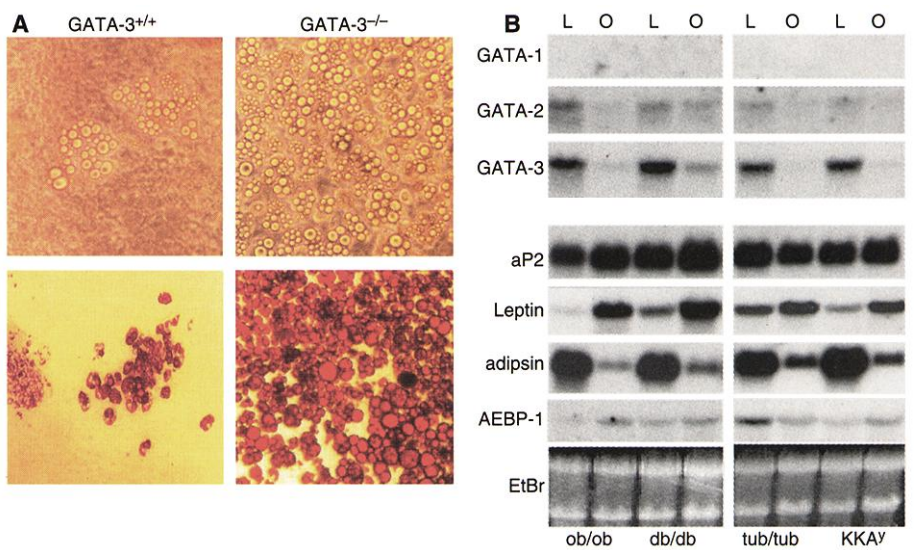

Fig. 5. GATA-3 deficiency results in enhanced capacity of adipocyte differentiation, and defective GATA expression is associated with obesity. (**A**) Wild-type and GATA-3–deficient ES cells were differentiated into adipocytes, as described (*26*), with the following modifications. ES cells were first cultured in suspension without retinoic acid treatment for 8 days to form embryonic bodies. These were then seeded on gelatin-coated plates and treated with insulin (5 μg/ml) for 10 days. After 3 days of treatment with dexamethasone (1 μM) and methylisobutylxanthine (0.5 mM), cells were maintained in insulin for nine additional days. Representative direct (upper panels) and Oil Red O–stained micrographs (lower panels) of wild-type and GATA-3–deficient ES cells are shown. (**B**) WAT samples were obtained from four different genetic models of murine obesity to determine GATA mRNA expression. The *db/db*, *KKA^{y} yellow*, and *tub/tub* mice and matched controls were from Jackson Laboratories (Bar Harbor, Maine). The *ob/ob* mice and the controls were from our own colony. The expression levels of adipsin, leptin, aP2, and AEBP-1 are shown as controls. L, lean; O, obese.

we investigated the expression of GATA-2 and GATA-3 in WAT samples from several different models of murine obesity. These experiments demonstrated a severe reduction in the adipose expression of both GATA-2 and GATA-3 in four independent genetic models of obesity, including *ob/ob*, *db/db*, *tub/tub*, and *KKA^{Y} yellow*, in comparison to matched lean littermates (Fig. 5B). This reduced expression was specific to GATA, because AEBP-1 mRNA expression was not regulated in the obese animals and the level of the Pref-1 in adipose tissue was very low.

Our data demonstrate that GATA-2 and GATA-3 are preadipocyte genes, which act as molecular gatekeepers by controlling the transition from preadipocytes to adipocytes. Despite substantial changes in the architecture and the molecular complexity of adipose tissue, the biology of GATA factors at this site has been preserved from the fruit fly to the mouse. In higher organisms with balanced energy homeostasis, only a portion of the preadipocyte pool is used to become differentiated adipocytes. The remainder of the preadipocytes remains quiescent. Under the appropriate conditions, such as imbalance between energy intake and output, these cells differentiate into adipocytes and expand adiposity. If these control points fail, the result would be increased adiposity and, consequently, a higher tendency for obesity. The opposite will result in a loss of adiposity. Therefore, it is not surprising that the cellular machinery is equipped with molecules to control the rate and extent of transition between preadipocytes and adipocytes. The data presented here indicate that GATA factors are important regulators of this homeostatic mechanism and they may serve as targets for therapeutic intervention in diseases such as lipodystrophies and obesity.

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25. The electrophoretic mobility shift assays were performed in a 20-μl reaction volume. Two microliters of GST-GATA fusion protein was incubated with 0.5-μg poly[d(I-C)] and ^{32}P-labeled DNA probe (4×10^4 cpm) in the presence or absence of 1 μg of specific or nonspecific competitors, at room temperature for 20 min. The sequence of the specific competitor is 5′-GATCTCCGGCAACTGATAAGGATTCCCTG-3′ (underlined sequence indicates GATA recognition site), and the sequence of the nonspecific competitor is 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′.
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27. We thank S. H. Orkin for GATA-1, GATA-2, and GATA-3; D. B. Wilson for GATA-4; M. S. Parmacek for GATA-5 and GATA-6; H. S. Sul for Pref-1; H. S. Ro for AEBP-1 cDNAs; J. R. Reddy for PPARγ; and F. Saatcioglu for estrogen and thyroid receptor response element–driven reporter constructs. Q.T. is a recipient of the National Research Service Award (F32DK09940). The work is supported partially by NIH grants to J.M.L. (R37AI29673) and G.S.H. (DK56894) and by the Biomedical Scholar award from the Pew Foundation to G.S.H.

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Proximity of Chromosomal Loci That Participate in Radiation-Induced Rearrangements in Human Cells

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Rearrangements involving the *RET* gene are common in radiation-associated papillary thyroid cancer (PTC). The *RET*/PTC1 type of rearrangement is an inversion of chromosome 10 mediated by illegitimate recombination between the *RET* and the *H4* genes, which are 30 megabases apart. Here we ask whether despite the great linear distance between them, *RET* and *H4* recombination might be promoted by their proximity in the nucleus. We used two-color fluorescence in situ hybridization and three-dimensional microscopy to map the positions of the *RET* and *H4* loci within interphase nuclei. At least one pair of *RET* and *H4* was juxtaposed in 35% of normal human thyroid cells and in 21% of peripheral blood lymphocytes, but only in 6% of normal mammary epithelial cells. Spatial contiguity of *RET* and *H4* may provide a structural basis for generation of *RET*/PTC1 rearrangement by allowing a single radiation track to produce a double-strand break in each gene at the same site in the nucleus.

Chromosomal rearrangements involving the *RET* gene are highly prevalent in radiation-induced thyroid tumors from children exposed to environmental radiation after the Chernobyl accident (*1–3*) and in thyroid cancers from patients with a history of medical external irradiation (*4*). They can also be detected 48 hours after exposing either human fetal thyroid explants or undifferentiated thyroid carcinoma cells to x-rays (*5*, *6*). Two common types of *RET* rearrangement in PTCs are intrachromosomal inversions that fuse the DNA sequence encoding the tyrosine kinase domain of *RET* with a portion of either the *H4* gene (*RET*/PTC1) or the *ELE1* gene (*RET*/PTC3) (*7*, *8*). In most *RET*/PTC3 tumors, the *ELE1-RET* fusion gene is formed by joining *ELE1* intron 5 to *RET* intron 11 by illegitimate recombination events, most of

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